Garland Science

Molecular Biology of THE CELL

Sixth Edition

The Problems Book

Also includes complete solutions to the end-of-chapter problems from *Molecular Biology of the Cell*, Sixth Edition

JOHN WILSON and TIM HUNT

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Garland Science
Taylor & Francis Group

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ISBN: 978-0-8153-4453-7 (paperback)

Published by Garland Science, Taylor & Francis Group, LLC, an informa business,
711 Third Avenue, New York, NY 10017, US
3 Park Square, Milton Park, Abingdon, OX14 4RN, UK

Printed in the United States of America

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

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Visit our website at http://www.garlandscience.com

We dedicate this book to the memory of our comrade, Julian Lewis.

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Preface

Welcome to *The Problems Book*, which aims to help students appreciate the ways in which an understanding of how cells work, as discussed in *Molecular Biology of the Cell*, Sixth Edition, by Alberts et al., can be further explored through experiments and simple calculations. As always, we hope to stimulate our readers to ask questions as well as to learn and digest the stories that "the big book" tells. In real life, knowledge and understanding come from research, which entails curiosity, puzzlement, doubt, criticism, and debate as well as performing experiments. Groping one's way through the fog of uncertainty during a project is a slow and often discouraging process; eureka moments (even if one is lucky) are few and far between. Nevertheless, those moments catch the essence of the drama, and we have tended to focus on them, where we have been able to cast them in the form of a problem. In this way, for student and teacher alike, we hope to encourage a questioning attitude to biology. Without curiosity there would be neither science nor scientists.

We have been making up problems together since 1985, and the revision leading to this new edition of *The Problems Book* has taken us more than four years. There are several new things about this edition. First, the book is now in color. As well as improving its look as a whole, we think this will improve the clarity and intelligibility of the figures. Second, we've added a new type of question, MCAT Style, modeled on the kind of problems that are found in most medical school admissions tests. These were drafted by Doug Kellogg at the University of California, Santa Cruz, and we think they make a great addition to the book. Indeed, we were pleasantly surprised to discover that these questions allowed us to frame problems in new and interesting ways. Elsewhere, we have done a considerable amount of pruning, partly to make space for these new problems and partly to eliminate problems that were showing their age or were no longer relevant to the parent text.

The organization of *The Problems Book* remains largely the same. There are Terms to Learn, Definitions, and True/False sections in every chapter. Next come Thought Problems, of which some are more challenging than others—they may be playful, or serious, but all are designed to make the reader think. Following these is a section called Calculations, designed to deal with quantitative aspects of cell biology. The calculations in this book are mostly very straightforward, usually involving no more than the interconversion of units, yet they provide a solid framework for thinking about the cell. Are cell-surface receptors sparse in the plasma membrane, or jam-packed? Do molecules diffuse across a cell slowly, or in the blink of an eye? Does chromatin occupy most of the nuclear volume, or just a tiny fraction? How fast could a tomato plant grow, theoretically? Numerical analysis of such questions is very important if one is to gain an understanding for the molecular basis of cell biology. The Data Handling section contains research-based problems. Our original brief was to compose problems based on experiments so as to allow readers to get a better feel for the way in which biological knowledge is obtained. It is tremendously important to keep asking, "How do we know that? What's the evidence?" or to wonder how one might go about finding something out. Often it's not at all obvious, often the initial breakthrough was a lucky chance observation, made while investigating some completely different

business. In fact, it takes most of us years of research experience to grasp the idea of how one simple fact "can illuminate a distant area, hitherto dark" (Boveri, 1902). Seeing how these tiny shards of evidence give rise to the big picture often involves considerable imagination as well as a certain discipline, to know how much weight the evidence will bear. We hope we have sometimes, at least, been able to capture the essence of how experiments lead to understanding. To do justice to the authors of the experiments we use in these problems, however, we strongly recommend recourse to the original papers, whose references we always provide.

A newly compiled section, Medical Links, contains problems of particular interest to health science students.

We hope that the organization and classification of problems will help both student and teacher to find what they are looking for. How should this book be used? We composed it by a process of constant dialogue and discussion, and we suspect that the most fruitful use of the problems will be to stimulate discussions in class, or between students. Tackling selected problems as homework will also surely help. Teachers have told us that they find ideas for exam questions here, and all the answers to our questions are now provided in *The Problems Book*, for many of these problems are difficult to answer and are not intended to be set as tests. Rather, we hope that readers will be intrigued (as we were) by the questions we ask, and after thinking a bit will want to see what the answer is, what form the discussion takes, and how to get at thinking about this particular kind of a problem.

The answers to the end-of-chapter problems in *Molecular Biology of the Cell*, Sixth Edition can also be found in the back of this book, including the answers to newly written problems for Chapters 21 through 24.

As always, we want to hear from our readers, for despite our best efforts, we do not always get things right. Please email John Wilson at jwilson@bcm.edu or Tim Hunt at tim.hunt@cancer.org.uk with your comments or queries, and we'll do our best to answer them.

Acknowledgments

It remains true that our rate of production, over the years, averages at about one chapter per year, but even this glacial progress would not have been possible without a tremendous amount of help from friends and colleagues whose names are recorded in previous editions of *The Problems Book*, which appeared in 1989, 1994, 2002, and 2008. As ever, we are indebted to Alastair Ewing who worked through all the new problems, discovering embarrassing mistakes and finding better, clearer, and more graceful ways of putting things. Denise Schanck has been a tower of strength, as always, and Emma Jeffcock a brilliant and startlingly efficient designer and friend. Allie Bochicchio coordinated our activities and kept us in good order. Mike Morales helped to set up an instant home-away-from-home during meetings in California, and his cheerful humor helped tremendously. Adam Sendroff, who took care of marketing and gave us audience feedback, was unfailingly supportive. We are especially grateful to all the authors of *Molecular Biology of the Cell*, who have been extremely helpful in the selection and refinement of problems that appear in the main text. We thank them most warmly for their suggestions. Once again, Nigel Orme has been a huge help with the illustrations, particularly in the business of adding color. The person who made the most important contribution to this edition, however, is Doug Kellogg of the University of California, Santa Cruz. Doug is an old friend who allowed himself to be persuaded to take on the task of composing MCAT-style questions, despite his heavy responsibilities as a teacher, researcher, and new father. We are very lucky, however, to have the loving support of our spouses and families, Lynda, Mary, Celia, and Aggie. They've put up heroically with our regular absences and preoccupations.

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A Couple of Useful Things to Know

Avogadro's Number (6.02×10^{23} molecules/mole)

Avogadro's number (N) is perhaps the most important constant in molecular sciences, and it appears again and again in this book. Do you know how it was determined? We didn't, or had forgotten if we ever knew. How can one measure the number of molecules in a mole? And who did it first? You will not find this information in modern biology books, partly because it is ancient history, and partly because it was the business of physicists; some pretty good physicists too, as we shall see.

Amadeo Avogadro had no idea how many molecules there were in 22.4 L of a gas. His hypothesis, presented in 1811, was simply that equal volumes of all gases contained the same number of molecules, irrespective of their size or density. Not until much later, when the reality of molecules was more widely accepted and the microscopic basis for the properties of gases was being worked out, were the first estimates attempted. An Austrian high school teacher called Josef Loschmidt used James Clerk Maxwell's recently developed kinetic theory of gases to estimate how many molecules there were in a cubic centimeter of air. Maxwell had derived an expression for the viscosity of a gas, which is proportional to the density of the gas, to the mean velocity of the molecules, and to their mean free path. The latter could be estimated if one knew the size and number of the molecules. Loschmidt simply made the assumption that when a gas was condensed into a liquid, its molecules were packed as closely as they could be, like oranges in a display on a fruit stand, and from this he was able to get a pretty accurate value for Avogadro's number. Not surprisingly, in Austria they often refer to N as "Loschmidt's number." In fact, it wasn't until 1909 that the term "Avogadro's number" was suggested by Jean Perrin, who won the 1926 Nobel Prize in Physics (his lecture is available on the Nobel web site, and his book entitled, simply, *Atoms* [*Les Atomes*, 1913, translated from the original French by D.L. Hammick, reprinted in 1990 by Ox Bow Press] is highly recommended—and accessible—reading. It has been called the finest book on physics of the twentieth century).

You may be surprised to discover, as we were, that estimating Avogadro's number was an important component of Albert Einstein's Ph.D. thesis. Abraham Pais's wonderful biography of Einstein, *Subtle is the Lord* (subtitled *The Science and the Life of Albert Einstein*, 1982, Oxford University Press), devotes Chapter 5, "The Reality of Molecules," to this period of the great physicist's life and work. Einstein found three independent ways to estimate N: from the viscosity of dilute sucrose solutions, from his analysis of Brownian motion, and from light-scattering by gases near the critical point, including the blueness of the sky. Because the sky is five million times less bright than direct sunlight, Avogadro's number is 6×10^{23}. Isn't that romantic?

But Einstein's was not the last word on the subject. Indeed, according to Pais, he made an "elementary but nontrivial mistake" in his thesis that was later corrected, and it was really Perrin who brought the whole field together with his experiments on Brownian motion. The Nobel presentation speech contains this line:

"His [Perrin's] measurements on the Brownian movement showed that Einstein's theory was in perfect agreement with reality. Through these measurements a new determination of Avogadro's number was obtained."

For most methods of counting molecules, neither the physics nor the math is easy to follow, but two are simple to understand. The first comes from radioactive decay, and another Nobel prize-winning physicist, Ernest Rutherford. When radium decays, it emits alpha particles, which are helium nuclei. If you can count the radioactive decay events with a Geiger counter and measure the volume of helium emitted, you can estimate Avogadro's number. The second way is much more modern. You can see large proteins and nucleic acids with the aid of an electron microscope, and count them directly!

Calculations and Unit Analysis

Many of the problems in this book involve calculations. Where the calculations are based on an equation (for example, the Nernst equation or the equation for the volume of a sphere), we provide the equation along with a brief explanation of symbols, and often their values. Many calculations, however, involve the conversion of information from one form into another, equivalent form. For example, if the concentration of a protein is 10^{-9} M, how many molecules of it would be present in a mammalian nucleus with a volume of 500 μm^3? Here, a concentration is given as M (moles/L), whereas the desired answer is molecules/nucleus; both values are expressed as "number/volume" and the problem is to convert one into the other.

Both kinds of calculation use constants and conversion factors that may or may not be included in the problem. The Nernst equation, for example, uses the gas constant R (8.3×10^{-3} kJ/K mole) and the Faraday constant F (96 kJ/V mole). And conversion of moles/L to molecules/nucleus requires Avogadro's number N (6.0×10^{23} molecules/mole). All of the constants, symbols, and conversion factors that are used in this book are listed in Tables 1–8 at the end of the book, on pages 963–966 (including the standard genetic code, the one-letter amino acid code, useful geometric formulas, and data on common radioisotopes used in biology).

For each type of calculation, we strongly recommend the powerful general strategy known as unit analysis (or dimensional analysis). If units (for example, moles/L) are included along with the numbers in the calculations, they provide an internal check on whether the numbers have been combined correctly. If you've made a mistake in your math, the units will not help, but if you've divided where you should have multiplied, for example, the units of the answer will be nonsensical: they will shout "error." Consider the conversion of 10^{-9} M (moles/L) to molecules/nucleus. In the conversion of moles to molecules, do you multiply 10^{-9} by 6×10^{23} (Avogadro's number) or do you divide by it? If units are included, the answer is clear.

$$\frac{10^{-9}\ \text{moles}}{\text{L}} \times \frac{6 \times 10^{23}\ \text{molecules}}{\text{mole}} = \frac{6 \times 10^{14}\ \text{molecules}}{\text{L}} \quad \textbf{YES}$$

$$\frac{10^{-9}\ \text{moles}}{\text{L}} \times \frac{\text{mole}}{6 \times 10^{23}\ \text{molecules}} = \frac{1.7 \times 10^{-33}\ \text{mole}^2}{\text{molecules L}} \quad \textbf{NO}$$

Similarly, in the conversion of liters to nuclei, the goal is to organize the conversion factors to transform the units to the desired form.

$$\frac{6 \times 10^{14}\ \text{molecules}}{\text{L}} \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{\text{mL}}{\text{cm}^3} \times \frac{\text{cm}^3}{(10^4\ \mu\text{m})^3} \times \frac{500\ \mu\text{m}^3}{\text{nucleus}} = \frac{300\ \text{molecules}}{\text{nucleus}}$$

If you do this calculation with pure numbers, you must worry at each step whether to divide or multiply. If you attach the units, however, the decision is obvious. It is important to realize that any set of (correct) conversion factors will give the same answer. If you are more comfortable converting liters to ounces, that's fine, so long as you know a string of conversion factors that will ultimately transform ounces to μm^3.

There are a few simple rules for handling units in calculations.

1. Quantities with different units cannot be added or subtracted. (You cannot subtract 3 meters from 10 kJ.)
2. Quantities with different units can be multiplied or divided; just multiply or divide the units along with the numbers. (You can multiply 3 meters times 10 kJ; the answer is 30 kJ meters.)
3. All exponents are unitless. (You can't use $10^{6\ \text{mL}}$.)
4. You cannot take the logarithm of a quantity with units.

Throughout this book, we have included the units for each element in every calculation. If the units are arranged so that they cancel to give the correct units for the answer, the numbers will take care of themselves.

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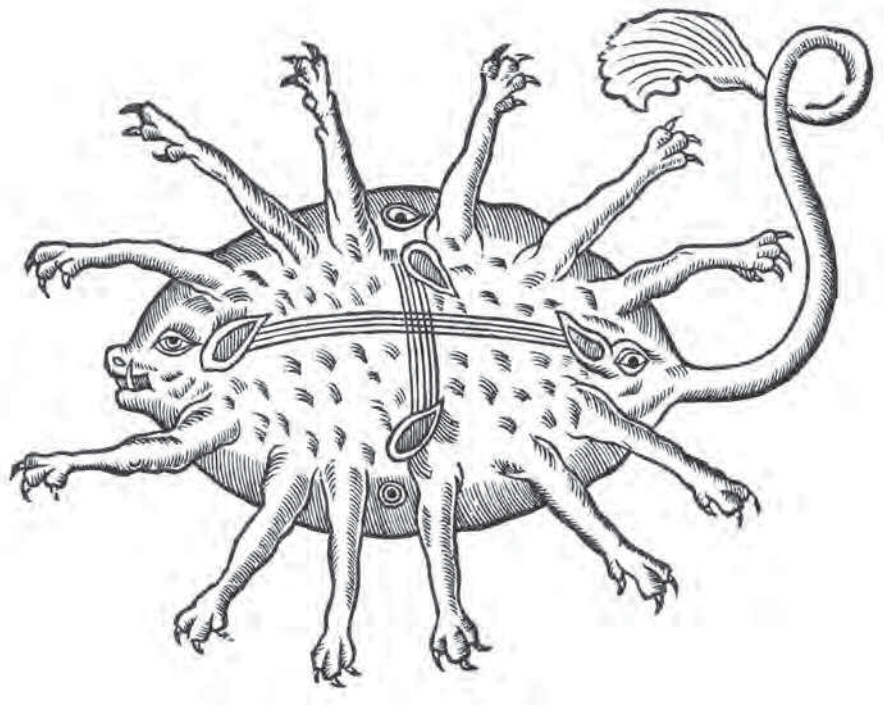


A Sea Creature Sighted Between Antibes and Nice in 1562.

We shall never know exactly what the person who drew this picture actually saw, but it is doubtful if he (or she) made it up completely. Anyone who has taken a histology course will know how very difficult it is to learn to see and abstract salient, accurate details from a completely unfamiliar scene. This is important for cell biologists; it is natural to interpret the unfamiliar in terms of what you already know, blinding you to the more truthful new. This theme runs through the opening pictures in this book, but if you are curious as to what the “sea creature” really looked like, turn to the Answers section.

CHAPTER 1

Cells and Genomes

IN THIS CHAPTER

THE UNIVERSAL FEATURES OF CELLS ON EARTH

TERMS TO LEARN

amino acid
DNA replication
enzyme
gene
genome
messenger RNA (mRNA)
nucleotide
plasma membrane
polypeptide
protein
ribonucleic acid (RNA)
transcription
translation

DEFINITIONS

Match each definition below with its term from the list above.

1–1 The selective barrier surrounding a living cell that enables the cell to concentrate nutrients, retain products, and excrete waste.

1–2 A protein that catalyzes a specific chemical reaction.

1–3 The copying of one strand of DNA into a complementary RNA sequence.

1–4 Process by which the sequence of nucleotides in an mRNA molecule directs the incorporation of amino acids into protein.

1–5 Region of DNA that controls a discrete hereditary characteristic of an organism, usually corresponding to a single protein (or set of alternative protein variants) or to a structural, catalytic, or regulatory RNA.

1–6 RNA molecule that specifies the amino acid sequence of a protein.

1–7 The building blocks of proteins.

1–8 The total genetic information of a cell or organism as embodied in its complete DNA sequence.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

1–9 Genes and their encoded proteins are co-linear; that is, the order of amino acids in proteins is the same as the order of the codons in the RNA and DNA.

1–10 DNA and RNA use the same four-letter alphabet.

THOUGHT PROBLEMS

1–11 "Life" is easy to recognize but difficult to define. The dictionary defines life as "The state or quality that distinguishes living beings or organisms from dead ones and from inorganic matter, characterized chiefly by metabolism, growth, and the ability to reproduce and respond to stimuli."

Biology textbooks usually elaborate slightly; for example, according to a popular text, living things

1. Are highly organized compared with natural inanimate objects.
2. Display homeostasis, maintaining a relatively constant internal environment.
3. Reproduce themselves.
4. Grow and develop from simple beginnings.
5. Take energy and matter from the environment and transform it.
6. Respond to stimuli.
7. Show adaptation to their environment.

Score a car, a cactus, and yourself with respect to these seven characteristics.

1–12 NASA has asked you to design a module that will identify signs of life on Mars. What will your module look for?

1–13 You have embarked on an ambitious research project: to create life in a test tube. You boil up a rich mixture of yeast extract and amino acids in a flask along with a sprinkling of the inorganic salts known to be essential for life. You seal the flask and allow it to cool. After several months, the liquid is as clear as ever, and there are no signs of life. A friend suggests that excluding air was a mistake, since most life as we know it requires oxygen. You repeat the experiment, but this time you leave the flask open to the atmosphere. To your great delight, the liquid becomes cloudy after a few days and under the microscope you see beautiful small cells that are clearly growing and dividing. Does this experiment prove that you managed to generate a novel life-form? How might you redesign your experiment to allow air into the flask, yet eliminate the possibility that contamination is the explanation for the results?

1–14 The genetic code (see Tables 7 and 8, page 966) specifies the entire set of codons that relate the nucleotide sequence of mRNA to the amino acid sequence of encoded proteins. Since the code was deciphered nearly four decades ago, some have claimed that it must be a frozen accident, while others have argued that it was shaped by natural selection.

A striking feature of the genetic code is its inherent resistance to the effects of mutation. For example, a change in the third position of a codon often specifies the same amino acid or one with similar chemical properties. But is the natural code more resistant to mutation (less susceptible to error) than other possible versions? The answer is an emphatic "Yes," as illustrated in **Figure 1–1**. Only one in a million computer-generated "random" codes is more error-resistant than the natural genetic code.

Does the extraordinary mutation resistance of the genetic code argue in favor of its origin as a frozen accident or as a result of natural selection? Explain your reasoning.

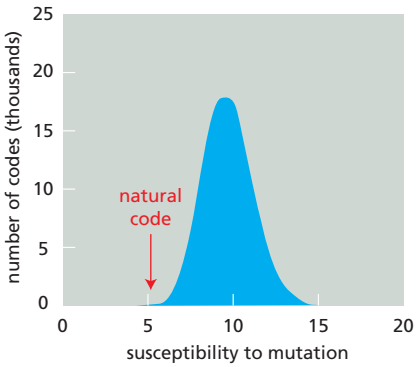


Figure 1–1 Susceptibility to mutation of the natural code shown relative to that of millions of other computer-generated alternative genetic codes (Problem 1–14). Susceptibility measures the average change in amino acid properties caused by random mutations in a genetic code. A small value indicates that mutations tend to cause only minor changes.

1–15 You have begun to characterize a sample obtained from the depths of the oceans on Europa, one of Jupiter's moons. Much to your surprise, the sample contains a life-form that grows well in a rich broth. Your preliminary analysis shows that it is cellular and contains DNA, RNA, and protein. When you show your results to a colleague, she suggests that your sample was contaminated with an organism from Earth. What approaches might you try to distinguish between contamination and a novel cellular life-form based on DNA, RNA, and protein?

1–16 In the 1940s, Erwin Chargaff made the remarkable observation that in samples of DNA from a wide range of organisms the mole percent of G [G/(A+T+C+G)] was equal to the mole percent of C, and the mole percents of A and T were equal. This was an essential clue to the structure of DNA. Nevertheless, Chargaff's "rules" were not universal. For example, in DNA from the virus ΦX174, which has a single-stranded genome, the mole percents are A = 24, C = 22, G = 23, and T = 31. What is the structural basis for Chargaff's rules, and how is it that DNA from ΦX174 doesn't obey the rules?

1–17 In 1944, at the beginning of his book *What is Life*, the great physicist Erwin Schrödinger (of cat fame) asked the following question: "How can the events *in time and space* which take place within the spatial boundary of a living organism be accounted for by physics and chemistry?" What would be your answer today? Do you think there are peculiar properties of living systems that disobey the laws of physics and chemistry?

1–18 Which of the following correctly describe the coding relationships (template → product) for replication, transcription, and translation?

A. DNA → DNA
B. DNA → RNA
C. DNA → protein
D. RNA → DNA
E. RNA → RNA
F. RNA → protein
G. Protein → DNA
H. Protein → RNA
I. Protein → protein

CALCULATIONS

1–19 An adult human is composed of about 10^{13} cells, all of which are derived by cell division from a single fertilized egg.

A. Assuming that all cells continue to divide (like bacteria in rich media), how many generations of cell divisions would be required to produce 10^{13} cells?
B. Human cells in culture divide about once per day. Assuming that all cells continue to divide at this rate during development, how long would it take to generate an adult organism?
C. Why is it, do you think, that adult humans take longer to develop than these calculations might suggest?

1–20 There are 21,000 protein-coding genes in the human genome. If you wanted to use a stretch of the DNA of each gene as a unique identification tag, roughly what minimum length of DNA sequence would you need? To be unique, the length of DNA in nucleotides would have to have a diversity (the number of different possible sequences) equivalent to at least 21,000 and would have to be present once in the haploid human genome (3.2×10^9 nucleotides). (Assume that A, T, C, and G are present in equal amounts in the human genome.)

1–21 Cell growth depends on nutrient uptake and waste disposal. You might imagine, therefore, that the rate of movement of nutrients and waste

products across the cell membrane would be an important determinant of the rate of cell growth. Is there a correlation between a cell's growth rate and its surface-to-volume ratio? Assuming that the cells are spheres, compare a bacterium (radius 1 μm), which divides every 20 minutes, with a human cell (radius 10 μm), which divides every 24 hours. Is there a match between the surface-to-volume ratios and the doubling times for these cells? [The surface area of a sphere = $4\pi r^2$; the volume = $(4/3)\pi r^3$.]

THE DIVERSITY OF GENOMES AND THE TREE OF LIFE

TERMS TO LEARN

archaea	homolog	paralog
bacteria	model organism	prokaryote
eukaryote	mutation	virus
gene family	ortholog	

DEFINITIONS

Match each definition below with its term from the list above.

1–22 A small packet of genetic material that has evolved as a parasite on the reproductive and biosynthetic machinery of host cells.

1–23 Organism selected for intensive study as a representative of a large group of species.

1–24 One of the two divisions of prokaryotes, typically found in hostile environments such as hot springs or concentrated brine.

1–25 The general term for genes that are related by descent.

1–26 Living organism composed of one or more cells with a distinct nucleus and cytoplasm.

1–27 Major category of living cells distinguished by the absence of a nucleus.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

1–28 The vast majority of CO_2 fixation into the organic compounds needed for further biosynthesis is carried out by phototrophs.

1–29 Each member of the human hemoglobin gene family, which consists of seven genes arranged in two clusters on different chromosomes, is an ortholog to all of the other members.

THOUGHT PROBLEMS

1–30 It is not so difficult to imagine what it means to feed on the organic molecules that living things produce. That is, after all, what we do. But what does it mean to "feed" on sunlight, as phototrophs do? Or, even stranger, to "feed" on rocks, as lithotrophs do? Where is the "food," for example, in the mixture of chemicals (H_2S, H_2, CO, Mn^+, Fe^{2+}, Ni^{2+}, CH_4, and NH_4^+) spewed forth from a hydrothermal vent?

1–31 At the bottom of the seas where hydrothermal vents pour their chemicals into the ocean, there is no light and little oxygen, yet giant (2-meter long) tube worms live there happily. These remarkable creatures have no mouth and no anus, living instead off the excretory products and dead cells of their symbiotic lithotrophic bacteria. These tube worms are bright red because they contain large amounts of hemoglobin, which is critical to the survival of their symbiotic bacteria, and, hence, the worms. This

specialized hemoglobin carries O_2 and H_2S. In addition to providing O_2 for the tube worm's oxidative metabolism, what role might this specialized hemoglobin play in the symbiotic relationship that is crucial for life in this hostile environment?

1–32 The overall reaction for the production of glucose ($C_6H_{12}O_6$) by oxygenic (oxygen-generating) photosynthesis,

$$6\ CO_2 + 6\ H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6\ O_2 \qquad \text{(Equation 1)}$$

was widely interpreted as meaning that light split CO_2 to generate O_2, and that the carbon was joined with water to generate glucose. In the 1930s, a graduate student at Stanford University, C.B. van Neil, showed that the stoichiometry for photosynthesis by purple sulfur bacteria was

$$6\ CO_2 + 12\ H_2S + \text{light} \rightarrow C_6H_{12}O_6 + 6\ H_2O + 12\ S \qquad \text{(Equation 2)}$$

On the basis of this stoichiometry, he suggested that the oxygen generated during oxygenic photosynthesis derived from water, not CO_2. His hypothesis was confirmed two decades later using isotopically labeled water. Yet how is it that the 6 H_2O in Equation 1 can give rise to 6 O_2? Can you suggest how Equation 1 might be modified to clarify exactly how the products are derived from the reactants?

1–33 How many possible different trees (branching patterns) can in theory be drawn to display the evolution of bacteria, archaea, and eukaryotes, assuming that they all arose from a common ancestor?

1–34 The genes for ribosomal RNA are highly conserved (relatively few sequence changes) in all organisms on Earth; thus, they have evolved very slowly over time. Were such genes "born" perfect?

1–35 Several prokaryotic genomes have been completely sequenced and their protein-coding genes have been counted. But how do you suppose one recognizes a gene in a string of Ts, As, Cs, and Gs?

1–36 Which one of the processes listed below is NOT thought to contribute significantly to the evolution of new genes? Why not?

A. Duplication of genes to create extra copies that can acquire new functions
B. Formation of new genes *de novo* from noncoding DNA in the genome
C. Horizontal transfer of DNA between cells of different species
D. Mutation of existing genes to create new functions
E. Shuffling of domains of genes by gene rearrangement

1–37 Genes participating in informational processes such as replication, transcription, and translation are transferred between species much less often than are genes involved in metabolism. The basis for this inequality is unclear at present, but one suggestion is that it relates to the underlying complexity of the two types of processes. Informational processes tend to involve large aggregates of different gene products, whereas metabolic reactions are usually catalyzed by enzymes composed of a single protein.

A. Archaea are more closely related to bacteria in their metabolic genes, but are more similar to eukaryotes in the genes involved in informational processes. In terms of evolutionary descent, do you think archaea separated more recently from bacteria or eukaryotes?
B. Why would the complexity of the underlying process—informational or metabolic—have any effect on the rate of horizontal gene transfer?

1–38 Why do you suppose that horizontal gene transfer is more prevalent in single-celled organisms than in multicellular organisms?

1–39 You are interested in finding out the function of a particular gene in the mouse genome. You have sequenced the gene, defined the portion that

codes for its protein product, and searched the appropriate databases; however, neither the gene nor the encoded protein resembles anything seen before. What types of information about the gene or the encoded protein would you like to know in order to narrow down the possible functions, and why? Focus on the information you want, rather than on the techniques you might use to get that information.

CALCULATIONS

1–40 Natural selection is such a powerful force in evolution because cells with even a small growth advantage quickly outgrow their competitors. To illustrate this process, consider a cell culture that initially contains 10^6 bacterial cells, which divide every 20 minutes. A single cell in this culture acquires a mutation that allows it to divide with a generation time of only 15 minutes. Assuming that there is an unlimited food supply and no cell death, how long would it take before the progeny of the mutated cell became predominant in the culture? The number of cells N in the culture at time t is described by the equation $N = N_0 \times 2^{t/G}$, where N_0 is the number of cells at zero time and G is the generation time. (Before you go through the calculation, make a guess: do you think it would take about a day, a week, a month, or a year?)

GENETIC INFORMATION IN EUKARYOTES

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

1–41 Eukaryotic cells contain either mitochondria or chloroplasts, but not both.

1–42 Most of the DNA sequences in a bacterial genome code for proteins, whereas most of the DNA sequences in the human genome do not.

1–43 The only horizontal gene transfer that has occurred in animals is from the mitochondrial genome to the nuclear genome.

THOUGHT PROBLEMS

1–44 Animal cells have neither cell walls nor chloroplasts, whereas plant cells have both. Fungal cells are somewhere in between; they have cell walls but lack chloroplasts. Are fungal cells more likely to be animal cells that gained the ability to make cell walls, or plant cells that lost their chloroplasts? This question represented a difficult issue for early investigators who sought to assign evolutionary relationships based solely on cell characteristics and morphology. How do you suppose that this question was eventually decided?

1–45 Giardiasis is an acute form of gastroenteritis caused by the protozoan parasite *Giardia lamblia*. *Giardia* is a fascinating eukaryote; it contains a nucleus but no mitochondria and no recognizable endoplasmic reticulum or Golgi apparatus—one of the very rare examples of such a cellular organization among eukaryotes. This organization might have arisen because *Giardia* is an ancient lineage that separated from the rest of eukaryotes before mitochondria were acquired and internal membranes were developed. Or it might be a stripped-down version of a more standard eukaryote that has lost these structures because they are not necessary in the parasitic lifestyle it has adopted. How might you use nucleotide sequence comparisons to distinguish between these alternatives?

1–46 Rates of evolution appear to vary in different lineages. For example, the rate of evolution in the rat lineage is significantly higher than in the

human lineage. These rate differences are apparent whether one looks at changes in nucleotide sequences that encode proteins and are subject to selective pressure or at changes in noncoding nucleotide sequences, which are not under obvious selection pressure. Can you offer one or more possible explanations for the slower rate of evolutionary change in the human lineage versus the rat lineage?

DATA HANDLING

1–47 It is difficult to obtain information about the process of gene transfer from the mitochondrial to the nuclear genome in animals because there are few differences among their mitochondrial genomes. The same set of 13 (or occasionally 12) protein genes is encoded in all the numerous animal mitochondrial genomes that have been sequenced. In plants, however, the situation is different, with quite a bit more variability in the sets of proteins encoded in mitochondrial genomes. Analysis of plants can thus provide valuable information on the process of gene transfer.

The respiratory gene *Cox2*, which encodes subunit 2 of cytochrome oxidase, was functionally transferred to the nucleus during flowering plant evolution. Extensive analyses of plant genera have pinpointed the time of appearance of the nuclear form of the gene and identified several likely intermediates in the ultimate loss from the mitochondrial genome. A summary of *Cox2* gene distributions between mitochondria and nuclei, along with data on their transcription, is shown in a phylogenetic context in **Figure 1–2**.

A. Assuming that transfer of the mitochondrial gene to the nucleus occurred only once (an assumption supported by the structures of the nuclear genes), indicate the point in the phylogenetic tree where the transfer occurred.

B. Are there any examples of genera in which the transferred gene and the mitochondrial gene both appear functional? Indicate them.

C. What is the minimum number of times that the mitochondrial gene has been inactivated or lost? Indicate those events on the phylogenetic tree.

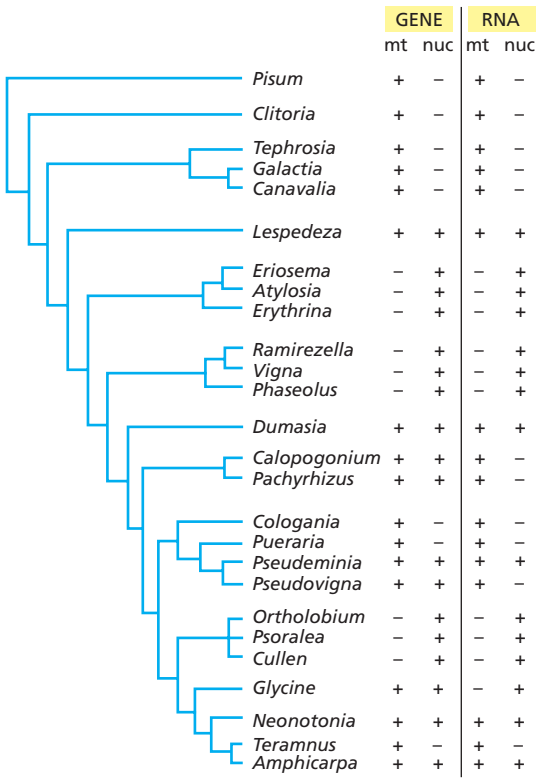


Figure 1–2 Summary of *Cox2* gene distribution and transcript data in a phylogenetic context (Problem 1–47). The presence of the intact gene or the functional transcript (RNA) is indicated by (+); the absence of the intact gene or the functional transcript is indicated by (–). mt, mitochondria; nuc, nuclei.

D. What is the minimum number of times that the nuclear gene has been inactivated or lost? Indicate those events on the phylogenetic tree.
E. Based on this information, propose a general scheme for transfer of mitochondrial genes to the nuclear genome.

1–48 Although stages in the process of mitochondrial gene transfer can be deduced from studies such as the one in the previous question, there is much less information on the mechanism by which the gene is transferred from mitochondria to the nucleus. Does a fragment of DNA escape the mitochondria and enter the nucleus? Or does the transfer somehow involve an RNA transcript of the gene as the intermediary? The *Cox2* gene provides a unique window on this question. In some species, it is found in the mitochondrial genome; in others, in the nuclear genome. It so happens that the initial transcript of the mitochondrial *Cox2* gene is modified by RNA editing, a process that changes several specific cytosines to uracils. How might this observation allow you to decide whether the informational intermediary in transfer was DNA or RNA? What do you think the answer is?

1–49 Some genes evolve rapidly, whereas others are highly conserved. But how can we tell whether a gene has evolved rapidly or has simply had a long time to diverge from its relatives? The most reliable approach is to compare several genes from the same two species, as shown for rat and human in **Table 1–1**. Two measures of rates of nucleotide substitution are indicated in the table. Nonsynonymous changes refer to single nucleotide changes in the DNA sequence that alter the encoded amino acid (ATC → TTC, which is I → F, for example). Synonymous changes refer to those that do not alter the encoded amino acid (ATC → ATT, which is I → I, for example). (As is apparent in the genetic code, see Tables 7 and 8 on page 966, individual amino acids are typically encoded by multiple codons.)

A. Why are there such large differences between the synonymous and nonsynonymous rates of nucleotide substitution?
B. Considering that the rates of synonymous changes are about the same for all three genes, how is it possible for the histone H3 gene to resist so effectively those nucleotide changes that alter the amino acid sequence?
C. In principle, a gene might be highly conserved because it exists in a "privileged" site in the genome that is subject to very low mutation rates. What feature of the data in Table 1–1 argues against this possibility for the histone H3 gene?

TABLE 1–1 Rates of nucleotide substitutions in three genes from rat and human (Problem 1–49).

Gene	Amino acids	Rates of change	
		Nonsynonymous	Synonymous
Histone H3	135	0.0	4.5
Hemoglobin α	141	0.6	4.4
Interferon γ	136	3.1	5.5

Rates are expressed as nucleotide changes per site per 10^9 years. The average rate of nonsynonymous changes for several dozen rat and human genes is about 0.8.

1–50 Plant hemoglobins were found initially in legumes, where they function in root nodules to lower the oxygen concentration so that the resident bacteria can fix nitrogen. These hemoglobins impart a characteristic pink color to the root nodules. When these genes were first discovered, it was

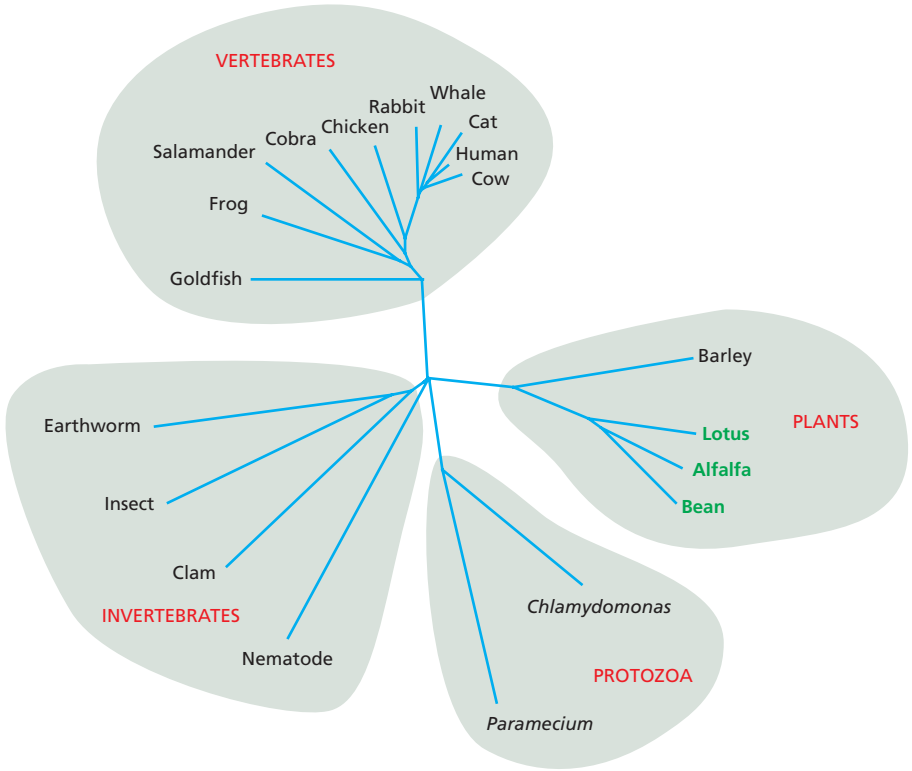


Figure 1–3 Phylogenetic tree for hemoglobin genes from a variety of species (Problem 1–50). The legumes are shown in *green*. The lengths of lines that connect the present-day species represent the evolutionary distances that separate them.

so surprising to find a gene typical of animal blood that it was hypothesized that the plant gene arose by horizontal transfer from some animal. Many more hemoglobin genes have now been sequenced, and a phylogenetic tree based on some of these sequences is shown in **Figure 1–3**.

A. Does this tree support or refute the hypothesis that the plant hemoglobins arose by horizontal gene transfer?

B. Supposing that the plant hemoglobin genes were originally derived from a parasitic nematode, for example, what would you expect the phylogenetic tree to look like?

MCAT STYLE

Passage 1 (Questions 1–51 to 1–53)

Molecules found in nature (termed "natural products") are a rich source of compounds with biological activities that are useful as drugs. Marine organisms are a particularly good source of natural products. Imagine that you are a marine biologist searching for new natural products for use as anticancer drugs. While diving at a coral reef near Tahiti, you notice an unusual sponge that you have never seen before. You collect a sample, take it back to your lab, make an extract, and find that it contains a compound that kills cancer cells, but not normal cells. You want to learn more about the active compound and how it is produced. Based on previous studies, you suspect that it may come from bacteria or archaea that live in a symbiotic relationship with the sponge, which is a eukaryote.

1–51 To define the source of the compound, you initially want to determine whether the sponge sample contains bacteria or archaea, and if so, how many different kinds. What would be the most informative way to do this?

A. Compare the sequences of the genes that encode ribosomal RNA for all the cells that are part of the sponge sample.

B. Culture each of the microorganisms and then test their susceptibility to antibiotics that kill bacteria or archaea.

C. Culture the microorganisms and classify them according to their nutritional requirements and biochemical pathways.
D. Examine the microorganisms under a microscope to see whether they look like bacteria or like archaea.

1–52 You are eventually able to culture multiple kinds of bacteria from the sponge. To your surprise, you discover that two very different bacteria produce the same anticancer compound. You sequence their genomes and find that the vast majority of their genes are either unique to each kind of bacterium or show significant sequence differences. However, several genes are nearly identical. You suspect that one or more of the nearly identical genes are involved in producing the compound. How is it that such divergent bacteria could have genes that are nearly identical?
A. The bacteria obtained the genes by horizontal transfer from the sponge.
B. The bacteria shared the genes by horizontal transfer of a plasmid.
C. The genes are from a common ancestor of the two kinds of bacteria.
D. The genes became nearly identical by convergent evolution.

1–53 You identify a gene that may play an essential role in the biosynthetic pathway that makes the compound. What would be the fastest way to gain clues to the function or biochemical activity of the protein encoded by this gene?
A. Compare the gene's sequence to all other gene sequences to see if it is similar to known genes.
B. Express and purify the protein encoded by the gene and study its enzyme functions *in vitro*.
C. Express the gene in *E. coli* and determine whether *E. coli* can now make the compound.
D. Make mutations in the gene and determine how they affect synthesis of the compound.

Cell Chemistry and Bioenergetics

CHAPTER 2

THE CHEMICAL COMPONENTS OF A CELL

TERMS TO LEARN

acid	electrostatic attraction	hydrophobic force
base	hydrogen bond	macromolecule
buffer	hydronium ion (H_3O^+)	pH scale
chemical group	hydrophilic	proton (H^+)
covalent bond	hydrophobic	van der Waals attraction

DEFINITIONS

Match each definition below with its term from the list above.

2–1 Force exerted by the hydrogen-bonded network of water molecules that brings two nonpolar surfaces together by excluding water between them.

2–2 Noncovalent bond in which an electropositive hydrogen atom is partially shared by two electronegative atoms.

2–3 Substance that releases protons when dissolved in water, forming a hydronium ion (H_3O^+).

2–4 Type of noncovalent bond that is formed at close range between nonpolar atoms.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

2–5 A 10^{-8} M solution of HCl has a pH of 8.

2–6 Strong acids bind protons strongly.

2–7 Most of the interactions between macromolecules could be mediated just as well by covalent bonds as by noncovalent bonds.

THOUGHT PROBLEMS

2–8 The mass of a hydrogen atom—and thus of a proton—is almost exactly 1 dalton. If protons and neutrons have virtually identical masses, and the mass of an electron is negligible, shouldn't all elements have atomic weights that are nearly integers? A perusal of the periodic table shows that this simple expectation is not true. Chlorine, for example, has an atomic weight of 35.5. How is it that elements can have atomic weights that are not integers?

2–9 C, H, and O account for 95% of the elements in living organisms (**Figure 2–1**). These atoms are present in the ratio C:2H:O, which is equivalent to the general formula for carbohydrates (CH_2O). Does this mean living organisms are mostly sugar? Why or why not?

IN THIS CHAPTER

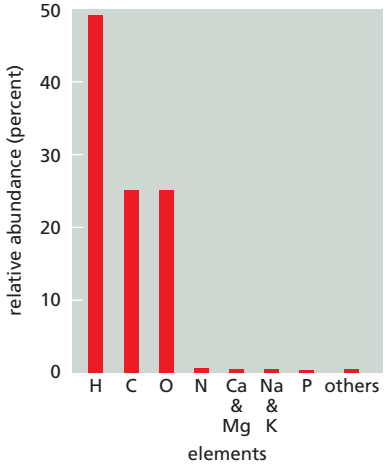


Figure 2–1 Abundance of elements in living organisms (Problem 2–9).

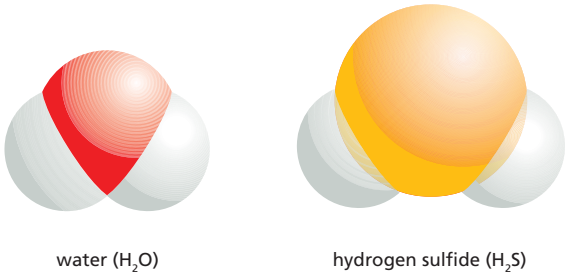


Figure 2–2 Space-filling models of H_2O and H_2S (Problem 2–11).

2–10 Order the following list of processes in terms of their energy content from smallest to largest.

A. ATP hydrolysis in cells.
B. Average thermal motions.
C. C–C bond.
D. Complete oxidation of glucose.
E. Noncovalent bond in water.

2–11 Oxygen and sulfur have similar chemical properties because both elements have six electrons in their outermost electron shells. Indeed, both oxygen and sulfur form molecules with two hydrogen atoms: water (H_2O) and hydrogen sulfide (H_2S) (**Figure 2–2**). Surprisingly, water is a liquid, yet H_2S is a gas, even though sulfur is much larger and heavier than oxygen. Propose an explanation for this striking difference.

2–12 What do you think the "p" in pH stands for?

2–13 Imagine that you dissolve some crystals of sodium chloride, potassium acetate, and ammonium chloride in separate beakers of water. Predict whether the pH values of the resulting solutions would be acidic, neutral, or basic. Explain your reasoning.

2–14 In solution at pH = 1, the amino acid glycine ($^+H_3NCH_2COOH$) has two ionizable groups: the carboxylic acid group (–COOH) and the basic amine group ($–NH_3^+$). Adding NaOH to this solution gives the titration curve shown in **Figure 2–3**.

A. Write the expressions ($HA \rightleftharpoons H^+ + A^-$) for dissociation of the carboxylic acid (–COOH) and amine groups ($–NH_3^+$).
B. Recall that pK is the pH at which exactly half of the carboxylic acid or amine groups are charged. Estimate the pK values for the carboxylate and amine groups of glycine.
C. Indicate the predominant ionic species of glycine at each of the points shown on the curve in Figure 2–3.
D. The isoelectric point of a solute is the pH at which it carries no net charge. Estimate the isoelectric point for glycine from the curve in Figure 2–3.

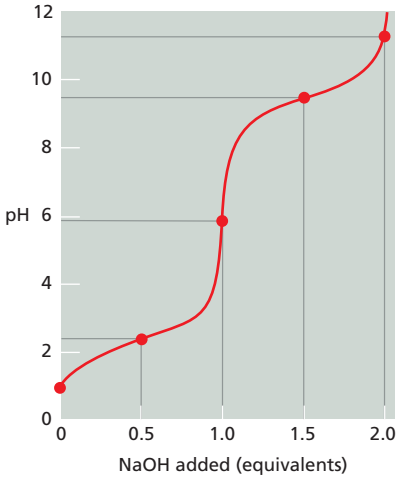


Figure 2–3 Titration of a solution of glycine (Problem 2–14). One equivalent of OH^- is the amount required to completely neutralize one acidic group.

2–15 If you want to order glycine from a chemical supplier, you have three choices: glycine, glycine sodium salt, and glycine hydrochloride. Write the structures of these three compounds.

2–16 From the pK values listed in **Table 2–1**, decide which amino acids were used in the titration curves shown in **Figure 2–4**.

2–17 During an all-out sprint, muscles metabolize glucose anaerobically, producing a high concentration of lactic acid, which lowers the pH of the blood and of the cytosol. The lower pH inside the cell decreases the efficiency of certain glycolytic enzymes, which reduces the rate of ATP production and contributes to the fatigue that sprinters experience well

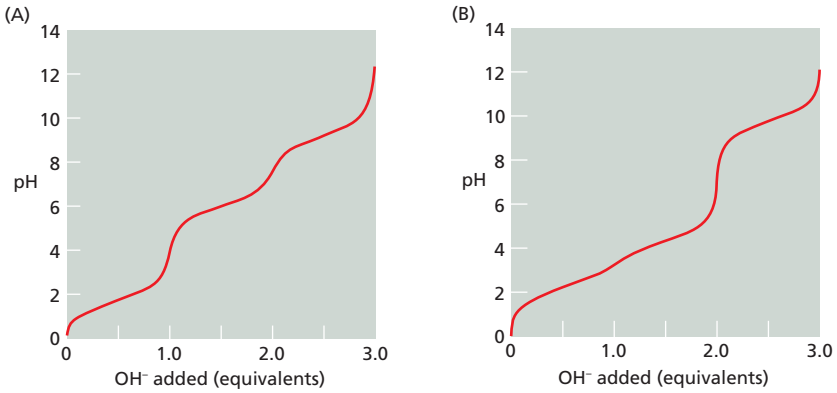


Figure 2–4 Titration curves for two amino acids (Problem 2–16).

TABLE 2–1 Values for the ionizable groups of several amino acids (Problem 2–16).

Amino acid	p*K* Values		
	–COOH	$-NH_3^+$	R Group
Leucine	2.4	9.6	
Proline	2.0	10.6	
Glutamate	2.2	9.7	4.3 (carboxyl)
Histidine	1.8	9.2	6.0 (imidazole)
Cysteine	1.8	10.8	8.3 (sulfhydryl)
Arginine	1.8	9.0	12.5 (guanidino)
Lysine	2.2	9.2	10.8 (amino)

before their fuel reserves are exhausted. The main blood buffer against pH changes is the bicarbonate/CO_2 system.

$$\underset{\text{(gas)}}{CO_2} \overset{p\mathit{K}_1 = 2.3}{\rightleftharpoons} \underset{\text{(dissolved)}}{CO_2} \overset{p\mathit{K}_2 = 3.8}{\rightleftharpoons} H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \overset{p\mathit{K}_3 = 10.3}{\rightleftharpoons} H^+ + CO_3^{2-}$$

To improve their performance, would you advise sprinters to hold their breath or to breathe rapidly for a minute immediately before the race? Explain your answer.

2–18 Aspirin is a weak acid (**Figure 2–5**) that is taken up into the bloodstream by diffusion through cells lining the stomach and the small intestine. Aspirin crosses the plasma membrane of a cell most effectively in its uncharged form; in its charged form it cannot cross the hydrophobic lipid bilayer of the membrane. The pH of the stomach is about 1.5 and that of the lumen of the small intestine is about 6.0. Is the majority of the aspirin absorbed in the stomach or in the intestine? Explain your reasoning.

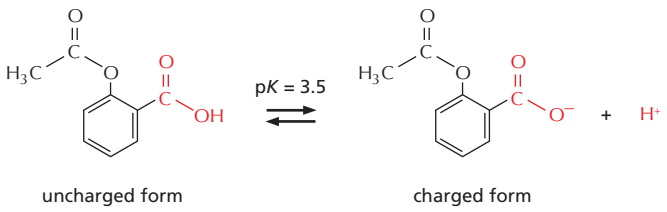


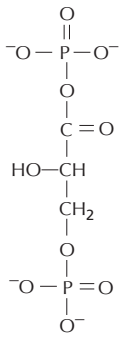
Figure 2–5 Aspirin (Problem 2–18).

2–19 What, if anything, is wrong with the following statement: "When NaCl is dissolved in water, the water molecules closest to the ions will tend to orient themselves so that their oxygen atoms point toward the sodium ions and away from the chloride ions." Explain your answer.

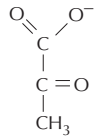
2–20 If noncovalent interactions are so weak in a water environment, how can they possibly be important for holding molecules together in cells?

2–21 The three molecules in **Figure 2–6** contain the seven most common reactive groups in biology. Most molecules in the cell are built from these functional groups. Indicate and name the functional groups in these molecules.

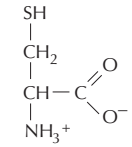
2–22 There are many different, chemically diverse ways in which small molecules can be linked to form polymers. For example, ethene (CH_2=CH_2) is used commercially to make the plastic polymer polyethylene (…–CH_2–CH_2–CH_2–CH_2–…). The individual subunits of the three major classes of biological macromolecules, however, are all linked by similar reaction mechanisms; namely, by condensation reactions that eliminate water. Can you think of any benefits that this chemistry offers and why it might have been selected in evolution?



1,3-bisphosphoglycerate



pyruvate



cysteine

Figure 2–6 Three molecules that illustrate the seven most common functional groups in biology (Problem 2–21). 1,3-Bisphosphoglycerate and pyruvate are intermediates in glycolysis and cysteine is an amino acid.

CALCULATIONS

2–23 To gain a better feeling for atomic dimensions, assume that the page on which this question is printed is made entirely of the polysaccharide cellulose (**Figure 2–7**). Cellulose is described by the formula $(C_6H_{12}O_6)_n$, where n is a large number that varies from one molecule to another. The atomic weights of carbon, hydrogen, and oxygen are 12, 1, and 16, respectively, and this page weighs 5 grams.

A. How many carbon atoms are there in this page?
B. In paper made of pure cellulose, how many carbon atoms would be stacked on top of each other to span the thickness of this page (the page is 21 cm × 27.5 cm × 0.07 mm)? (Rather than solving three simultaneous equations for the carbon atoms in each dimension, you might try a shortcut. Determine the linear density of carbon atoms by calculating the number of carbon atoms on the edge of a cube with the same volume as this page, and then adjust that number to the thickness of the page.)
C. Now consider the problem from a different angle. Assume that the page is composed only of carbon atoms, which have a van der Waals radius of 0.2 nm. How many carbon atoms stacked end to end at their van der Waals contact distance would it take to span the thickness of the page?
D. Compare your answers from parts B and C and explain any differences.

2–24 In the United States, the concentration of glucose in blood is commonly reported in milligrams per deciliter (dL = 100 mL). Over the course of a day in a normal individual the circulating levels of glucose vary around a mean of about 90 mg/dL (**Figure 2–8**). What would this value be if it were expressed as a molar concentration of glucose in blood, which is the way it is typically reported in the rest of the world?

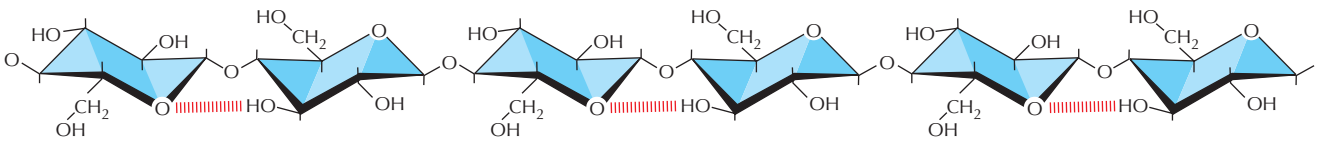


Figure 2–7 Structure of the polysaccharide cellulose (Problem 2–23).

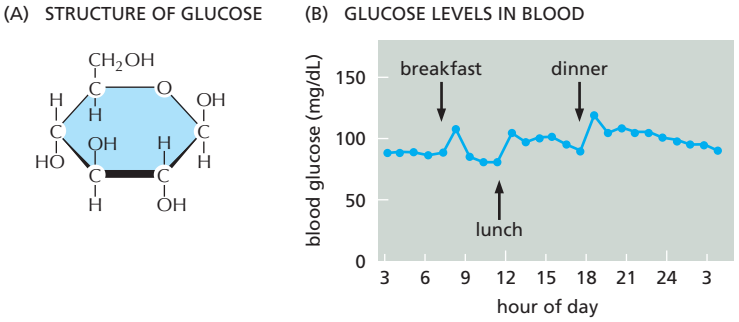


Figure 2–8 Circulating blood glucose (Problem 2–24). (A) Structure of glucose. (B) Typical variation in blood glucose over the course of a day.

2–25 Imagine that you have a beaker of pure water at neutral pH (pH 7.0).

A. What is the concentration of H_3O^+ ions and how were they formed?
B. What is the molarity of pure water? (Hint: 1 liter of water weighs 1 kg.)
C. What is the ratio of H_3O^+ ions to H_2O molecules?

2–26 By a convenient coincidence the ion product of water, $K_w = [H^+][OH^-]$, is a nice round number: $1.0 \times 10^{-14}\ M^2$.

A. Why is a solution at pH 7.0 said to be neutral?
B. What is the H^+ concentration and pH of a 1 mM solution of NaOH?
C. If the pH of a solution is 5.0, what is the concentration of OH^- ions?

2–27 The Henderson–Hasselbalch equation

$$\text{pH} = \text{p}K + \log \frac{[A^-]}{[HA]}$$

is a useful transformation of the equation for dissociation of a weak acid, HA:

$$K = \frac{[H^+][A^-]}{[HA]}$$

A. It is instructive to use the Henderson–Hasselbalch equation to determine the extent of dissociation of an acid at pH values above and below the pK. For the pH values listed in **Table 2–2**, fill in the values for log $[A^-]/[HA]$ and $[A^-]/[HA]$, and indicate the percentage of the acid that has dissociated.

TABLE 2–2 Dissociation of a weak acid at pH values above and below the pK (Problem 2–27).

pH	$\log \frac{[A^-]}{[HA]}$	$\frac{[A^-]}{[HA]}$	% Dissociation
pK +4			
pK +3			
pK +2			
pK +1			
pK			
pK –1			
pK –2			
pK –3			
pK –4			

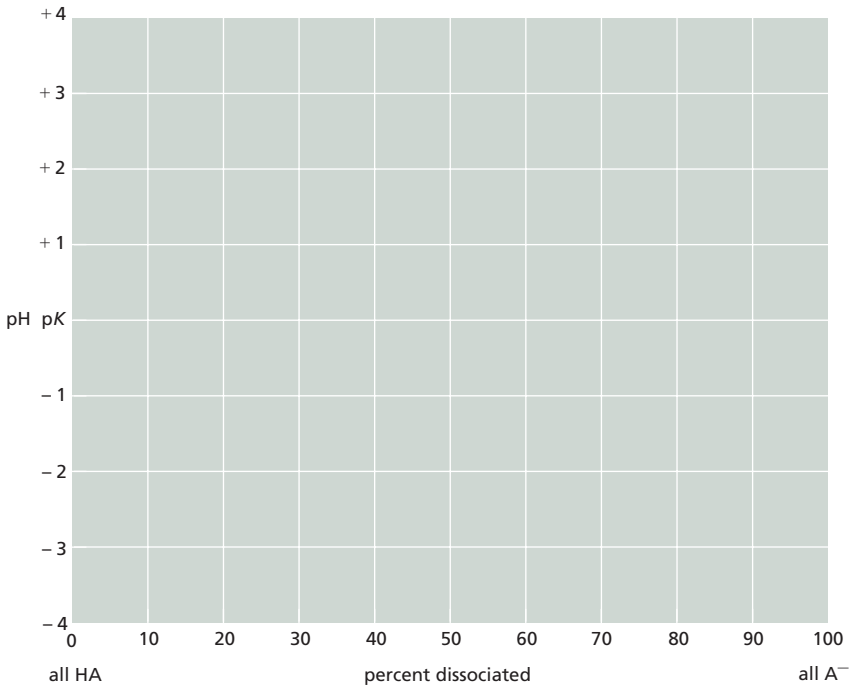


Figure 2–9 Graph for plotting values from Table 2–2 (Problem 2–27).

B. Using the graph in **Figure 2–9**, sketch the relationship between pH of the solution and the fractional dissociation of a weak acid. Will the shape of this curve be the same for all weak acids?

2–28 Cells maintain their cytosolic pH in a narrow range around pH 7.0 by using a variety of weak acids to buffer against changes in pH. This is essential because a large number of processes in cells generate or consume H^+ ions. Weak acids resist changes in pH—the definition of a buffer—most effectively within about one pH unit on either side of their p*K* values. Ionization of phosphoric acid provides an important buffering system in cells. Phosphoric acid has three ionizable protons, each with a unique p*K*.

$$H_3PO_4 \overset{pK = 2.1}{\rightleftharpoons} H^+ + H_2PO_4^- \overset{pK = 6.9}{\rightleftharpoons} H^+ + HPO_4^{2-} \overset{pK = 12.4}{\rightleftharpoons} H^+ + PO_4^{3-}$$

A. Using the values derived in Problem 2–27, estimate how much of each of the four forms of phosphate (H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}), as a percentage of the total, are present in the cytosol of cells at pH 7. (No calculators permitted.)

B. What is the ratio of $[HPO_4^{2-}]$ to $[H_2PO_4^-]$ ($[A^-]/[HA]$) in the cytosol at pH 7? If the cytosol is 1 mM phosphate (sum of all forms), what are the concentrations of $H_2PO_4^-$ and HPO_4^{2-} in the cytosol? (Calculators permitted.)

2–29 Inside cells, the two most important buffer systems are provided by phosphate and proteins. The quantitative aspects of a buffer system pertain to both the effective buffering range (how near the pH is to the p*K* for the buffer) and the overall concentration of the buffering species (which determines the number of protons that can be handled). As discussed in Problem 2–28, $H_2PO_4^- \rightleftharpoons HPO_4^{2-}$ has a p*K* of 6.9 with an overall intracellular phosphate concentration of about 1 mM. In red blood cells, the concentration of globin chains (molecular weight = 15,000) is about 100 mg/mL and each has 10 histidines, with p*K* values between 6.5 and 7.0. Which of these two buffering systems do you think is quantitatively the more important in red blood cells, and why do you think so?

2–30 The most important buffer in the bloodstream is the bicarbonate/CO_2 system. It is much more important than might be expected from its pK because it is an open system in which the CO_2 is maintained at a relatively constant value by exchange with the atmosphere. (By contrast, the buffering systems described in Problem 2–29 are closed systems with no exchange.) The equilibria involved in the bicarbonate/CO_2 buffering system are

$$CO_2(gas) \rightleftharpoons CO_2(dis) \overset{pK_1 = 2.3}{\rightleftharpoons} H_2CO_3 \overset{pK_2 = 3.8}{\rightleftharpoons} H^+ + HCO_3^- \overset{pK_3 = 10.3}{\rightleftharpoons} H^+ + CO_3^{2-}$$

pK_3 ($HCO_3^- \rightleftharpoons H^+ + CO_3^{2-}$) is so high that it never comes into play in biological systems. pK_2 ($H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$) seems much too low to be useful, but it is influenced by the dissolved CO_2 [CO_2(dis)], which is directly proportional to the partial pressure of CO_2 in the gas phase. The dissolved CO_2 in turn is kept in equilibrium with H_2CO_3 by the enzyme carbonic anhydrase.

$$K_1 = \frac{[H_2CO_3]}{[CO_2(dis)]} = 5 \times 10^{-3}, \text{ or } pK_1 = 2.3$$

The equilibrium for hydration of CO_2(dis) can be combined with the equilibrium for dissociation of H_2CO_3 to give a pK' for CO_2(dis) $\rightleftharpoons H^+ + HCO_3^-$

$$K' = \frac{[H^+][HCO_3^-]}{[CO_2(dis)]} = K_1 \times K_2$$

$$pK' = pK_1 + pK_2 = 2.3 + 3.8 = 6.1$$

Even the pK' of 6.1 seems too low to maintain the blood pH around 7.4, yet this open system is very effective, as can be illustrated by a few calculations. The total concentration of carbonate in its various forms, but almost entirely CO_2(dis) and HCO_3^-, is about 25 mM.

A. Using the Henderson–Hasselbalch equation, calculate the ratio of HCO_3^- to CO_2(dis) at pH 7.4. What are the concentrations of HCO_3^- and CO_2(dis)?
B. What would the pH be if 5 mM H^+ were added under conditions where CO_2 was not allowed to leave the system; that is, if the concentration of CO_2(dis) was not maintained at a constant value?
C. What would the pH be if 5 mM H^+ were added under conditions where CO_2 was permitted to leave the system; that is, if the concentration of CO_2(dis) was maintained at a constant value?

2–31 The proteins in a mammalian cell account for 18% of its net weight. If the density of a typical mammalian cell is about 1.1 g/mL and the volume of the cell is 4×10^{-9} mL, what is the concentration of protein in mg/mL?

DATA HANDLING

2–32 The ionizable groups in amino acids can influence one another, as shown by the pK values for the carboxyl and amino groups of alanine and various oligomers of alanine (**Figure 2–10**). Suggest an explanation for why the pK of the carboxyl group increases with oligomer length, while that of the amino group decreases.

2–33 A histidine side chain is known to have an important role in the catalytic mechanism of an enzyme; however, it is not clear whether histidine is required in its protonated (charged) or unprotonated (uncharged) state. To answer this question you measure enzyme activity over a range of pH, with the results shown in **Figure 2–11**. Which form of histidine is required for enzyme activity?

CHEMICAL FORMULAS	pK VALUES	
	$-COO^-$	$^+H_3N-$
Ala $^+H_3N-CH(CH_3)-COO^-$	2.34	9.69
Ala_2 $^+H_3N-CH(CH_3)-C(=O)-NH-CH(CH_3)-COO^-$	3.12	8.30
Ala_3 $^+H_3N-CH(CH_3)-C(=O)-NH-CH(CH_3)-C(=O)-NH-CH(CH_3)-COO^-$	3.39	8.03
Ala_4 $^+H_3N-CH(CH_3)-C(=O)-NH-CH(CH_3)-C(=O)-NH-CH(CH_3)-C(=O)-NH-CH(CH_3)-COO^-$	3.42	7.94

Figure 2–10 p*K* values for the carboxyl and amino groups in oligomers of alanine (Problem 2–32).

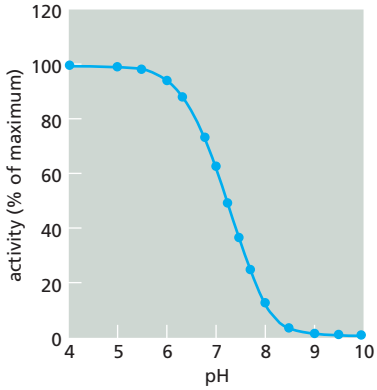


Figure 2–11 Enzyme activity as a function of pH (Problem 2–33).

MEDICAL LINKS

2–34 The drug thalidomide was once prescribed as a sedative to help with nausea during the early stages of pregnancy. One of its optical isomers, (*R*)-thalidomide (**Figure 2–12**), is the active agent responsible for its sedative effects. It was synthesized, however, as a mixture of both optical isomers—a not uncommon practice that usually causes no problems. Unfortunately, the other optical isomer is a teratogen that led to a horrific series of birth defects characterized by malformed or absent limbs. On the structural formula in Figure 2–12A, identify the carbon that is responsible for its optical activity (its chiral center) and sketch the structure of the teratogenic form of thalidomide.

2–35 The molecular weight of ethanol (CH_3CH_2OH) is 46 and its density is 0.789 g/cm^3.

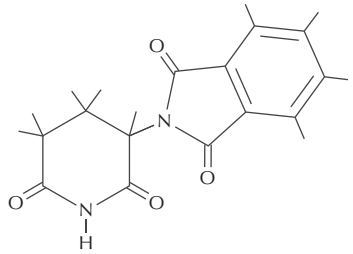
A. What is the molarity of ethanol in beer that is 5% ethanol by volume? [Alcohol content of beer varies from about 4% (lite beer) to 8% (stout beer).]

B. The legal limit for a driver's blood alcohol content varies, but 80 mg of ethanol per 100 mL of blood (usually referred to as a blood alcohol level of 0.08) is typical. What is the molarity of ethanol in a person at this legal limit?

C. How many 12-oz (355-mL) bottles of 5% beer could a 70-kg person drink and remain under the legal limit? A 70-kg person contains about 40 liters of water. Ignore the metabolism of ethanol, and assume that the water content of the person remains constant.

D. Ethanol is metabolized at a constant rate of about 120 mg per hour per kg body weight, regardless of its concentration. If a 70-kg person were at twice the legal limit (160 mg/100 mL), how long would it take for their blood alcohol level to fall below the legal limit?

(A) THALIDOMIDE CHEMICAL FORMULA



(B) THALIDOMIDE SPACE-FILLING MODEL

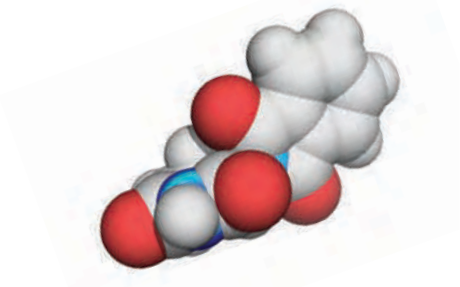


Figure 2–12 The structure of the sedative (*R*)-thalidomide (Problem 2–34).

CATALYSIS AND THE USE OF ENERGY BY CELLS

TERMS TO LEARN

acetyl CoA
activated carrier
activation energy
ADP
aerobic respiration
ATP
catalyst
coupled reaction
diffusion
entropy
enzyme
equilibrium
equilibrium constant (*K*)
free energy (*G*)
free-energy change (ΔG)
metabolism
NAD^+/NADH
$NADP^+$/NADPH
oxidation
reduction
standard free-energy change (ΔG°)
substrate

DEFINITIONS

Match each definition below with its term from the list above.

2–36 Extra energy that must be possessed by atoms or molecules in addition to their ground-state energy in order to undergo a particular chemical reaction.

2–37 Free-energy change of two reacting molecules at standard temperature and pressure when all components are present at a concentration of 1 mole per liter.

2–38 Loss of electrons from an atom, as occurs during the addition of oxygen to a molecule or when a hydrogen is removed.

2–39 Molecule on which an enzyme acts.

2–40 Net drift of molecules in the direction of lower concentration due to random thermal movement.

2–41 Protein that catalyzes a specific chemical reaction.

2–42 Linked pair of chemical reactions in which the free energy released by one of the reactions serves to drive the other.

2–43 State at which there is no net change in a system. In a chemical reaction, this state is reached when the forward and reverse rates are equal.

2–44 The energy that can be extracted from a system to drive reactions. Takes into account changes in both energy and entropy.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

2–45 Animals and plants use oxidation to extract energy from food molecules.

2–46 If an oxidation occurs in a reaction, it must be accompanied by a reduction.

2–47 Linking the energetically unfavorable reaction A $\longrightarrow$ B to a second, favorable reaction B $\longrightarrow$ C will shift the equilibrium constant for the first reaction.

THOUGHT PROBLEMS

2–48 The organic chemistry of living cells is said to be special for two reasons: it occurs in an aqueous environment and it accomplishes some very complex reactions. But do you suppose it's really all that much different from the organic chemistry carried out in the top laboratories in the world? Why or why not?

2–49 Distinguish between catabolic and anabolic pathways of metabolism, and indicate in a general way how such pathways are linked to one another in cells.

2–50 The second law of thermodynamics states that systems will change spontaneously toward arrangements with greater entropy (disorder). Living systems are so intricately ordered, however, it seems they must surely violate the second law. Explain briefly—and in a way your parents could understand—how life is fully compatible with the laws of thermodynamics.

2–51 In the reaction $2\ Na + Cl_2 \rightarrow 2\ Na^+ + 2\ Cl^-$, what is being oxidized and what is being reduced? How can you tell?

2–52 If a cell in mitosis is cooled to 0°C, the microtubules in the spindle depolymerize into tubulin subunits. The same is true for microtubules made from pure tubulin in a test tube; they assemble readily at 37°C, but disassemble at low temperature. In fact, many protein assemblies that are held together by noncovalent bonds show the same behavior: they disassemble when cooled. This behavior is governed by the basic thermodynamic equation

$$\Delta G = \Delta H - T\Delta S$$

where ΔH is the change in enthalpy (chemical-bond energy), ΔS is the change in entropy (disorder of the system), and T is the absolute temperature.

A. The change in free energy (ΔG) must be negative for the reaction (tubulin subunits → microtubules) to proceed at high temperature. At low temperature, ΔG must be positive to permit disassembly; that is, to favor the reverse reaction. Decide what the signs (positive or negative) of ΔH and ΔS must be, and show how your choices account for polymerization of tubulin at high temperature and its depolymerization at low temperature. (Assume that the ΔH and ΔS values themselves do not change with temperature.)

B. Polymerization of tubulin subunits into microtubules at body temperature clearly occurs with an increase in the orderliness of the subunits (**Figure 2–13**). Yet tubulin polymerization occurs with an increase in entropy (decrease in order). How can that be?

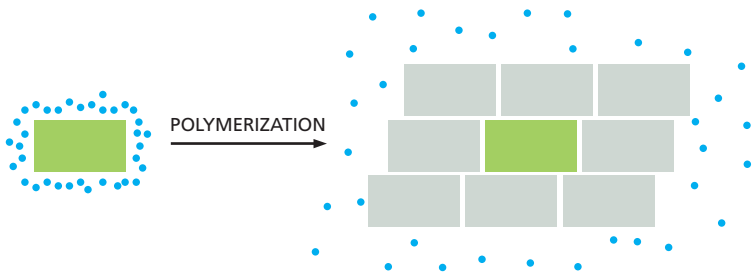


Figure 2–13 Polymerization of tubulin subunits into a microtubule (Problem 2–52). The fates of one subunit (*green*) and its associated water molecules (*blue spheres*) are shown. Additional tubulin subunits in the microtubule are *lightly shaded*; their associated water molecules are not shown.

2–53 Discuss the statement: "The criterion for whether a reaction proceeds spontaneously is ΔG not $\Delta G°$, because ΔG takes into account the concentrations of the substrates and products."

2–54 At a particular concentration of substrates and products the reaction below has a negative ΔG.

$$A + B \rightarrow C + D \quad \Delta G = -20\ kJ/mole$$

At the same concentrations, what is ΔG for the reverse reaction?

$$C + D \rightarrow A + B$$

2–55 The values for $\Delta G°$ and for ΔG in cells have been determined for many different metabolic reactions. What information do these values provide about the rates of these reactions?

2–56 Thermodynamically, it is perfectly valid to consider the cellular phosphorylation of glucose to produce glucose 6-phosphate (G6P) as the sum of two reactions.

$$\begin{array}{lll} (1) & \text{glucose} + P_i \rightarrow \text{G6P} + H_2O & \Delta G° = 13.8 \text{ kJ/mole} \\ (2) & \text{ATP} + H_2O \rightarrow \text{ADP} + P_i & \Delta G° = -30.5 \text{ kJ/mole} \\ \hline \text{NET:} & \text{glucose} + \text{ATP} \rightarrow \text{G6P} + \text{ADP} & \end{array}$$

But biologically it makes no sense at all. Hydrolysis of ATP (reaction 2) in one part of the cell can have no effect on phosphorylation of glucose (reaction 1) elsewhere in the cell, given that [ATP], [ADP], and [P_i] are maintained within narrow limits. How does the cell manage to link these two reactions to achieve the phosphorylation of glucose?

2–57 Each phosphoanhydride bond between the phosphate groups in ATP is a high-energy linkage with a $\Delta G°$ value of –30.5 kJ/mole. Hydrolysis of this bond in cells normally liberates usable energy in the range of 45 to 55 kJ/mole. Why do you think a range of values for released energy is given for ΔG, rather than a precise number, as for $\Delta G°$?

2–58 Consider the effects of two enzymes. Enzyme A catalyzes the reaction

$$\text{ATP} + \text{GDP} \rightleftharpoons \text{ADP} + \text{GTP}$$

whereas enzyme B catalyzes the reaction

$$\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH}$$

Discuss whether the enzymes would be beneficial or detrimental to cells.

2–59 Match the activated carriers below with the group carried in high-energy linkage.

A. Acetyl CoA	1. acetyl group
B. *S*-Adenosylmethionine	2. carboxyl group
C. ATP	3. electrons and hydrogens
D. Carboxylated biotin	4. glucose
E. NADH, NADPH, $FADH_2$	5. methyl group
F. Uridine diphosphate glucose	6. phosphate

2–60 Which of the following reactions will occur only if coupled to a second, energetically favorable reaction?

A. glucose + $O_2 \rightarrow CO_2 + H_2O$
B. $CO_2 + H_2O \rightarrow$ glucose + O_2
C. nucleoside triphosphate + $DNA_n \rightarrow DNA_{n+1} + 2\ P_i$
D. nucleosides → nucleoside triphosphates
E. ADP + $P_i \rightarrow$ ATP

CALCULATIONS

2–61 If an uncatalyzed reaction occurred at the rate of 1 event per century, and if an enzyme speeded up the rate by a factor of 10^{14}, how many seconds would it take the enzyme to catalyze one event?

2–62 "Diffusion" sounds slow—and over everyday distances it is—but on the scale of a cell it is very fast. The average instantaneous velocity of a particle in solution—that is, the velocity between its very frequent collisions—is

$$v = (kT/m)^{1/2}$$

where $k = 1.38 \times 10^{-16}$ g cm^2/K sec^2, T = temperature in K (37°C is 310 K), and m = mass in g/molecule.

Calculate the instantaneous velocity of a water molecule (molecular mass = 18 daltons), a glucose molecule (molecular mass = 180 daltons), and a myoglobin molecule (molecular mass = 15,000 daltons) at 37°C. Just for fun, convert these numbers into kilometers/hour. Before you do any calculations, you might try to guess whether the molecules are moving at a slow crawl (<1 km/hr), an easy walk (5 km/hr), or a record-setting sprint (40 km/hr).

2–63 The instantaneous velocity tells you little about the time it takes for a molecule to move cellular distances because its trajectory is constantly altered by collisions with other molecules in solution (**Figure 2–14**). The average time it takes for a molecule to travel x cm by diffusion in three dimensions is

$$t = x^2/6D$$

where t is the time in seconds and D is the diffusion coefficient, which is a constant that depends on the size and shape of the particle. Glucose and myoglobin, for example, have diffusion coefficients of about 5×10^{-6} cm^2/sec and 5×10^{-7} cm^2/sec, respectively. Calculate the average time it would take for glucose and myoglobin to diffuse a distance of 20 μm, which is approximately the width of a mammalian cell.

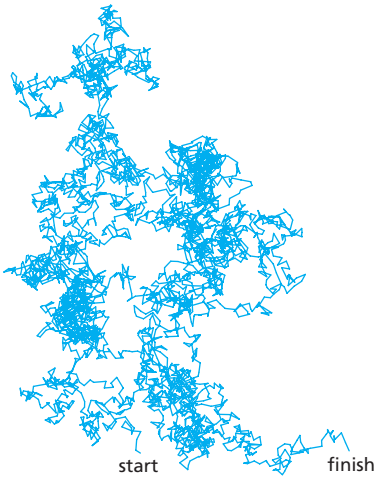


Figure 2–14 A two-dimensional, simulated random walk of a molecule in solution (Problem 2–63).

2–64 Phosphoglucose isomerase catalyzes the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P):

$$\text{G6P} \rightleftharpoons \text{F6P}$$

The ΔG for this reaction is given by the equation

$$\Delta G = \Delta G^\circ + 2.3\, RT \log \frac{[\text{F6P}]}{[\text{G6P}]}$$

where $R = 8.3 \times 10^{-3}$ kJ/K mole and $T = 310$ K. A useful number to remember is that $2.3\, RT = 5.9$ kJ/mole at 37°C, which is body temperature.

A. At equilibrium, [F6P]/[G6P] is equal to the equilibrium constant (K) for the reaction. Rewrite the above equation for the reaction at equilibrium.
B. At equilibrium, the ratio of [F6P] to [G6P] is observed to be 0.5. At this equilibrium ratio, what are the values of ΔG and ΔG°?
C. Inside a cell, the value of ΔG for this reaction is –2.5 kJ/mole. What is the ratio of [F6P] to [G6P]? What is ΔG°?

2–65 A 70-kg adult human (154 lb) could meet his or her entire energy needs for one day by eating 3 moles of glucose (540 g). (We don't recommend this.) Each molecule of glucose generates 30 molecules of ATP when it is oxidized to CO_2. The concentration of ATP is maintained in cells at about 2 mM, and a 70-kg adult has about 25 L of intracellular fluid. Given that the ATP concentration remains constant in cells, calculate how many times per day, on average, each ATP molecule in the body is hydrolyzed and resynthesized.

DATA HANDLING

2–66 The polymerization of subunits into a pentameric ring is shown in **Figure 2–15**. The equilibrium constants for association of a subunit at each step in the assembly of the tetramer (that is, K_1, K_2, and K_3) are approximately equal at 10^6 M^{-1}. The equilibrium constant for association of the final subunit in the ring (K_4), however, is $>10^{12}$ M^{-1}. Why is association of the final subunit so much more highly favored than association of the initial subunits? Why do you suppose the equilibrium constant for the

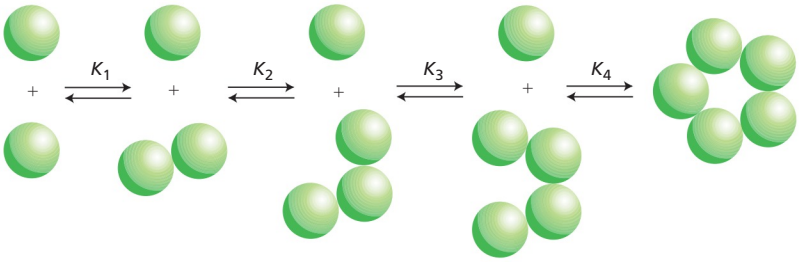


Figure 2–15 Polymerization of subunits into a pentameric ring (Problem 2–66).

association of the final subunit is approximately the square of the equilibrium constants for the earlier steps?

2–67 Red blood cells obtain energy in the form of ATP by converting glucose to pyruvate via the glycolytic pathway (**Figure 2–16**). The values of $\Delta G°$ and ΔG (in kJ/mole) have been calculated for each of the steps in glycolysis in red blood cells that are actively metabolizing glucose, as summarized in **Table 2–3**. The $\Delta G°$ values are based on the known equilibrium constants for the reactions; the ΔG values are calculated from the $\Delta G°$ values and actual measurements of concentrations of the intermediates in red blood cells.

The flow of metabolites through a metabolic pathway can occur *only* when the ΔG value for *each* step is negative. This is a true statement. Despite this assertion, three reactions in red blood cell glycolysis have slightly positive ΔG values. What do you suppose is the explanation for the results in the table?

TABLE 2–3 The reactions of glycolysis in red blood cells and their associated $\Delta G°$ and ΔG values in kJ/mole (Problem 2–67).

Step	Reaction	$\Delta G°$	ΔG
1	GLC + ATP ⟶ G6P + ADP + H^+	–16.7	–33.4
2	G6P ⟶ F6P	+1.7	–2.5
3	F6P + ATP ⟶ F1,6BP + ADP + H^+	–14.2	–22.2
4	F1,6BP ⟶ DHAP + G3P	+23.8	–1.3
5	DHAP ⟶ G3P	+7.5	+2.5
6	G3P + P_i + NAD^+ ⟶ 1,3BPG + NADH + H^+	+6.3	–1.7
7	1,3BPG + ADP ⟶ 3PG + ATP	–18.8	+1.3
8	3PG ⟶ 2PG	+4.6	+0.8
9	2PG ⟶ PEP + H_2O	+1.7	–3.3
10	PEP + ADP + H^+ ⟶ PYR + ATP	–31.4	–16.7

GLC = glucose; G6P = glucose 6-phosphate; F6P = fructose 6-phosphate; F1,6BP = fructose 1,6-bisphosphate; DHAP = dihydroxyacetone phosphate; G3P = glyceraldehyde 3-phosphate; 1,3BPG = 1,3-bisphosphoglycerate; 3PG = 3-phosphoglycerate; 2PG = 2-phosphoglycerate; PEP = phosphoenolpyruvate; PYR = pyruvate.

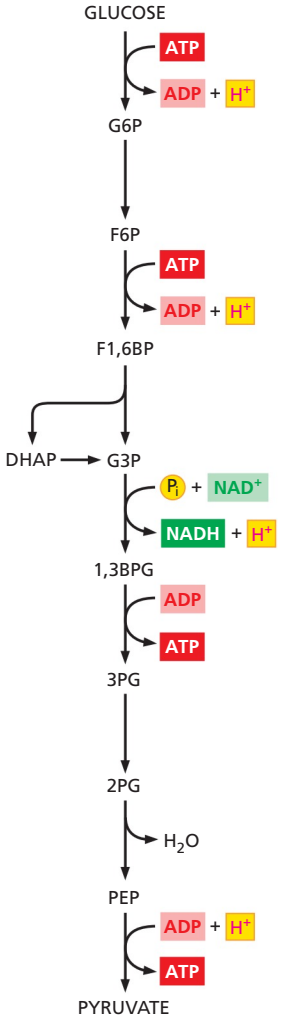


Figure 2–16 The glycolytic pathway (Problem 2–67). See Table 2–3 for the key to abbreviations.

HOW CELLS OBTAIN ENERGY FROM FOOD

TERMS TO LEARN

citric acid cycle	fermentation	nitrogen fixation
electron-transport chain	glycogen	oxidative phosphorylation
FAD/$FADH_2$	glycolysis	starch
fat	GTP	

DEFINITIONS

Match each definition below with its term from the list above.

2–68 Central metabolic pathway found in aerobic organisms, which oxidizes acetyl groups derived from food molecules to CO_2 and H_2O. In eukaryotic cells, it occurs in the mitochondria.

2–69 Energy-storage lipids in cells that are composed of triacylglycerols (triglycerides), which are fatty acids esterified with glycerol.

2–70 Polysaccharide composed exclusively of glucose units used to store energy in animal cells. Granules of it are especially abundant in liver and muscle cells.

2–71 Process in bacteria and mitochondria in which ATP formation is driven by the transfer of electrons from food molecules to molecular oxygen.

2–72 Series of electron carrier molecules along which electrons move from a higher to a lower energy level to a final acceptor molecule, with the associated production of ATP.

2–73 Ubiquitous metabolic pathway in the cytosol in which sugars are partially metabolized to produce ATP.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

2–74 Because glycolysis is only a prelude to the oxidation of glucose in mitochondria, which yields 15-fold more ATP, glycolysis is not really important for human cells.

2–75 The reactions of the citric acid cycle do not directly require the presence of oxygen.

THOUGHT PROBLEMS

2–76 Match the polymeric molecules in food with the monomeric subunits into which they are digested before they can be oxidized to produce energy.

A. fats	1. amino acids
B. polysaccharides	2. fatty acids
C. proteins	3. glycerol
	4. sugars

2–77 From a chemical perspective, the glycolytic pathway (see Figure 2–16) can be thought of as occurring in two stages. The first stage from glucose to glyceraldehyde 3-phosphate (G3P) prepares glucose so that its cleavage yields G3P and an equivalent three-carbon fragment, which is then converted into G3P. The second stage—from G3P to pyruvate—harvests energy in the form of ATP and NADH.

A. Write the balanced equation for the first stage of glycolysis (glucose → G3P).

B. Write the balanced equation for the second stage of glycolysis (G3P → pyruvate).
C. Write the balanced equation for the overall pathway (glucose → pyruvate).

2–78 At first glance, fermentation of pyruvate to lactate appears to be an optional add-on reaction to glycolysis (**Figure 2–17**). After all, couldn't cells growing in the absence of oxygen simply discard pyruvate as a waste product? In the absence of fermentation, which products derived from glycolysis would accumulate in cells under anaerobic conditions? Could the metabolism of glucose via the glycolytic pathway continue in the absence of oxygen in cells that cannot carry out fermentation? Why or why not?

2–79 In the absence of oxygen, cells consume glucose at a high, steady rate. When oxygen is added, glucose consumption drops precipitously and is then maintained at the lower rate. Why is glucose consumed at a high rate in the absence of oxygen and at a low rate in its presence?

2–80 Arsenate (AsO_4^{3-}) is chemically very similar to phosphate (PO_4^{3-}) and is used as an alternative substrate by many phosphate-requiring enzymes. In contrast to phosphate, however, the anhydride bond between arsenate and a carboxylic acid group is very quickly hydrolyzed in water. Knowing this, suggest why arsenate is a compound of choice for murderers, but not for cells. Formulate your explanation in terms of the step in glycolysis at which 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate, generating ATP (**Figure 2–18**).

2–81 The liver provides glucose to the rest of the body between meals. It does so by breaking down glycogen, forming glucose 6-phosphate in the penultimate step. Glucose 6-phosphate is converted to glucose by splitting off the phosphate ($\Delta G°$ = –13.8 kJ/mole). Why do you suppose the liver removes the phosphate by hydrolysis, rather than reversing the reaction by which glucose 6-phosphate is formed from glucose (glucose + ATP → G6P + ADP, $\Delta G°$ = –16.7 kJ/mole)? By reversing this reaction, the liver could generate both glucose *and* ATP.

2–82 What, if anything, is wrong with the following statement: "The oxygen consumed during the oxidation of glucose in animal cells is returned as CO_2 to the atmosphere." How might you support your answer experimentally?

CALCULATIONS

2–83 If a cell hydrolyzes and replaces 10^9 ATP molecules per minute, how long will it take for a cell to consume its own volume (1000 μm^3) of oxygen? Roughly 90% of the ATP in the cell is regenerated by oxidative phosphorylation. Assume that 5 molecules of ATP are regenerated by each

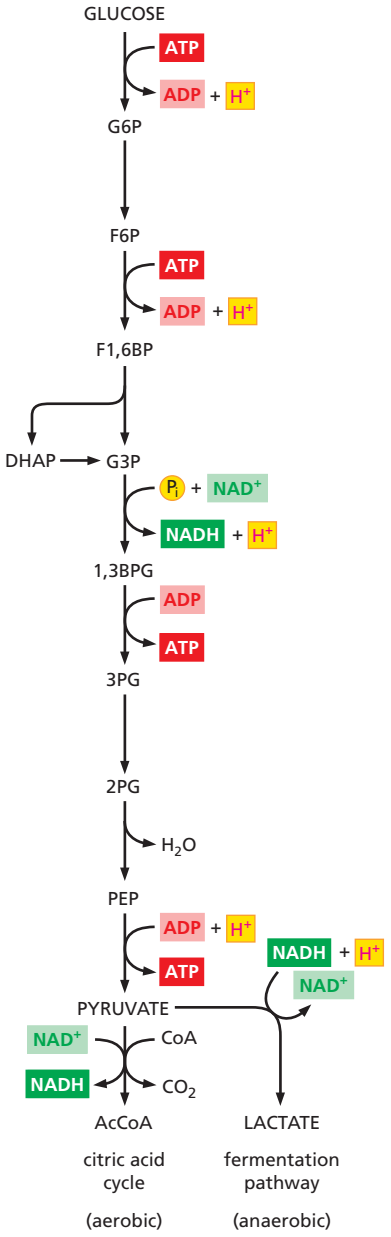


Figure 2–17 Fermentation of pyruvate to lactate (Problem 2–78). AcCoA, acetyl CoA. See Table 2-3 for the key to the rest of the abbreviations.

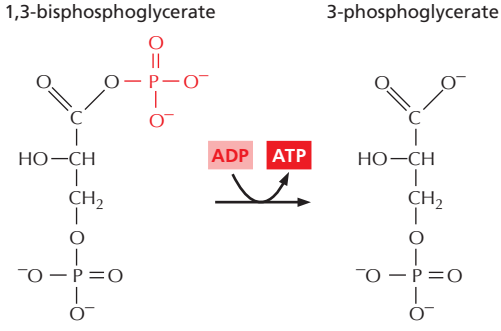


Figure 2–18 Conversion of 1,3-bisphosphoglycerate and ADP to 3-phosphoglycerate and ATP (Problem 2–80).

molecule of oxygen (O_2) that is converted to water. Recall that a mole of a gas occupies 22.4 L.

2–84 Assuming that there are 5×10^{13} cells in the human body and that ATP is turning over at a rate of 10^9 ATP molecules per minute in each cell, how many watts is the human body consuming? (A watt is a J per second.) Assume that hydrolysis of ATP yields 50 kJ/mole.

2–85 Does a Snickers™ candy bar (65 g, 1360 kJ) provide enough energy to climb from Zermatt (elevation 1660 m) to the top of the Matterhorn (4478 m, **Figure 2–19**), or might you need to stop at Hörnli Hut (3260 m) to eat another one? Imagine that you and your gear have a mass of 75 kg, and that all of your work is done against gravity (that is, you're just climbing straight up).

$$\text{work (J)} = \text{mass (kg)} \times g\,(\text{m/sec}^2) \times \text{height gained (m)}$$

where g is acceleration due to gravity (9.8 m/sec^2). One joule is 1 kg m^2/sec^2.

What assumptions made here will greatly underestimate how much candy you need?

Figure 2–19 The Matterhorn (Problem 2–85).

2–86 Muscles contain creatine phosphate (CP) as an energy buffer to maintain the levels of ATP in the initial stages of exercise. Creatine phosphate can transfer its phosphate to ADP to generate creatine (C) and ATP, with a $\Delta G°$ of –13.8 kJ/mole.

$$\text{CP} + \text{ADP} \longrightarrow \text{C} + \text{ATP} \quad \Delta G° = -13.8 \text{ kJ/mole}$$

A. In a resting muscle, [ATP] = 4 mM, [ADP] = 0.013 mM, [CP] = 25 mM, and [C] = 13 mM. What is the ΔG for this reaction in resting muscle? Does this value make sense to you? Why or why not?
B. Consider an initial stage in vigorous exercise, when 25% of the ATP has been converted to ADP. Assuming that no other concentrations have changed, what is the ΔG for the reaction at this stage in exercising muscle? Does this value make sense?
C. If the ATP in muscle could be completely hydrolyzed (in reality it never is), it would power an all-out sprint for about 1 second. If creatine phosphate could be completely hydrolyzed to regenerate ATP, how long could a sprint be powered? Where do you suppose the energy comes from to allow a runner to finish a 200-meter sprint?

DATA HANDLING

2–87 In 1904, Franz Knoop performed what was probably the first successful labeling experiment to study metabolic pathways. He fed many different fatty acids labeled with a terminal benzene ring to dogs and analyzed their urine for excreted benzene derivatives. Whenever the fatty acid had an even number of carbon atoms, phenylacetate was excreted (**Figure 2–20A**). Whenever the fatty acid had an odd number of carbon atoms, benzoate was excreted (**Figure 2–20B**).

From these experiments Knoop deduced that oxidation of fatty acids to CO_2 and H_2O involved the removal of two-carbon fragments from the carboxylic acid end of the chain. Can you explain the reasoning that led him to conclude that two-carbon fragments, as opposed to any other number, were removed, and that degradation was from the carboxylic acid end, as opposed to the other end?

2–88 In 1937, Hans Krebs deduced the operation of the citric acid cycle (**Figure 2–21**) from careful observations on the oxidation of carbon compounds in minced preparations of pigeon flight muscle. (Pigeon breast is a rich source of mitochondria, but the function of mitochondria was

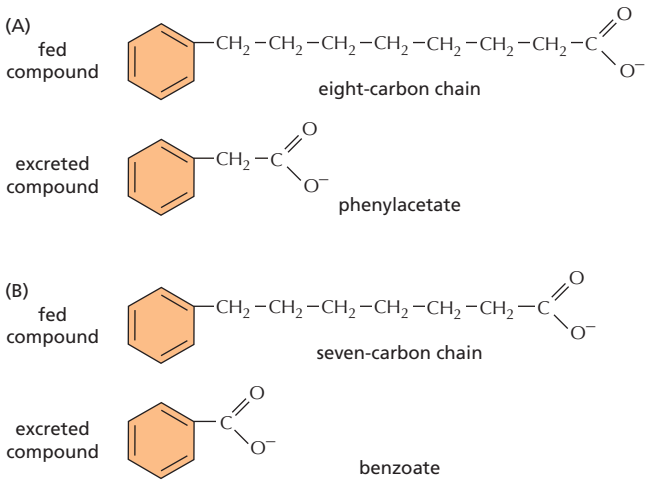


Figure 2–20 The original labeling experiment to analyze fatty acid oxidation (Problem 2–87). (A) Fed and excreted derivatives of an even-number fatty acid chain. (B) Fed and excreted derivatives of an odd-number fatty acid chain.

unknown at the time.) In one set of experiments, Krebs found that addition of a small amount of citrate resulted in a much larger increase in the consumption of oxygen than could be accounted for by the oxidation of citrate (Table 2–4). This surprising observation ultimately led to the description of the citric acid cycle.

A. If citrate were an intermediate in a linear pathway of oxidation, would you expect that addition of a small amount would lead to a large increase in oxygen consumption? Why or why not?

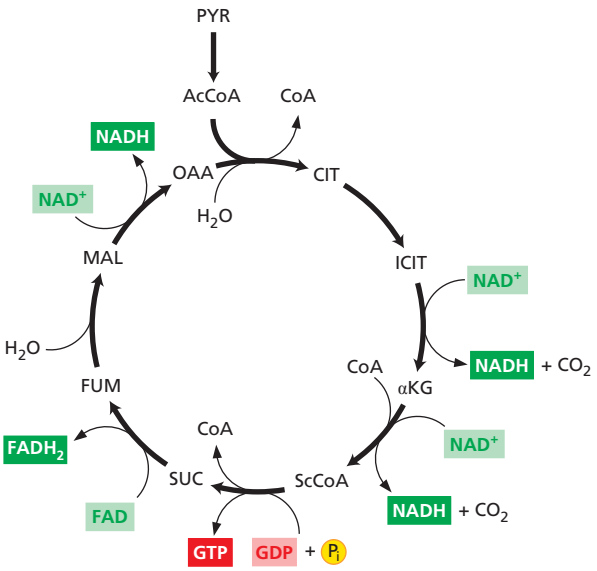


Figure 2–21 The citric acid cycle (Problem 2–88). PYR = pyruvate, AcCoA = acetyl coenzyme A, CIT = citrate, ICIT = isocitrate, αKG = α-ketoglutarate, ScCoA = succinyl coenzyme A, SUC = succinate, FUM = fumarate, MAL = malate, and OAA = oxaloacetate.

TABLE 2–4 Respiration in minced pigeon breast in the presence and absence of citrate (Problem 2–88).

Time (minutes)	Oxygen consumption (mmol)		
	No citrate	3 mmol citrate	Difference
30	29	31	2
60	47	68	21
90	51	87	36
150	53	93	40

B. How does the operation of the citric acid cycle explain the high level of oxygen consumption after addition of a small amount of citrate?
C. Toward the end of the paper, Krebs states, "While the citric acid cycle thus seems to occur generally in animal tissues, it does not exist in yeast or in *E. coli*, for yeast and *E. coli* do not oxidize citric acid at an appreciable rate." Why do you suppose Krebs got this point wrong?

2–89 Pathways for synthesis of amino acids in microorganisms were worked out in part by cross-feeding experiments among mutant organisms that were defective for individual steps in the pathway. Results of cross-feeding experiments for three mutants defective in the tryptophan pathway—*TrpB*$^-$, *TrpD*$^-$, and *TrpE*$^-$—are shown in **Figure 2–22A**. The mutants were streaked on a Petri dish and allowed to grow briefly in the presence of a very small amount of tryptophan, producing three pale streaks. As shown, heavier growth was observed at points where some streaks were close to other streaks. These spots of heavier growth indicate that one mutant can cross-feed (supply an intermediate) to the other one.

A. From the pattern of cross-feeding shown in Figure 2–22A, deduce the order of the steps controlled by the products of the *TrpB*, *TrpD*, and *TrpE* genes. Explain your reasoning.
B. If accumulated intermediates at the block are responsible for the cross-feeding phenomenon, it should be possible to grow individual mutants on some intermediates. The three mutants were tested for growth on tryptophan and intermediates in the pathway (**Figure 2–22B**), with the results shown in **Table 2–5**. Use this information to arrange the defective genes relative to the tryptophan pathway.

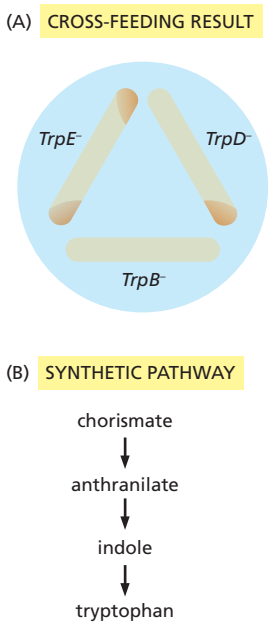


Figure 2–22 Defining the pathway for tryptophan synthesis using cross-feeding experiments (Problem 2–89). (A) Results of a cross-feeding experiment among mutants defective for steps in the tryptophan biosynthetic pathway. *Orange areas* on the Petri dish show regions of heavy cell growth. (B) The tryptophan biosynthetic pathway. Several steps precede chorismate in the pathway and there are several steps between anthranilate and indole.

TABLE 2–5 Growth of mutants on intermediates in the pathway for tryptophan biosynthesis (Problem 2–89).

Strain	Growth on minimal medium supplemented with				
	None	Chorismate	Anthranilate	Indole	Tryptophan
Wild type	+	+	+	+	+
TrpB$^-$	–	–	–	–	+
TrpD$^-$	–	–	–	+	+
TrpE$^-$	–	–	+	+	+

MCAT STYLE

Passage 1 (Questions 2–90 to 2–93)

Otto Warburg was one of the pioneering scientists who elucidated the biochemical basis of glycolysis in the early 1900s. He also made a puzzling discovery about cancer cells that remains interesting and relevant today. Normal differentiated cells, which do not proliferate, rely primarily upon aerobic respiration to generate ATP, except when they experience decreased levels of oxygen, in which case they switch to glycolysis. In contrast, cancer cells rely almost entirely upon glycolysis for producing ATP, regardless of the presence of oxygen. Cancer cells can increase the rate of glycolysis up to 200-fold relative to normal differentiated cells. This phenomenon, known as the Warburg effect, is still poorly understood. It is also a focus of renewed interest because it suggests a unique attribute of cancer cells that could be exploited to kill them selectively.

2–90 Which of the following properties of cancer cells would have provided evidence for the Warburg effect?

I. Increased oxidation of pyruvate
II. Increased release of CO_2
III. Increased release of lactate

A. I
B. II
C. III
D. I and II

2–91 You want to develop a drug that specifically kills cancer cells. Knowing about the Warburg effect, you hypothesize that cancer cells may be especially sensitive to inhibitors of glycolysis because they are uniquely dependent upon a high rate of glycolysis. Which one of the following would be a good target for drug development?

A. Acetyl CoA
B. Isocitrate dehydrogenase
C. Pyruvate kinase
D. The electron-transport chain

2–92 Cancer cells undergo rapid unrestrained proliferation. The increased reliance of cancer cells upon glycolysis therefore seems paradoxical, since glycolysis generates only 2 ATP from each glucose molecule, whereas complete oxidative respiration of glucose generates up to 36 ATPs per molecule. Which of the following best explains why glycolysis may be advantageous to rapidly proliferating cancer cells?

A. Glycolysis generates NADH that can be used as a source of reducing power for macromolecular synthesis.
B. Glycolysis produces intermediates that can be used to generate macromolecules needed for cell growth.
C. Relative to aerobic respiration, glycolysis produces more NADH, which can be used as an energy source.
D. Unlike aerobic respiration, glycolysis produces fatty acids, which can be stored as a source of energy.

2–93 The Warburg effect is exploited in an imaging technique that is commonly used to detect tumors. An individual is dosed with a molecule labeled with a radioactive isotope of fluorine (^{18}F). The labeled molecule is preferentially taken up by cancer cells and is detected by positron emission tomography (PET scanning). Which of the following molecules would you label with ^{18}F to detect tumors via the Warburg effect?

A. Acetyl CoA
B. Glucose
C. Lactate
D. Pyruvate

Passage 2 (Questions 2–94 to 2–95)

Extremophiles are microorganisms that can survive and proliferate in extreme environments. One fascinating group of extremophiles, called lithotrophs, are remarkable organisms that are found deep beneath the Earth's surface, living on rocks under anaerobic conditions and surviving on CO_2 as their sole source of carbon.

2–94 Imagine that you are studying a newly discovered lithotroph and are trying to determine what it uses as a source of electrons for reducing CO_2 and for producing energy. Which one of the following conditions must be met for a molecule to serve as a useful electron donor?

A. Oxidation of the molecule occurs with a decrease in free energy
B. Oxidation of the molecule occurs with an increase in free energy
C. Reduction of the molecule occurs with a decrease in free energy
D. Reduction of the molecule occurs with an increase in free energy

2–95 Which of the following is most likely to be used by the lithotrophs as a source of electrons for reducing CO_2 to form useful organic molecules and as an energy source for generating ATP?

A. Oxidation of glucose
B. Oxidation of H_2S
C. Reduction of H_2
D. Reduction of NO_2

CHAPTER 3

Proteins

IN THIS CHAPTER

THE SHAPE AND STRUCTURE OF PROTEINS

TERMS TO LEARN

α helix
amyloid fibril
β sheet
binding site
coiled-coil
conformation
polypeptide backbone
primary structure
prion disease
protein
protein domain
protein subunit
quaternary structure
secondary structure
side chain
tertiary structure

DEFINITIONS

Match the definition below with its term from the list above.

3–1 Three-dimensional relationship of the different polypeptide chains in a multisubunit protein or protein complex.

3–2 Common folding pattern in proteins in which a linear sequence of amino acids folds into a right-handed coil stabilized by internal hydrogen-bonding between backbone atoms.

3–3 The amino acid sequence of a protein.

3–4 A region on the surface of a protein that can interact with another molecule through noncovalent bonding.

3–5 Self-propagating, stable aggregate made up of identical polypeptide chains layered into a continuous stack of β sheets.

3–6 The chain of repeating carbon and nitrogen atoms, linked by peptide bonds, in a protein.

3–7 Common structural motif in proteins in which different sections of the polypeptide chain run alongside each other and are joined together by hydrogen-bonding between atoms of the polypeptide backbone.

3–8 Portion of a protein that has a tertiary structure of its own.

3–9 Regular local folding patterns in a protein, including α helix and β sheet.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

3–10 Each strand in a β sheet is a helix with two amino acids per turn.

3–11 Intrinsically disordered regions of proteins can be identified using bioinformatic methods to search genes for encoded amino acid sequences that possess high hydrophobicity and low net charge.

3–12 Loops of polypeptide that protrude from the surface of a protein often form the binding sites for other molecules.

3–13 Prion diseases can spread from one organism to another, providing that the second organism eats tissue that contains the gene encoding the protein involved in formation of amyloid fibrils.

THOUGHT PROBLEMS

3–14 Why do you suppose that only L-amino acids and not a random mixture of L- and D-amino acids are used to make proteins?

3–15 When egg white is heated, it hardens. This cooking process cannot be reversed, but hard-boiled egg white can be dissolved by heating it in a solution containing a strong detergent (such as sodium dodecyl sulfate) together with a reducing agent, like 2-mercaptoethanol. Neither reagent alone has any effect.

A. Why does boiling an egg white cause it to harden?
B. Why does it require both a detergent and a reducing agent to dissolve the hard-boiled egg white?

3–16 Although α helices are common components of polypeptide chains, they need to be of a certain minimum length. To find out how chain length affects α-helix formation, you measure the circular dichroism (a measure of helicity) for a series of peptides of increasing length (**Figure 3–1**). Why is there essentially no helix formation until the chain is at least six amino acids long?

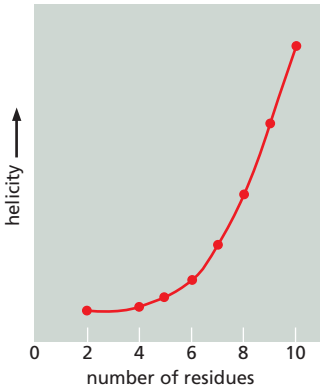


Figure 3–1 Helicity of various peptides of increasing length (Problem 3–16).

3–17 The uniform arrangement of the backbone carbonyl oxygens and amide nitrogens in an α helix gives the helix a net dipole, so that it carries a partial positive charge at the amino end and a partial negative charge at the carboxyl end. Where would you expect the ends of α helices to be located in a protein? Why?

3–18 α Helices are often embedded in a protein so that one side faces the surface and the other side faces the interior. Such helices are often termed amphiphilic because the surface side is hydrophilic and the interior side is hydrophobic. A simple way to decide whether a sequence of amino acids might form an amphiphilic helix is to arrange the amino acids around what is known as a "helix-wheel projection" (**Figure 3–2**). If the hydrophobic and hydrophilic amino acids are segregated on opposite sides of the wheel, the helix is amphiphilic. Using the helix-wheel projection, decide which of the three peptides in Figure 3–2 might form an amphiphilic helix. (The mnemonic "FAMILY VW" will help you recognize hydrophobic amino acids.)

3–19 Examine the segment of β sheet shown in **Figure 3–3**. For each strand of the sheet decide whether it is parallel or antiparallel to each of its neighbors.

3–20 Like α helices, β sheets often have one side facing the surface of the protein and one side facing the interior, giving rise to an amphiphilic

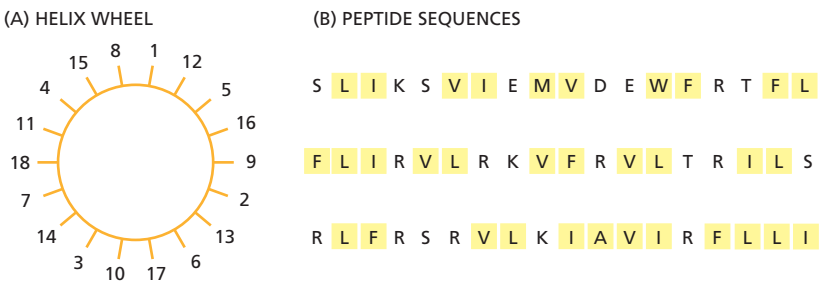


Figure 3–2 Helix-wheel projection (Problem 3–18). (A) Helix wheel. The circle (wheel) represents the helix as viewed from one end. Numbers show the positions of the amino acid side chains, as projected on the wheel. The positions of the first 18 amino acids are shown; amino acid 19 would occupy the same position as amino acid 1. Amino acid 1 is closest to the reader; amino acid 18 is farthest away. (B) Peptide sequences. The N-termini are shown at the left; hydrophobic amino acids are highlighted in *yellow*; hydrophilic amino acids are *unmarked*. (See Table 8, page 966, for one-letter amino acid code.)

sheet with one hydrophobic surface and one hydrophilic surface. From the sequences listed below, pick the one that could form a strand in an amphiphilic β sheet. Think about the way side chains are arranged in a strand of a β sheet. (See Table 8, page 966, for one-letter amino acid code; the mnemonic in Problem 3–18 might also be helpful.)

A. A L S C D V E T Y W L I
B. D K L V T S I A R E F M
C. D S E T K N A V F L I L
D. T L N I S F Q M E L D V
E. V L E F M D I A S V L D

3–21 Several different protein folds are represented in schematic form in **Figure 3–4**. These diagrams preserve the topology of the protein and allow one to decide, for example, whether a protein is folded in a new way or is an example of a protein fold that is already known. These diagrams also permit a ready demonstration of a fundamental principle of protein folding. For each of these folds, imagine that you could grasp the N- and C-termini and pull them apart. Would any of the illustrated folds produce a knot when fully stretched out?

3–22 It is a common observation that antiparallel strands in a β sheet are connected by short loops, but that parallel strands are connected by α helices. Why do you think this is?

3–23 In 1968, Cyrus Levinthal pointed out a complication in protein folding that is widely known as the Levinthal paradox. He argued that because there are astronomical numbers of conformations open to a protein in the denatured state, it would take a very long time for a protein to search through all the possibilities to find the correct one, even if it tested each possible conformation exceedingly rapidly. Yet denatured proteins typically take less than a second to fold inside the cell or in the test tube. How do you suppose that proteins manage to fold so quickly?

3–24 Comparison of a homeodomain protein from yeast and from *Drosophila* shows that only 17 of 60 amino acids are identical. How is it possible for a protein to change over 70% of its amino acids and still fold in the same way?

3–25 Often, the hard part of protein structure determination by x-ray diffraction is getting good crystals. In difficult cases, there are two common approaches for obtaining crystals: (1) using fragments of the protein and (2) trying homologous proteins from different species.

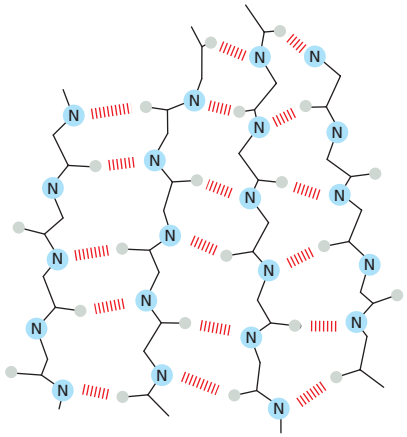


Figure 3–3 A segment of β sheet from the interior of thioredoxin (Problem 3–19). Amide nitrogens are indicated by *circled* Ns; hydrogen bonds are shown as *red lines.*

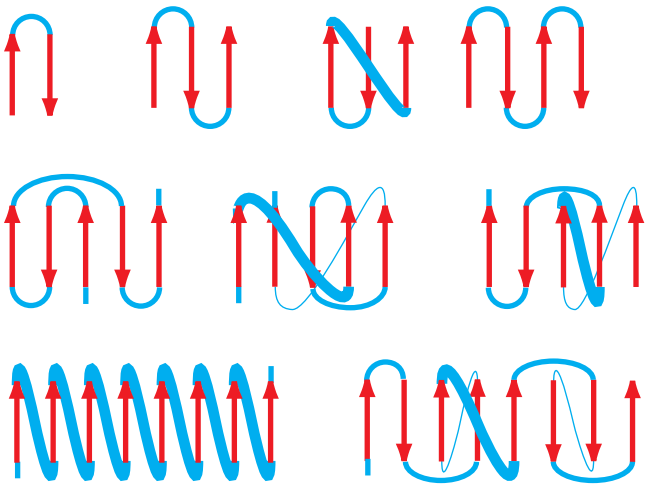


Figure 3–4 Topological representations of several protein folds (Problem 3–21). *Vertical red arrows* represent strands in β sheets; *blue connectors* may be loops or helices. *Thick blue diagonal lines* are above the plane of the page; *thin blue lines* lie below the plane of the page.

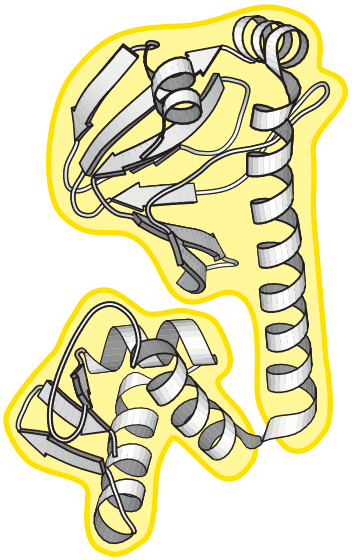


Figure 3–5 Catabolite activator protein from *E. coli* (Problem 3–25). *Yellow shading* indicates its domain structure.

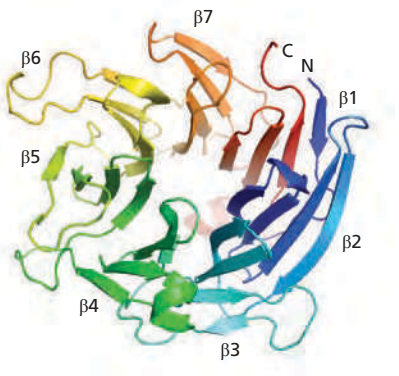


Figure 3–6 The kelch repeat domain of galactose oxidase from *D. dendroides* (Problem 3–27). The seven individual β propellers are *color coded* and labeled. The N- and C-termini are indicated by N and C.

A. Examine the protein in **Figure 3–5**. Where would you cleave this protein to obtain fragments that might be expected to fold properly and perhaps form crystals?
B. Why do you suppose that homologous proteins from different species might differ in their ability to form crystals?

3–26 A common strategy for identifying distantly related homologous proteins is to search the database using a short signature sequence indicative of the particular protein function. Why is it better to search with a short sequence than with a long sequence? Don't you have more chances for a "hit" in the database with a long sequence?

3–27 The so-called kelch motif consists of a four-stranded β sheet, which forms what is known as a β propeller. It is usually found to be repeated four to seven times, forming a kelch repeat domain in a multidomain protein. One such kelch repeat domain is shown in **Figure 3–6**. Would you classify this domain as an "in-line" or "plug-in" type domain?

3–28 Examine the three protein monomers in **Figure 3–7**. From the arrangement of complementary binding surfaces, which are indicated by similarly shaped protrusions and invaginations, decide which monomer would assemble into a ring, which would assemble into a chain, and which would assemble into a sheet.

3–29 Cro is a bacterial gene regulatory protein that binds to DNA to turn genes off. It is a symmetrical "head-to-head" dimer. Each of the two subunits of the dimer recognizes a particular short sequence of nucleotides in DNA. If the sequence of nucleotides recognized by one subunit is represented as an arrow (→), so that the "head" of the arrow corresponds to DNA recognized by the "head" of the subunit, which of the following sequences in DNA represents the binding site for the Cro dimer?

A. →→
B. →←
C. ←←
D. ←→
E. Could be more than one of the above

3–30 Why is it that there are numerous examples of "head-to-head" and "tail-to-tail" dimers, but few, if any, examples of "head-to-tail" dimers?

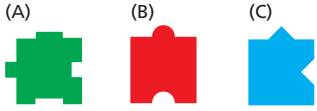


Figure 3–7 Three protein monomers (Problem 3–28).

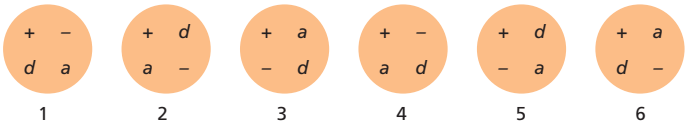


Figure 3–8 Binding surfaces for six different proteins (Problem 3–31). In each case, the bulk of the protein is below the plane of the page.

3–31 Proteins bind to one another via weak interactions across complementary surfaces. Oppositely charged amino acids are apposed, as are hydrogen-bond donors and acceptors, and protrusions match invaginations so that van der Waals contacts can be maximized. When two copies of a protein bind to form a "head-to-head" dimer, they use the same binding surface. Examine the binding surfaces of the six proteins shown in **Figure 3–8**, where charged amino acids are indicated by + and –, and hydrogen-bond donors and acceptors are indicated by *d* and *a*. (Protrusions and invaginations—three-dimensional shapes—are not represented in the binding surfaces in Figure 3–8 just because it is difficult to do so, but their absence does not change the general principles derived from this problem.) In which cases could two copies of one protein form a "head-to-head" dimer in which the charges and hydrogen-bonding groups are appropriately matched? Can you spot any common feature of the surfaces that allows such dimers to form?

3–32 Nuclear lamin C is a member of the intermediate filament family. Thus, it should show regions of the coiled-coil heptad repeat motif AbcDefg, where A and D are hydrophobic amino acids and b, c, e, f, and g can be almost any amino acid. The sequence of nuclear lamin C is shown in **Figure 3–9** with potential coiled-coil regions highlighted. Examine the segment marked "coil 1A." How well does it conform to the heptad repeat? (Don't forget the mnemonic FAMILY VW.)

coil 1A

METPSQRRATRSGAQASSTPLSPTRITRLQEKED**LQELNDRLAVYIDRVRSLETENA**

coil 1B

GLRLRITESEEVVSREVSGIKAA**YEAELGDARKTLDSVAKERARLQLELSKVREEFK**

ELKARNTKKEGDLIAAQARLKDLEALLNSKEAALSTALSEKRTLEGELHDLRGQVAK

LEAALGEAKKQLQDEMLRRVDAENRLQTMKEELDFQKNIYSEELRETKRRHETRLVE

coil 2

IDNGKQREFESRLAD**ALQQLRAQHEDQVEQYKKELEKTYSAKLDNARQSAERNSNLV**

GAAHEELQQSRIRIDSLSAQLSQLQKQLAAKEAKLRDLEDSLARERDTSRRLLAEKE

REMAEMRARMQQQLDEYQQLLDIKLALDMQIHAYRKLLEGEEERLRLSPSPTSQRSR

GRASSHSSQTQGGGSVTKKRKLESTESRSSPSQHARTSGRVAVEEVDEEGKFVRLRN

KSNEDQSMGNWQIKRQNGDDPLLTYRFPPKFTLKAGQVVTIWAAGAGATHSPPTDLV

WKAQNTWGCGNSLRTALINSTGEEVAMRKLVRSVTVVEDDEDEDGDDLLHHHHVSGS

RR

Figure 3–9 The amino acid sequence of nuclear lamin C (Problem 3–32).

CALCULATIONS

3–33 Typical proteins have a stability ranging from 30 to 60 kJ/mole at 37°C. Stability is a measure of the equilibrium between the folded and unfolded forms of the protein:

$$\text{folded [F]} \leftrightarrow \text{unfolded [U]}, K = [\text{U}]/[\text{F}]$$

For a protein with a stability of 41.5 kJ/mole, calculate the fraction of unfolded protein that would exist at equilibrium at 37°C. At equilibrium,

$$\Delta G^\circ = -RT\ln K = -2.3RT\log K,$$

where $R = 8.3 \times 10^{-3}$ kJ/(mole K) and T is temperature in K (37°C = 310 K).

3–34 Consider the following statement. "To produce one molecule of each possible kind of polypeptide chain, 300 amino acids in length, would require more atoms than exist in the universe." Given the size of the universe, do you suppose this statement could possibly be correct? Since counting atoms is a tricky business, consider the problem from the standpoint of mass. The mass of the observable universe is estimated to be about 10^{80} grams, give or take an order of magnitude or so. Assuming that the average mass of an amino acid is 110 daltons, what would be the mass of one molecule of each possible kind of polypeptide chain 300 amino acids in length? Is this greater than the mass of the universe?

DATA HANDLING

3–35 Most proteins denature at both high and low pH. At high pH, the ionization of internal tyrosines is thought to be the main destabilizing influence, whereas at low pH, the protonation of buried histidines (**Figure 3–10A**) is the likely culprit. A titration curve for the unfolding of the enzyme ribonuclease is shown in **Figure 3–10B**. Superimposed on it is the expected titration curve for the ionization of a histidine side chain with a pK of about 4, which is typical for a buried histidine (the pK for the side chain of the free amino acid is 6). The titration curve for denaturation is clearly much steeper than that for the side chain. Given the discrepancy between the titration curves for protein unfolding and histidine protonation, how can it be true that protonation of histidine causes protein unfolding?

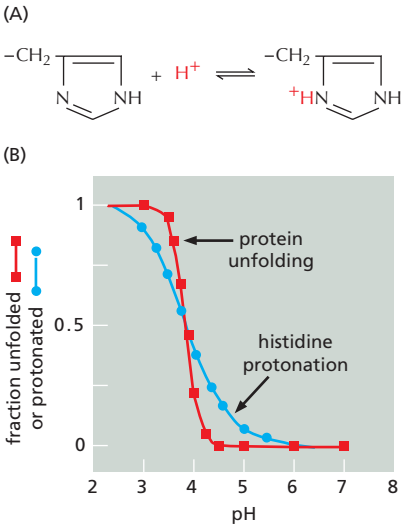


Figure 3–10 Denaturation of proteins (Problem 3–35). (A) Histidine protonation. (B) Titration curves for protein unfolding and histone protonation.

3–36 Titin, which has a molecular weight of about 3×10^6, is the largest polypeptide yet described. Titin molecules extend from muscle thick filaments to the Z disc; they are thought to act as springs to keep the thick filaments centered in the sarcomere. Titin is composed of a large number of repeated immunoglobulin (Ig) sequences of 89 amino acids, each of which is folded into a domain about 4 nm in length (**Figure 3–11A**).

You suspect that the springlike behavior of titin is caused by the sequential unfolding (and refolding) of individual Ig domains. You test this hypothesis using the atomic force microscope, which allows you to pick up one end of a protein molecule and pull with an accurately measured force. For a fragment of titin containing seven repeats of the Ig domain, this experiment gives the sawtooth force-versus-extension curve shown in **Figure 3–11B**. If the experiment is repeated in a solution of 8 M urea (a protein denaturant), the peaks disappear and the measured extension becomes much longer for a given force. If the experiment is repeated after the protein has been cross-linked by treatment with glutaraldehyde, once again the peaks disappear but the extension becomes much smaller for a given force.

A. Are the data consistent with your hypothesis that titin's springlike behavior is due to the sequential unfolding of individual Ig domains? Explain your reasoning.

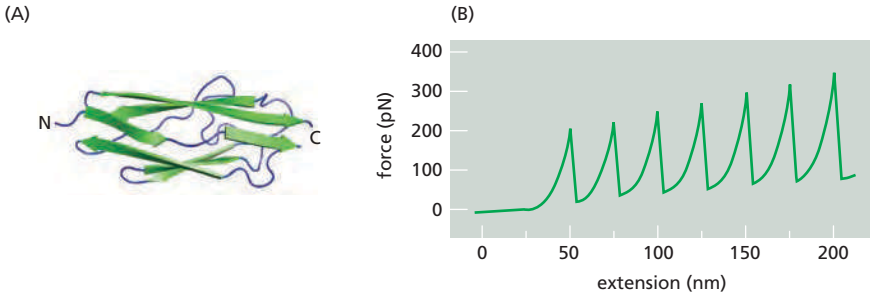


Figure 3–11 Springlike behavior of titin (Problem 3–36). (A) The structure of an individual Ig domain. (B) Force in piconewtons versus extension in nanometers obtained by atomic force microscopy.

B. Is the extension for each putative domain-unfolding event the magnitude you would expect? (In an extended polypeptide chain, amino acids are spaced at intervals of 0.34 nm.)
C. Why is each successive peak in Figure 3–11B a little higher than the one before?
D. Why does the force collapse so abruptly after each peak?

3–37 You are skeptical of the blanket statement that cysteines in intracellular proteins are not involved in disulfide bonds, while in extracellular proteins they are. To test this statement you carry out the following experiment. As a source of intracellular protein you use reticulocytes, which have no internal membranes and, thus, no proteins from the endoplasmic reticulum (ER) or other membrane-enclosed compartments. As examples of extracellular proteins, you use bovine serum albumin (BSA), which has 37 cysteines, and insulin, which has 6. You denature the soluble proteins from a reticulocyte lysate and the two extracellular proteins so that all cysteines are exposed. To probe the status of cysteines, you treat the proteins with *N*-ethylmaleimide (NEM), which reacts covalently with the –SH groups of free cysteines, but not with sulfur atoms in disulfide bonds. In the first experiment, you treat the denatured proteins with radiolabeled NEM, then break any disulfide bonds with dithiothreitol (DTT) and react a second time with unlabeled NEM. In the second experiment, you do the reverse: you first treat the denatured proteins with unlabeled NEM, then break disulfide bonds with DTT and treat with radiolabeled NEM. The proteins are separated according to size by electrophoresis on a polyacrylamide gel (**Figure 3–12**).

A. Do any cytosolic proteins have disulfide bonds?
B. Do the extracellular proteins have any free cysteine –SH groups?
C. How do you suppose the results might differ if you used lysates of cells that have internal membrane-enclosed compartments?

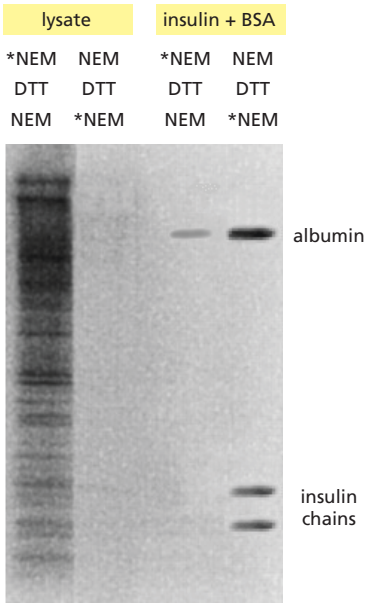


Figure 3–12 Test for disulfide bonds in cytosolic and extracellular proteins (Problem 3–37). The order of treatment with NEM and DTT is indicated at the top of each lane; *NEM indicates radiolabeled NEM.

PROTEIN FUNCTION

TERMS TO LEARN

active site
allosteric protein
antibody
antigen
catalyst
coenzyme
equilibrium constant (*K*)
enzyme
feedback inhibition
GTP-binding protein (GTPase)
ligand
linkage
lysozyme
motor protein
protein kinase
protein phosphatase
proteomics
regulatory site
scaffold protein
substrate
transition state
ubiquitin
ubiquitin ligase

DEFINITIONS

Match the definition below with its term from the list above.

3–38 A protein that serves both to link together a set of interacting proteins and to position them at a specific location in a cell.

3–39 Type of metabolic regulation in which the activity of an enzyme acting near the beginning of a reaction pathway is reduced by a product of the pathway.

3–40 Protein produced by the immune system in response to a foreign molecule or invading microorganism.

3–41 Region of an enzyme surface to which a substrate molecule binds in order to undergo a catalyzed reaction.

3–42 A protein catalyst that speeds up a reaction, often by a factor of a million or more, without itself being changed.

3–43 The first example of a special family of small proteins whose members are covalently attached to other proteins to influence their activity or fate.

3–44 Mutual effect of the binding of one ligand on the binding of another that is a central feature of the behavior of all allosteric proteins.

3–45 Enzyme that transfers the terminal phosphate group of ATP to a specific amino acid in a target protein.

3–46 Rate-limiting structure that forms transiently in the course of a chemical reaction and has the highest free energy of any reaction intermediate.

3–47 Protein that changes its conformation (and often its activity) when it binds a regulatory molecule or when it is covalently modified.

3–48 A term often used to describe research focused on the simultaneous analysis of large numbers of proteins.

3–49 Small molecule that is tightly associated with a protein catalyst and participates in the chemical reaction, often by forming a covalent bond to the substrate.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

3–50 The tendency for an amino acid side-chain group such as –COOH to release a proton, its p*K*, is the same for the amino acid in solution and for the amino acid in a protein.

3–51 For a family of related genes that do not match genes of known function in the sequence database, it should be possible to deduce their function by using "evolutionary tracing" to see where conserved amino acids cluster on their surfaces.

3–52 Higher concentrations of enzyme give rise to a higher turnover number.

3–53 Enzymes that undergo cooperative allosteric transitions invariably consist of symmetric assemblies of multiple subunits.

3–54 Continual addition and removal of phosphates by protein kinases and protein phosphatases is wasteful of energy—since their combined action consumes ATP—but it is a necessary consequence of effective regulation by phosphorylation.

3–55 Conformational changes in proteins never exceed a few tenths of a nanometer.

THOUGHT PROBLEMS

3–56 Antarctic notothenioid fish (**Figure 3–13**) avoid freezing in their perpetually icy environment because of an antifreeze protein that circulates in their blood. This evolutionary adaptation has allowed the Notothenioidei suborder to rise to dominance in the freezing Southern Ocean. It is said that all proteins function by binding to other molecules. To what ligand do you suppose antifreeze proteins bind to keep the fish from freezing? Or do you think this might be an example of a protein that functions in the absence of any molecular interaction?

3–57 How does the protein environment surrounding an amino acid side chain affect its chemical properties? Consider the carboxyl group on an aspartate side chain in the following environments in a protein. Rank order these environments from the highest to the lowest proportion of

Dissostichus eleginoides, the Chilean sea bass

Pagothenia borchgrevinki

Figure 3–13 Two notothenioid fish (Problem 3–56). The notothenioid family now dominates Antarctica's continental shelf, accounting for 50% of the species and 95% of the biomass of fish. The Chilean sea bass is commonly served in restaurants.

carboxyl groups in the –COO^- form; that is, in terms of their pK_as. Explain your ranking.

1. An aspartate side chain on the surface of a protein with no other ionizable groups nearby.
2. An aspartate side chain buried in a hydrophobic pocket on the surface of a protein.
3. An aspartate side chain in a hydrophobic pocket adjacent to a glutamate side chain.
4. An aspartate side chain in a hydrophobic pocket adjacent to a lysine side chain.

3–58 Aminoacyl-tRNA synthetases attach specific amino acids to their appropriate tRNAs in preparation for protein synthesis. The synthetase that attaches valine to $tRNA^{Val}$ must be able to discriminate valine from threonine, which differ only slightly in structure: valine has a methyl group where threonine has a hydroxyl group (**Figure 3–14**). Valyl-tRNA synthetase achieves this discrimination in two steps. In the first, it uses a binding pocket whose contours allow valine or threonine (but not other amino acids) to bind, but the binding of valine is preferred. This site is responsible for coupling the amino acid to the tRNA. In the second step, the enzyme checks the newly made aminoacyl-tRNA using a second binding site that is very specific for threonine and hydrolyzes it from the tRNA. How do you suppose it is that the second binding site can be very specific for threonine, whereas the first binding site has only a moderate specificity for valine?

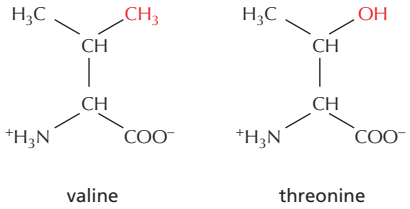


Figure 3–14 Structures of valine and threonine (Problem 3–58).

3–59 You have raised a specific, high-affinity monoclonal antibody against the enzyme you are working on, and have identified its interaction site as a stretch of six amino acids in the enzyme. Your advisor suggests that you could use the antibody to purify the enzyme by affinity chromatography. This technique would involve attaching the antibody to the inert matrix of a column, passing a crude cell lysate over the column, allowing the antibody to bind your enzyme but not other proteins, and finally eluting your enzyme by washing the column with a solution containing the six-amino-acid peptide corresponding to the binding site. The principal advantage of affinity chromatography is that it allows a rapid, one-step purification under mild conditions that retain enzyme activity.

In a preliminary experiment, you show that if you incubate the antibody with the peptide corresponding to the binding site it will no longer bind to your enzyme, demonstrating that the antibody binds the peptide. Encouraged, you bind the antibody to the column and show that it completely removes your enzyme from the crude cell lysate. When you try to elute your enzyme with a solution containing a high concentration of the peptide, however, you find that none of your enzyme comes off the column. What could have gone wrong? (Think about what must happen for the enzyme to come off the column.)

3–60 Examine **Figure 3–15**, which compares the energetics of a catalyzed and uncatalyzed reaction during the progress of the reaction from substrate (S) to product (P). The highest peak in such a diagram corresponds to the transition state, which is an unstable, high-energy arrangement of substrate atoms that is intermediate between substrate and product. The free energy required to surmount this barrier to the reaction is termed the activation energy. Enzymes function by lowering the activation energy, thereby allowing a more rapid approach to equilibrium.

With this diagram in mind, consider the following question. Suppose the enzyme in the diagram were mutated in such a way that its affinity for the substrate was increased by a factor of 100. Assume that there was no other effect beyond increasing the depth of the trough labeled ES

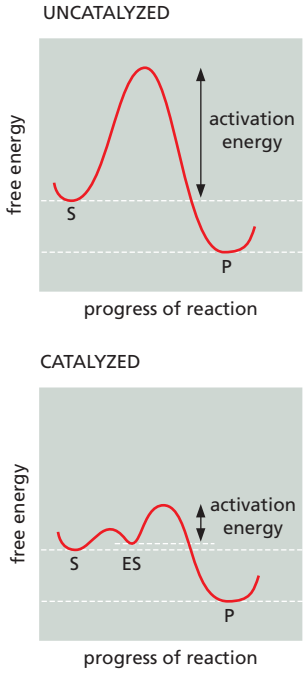


Figure 3–15 Catalyzed and uncatalyzed reactions showing the free energy at various stages in the progress of the reaction (Problem 3–60).

(enzyme–substrate complex) in Figure 3–15. Would you expect the rate of the reaction catalyzed by the altered enzyme to be faster, slower, or equal to the reaction rate catalyzed by the normal enzyme?

3–61 Which one of the following properties of an enzyme is responsible for its saturation behavior; that is, a maximum rate insensitive to further increases in substrate concentration?

A. The enzyme does not change the overall equilibrium constant for a reaction.
B. The enzyme lowers the activation energy of a chemical reaction.
C. The enzyme is a catalyst that is not consumed by the reaction.
D. The enzyme has a fixed number of active sites where substrate binds.
E. The product of the enzyme reaction usually inhibits the enzyme.

3–62 The Michaelis constant, K_m, is often spoken of as if it were a measure of the affinity of the enzyme for the substrate: the lower the K_m, the higher the binding affinity. This would be true if K_m were the same as K_d (the equilibrium constant for the dissociation reaction), but it is not. For an enzyme-catalyzed reaction

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

$$K_m = \frac{(k_{-1} + k_{cat})}{k_1}$$

A. In terms of these rate constants, what is K_d for dissociation of the ES complex to E + S?
B. Under what conditions is K_m approximately equal to K_d?
C. Does K_m consistently overestimate or underestimate the binding affinity? Or does it sometimes overestimate and sometimes underestimate the binding affinity?

3–63 You are trying to determine whether it is better to purify an enzyme from its natural source or to express the gene in bacteria and then purify it. You purify the enzyme in the same way from both sources and show that each preparation gives a single band by denaturing gel electrophoresis, a common measure of purity. When you compare the kinetic parameters, you find that both enzymes have the same K_m but the enzyme from bacteria has a 10-fold lower V_{max}. Propose possible explanations for this result.

3–64 The enzyme hexokinase adds a phosphate to D-glucose but ignores its mirror image, L-glucose. Suppose that you were able to synthesize hexokinase entirely from D-amino acids, which are the mirror image of the normal L-amino acids.

A. Assuming that the "D" enzyme would fold to a stable conformation, what relationship would you expect it to bear to the normal "L" enzyme?
B. Do you suppose the "D" enzyme would add a phosphate to L-glucose, and ignore D-glucose?

3–65 In 1948, Linus Pauling proposed what is now considered to be a key aspect of enzyme function.

"I believe that an enzyme has a structure closely similar to that found for antibodies, but with one important difference, namely, that the surface configuration of the enzyme is not so closely complementary to its specific substrate as is that on an antibody, but is instead complementary to an unstable molecule with only transient existence—namely, the 'activated complex' [transition state, in modern parlance] for the reaction that is catalyzed by the enzyme. The mode of action of an enzyme would then be the following: the enzyme would show a small power of

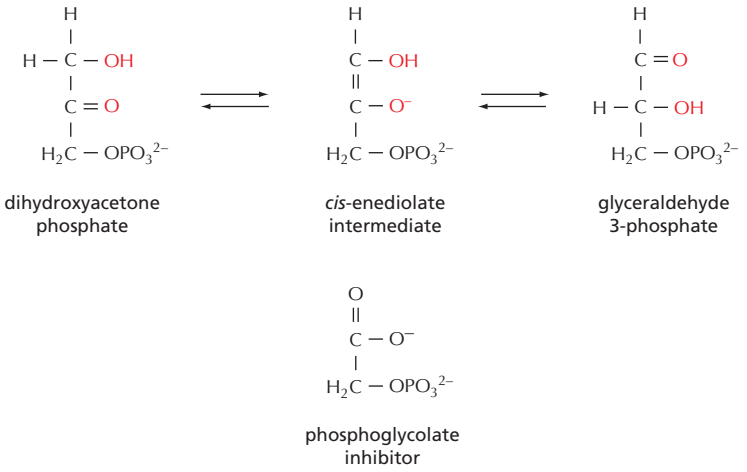


Figure 3–16 The reaction catalyzed by triosephosphate isomerase, and the enzyme inhibitor, phosphoglycolate (Problem 3–65).

attraction for the substrate molecule or molecules, which would become attached to it in its active surface region. This substrate molecule, or these molecules, would then be strained by the forces of attraction to the enzyme, which would tend to deform it into the configuration for the activated complex, for which the power of attraction by the enzyme is the greatest.... The assumption made above that the enzyme has a configuration complementary to the activated complex, and accordingly has the strongest power of attraction for the activated complex, means that the activation energy for the reaction is less in the presence of the enzyme than in its absence, and accordingly that the reaction would be speeded up by the enzyme."

The enzyme triosephosphate isomerase catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate through a *cis*-enediolate intermediate (**Figure 3–16**). Phosphoglycolate (Figure 3–16) is a competitive inhibitor of triosephosphate isomerase with a K_d of 7 μM. The normal substrates for the enzyme have a K_d of about 100 μM. Do you think that phosphoglycolate is a transition-state analog? Why or why not?

3–66 The mechanism for lysozyme cleavage of its polysaccharide substrate requires Glu35 in its nonionized form, whereas the nearby Asp52 must be ionized (**Figure 3–17**). The p*K* values for the side-chain carboxyl groups on the two amino acids in solution are virtually identical.

A. How can one carboxyl group be charged and the other uncharged in the active site of lysozyme?

B. The pH optimum for lysozyme is about 5. Why do you suppose that the activity decreases above and below this optimum?

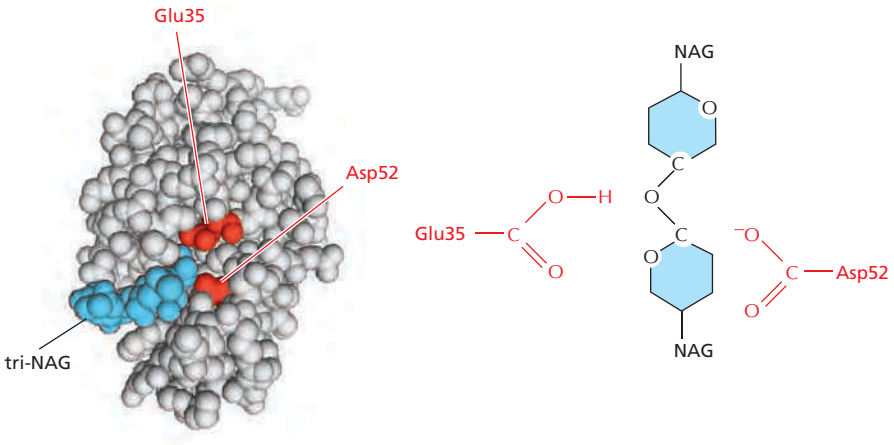


Figure 3–17 Forms of Glu35 and Asp52 required for polysaccharide cleavage by lysozyme (Problem 3–66). In the space-filling model, the positions of Glu35 and Asp52 are shown relative to the trisaccharide of *N*-acetylglucosamine (NAG) units, tri-NAG, which is not quite long enough to be cleaved. In the schematic diagram, the positions of Glu35 and Asp52 are shown relative to the glycosidic bond to be cleaved in a polysaccharide composed of NAG residues.

Figure 3–18 Schematic diagram of the metabolic pathway for synthesis of AMP and GMP from R5P (Problem 3–70).

3–67 How do you suppose that a molecule of hemoglobin is able to bind oxygen efficiently in the lungs, and yet release it efficiently in the tissues?

3–68 If you were in charge of enzyme design for a cell, for what circumstances might you design an enzyme that had a K_m much, much lower than the prevailing substrate concentration ([S] >> K_m)? A K_m around the prevailing substrate concentration ([S] ≈ K_m)? A K_m much, much higher than the prevailing substrate concentration ([S] << K_m)?

3–69 Which of the following does NOT describe a mechanism that cells use to regulate enzyme activities?

A. Cells control enzyme activity by phosphorylation and dephosphorylation.
B. Cells control enzyme activity by the binding of small molecules.
C. Cells control the rates of diffusion of substrates to enzymes.
D. Cells control the rates of enzyme degradation.
E. Cells control the rates of enzyme synthesis.
F. Cells control the targeting of enzymes to specific organelles.

3–70 Synthesis of the purine nucleotides AMP and GMP proceeds by a branched pathway starting with ribose 5-phosphate (R5P), as shown schematically in **Figure 3–18**. Using the principles of feedback inhibition, propose a regulatory strategy for this pathway that ensures an adequate supply of both AMP and GMP and minimizes the buildup of the intermediates (*A*–*I*) when supplies of AMP and GMP are adequate.

3–71 Pathways devoted to the synthesis of specific bioproducts such as purines are commonly regulated via feedback inhibition by the final product. By contrast, the flow of metabolites through the web of pathways devoted to overall energy metabolism—production and utilization of ATP, as well as the buildup and breakdown of internal fuel reserves—is regulated by metabolites whose concentrations reflect the energy status of the cell. ATP-like signal metabolites (such as ATP and NADH) tend to accumulate when the cell is slowly consuming ATP to meet its energy needs; AMP-like signal metabolites (such as AMP, ADP, inorganic phosphate, and NAD^+) tend to accumulate when the cell is rapidly using ATP.

Consider the pathways for the synthesis and breakdown of glycogen, the main fuel reserve in muscle cells (**Figure 3–19**). The synthetic pathway is controlled by glycogen synthase, whereas the breakdown pathway is controlled by glycogen phosphorylase. In resting muscle, which type of signal metabolite would be expected to accumulate? How would those signal metabolites be expected to affect the activity of the two regulated enzymes of glycogen metabolism? What about in exercising muscle?

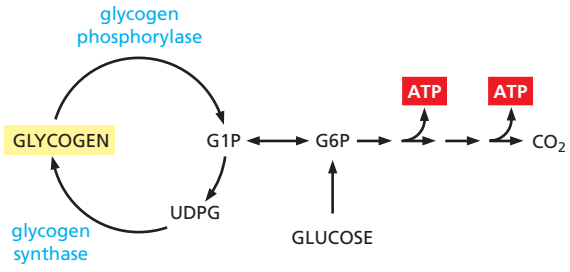


Figure 3–19 Pathways for glycogen synthesis and its breakdown to glucose 6-phosphate, which is an intermediate along the pathway for glucose metabolism to CO_2 (Problem 3–71). G6P stands for glucose 6-phosphate, G1P for glucose 1-phosphate, and UDPG for uridine diphosphoglucose.

3–72 The enzyme glycogen phosphorylase uses phosphate as a substrate to split off glucose 1-phosphate from glycogen, which is a polymer of glucose. Glycogen phosphorylase in the absence of any ligands is a dimer that exists in two conformations: a predominant one with low enzymatic activity and a rarer one with high activity. Both phosphate, a substrate that binds to the active site, and AMP, an activator that binds to an allosteric site, alter the conformational equilibrium by binding preferentially to one conformation. To which conformation of the enzyme would you expect phosphate to bind, and why? To which conformation would you expect AMP to bind, and why? How does the binding of either molecule alter the activity of the enzyme?

3–73 Monod, Wyman, and Changeux (MWC) originally explained the kinetic behavior of allosteric enzymes using four postulates, as summarized below.

1. All subunits in the allosteric enzyme have identical conformations and are arranged symmetrically.
2. Each subunit carries a binding site for each ligand.
3. The allosteric enzyme can exist in at least two conformations that conserve its overall symmetry. The different conformations may have very different affinities for the ligands, which may be bound to any combination of subunits.
4. The binding affinity of a ligand depends only on the conformational state of the allosteric enzyme and not on the occupancy of neighboring sites.

A subset of all the arrangements of subunits in an allosteric enzyme composed of four identical subunits is shown in **Figure 3–20**. Each subunit has two possible conformations and *dark blue* subunits have a bound ligand. Assuming that the ligand binds much more tightly to one conformation of subunit (*circle*), decide which of the tetrameric species are consistent with the MWC postulates. What would your answer be if the ligand bound equally well to each of the two conformations of subunit?

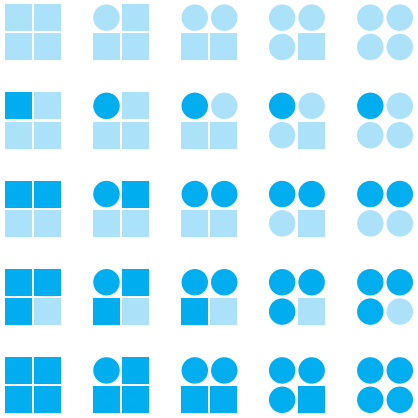


Figure 3–20 A subset of all the possible arrangements of a tetramer, composed of subunits with either of two conformations (Problem 3–73). *Circles* and *squares* represent the two conformations of the subunits; *dark blue* indicates a subunit with a bound ligand.

3–74 Many proteins inside cells are regulated by phosphorylation and dephosphorylation. Consider a metabolic reaction catalyzed by an enzyme that is fully active when not phosphorylated and completely inactive when it is phosphorylated (**Figure 3–21**). Inside the cell, the rate of this metabolic reaction can vary continuously from very fast to very slow (or zero) at any given substrate concentration. How is it that the reaction in the cell can proceed at any rate between very fast and zero, even though individual enzyme molecules are either fully on or completely turned off?

3–75 Motor proteins generally require ATP (or GTP) hydrolysis to ensure unidirectional movement.

A. In the absence of ATP, would you expect a motor protein to stop moving, to wander back and forth, to move in reverse, or to continue moving forward but more slowly?

B. Assume that the concentrations of ATP, ADP, and phosphate were adjusted so that the free-energy change for ATP hydrolysis by the motor protein was equal to zero (instead of very negative, as it is normally). Under these conditions, would you expect a motor protein to stop moving, to wander back and forth, to move in reverse, or to continue moving forward but more slowly?

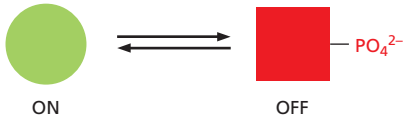


Figure 3–21 Phosphorylated and nonphosphorylated states of a metabolic enzyme (Problem 3–74).

CALCULATIONS

3–76 An antibody binds to another protein with an equilibrium constant, K, of $5 \times 10^9\ M^{-1}$. When it binds to a second, related protein, it forms three fewer hydrogen bonds, reducing its binding affinity by 11.9 kJ/mole.

What is the K for its binding to the second protein? (Free-energy change is related to the equilibrium constant by the equation $\Delta G° = -2.3\ RT \log K$, where R is 8.3×10^{-3} kJ/(mole K) and T is 310 K.)

3–77 The equilibrium constant for a reaction like that of antibody (Ab) binding to a protein (Pr) to form an antibody–protein complex (Ab–Pr) is equal to the ratio of the association rate constant, k_{on}, to the dissociation rate constant, k_{off} ($K = [\text{Ab–Pr}]/([\text{Ab}][\text{Pr}]) = k_{on}/k_{off}$). Recall that the association rate ($k_{on}[\text{Ab}][\text{Pr}]$) equals the dissociation rate ($k_{off}[\text{Ab–Pr}]$) at equilibrium. Consider two such reactions. The first has an on rate constant of $10^5\ M^{-1}\ sec^{-1}$ and an off rate constant of $10^{-3}\ sec^{-1}$ at 37°C. The second has an on rate constant of $10^3\ M^{-1}\ sec^{-1}$ and an off rate of $10^{-5}\ sec^{-1}$ at 37°C.

A. What are the equilibrium constants for these two reactions?
B. At equal concentrations of antibody and protein, which of these reactions will reach its equilibrium point more quickly?
C. You wish to use an antibody to purify the protein you are studying. You are concerned that the complex may fall apart in the time it takes you to isolate it, and you are unsure how the off rates relate to the half-time for dissociation; that is, the time at which half the complex will have dissociated. It can be shown that the fraction of complex remaining at time t ($[\text{Ab–Pr}]_t$) relative to that present initially ($[\text{Ab–Pr}]_0$) is

$$\frac{[\text{Ab–Pr}]_t}{[\text{Ab–Pr}]_0} = e^{-k_{off}t}$$

an equation more easily dealt with in its logarithmic form,

$$2.3 \log \frac{[\text{Ab–Pr}]_t}{[\text{Ab–Pr}]_0} = -k_{off}t$$

Using this relationship, decide how long it will take for half of the Ab–Pr complexes in each of the above two reactions to dissociate. Neglect any contribution from new complex being formed in the association reaction.

3–78 Consider an uncatalyzed reaction, $A \rightleftharpoons B$. The rate constants for the forward and reverse reactions are $k_f = 10^{-4}\ sec^{-1}$ and $k_r = 10^{-7}\ sec^{-1}$. Thus, the rates or velocities (v) of the forward and reverse reactions are

$$v_f = k_f [A] \qquad \text{and} \qquad v_r = k_r [B]$$

The overall reaction rate is

$$v = v_f - v_r = k_f [A] - k_r [B]$$

A. What is the overall reaction rate at equilibrium?
B. What is the value of the equilibrium constant, K?
C. You now add an enzyme that increases k_f by a factor of 10^9. What will the value of the equilibrium constant be with the enzyme present? What will the value of k_r be?

3–79 Many enzymes obey simple Michaelis–Menten kinetics, which are summarized by the equation

$$\text{rate} = \frac{V_{max}[S]}{[S] + K_m}$$

where V_{max} = maximum velocity, [S] = concentration of substrate, and K_m = the Michaelis constant.

It is instructive to plug a few values of [S] into the equation to see how rate is affected. What are the rates for [S] equal to zero, equal to K_m, and equal to infinite concentration?

3–80 Suppose that the enzyme you are studying is regulated by phosphorylation. When the enzyme is phosphorylated, the K_m for its substrate

increases by a factor of 3, but V_{max} is unaltered. At a concentration of substrate equal to the K_m for the unphosphorylated enzyme, decide whether phosphorylation activates the enzyme or inhibits it? Explain your reasoning.

3–81 For an enzyme that follows Michaelis–Menten kinetics, by what factor does the substrate concentration have to increase to change the rate of the reaction from 20% to 80% V_{max}?

A. A factor of 2
B. A factor of 4
C. A factor of 8
D. A factor of 16
E. The factor required cannot be calculated without knowing K_m

3–82 The "turnover number," or k_{cat}, for an enzyme is the number of substrate molecules converted into product by an enzyme molecule per unit time when the enzyme is fully saturated with substrate. The maximum rate of a reaction, V_{max}, equals k_{cat} times the concentration of enzyme. (Remember that the maximum rate occurs when all of the enzyme is present as the ES complex.) Carbonic anhydrase catalyzes the hydration of CO_2 to form H_2CO_3. Operating at its maximum rate, 10 μg of pure carbonic anhydrase (M_r 30,000) in 1 mL hydrates 0.90 g of CO_2 in 1 minute. What is the turnover number for carbonic anhydrase?

3–83 Rous sarcoma virus (RSV) carries an oncogene called *Src*, which encodes a continuously active protein tyrosine kinase that leads to unchecked cell proliferation. Normally, Src carries an attached fatty acid (myristoylate) group that allows it to bind to the cytoplasmic side of the plasma membrane. A mutant version of Src that does not allow attachment of myristoylate does not bind to the membrane. Infection of cells with RSV encoding either the normal or the mutant form of Src leads to the same high level of protein tyrosine kinase activity, but the mutant Src does not cause cell proliferation.

A. Assuming that the normal Src is all bound to the plasma membrane and that the mutant Src is distributed throughout the cytoplasm, calculate their relative concentrations in the neighborhood of the plasma membrane. For the purposes of this calculation, assume that the cell is a sphere with a radius (r) of 10 μm and that the mutant Src is distributed throughout the cell, whereas the normal Src is confined to a 4-nm-thick layer immediately beneath the membrane. [For this problem, assume that the membrane has no thickness. The volume of a sphere is $(4/3)\pi r^3$.]
B. The target (X) for phosphorylation by Src resides in the membrane. Explain why the mutant Src does not cause cell proliferation.

DATA HANDLING

3–84 The binding of platelet-derived growth factor (PDGF) to the PDGF receptor stimulates phosphorylation of eight tyrosines in the receptor's cytoplasmic domain. The enzyme phosphatidylinositol 3′-kinase (PI 3-kinase) binds to one or more of the phosphotyrosines through its SH2 domains and is thereby activated. To identify the activating phosphotyrosines, you synthesize eight pentapeptides that contain the critical tyrosines (at the N-terminus) in their phosphorylated or unphosphorylated forms. You then mix an excess of each of the various pentapeptides with phosphorylated PDGF receptor and PI 3-kinase. Immunoprecipitation of the PDGF receptor will bring down any bound PI 3-kinase, which can be assayed by its ability to add ^{32}P-phosphate to its substrate (**Figure 3–22**).

A. Do your results support the notion that PI 3-kinase binds to phosphotyrosines in the activated PDGF receptor? Why or why not?

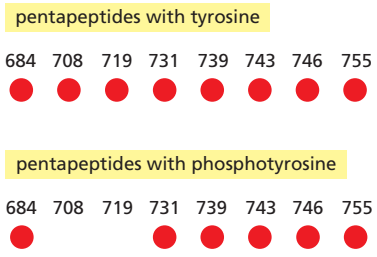


Figure 3–22 Assay for PI 3-kinase in immunoprecipitates of the PDGF receptor (Problem 3–84). Pentapeptides are indicated by numbers that refer to the position of tyrosine in the PDGF receptor. *Red circles* indicate incorporation of ^{32}P-phosphate into the substrate for PI 3-kinase.

B. The amino acid sequences of the PDGF receptor pentapeptides tested above (numbered according to the position of tyrosine in the PDGF receptor) and of peptide segments that are known to bind to PI 3-kinase in other activated receptors are shown below.

684	YSNAL	YMMMR	(FGF receptor)
708	YMDMS	YTHMN	(insulin receptor)
719	YVPML	YEVML	(hepatocyte growth factor receptor)
731	YADIE	YMDMK	(steel factor receptor)
739	YMAPY	YVEMR	(CSF-1 receptor)
743	YDNYE		
746	YEPSA		
755	YRATL		

What are the common features of peptide segments that form binding sites for PI 3-kinase?

C. Which of the three common types of protein–protein interaction—surface–string, helix–helix, or surface–surface—does the binding of PI 3-kinase with the PDGF receptor most likely illustrate?

3–85 A common method for determining the equilibrium for binding of a ligand (L) to a protein (Pr) is to use gel electrophoresis to separate the bound (Pr–L) and free forms of the ligand. By convention, the equilibrium is usually considered for the dissociation reaction (Pr–L $\rightarrow$ Pr + L), rather than the association reaction (Pr + L $\rightarrow$ Pr–L), and thus the equilibrium constant is referred to as the dissociation constant, K_d. This method was used to show that the protein, SmpB, binds specifically to a special species of tRNA (tmRNA) that is used in bacteria to eliminate the incomplete proteins made from truncated mRNAs. In this experiment, tmRNA was labeled and included in the binding reactions at a concentration of 0.1 nM (10^{-10} M). Purified SmpB protein was included at a range of concentrations and the mixture was incubated until the binding reaction was at equilibrium. Free and bound tmRNA were then separated by electrophoresis and made visible by autoradiography (**Figure 3–23**). (Note that this method requires that the off rate be slow enough that the complex will not dissociate during the time it takes to carry out the electrophoresis, usually a few hours.)

A. Consider the equation K_d = [Pr][L]/[Pr–L]. When the concentrations of bound and free ligand are equal, what is the relationship between the concentration of free protein and the K_d?

B. By visual inspection of Figure 3–23, estimate the K_d. Do you have to worry about the concentration of bound protein in this experiment? Why or why not?

C. The concentration of labeled tmRNA in these experiments was 100 pM. Would the results have been the same if tmRNA had been used at 100 nM? At 100 μM?

3–86 If the data in Figure 3–23 are plotted as fraction tmRNA bound versus SmpB concentration, one obtains a symmetrical S-shaped curve as shown in **Figure 3–24**. This curve is a visual display of a very useful relationship between K_d and concentration, which has broad applicability. The expression for fraction of ligand bound is derived from the equation

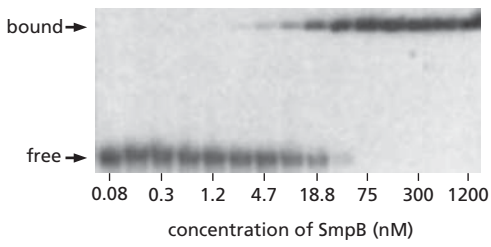


Figure 3–23 Assay of the binding of purified SmpB protein to ^{32}P-labeled tmRNA (Problem 3–85). From left to right across the gel, the experiment in each successive lane used a 2-fold increase in concentration of SmpB protein; concentrations in every other lane are indicated.

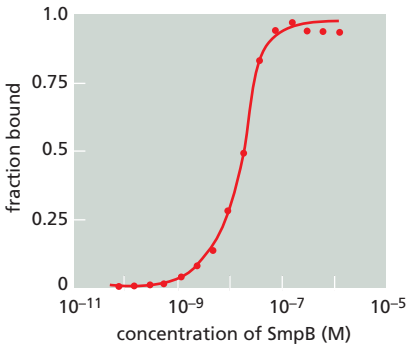


Figure 3–24 Fraction of tmRNA bound versus SmpB concentration (Problem 3–86).

for K_d by substituting ($[L]_{TOT}$ – [L]) for [Pr–L] and rearranging. Because the total concentration of ligand ($[L]_{TOT}$) is equal to the free ligand ([L]) plus bound ligand ([Pr–L]),

$$\text{fraction bound} = \frac{[\text{Pr–L}]}{[\text{L}]_{TOT}} = \frac{[\text{Pr}]}{[\text{Pr}] + K_d}$$

(An equivalent relationship in terms of the fraction of protein bound can be derived in an analogous way; it is $[\text{Pr–L}]/[\text{Pr}]_{TOT} = [\text{L}]/([\text{L}] + K_d)$.)

Using this relationship, calculate the fraction of ligand bound for protein concentrations expressed in terms of K_d, using Table 3–1.

TABLE 3–1 Fraction of ligand bound versus protein concentration (Problem 3–86).

Protein concentration	Fraction bound (%)
$10^4 K_d$	
$10^3 K_d$	
$10^2 K_d$	
$10^1 K_d$	
K_d	
$10^{-1} K_d$	
$10^{-2} K_d$	
$10^{-3} K_d$	
$10^{-4} K_d$	

3–87 The rates of production of product, P, from substrate, S, catalyzed by enzyme, E, were measured under conditions in which very little product was formed. The results are summarized in Table 3–2.

A. Why is it important to measure rates of product formation under conditions in which very little product is formed?

B. Plot these data as rate versus substrate concentration. Is this plot a rectangular hyperbola as expected for an enzyme that obeys Michaelis–Menten kinetics? What would you estimate as the K_m and V_{max} values for this enzyme?

C. To obtain more accurate values for the kinetic constants, the Lineweaver–Burk transformation of the Michaelis–Menten equation is often used so that the data can be plotted as a straight line.

Michaelis–Menten equation:

$$\text{rate} = \frac{V_{max}[\text{S}]}{[\text{S}] + K_m}$$

Lineweaver–Burk equation:

$$\frac{1}{\text{rate}} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[\text{S}]} + \frac{1}{V_{max}}$$

This equation has the form of a straight line, $y = ax + b$. Thus, when 1/rate (y) is plotted versus 1/[S] (x), the slope of the line equals K_m/V_{max} (a) and the y intercept is $1/V_{max}$ (b). Furthermore, it can be shown that the x intercept is equal to $-1/K_m$.

Plot 1/rate versus 1/[S] and determine the kinetic parameters K_m and V_{max}. (The values for 1/rate and 1/[S] are shown in Table 3–2.)

TABLE 3–2 Initial rates of product formation at various substrate concentrations (Problem 3–87).

Rate (μmol/min)	[S] (μM)
0.15	0.08
0.21	0.12
0.7	0.54
1.1	1.23
1.3	1.82
1.5	2.72
1.7	4.94
1.8	10.00
1/Rate (min/μmol)	**1/[S] (1/μM)**
6.7	12.5
4.8	8.3
1.4	1.9
0.91	0.81
0.77	0.55
0.67	0.37
0.59	0.20
0.56	0.10

3–88 Lysozyme achieves its antibacterial effect by cleaving the polysaccharide chains that form the bacterial cell wall. In the absence of this rigid mechanical support, the bacterial cell literally explodes due to its high internal osmotic pressure. The cell wall polysaccharide is made up of alternating sugars, *N*-acetylglucosamine (NAG) and *N*-acetylmuramate (NAM), linked together by glycosidic bonds (Figure 3–25). Lysozyme normally cleaves after NAM units in the chain (that is, between NAM and

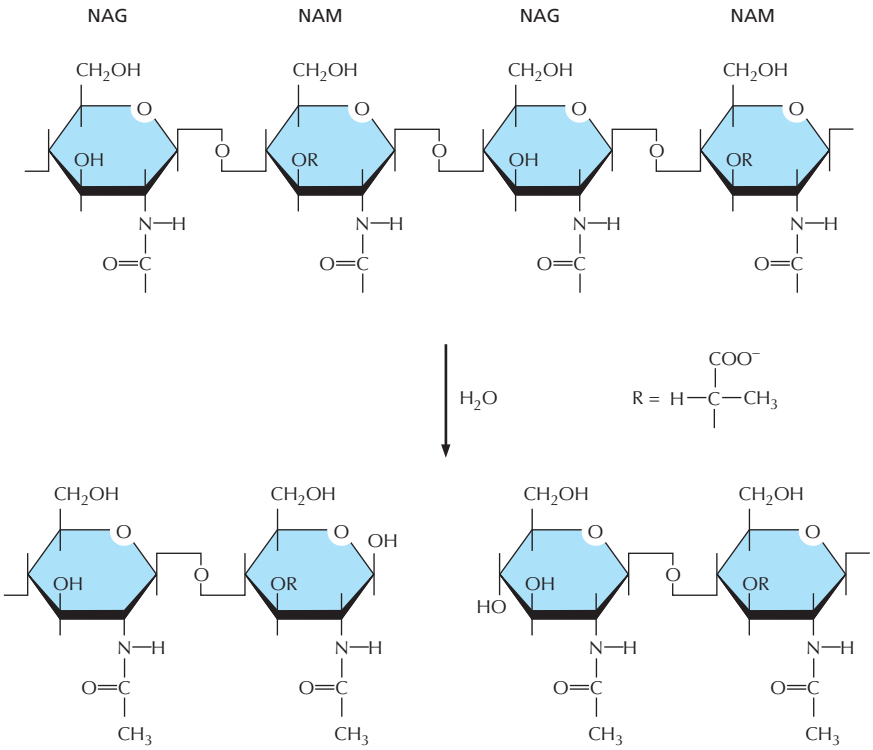


Figure 3–25 Arrangement of NAM and NAG in the bacterial cell-wall polysaccharide (Problem 3–88).

NAG), but will also cleave artificial substrates composed entirely of NAG units. When the crystal structure of lysozyme bound to a chain of three NAG units (tri-NAG) was solved, it was discovered that the binding cleft in lysozyme included six sugar-binding sites, A through F, and that tri-NAG filled the first three of these sites. From the crystal structure it was not apparent, however, which of the five bonds between the six sugars was the one that was normally cleaved. Tri-NAG is not cleaved by lysozyme, although longer NAG polymers are. It was clear from modeling studies that NAM is too large to fit into site C. Where are the catalytic groups responsible for cleavage located relative to the six sugar-binding sites?

A. Between sites A and B
B. Between sites B and C
C. Between sites C and D
D. Between sites D and E
E. Between sites E and F

3–89 Aspartate transcarbamoylase (ATCase) is an allosteric enzyme with six catalytic and six regulatory subunits. It exists in two conformations: one with low enzymatic activity and the other with high activity. In the absence of any ligands, the low-activity conformation predominates. Malate is an inhibitor of ATCase that binds in the active site at the position where the substrate aspartate normally binds. A very peculiar effect of malate is observed when the activity of ATCase is measured at low aspartate concentrations: there is an *increase* in ATCase activity at very low malate concentrations, but then the activity decreases at higher concentrations (**Figure 3–26**).

A. How is it that malate, a bona fide inhibitor, can increase ATCase activity under these conditions?
B. Would you expect malate to have the same peculiar effect if the measurements were made in the presence of a high concentration of aspartate? Why or why not?

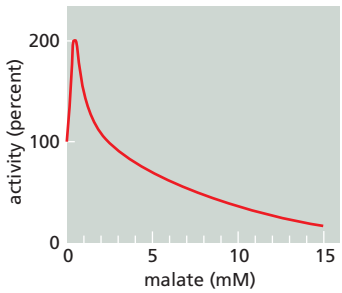


Figure 3–26 The activity of ATCase with increasing concentrations of the inhibitor malate (Problem 3–89). These measurements were made at an aspartate concentration well below its K_m.

TABLE 3–3 The observed dissociation constants (K_d values) of Cdk2 for ATP, ADP, cyclin A, and histone H1 (Problem 3–90).

	K_d (μM)			
	ATP	ADP	Cyclin A	Histone H1
Cdk2	0.25	1.4	0.05	not detected
P-Cdk2	0.12	6.7	0.05	100
Cdk2 + cyclin A				1.0
P-Cdk2 + cyclin A				0.7

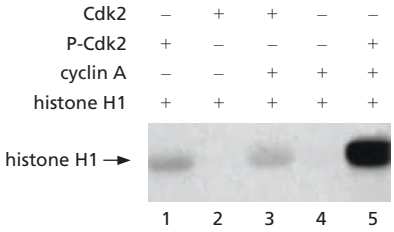


Figure 3–27 Phosphorylation of histone H1 by various combinations of Cdk2 and cyclin A (Problem 3–90). The amount of radioactive phosphate attached to histone H1 in lanes 1 and 3 is 0.3% and 0.2%, respectively, of that in lane 5.

3–90 Cyclin-dependent protein kinase 2 (Cdk2) regulates critical events in the progression of the cell cycle in mammalian cells. Cdk2 can form a complex with cyclin A and can be phosphorylated by another protein kinase to produce P-Cdk2. To determine the roles of cyclin A and phosphorylation in the function of Cdk2, you purify nonphosphorylated and phosphorylated Cdk2. You mix these two forms of Cdk2 and cyclin A in various combinations with ^{32}P-ATP and assay for phosphorylation of histone H1 (**Figure 3–27**). You also measure the binding affinity of various forms of Cdk2 for ATP, ADP, cyclin A, and histone H1 (**Table 3–3**).

A. From Figure 3–27, what is required for Cdk2 to phosphorylate histone H1 efficiently?

B. How do the requirements identified in part A specifically affect the function of Cdk2 relative to its target, histone H1 (Table 3–3 and Figure 3–27)?

C. The usual intracellular concentrations of ATP and ADP are in the range 0.1 to 1 mM. Assume that the binding of cyclin A to Cdk2 or P-Cdk2 does not alter the affinities of either form of Cdk2 for ATP and ADP. Is it likely that the observed changes in affinity for ATP and ADP are important for Cdk2 function? Why or why not?

3–91 β-Catenin is a target for phosphorylation by glycogen synthase kinase 3 and also a substrate for ubiquitylation, which triggers degradation in proteasomes. Treatment of mouse fibroblasts with a proteasome inhibitor, ALLN, increases the stability of β-catenin and causes the appearance of new, slower migrating forms of the protein on sodium dodecyl sulfate (SDS) polyacrylamide gels (**Figure 3–28**, lanes 1 and 2). Do these new bands represent phosphorylated proteins, which often run more slowly on such gels, or do they arise by addition of ubiquitin, which would increase their size? To test for phosphorylation, you treat samples with a protein phosphatase that efficiently removes phosphates from proteins and run them on gels (lanes 3 and 4). To test for ubiquitylation, you express in cells a modified form of ubiquitin that carries six histidine residues at its C-terminus, allowing its easy purification via Ni^{2+}-column chromatography. You treat these cells with ALLN (or not), and then purify His-ubiquitylated (His-Ub) proteins before running them on gels (lanes 5 and 6). In all cases, you detect β-catenin specifically, using antibodies directed against it.

Are the slower migrating forms of β-catenin due to phosphorylation or ubiquitylation? Explain your answer.

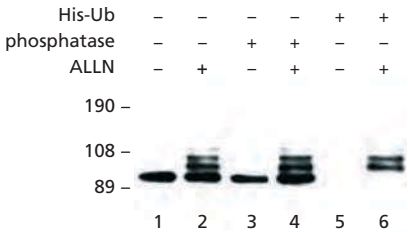


Figure 3–28 Electrophoretic analysis of β-catenin (Problem 3–91). β-Catenin was detected using β-catenin-specific antibodies. Size markers in kilodaltons are shown on the left.

3–92 Genome sequencing has revealed a surprisingly large number of protein tyrosine phosphatases (PTPs), very few of which have known roles in the life of a cell. PTP1B was the very first member of the PTP family to be discovered, and its three-dimensional structure and catalytic mechanism are well defined. PTP1B will dephosphorylate almost any phosphotyrosine-containing protein or peptide in the test tube, but in the cell it is

TABLE 3–4 Kinetic parameters of purified PTP1B mutants (Problem 3–92).

Enzyme	V_{max} [nmol/(min/mg)]	K_m (nM)	k_{cat} (min^{-1})
Wild type	60,200	102	2244
Tyr46 → Leu	4160	1700	155
Glu115 → Ala	5700	45	212
Lys120 → Ala	19,000	80	708
Asp181 → Ala	0.61	126	0.023
His214 → Ala	700	20	26
Cys215 → Ser	no activity		
Arg221 → Lys	11	80	0.41
Arg221 → Met	3.3	1060	0.12

likely to have better-defined targets. One strategy for identifying a binding target is to incubate the known protein with a cell lysate, and then isolate the known protein and identify any proteins that are bound to it. An enzyme such as PTP1B, however, binds and releases its target as part of its catalytic cycle. You reason that if you interfere with catalysis you might be able to increase the dwell time of the substrate, making it stable enough for isolation. To this end, you make several mutants of PTP1B by changing specific amino acids in the active site, and measure their kinetic parameters (**Table 3–4**).

You pick mutant Cys215 → Ser (C215S) for study because the –SH group of Cys215 initiates a nucleophilic attack on the phosphotyrosine, releasing the phosphate. You pick a second mutant—mutant 2—because it has promising kinetic properties. You use genetic engineering tricks to fuse these two mutants and the wild-type enzyme to glutathione *S*-transferase (GST), so that each of the three proteins can be rapidly purified by precipitation with glutathione-Sepharose. You express the GST-tagged proteins, precipitate them from cell lysates, separate the precipitated proteins by gel electrophoresis, and identify phosphotyrosine-containing proteins using anti-phosphotyrosine antibodies. As shown in **Figure 3–29**, GST-mutant 2 bound two phosphotyrosine-containing proteins (the two dark bands in lane 3), whereas GST-C215S bound none.

A. Which mutant PTP1B in Table 3–4 is likely to correspond to GST-mutant 2? Why do you think this protein gave a successful result?
B. Why might GST-C215S have failed to precipitate any phosphotyrosine-containing proteins?

MEDICAL LINKS

3–93 The Ras protein is a GTPase that functions in many growth-factor signaling pathways. In its active form, with GTP bound, it transmits a downstream signal that leads to cell proliferation; in its inactive form, with GDP bound, the signal is not transmitted. Mutations in the gene for Ras are found in many cancers. Of the choices below, which alteration of Ras activity is most likely to contribute to the uncontrolled growth of cancer cells?

A. A mutation that prevents Ras from being made.
B. A mutation that increases the affinity of Ras for GDP.

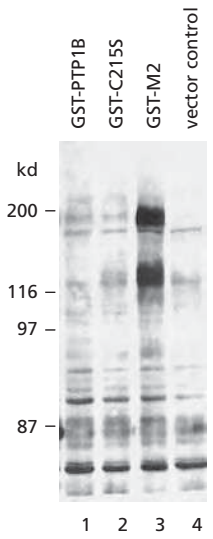


Figure 3–29 Phosphotyrosine-containing proteins precipitated with GST-tagged PTP1B enzymes (Problem 3–92). GST-PTP1B is the fused wild-type protein; GST-M2 is the fused mutant 2 protein. The positions of "marker" proteins of known molecular masses are indicated on the left.

C. A mutation that decreases the affinity of Ras for GTP.
D. A mutation that decreases the affinity of Ras for its downstream targets.
E. A mutation that decreases the rate of hydrolysis of GTP by Ras.

3–94 The activity of Ras is carefully regulated by two other proteins, a guanine nucleotide exchange factor (GEF) that stimulates the uptake of GTP by Ras, and a GTPase-activating protein (GAP) that stimulates the hydrolysis of GTP by Ras. The activities of these regulatory proteins are in turn also regulated. Which of the following changes in GAP and GEF proteins might cause a cell to proliferate excessively?

A. A nonfunctional GAP
B. A permanently active GAP
C. A nonfunctional GEF
D. A permanently active GEF

MCAT STYLE

Passage 1 (Questions 3–95 to 3–99)

E2F protein binds specific DNA sequences via its DNA-binding domain and activates transcription of genes that promote cell division. Another protein called Rb binds to E2F and inhibits it, thereby preventing cell division. When the cell receives a signal to initiate cell division, a protein kinase called Cdk binds Rb and phosphorylates it. Cdk binds to Rb via a binding site that is separate from the kinase catalytic site. Phosphorylation of Rb causes the Rb–E2F complex to dissociate, releasing E2F to activate transcription of cell-division genes. Rb is thus an inhibitor of cell division. Inactivation of Rb by mutation or deletion is an important event in the genesis of many kinds of cancer.

3–95 Which of the following best describes the defining features of a protein domain?

A. A discrete structural and functional unit within a protein
B. A group of proteins that share related functions
C. An evolutionarily conserved sequence of amino acids
D. The region of the cell in which a protein functions

3–96 Imagine a mutation that inactivates the site on Cdk that binds to Rb. What would be the effect of this mutation on the reaction in which Cdk phosphorylates Rb?

A. A large decrease in K_m
B. A large increase in K_m
C. A large decrease in V_{max}
D. A large increase in V_{max}

3–97 Which one of the following amino acids can be phosphorylated?

A. Alanine
B. Leucine
C. Lysine
D. Serine

3–98 Phosphorylation can affect the structure or function of a protein in which of the following ways?

I. Charge attraction between the phosphate group and positively charged amino acids causes a conformational change in the protein.
II. Charge repulsion between the phosphate group and negatively charged amino acids causes a conformational change in the protein.
III. The phosphate group creates a binding site for another protein.

A. I
B. III
C. I and II
D. I, II, and III

3–99 Which of the following hypotheses provides the most likely explanation for how phosphorylation of Rb causes its dissociation from E2F?

A. Phosphorylation of Rb causes E2F to change conformation so that it dissociates from Rb.
B. Phosphorylation of Rb causes it to undergo a conformational change that blocks the E2F binding site.
C. Phosphorylation of Rb inactivates the Rb binding site for Cdk.
D. Phosphorylation of Rb leads to a charge repulsion between the phosphate group and a positively charged pocket on E2F.

Passage 2 (Questions 3–100 to 3–103)

Steroid hormones such as estrogen, testosterone, and cortisol are small, diffusible signaling molecules that control diverse aspects of cell physiology. Steroid hormones signal to cells via receptor proteins. Typically, a steroid hormone diffuses across the cell membrane and binds to a receptor protein in the cytoplasm. Binding of the steroid hormone to the receptor protein causes the receptor to bind to transcription factor proteins that control transcription. In this manner, steroid hormones activate or repress genes that control cellular physiology.

3–100 Which of the following best describes how a steroid hormone might cause the receptor to bind to proteins that control transcription?

A. The steroid hormone acts catalytically to promote the binding reaction between the receptor and the transcription factor.
B. The steroid hormone decreases the K_m of the binding reaction between the receptor and the transcription factor.
C. The steroid hormone induces the receptor to undergo a conformational change that exposes a binding site for the transcription factor.
D. The steroid hormone promotes positive feedback to ensure that more and more receptor binds to the transcription factor.

3–101 What kinds of interaction are likely to be responsible for binding of the receptor protein to the transcription factor protein?

I. Hydrophobic forces
II. Noncovalent bonds
III. Covalent bonds

A. I
B. III
C. I and II
D. I, II, and III

3–102 All of the following are correct statements about common features of binding interactions between proteins EXCEPT:

A. At equilibrium, proteins often undergo frequent association and dissociation.
B. Binding reactions usually require an input of energy from ATP hydrolysis.
C. Some proteins, once they have bound one another, remain tightly associated.
D. The rate of binding does not usually depend on the strength of the interaction.

3–103 Which one of the following approaches would provide information about the strength of the interaction between the steroid hormone and its receptor?

A. Measure the equilibrium constant
B. Measure the rate of association
C. Measure the rate of dissociation
D. Measure the V_{max} for the binding reaction

DNA, Chromosomes, and Genomes

CHAPTER 4

THE STRUCTURE AND FUNCTION OF DNA

TERMS TO LEARN

antiparallel	deoxyribonucleic acid (DNA)	genome
base pair	double helix	template
complementary	gene	

DEFINITIONS

Match each definition below with its term from the list above.

4–1 The totality of the genetic information carried in the DNA of a cell or an organism.

4–2 The three-dimensional structure of DNA, in which two DNA chains held together by hydrogen bonds between the bases are coiled around one another.

4–3 Information-containing element that controls a discrete hereditary characteristic.

4–4 Describes the relative orientation of the two strands in a DNA helix; the polarity of one strand is oriented in the opposite direction to that of the other.

4–5 Two nucleotides in an RNA or DNA molecule that are held together by hydrogen bonds.

TRUE/FALSE

Decide whether this statement is true or false, and then explain why.

4–6 Human cells do not contain any circular DNA molecules.

THOUGHT PROBLEMS

4–7 The start of the coding region for the human β-globin gene reads 5′-ATGGTGCAC-3′. What is the sequence of the complementary strand for this segment of DNA?

4–8 Upon returning from a recent trip abroad, you explain to the customs agent that you are bringing in a sample of DNA, deoxyribonucleic acid. He is aghast that you want to bring an acid into his country. What is the acid in DNA? Should the customs agent be wary?

4–9 The chemical structures for an A-T and a G-C base pair are shown in **Figure 4–1**, along with their points of attachment to the sugar-phosphate backbones.

IN THIS CHAPTER

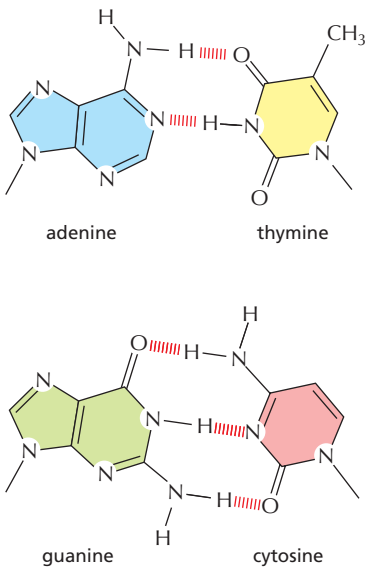


Figure 4–1 An A-T and a G-C base pair, as viewed along the helix axis (Problem 4–9). Each base is attached to its deoxyribose sugar via the line extending from the ring nitrogen.

A. Indicate the positions of the major and minor grooves of the DNA helix on these representations.
B. Draw the structure of a T-A base pair in the same way as in Figure 4–1.
C. Do the same chemical moieties (for example, the methyl group of thymine) always project into the same groove?

4–10 Examine the space-filling models of the base pairs shown in **Figure 4–2**. Each base pair includes two bases, two deoxyribose sugars, and two phosphates. Can you identify the locations of the purine base, the pyrimidine base, the sugars, and the phosphates? Identify each base pair as C-G, G-C, T-A, or A-T.

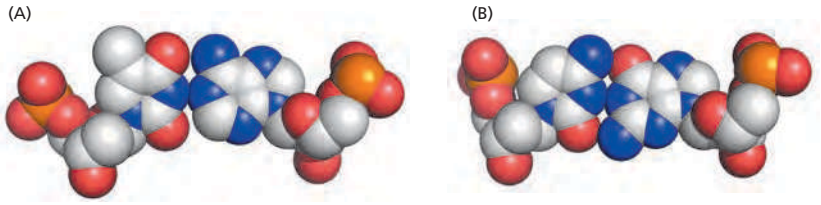


Figure 4–2 Space-filling models of two base pairs (Problem 4–10). Carbon and phosphorus atoms are *gray* and *orange*, respectively, nitrogen atoms are *blue*, and oxygen atoms are *red*. No hydrogen atoms are shown.

4–11 DNA isolated from the bacterial virus M13 contains 25% A, 33% T, 22% C, and 20% G. Do these results strike you as peculiar? Why or why not? How might you explain these values?

4–12 A segment of DNA from the interior of a single strand is shown in **Figure 4–3**. What is the polarity of this DNA from top to bottom?

4–13 DNA forms a right-handed helix. Pick out the right-handed helix from those shown in **Figure 4–4**.

CALCULATIONS

4–14 Human DNA contains 20% C on a molar basis. What are the mole percents of A, G, and T?

4–15 The diploid human genome comprises 6.4×10^9 bp and fits into a nucleus that is 6 μm in diameter.
A. If base pairs occur at intervals of 0.34 nm along the DNA helix, what is the length of DNA in a human cell?
B. If the diameter of the DNA helix is 2.4 nm, what fraction of the volume of the nucleus is occupied by DNA? [Volume of a sphere is $(4/3)\pi r^3$ and volume of a cylinder is $\pi r^2 h$.]

4–16 One gram of cultured human cells contains about 10^9 cells and occupies roughly 1 mL. If the average molecular mass of a base pair is 660 daltons and each cell contains 6.4×10^9 bp, what mass of DNA is present in this one-gram sample? If all the DNA molecules in the sample were laid end-to-end to form a single thread, do you suppose it would be long enough to reach from the Earth to the Moon (385,000 kilometers)?

DATA HANDLING

4–17 Bacteriophage T4 attaches to its bacterial host and injects its DNA to initiate an infection that ultimately releases hundreds of progeny virus. In 1952, Alfred Hershey and Martha Chase radiolabeled the DNA of bacteriophage T4 with $^{32}PO_4^{3-}$ and the proteins with ^{35}S-methionine. They then mixed the labeled bacteriophage with bacteria and after a brief time agitated the mixture vigorously in a blender to detach T4 from the bacteria. They then separated the phage from the bacteria by centrifugation. They demonstrated that the bacteria contained 30% of the ^{32}P label but

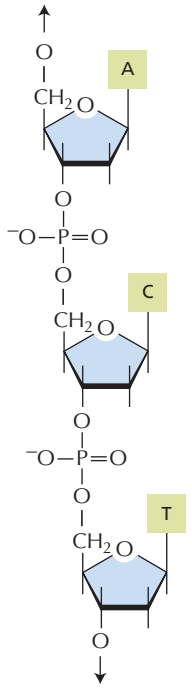


Figure 4–3 Three nucleotides from the interior of a single strand of DNA (Problem 4–12). *Arrows* at the ends of the DNA strand indicate that the structure continues in both directions.

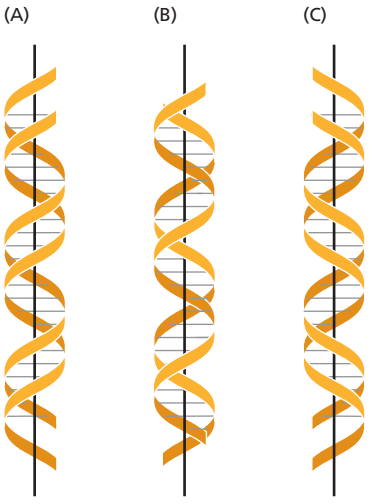


Figure 4–4 Three "DNA" helices (Problem 4–13).

virtually none of the ^{35}S label. When new bacteriophage were released from these bacteria, they were also found to contain ^{32}P but no ^{35}S. How does this experiment demonstrate that DNA rather than protein is the genetic material? (Note that bacteriophage T4 contains only protein and DNA.)

CHROMOSOMAL DNA AND ITS PACKAGING IN THE CHROMATIN FIBER

TERMS TO LEARN

cell cycle	histone	karyotype
centromere	histone H1	nucleosome
chromatin	homologous chromosome (homolog)	replication origin
chromosome	intron	telomere
exon		

DEFINITIONS

Match each definition below with its term from the list above.

4–18 Full set of chromosomes of a cell arranged with respect to size, shape, and number.

4–19 Constricted region of a mitotic chromosome that holds sister chromatids together.

4–20 Any one of a group of small abundant proteins, rich in arginine and lysine, that form the primary level of chromatin organization.

4–21 Structure composed of a very long DNA molecule and associated proteins that carries part (or all) of the hereditary information of an organism.

4–22 The orderly sequence of events by which a cell duplicates its contents and divides into two.

4–23 Complex of DNA, histones, and non-histone proteins found in the nucleus of a eukaryotic cell.

4–24 One of the two copies of a particular chromosome in a diploid cell, each copy being derived from a different parent.

4–25 Beadlike structure in eukaryotic chromatin, composed of a short length of DNA wrapped around a core of histone proteins.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

4–26 Human females have 23 different chromosomes, whereas human males have 24.

4–27 In the living cell, chromatin usually adopts the extended "beads-on-a-string" form.

4–28 The four core histones are relatively small proteins with a very high proportion of positively charged amino acids; the positive charge helps the histones bind tightly to DNA, regardless of its nucleotide sequence.

4–29 Nucleosomes bind DNA so tightly that they cannot move from the positions where they are first assembled.

THOUGHT PROBLEMS

4–30 In the 1950s, the techniques for isolating DNA from cells all yielded molecules of about 10,000 to 20,000 base pairs. We now know that the DNA

molecules in all cells are very much longer. Why do you suppose such short pieces were originally isolated?

4–31 Consider the following statement: a human cell contains 46 molecules of DNA in its nucleus. Do you agree with it? Why or why not?

4–32 Chromosome 3 in orangutans differs from chromosome 3 in humans by two inversion events that occurred in the human lineage (**Figure 4–5**). Draw the intermediate chromosome that resulted from the first inversion and explicitly indicate the segments included in each inversion.

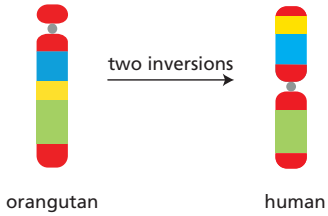


Figure 4–5 Chromosome 3 in orangutans and humans (Problem 4–32). Differently *colored blocks* indicate segments of the chromosomes that are homologous in DNA sequence.

4–33 Define a "gene."

4–34 List the three specialized DNA sequences and their functions that act to ensure that the number and morphology of chromosomes are constant from one generation of a cell to the next.

4–35 Histone proteins are among the most highly conserved proteins in eukaryotes. Histone H4 proteins from a pea and a cow, for example, differ in only 2 of 102 amino acids. However, comparison of the two *gene* sequences shows many more differences. These observations indicate that mutations that change amino acids must be selected against. Why do you suppose that most amino acid-altering mutations in histone genes are deleterious?

4–36 Duplex DNA composed entirely of CTG/CAG trinucleotide repeats (5′-CTG in one strand and 5′-CAG in the other strand) is unusually flexible. If 75 CTG/CAG repeats are incorporated into a much longer DNA molecule and mixed with histone octamers, the first nucleosome that assembles nearly always includes the CTG/CAG repeat region. Can you suggest a reason why CTG/CAG repeats might be such effective elements for positioning nucleosomes? (Consider what the energy of the binding interaction between the histone octamers and the DNA must accomplish.)

CALCULATIONS

4–37 At mitosis, human chromosome 1 is condensed to a form that measures only 10 μm in length.

A. Given that this chromosome contains 2.8×10^8 bp, how compacted is the DNA molecule in chromosome 1 at mitosis relative to its fully extended length as naked DNA? (Recall that a DNA base pair is 0.34 nm in length.)

B. A single nucleosome core particle is 11 nm long and contains 147 bp of DNA (0.34 nm/bp). What packing ratio (DNA length to nucleosome length) has been achieved by wrapping DNA around the histone octamer? What fraction of the condensation that occurs at mitosis does this first level of packing represent?

C. Assuming that the 30-nm chromatin fiber contains about 20 nucleosomes (200 bp/nucleosome) per 50 nm of length, calculate the degree of compaction of DNA associated with this level of chromatin structure. What fraction of the condensation that occurs at mitosis does this level of DNA packing represent?

4–38 The total number of protein-coding genes in the human genome can be calculated in several ways. It is important to remember that all such numbers are estimates at present, because it is difficult to identify a gene just from the DNA sequence. Chromosome 22 has about 700 genes in 48 Mb of sequence, which represents 1.5% of the 3200 Mb in the haploid genome. Using these numbers, how many genes would you estimate for the haploid human genome? If your estimate is significantly larger or smaller than the accepted value of approximately 21,000 protein-coding genes, suggest possible explanations for the discrepancy.

4–39 The 700 genes on chromosome 22 (48 Mb) average 19,000 bp in length and contain an average of 5.4 exons, each of which averages 266 bp. On average, what fraction of each gene sequence is converted to mRNA? What fraction of the whole chromosome do genes occupy?

4–40 Assuming that the histone octamer forms a cylinder 9 nm in diameter and 5 nm in height and that the human genome forms 32 million nucleosomes, what volume of the nucleus (6 μm in diameter) is occupied by histone octamers? [Volume of a cylinder is $\pi r^2 h$; volume of a sphere is $(4/3)\pi r^3$.] What fraction of the nuclear volume do the DNA and the histone octamers occupy (see Problem 4–15)?

DATA HANDLING

4–41 One way to demonstrate that a chromosome contains a single DNA molecule is to use a technique called pulsed-field gel electrophoresis, which can separate DNA molecules up to 10^7 bp in length. Ordinary gel electrophoresis cannot separate such long molecules because the steady electric field stretches them out so they travel end-first through the gel matrix at a rate that is independent of their length. If the electric field is changed periodically, however, the DNA molecules are forced to re-orient to the new field before continuing their snakelike movement through the gel. The time for re-orientation depends on length, so that longer molecules move more slowly through the gel.

The results of pulsed-field gel electrophoresis of the DNA from the yeast *Saccharomyces cerevisiae* are shown in **Figure 4–6**. How many chromosomes does *S. cerevisiae* have?

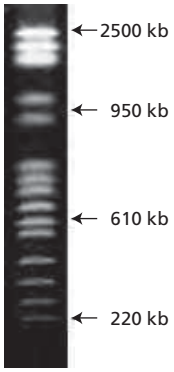


Figure 4–6 Pulsed-field gel electrophoresis of *S. cerevisiae* chromosomes (Problem 4–41). To minimize the handling of DNA, which would surely break it, the cells themselves are placed at the top of the gel and gently opened by addition of a lysis buffer. The DNA molecules in this gel have been exposed to the dye ethidium bromide, which fluoresces under ultraviolet light when it is bound to DNA. This treatment allows the DNA—otherwise invisible—to be seen.

4–42 A classic experiment attached telomeres from *Tetrahymena* to a linearized yeast plasmid, allowing the plasmid to grow as a linear molecule—that is, as an artificial chromosome (**Figure 4–7**). A circular 9-kb plasmid was constructed to contain a yeast origin of replication (*Ars1*) and the yeast *Leu2* gene. Cells that are missing the chromosomal *Leu2* gene, but have taken up the plasmid, can grow in medium lacking leucine. The plasmid was linearized with BglII, which cuts once (Figure 4–7), and then mixed with 1.5-kb *Tetrahymena* telomere fragments generated by cleavage with BamHI (Figure 4–7). The mixture was incubated with DNA ligase and the two restriction nucleases, BglII and BamHI. The ligation products included molecules of 10.5 kb and 12 kb in addition to the original components. The 12-kb band was purified and transformed into yeast, which were then selected for growth in the absence of leucine. Samples of DNA from one transformant were digested with HpaI, PvuII, or PvuI and the fragments were separated by gel electrophoresis and visualized after hybridization to a plasmid-specific probe (**Figure 4–8**).

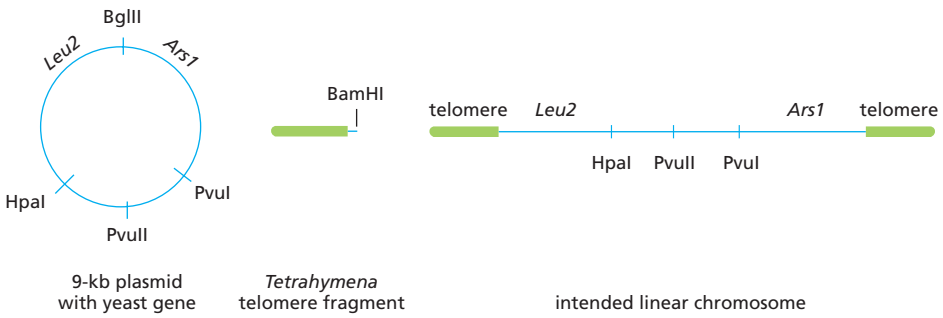


Figure 4–7 Structure of 9-kb plasmid, telomere fragment, and the intended linear chromosome with *Tetrahymena* telomeres (Problem 4–42). The sites of unique cutting by restriction enzymes are indicated.

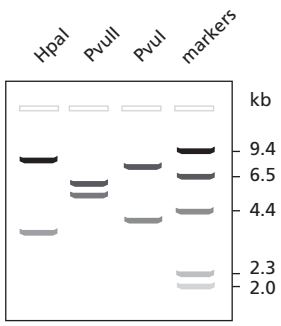


Figure 4–8 Autoradiograph of restriction analysis of plasmid structure (Problem 4–42). Marker DNAs of known sizes are shown on the *right*.

A. How do the results of the analysis in Figure 4–8 distinguish between the intended linear form of the plasmid in the transformed yeast and the more standard circular form?

B. Explain how ligation of the DNA fragments in the presence of the restriction nucleases BamHI and BglII ensures that you get predominantly the linear construct you want. The recognition site for BglII is 5′-A*GATCT-3′ and for BamHI is 5′-G*GATCC-3′, where the asterisk (*) is the site of cutting.

4–43 Nucleosomes can be assembled onto defined DNA segments. When a particular 225-bp segment of human DNA was used to assemble nucleosomes and then incubated with micrococcal nuclease, which digests DNA that is not located within the nucleosome, uniform fragments 147 bp in length were generated. Subsequent digestion of these fragments with a restriction enzyme that cuts once within the original 225-bp sequence produced two well-defined bands at 37 bp and 110 bp. Why do you suppose two well-defined fragments were generated by restriction digestion, rather than a range of fragments of different sizes? How would you interpret this result?

4–44 You have been sent the first samples of a newly discovered Martian microorganism for analysis of its chromatin. The cells resemble Earthly eukaryotes and are composed of similar molecules, including DNA, which is located within a nucleus-like structure in the cell. One member of your team has identified two basic histone-like proteins associated with the DNA in roughly an equal mass ratio with the DNA. You isolate nuclei from the cells and treat them with micrococcal nuclease for various times. You then extract the DNA and run it on an agarose gel alongside DNA from rat-liver nuclei that had been briefly digested with micrococcal nuclease. As shown in **Figure 4–9**, the digest of rat-liver nuclei gives a standard ladder of nucleosomes, but the digest of the Martian organism gives a smear of products with a nuclease-resistant limit of about 300 nucleotides. As a control, you isolate the Martian DNA free of all protein and digest it with micrococcal nuclease: it is completely susceptible, giving predominantly mono- and dinucleotides.

Do these results suggest that the Martian organism has nucleosome-like structures in its chromatin? If so, what can you deduce about their spacing along the DNA?

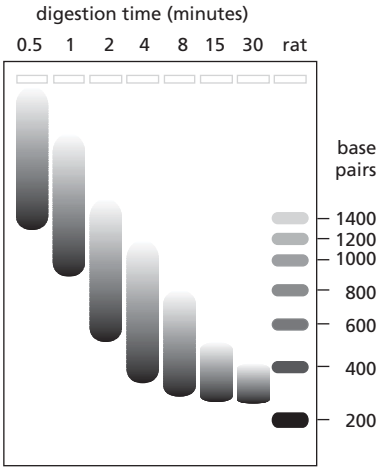


Figure 4–9 Micrococcal digest of chromatin from a Martian organism (Problem 4–44). The results of digestion of rat-liver chromatin are shown on the *right.*

4–45 Moving nucleosomes out of the way is important for turning genes on. In yeast the SWI/SNF complex, which is the founding member of the ATP-dependent chromatin remodeling complexes, is required for both activating and repressing gene transcription. How does it work? In principle, it could slide nucleosomes along the chromosome, knock them off the DNA, or transfer them from one duplex to another.

To investigate this problem, you assemble a nucleosome on a 189-bp segment of labeled DNA, which you then ligate to a longer piece of DNA tethered to a magnetic bead, as shown in **Figure 4–10A**. You incubate this substrate with the SWI/SNF complex either before or after you cut the DNA with NheI, which cleaves near the nucleosome, or with EcoRI, which cleaves near the bead. You use a magnet to separate the DNA that is still attached to the bead from the released DNA. If the nucleosome is present, the released DNA will run with a slower mobility on an agarose gel than if it is absent. Incubation with the SWI/SNF complex in the presence of ATP, followed by NheI digestion (NheI 2nd), releases most of the label as the free DNA fragment (**Figure 4–10B**, lane 6). By contrast, incubation with SWI/SNF after cleavage with NheI (NheI 1st) releases most of the label as nucleosome-bound DNA (lane 4). Similar experiments with EcoRI digestion showed that incubation with SWI/SNF released most of the label as nucleosome-bound DNA regardless of whether EcoRI cleavage preceded or followed the incubation.

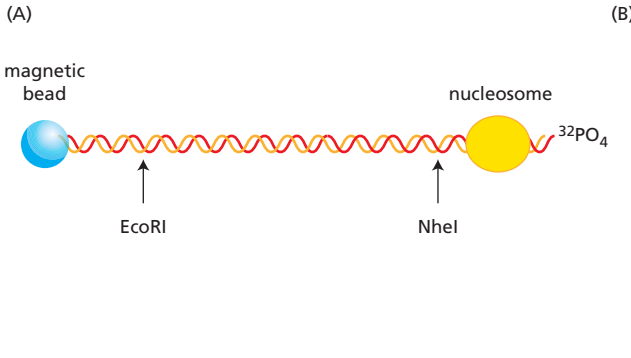


Figure 4–10 The action of the SWI/SNF complex on a nucleosome (Problem 4–45). (A) Nucleosome-containing substrate tethered to a magnetic bead. Sites of cleavage by the restriction enzymes NheI and EcoRI are indicated. (B) Results of incubation with the SWI/SNF complex before (NheI 2nd) or after (NheI 1st) cleavage with NheI. In the absence of cleavage (lane 1), the labeled substrate does not enter the gel.

Do your results distinguish among the three possible mechanisms for SWI/SNF action—nucleosome sliding, release, or transfer? Explain how your results argue for or against each of these mechanisms.

MEDICAL LINKS

4–46 An abnormal human karyotype is shown in **Figure 4–11**. This particular karyotype is found in the cancer cells of more than 90% of patients with chronic myelogenous leukemia. Arrows indicate two abnormal chromosomes. Describe the event that led to this abnormal karyotype. Is this patient male or female?

CHROMATIN STRUCTURE AND FUNCTION

TERMS TO LEARN

epigenetic inheritance
euchromatin
heterochromatin
position effect variegation

DEFINITIONS

Match each definition below with its term from the list above.

4–47 Less condensed region of an interphase chromosome that stains diffusely.

4–48 Form of transmission of information from cell to cell, or from parent to progeny, that is not encoded in DNA.

4–49 Difference in gene expression that depends on the location of the gene on the chromosome.

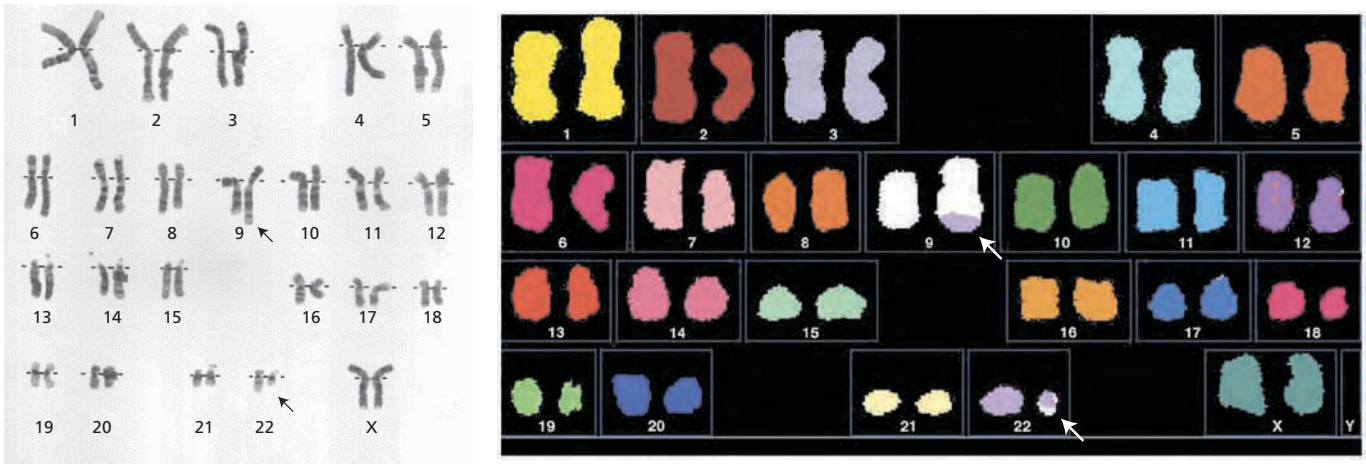


Figure 4–11 Two karyotypes illustrating the typical alteration seen in chronic myelogenous leukemia (Problem 4–46).

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

4–50 Deacetylation of histone tails allows nucleosomes to pack together into tighter arrays, which usually reduces gene expression.

4–51 Histone variants are often inserted into already formed chromatin.

THOUGHT PROBLEMS

4–52 Phosphorylation of serines and methylation and acetylation of lysines in histone tails affect the stability of chromatin structure above the nucleosome level and have important consequences for gene expression. Draw the structures for serine and phosphoserine, as well as those for lysine, monomethylated lysine, and acetylated lysine. Which modifications alter the net charge on a histone tail? Would you expect the changes in charge to increase or decrease the ability of the tails to interact with DNA?

4–53 In contrast to histone acetylation, which always correlates with gene activation, histone methylation can lead to either transcriptional activation or repression. How do you suppose that the same modification—methylation—can mediate different biological outcomes?

4–54 Why is a chromosome with two centromeres (a dicentric chromosome) said to be unstable? Wouldn't a back-up centromere be a good thing for a chromosome, giving it two chances to form a kinetochore and attach to microtubules during mitosis? Wouldn't that help to ensure that the chromosome didn't get left behind at mitosis—sort of like using a belt and braces to keep your pants up?

DATA HANDLING

4–55 The first paper to demonstrate different chromatin structures in active and inactive genes used nucleases to probe the globin loci in chicken red blood cells, which express globin mRNA, and in chicken fibroblasts, which do not. Isolated nuclei from these cells were treated with either micrococcal nuclease or DNase I, and then DNA was prepared and hybridized in vast excess to a ^{3}H-thymidine-labeled globin cDNA. (A cDNA is a DNA molecule made as a copy of mRNA.) If the nuclear DNA has not been degraded, it will hybridize to the globin cDNA and protect it from digestion by S1 nuclease, which is specific for single strands of DNA.

Digestion of red-cell nuclei or fibroblast nuclei with micrococcal nuclease (so that about 50% of the DNA was degraded) yielded DNA samples that still protected greater than 90% of the cDNA from subsequent digestion with S1 nuclease. Similarly, digestion of fibroblast nuclei with DNase I (so that less than 20% was degraded) yielded DNA that protected greater than 90% of the cDNA. An identical digestion of red-cell nuclei with DNase I, however, yielded DNA that protected only about 25% of the cDNA. These results are summarized in **Table 4–1**.

When nucleosome monomers were isolated from red blood cells by digestion with micrococcal nuclease, their DNA protected more than 90% of globin cDNA. If the monomers were first treated with DNase I, the isolated DNA protected only 25% of globin cDNA. If the monomers were briefly treated with trypsin to remove 20–30 amino acids from the N-terminus of each histone, digestion of the modified nucleosomes with *micrococcal nuclease* yielded DNA that protected only 25% of globin cDNA (Table 4–1).

A. Which nuclease—micrococcal nuclease or DNase I—digests chromatin that is being expressed (active chromatin)? How can you tell?

TABLE 4–1 Protection of globin cDNA by untreated and nuclease-treated chromatin samples (Problem 4–55).

Source of DNA	Protected globin cDNA after treatment		
	none	micrococcal	DNase I
Fibroblast nuclei	93%	91%	91%
Red-cell nuclei	95%	92%	25%
Red-cell nucleosomes		91%	25%
Red-cell nucleosomes (trypsin)		25%	

B. Does trypsin treatment of nucleosome monomers appear to render a random population or a specific population of nucleosomes sensitive to micrococcal nuclease? How can you tell?

C. Is the alteration that distinguishes active chromatin from bulk chromatin a property of individual nucleosomes, or is it related to the way nucleosome monomers are packaged into higher-order structures within the cell nucleus?

4–56 HP1 proteins, a family of proteins found in heterochromatin, are implicated in gene silencing and chromatin structure. The three proteins in humans—HP1α, HP1β, and HP1γ—share a highly conserved domain that directs their localization to chromatin. To determine whether these proteins could bind to the histone H3 N-terminus, you have covalently attached to separate beads various versions of the H3 N-terminal peptide—unmodified, Lys9-dimethylated (K9-Me), and Ser10-phosphorylated (S10-P)—along with an unmodified tail from histone H4. This arrangement allows you to incubate the beads with various proteins, wash away unbound proteins, and then elute bound proteins for assay by Western blotting. The results of your "pull-down" assay for the HP1 proteins are shown in **Figure 4–12**, along with the results from several control proteins, including Pax5, which is a gene regulatory protein, polycomb protein Pc1, which is known to bind to histones, and Suv39h1, a histone methyl transferase.

Based on these results, which of the proteins tested bind to the unmodified tails of histones? Do any of the HP1 proteins and control proteins selectively bind to any of the various histone N-terminal peptides? What histone modification would you predict would be found in heterochromatin?

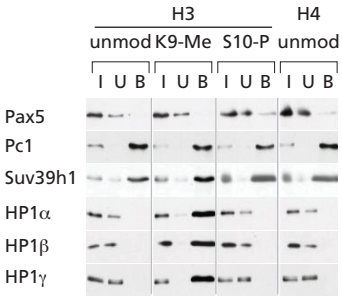


Figure 4–12 Pull-down assays to determine binding specificity of HP1 proteins (Problem 4–56). Each protein at the *left* was detected by immunoblotting using a specific antibody after separation by SDS polyacrylamide-gel electrophoresis. For each histone N-terminal peptide, the total input protein (I), the unbound protein (U), and the bound protein (B) are indicated.

4–57 Look at the two yeast colonies in **Figure 4–13**. Each of these colonies contains about 100,000 cells descended from a single yeast cell, originally

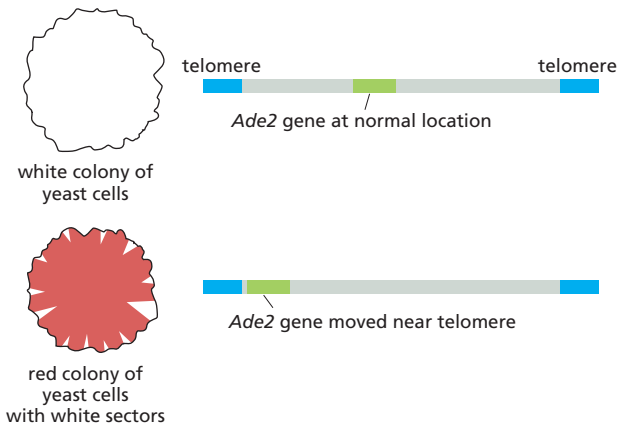


Figure 4–13 Position effect on expression of the yeast *Ade2* gene (Problem 4–57). The *Ade2* gene codes for one of the enzymes of adenosine biosynthesis, and the absence of the *Ade2* gene product leads to the accumulation of a red pigment. Therefore a colony of cells that express *Ade2* is *white,* and one composed of cells in which the *Ade2* gene is not expressed is *red.*

somewhere in the middle of the clump. A white colony arises when the *Ade2* gene is expressed from its normal chromosomal location. When the *Ade2* gene is moved to a location near a telomere, it is packed into heterochromatin and inactivated in most cells, giving rise to colonies that are mostly red. In these largely red colonies, white sectors fan out from the middle of the colony. In both the red and white sectors, the *Ade2* gene is still located near telomeres. Explain why white sectors have formed near the rim of the red colony. Based on the patterns observed, what can you conclude about the propagation of the transcriptional state of the *Ade2* gene from mother to daughter cells in this experiment?

4–58 High-density DNA microarrays can be used to analyze changes in expression of all the genes in the yeast genome in response to various perturbations. The effects of depletion of histone H4 and deletion of the *Sir3* gene on expression of all yeast genes were analyzed in this way, as summarized on the yeast chromosomes illustrated in **Figure 4–14**. Depletion of histone H4 was achieved in a strain in which the gene was engineered to respond to galactose. In the absence of galactose, the gene is turned off and depletion of histone H4 is evident within 6 hours, leading to a decreased density of nucleosomes throughout the genome. Deletion of *Sir3* removes a critical component of the Sir protein complex that binds to telomeres and is responsible for deacetylation of telomeric nucleosomes.

A. Depletion of histone H4 significantly increased expression of 15% of all yeast genes (Figure 4–14A, black bars). Does loss of histone H4 increase expression of a greater fraction of genes near telomeres than in the rest of the genome? Explain how you arrived at your conclusion.

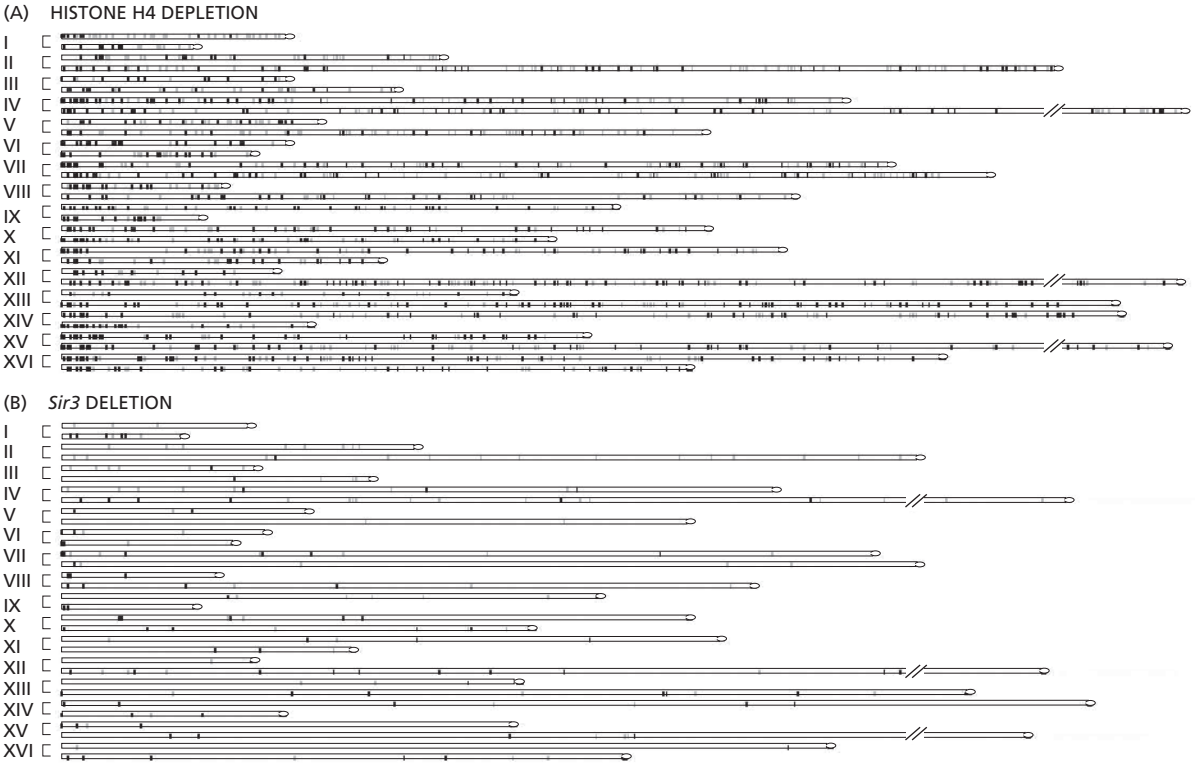


Figure 4–14 Changes in expression of the genes on chromosomes I–XVI of yeast (Problem 4–58). (A) In response to depletion of histone H4. (B) In response to deletion of *Sir3*. *Black bars* indicate genes whose expression was increased relative to wild-type yeast threefold or more. The very light *gray bars* show genes whose expression was decreased by threefold or more; they are not relevant to this problem but have been added for the sake of completeness. Chromosomes were split at their centromeres so that all their telomeres could be aligned at the left; *brackets* indicate the pairs of arms that make up individual chromosomes. Three chromosome arms have been shortened to fit into the figure, as indicated by diagonal lines.

B. Deletion of the *Sir3* gene significantly increased expression of 1.5% of all yeast genes (Figure Figure 4–14B, black bars). Does the absence of Sir3 protein increase expression of a greater fraction of genes near telomeres than in the rest of the genome? Explain how you arrived at your conclusion.

C. If you concluded that either the depletion of histone H4 or the deletion of the *Sir3* gene, or both, preferentially increased expression of genes near telomeres, propose a mechanism for how that might happen.

4–59 A classic paper examined the arrangement of nucleosomes around the centromere (CEN3) of yeast chromosome III. Because centromeres are the chromosome attachment sites for microtubules, it was unclear whether they would have the usual arrangement of nucleosomes. This study used plasmids into which were cloned various lengths of the native chromosomal DNA around the centromere (**Figure 4–15**). Chromatin from native yeasts and from yeasts that carried individual plasmids was treated briefly with micrococcal nuclease, and then the DNA was deproteinized and digested with the restriction enzyme BamHI, which cuts the DNA only once (Figure 4–15). The digested DNA was fractionated by gel electrophoresis and analyzed by Southern blotting using a segment of radiolabeled centromeric DNA as a hybridization probe (Figure 4–15). This procedure (called indirect end labeling) allows visualization of all the DNA fragments that include the DNA immediately to the right of the BamHI cleavage site. As a control, a sample of naked DNA from the same region was treated with micrococcal nuclease and subjected to the same analysis. An autoradiogram of the results is shown in **Figure 4–16**.

A. When the digestion with BamHI was omitted, a regular, though much less distinct, set of dark bands was apparent. Why does digestion with BamHI make the pattern so much clearer and easier to interpret?

B. Draw a diagram showing the micrococcal-nuclease-sensitive sites on the chromosomal DNA and the arrangement of nucleosomes along the chromosome. What is special about the centromeric region?

C. What is the purpose of including a naked DNA control in the experiment?

D. The autoradiogram in Figure 4–16 shows that the native chromosomal DNA yields a regularly spaced pattern of bands beyond the centromere; that is, the bands at 600 nucleotides and above are spaced at 160-nucleotide intervals. Does this regularity result from the lining up of nucleosomes at the centromere, like cars at a stoplight? Or, is the regularity an intrinsic property of the DNA sequence itself? Explain how the results with plasmids 1, 2, and 3 decide the issue.

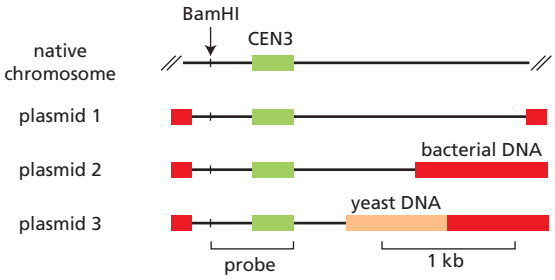


Figure 4–15 Diagrams of the structures of the native chromosome and three plasmids (Problem 4–59). The native chromosome is linear; its true ends extend well beyond the positions marked by the diagonal lines. The plasmids, which are circular, are shown here as linear molecules for ease of comparison. The native yeast sequences around the centromere are shown as *thin lines.* Bacterial DNA sequences in the plasmids are shown as *red rectangles.* The yeast DNA in plasmid 3, which is shown as a *pink rectangle,* is a segment of yeast chromosomal DNA far removed from the centromere.

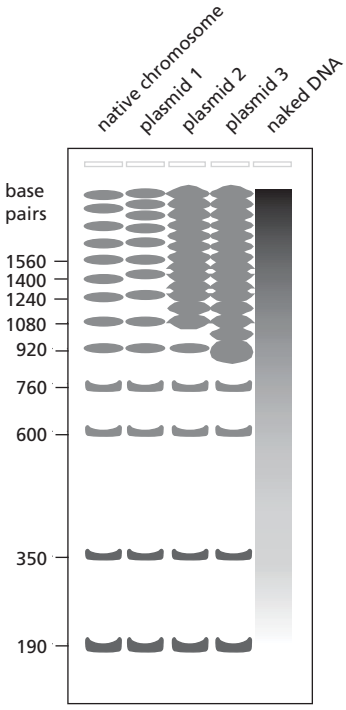


Figure 4–16 Results of micrococcal-nuclease digestion of DNA around CEN3 (Problem 4–59). Approximate lengths of DNA fragments in nucleotide pairs are indicated on the *left* of the autoradiogram.

THE GLOBAL STRUCTURE OF CHROMOSOMES

TERMS TO LEARN

lampbrush chromosome
mitotic chromosome
nucleolus
polytene chromosome

DEFINITIONS

Match each definition below with its term from the list above.

4–60 Giant chromosome in which the DNA has undergone repeated replication without separation into new chromosomes.

4–61 Paired chromosomes in meiosis in immature amphibian eggs, in which the chromatin forms large stiff loops extending out from the linear axis of the chromosome.

4–62 Highly condensed, duplicated chromosome with the two new chromosomes still held together at the centromere as sister chromatids.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

4–63 In lampbrush chromosomes of amphibian oocytes, most of the DNA is in the loops, which are actively transcribed, while the rest remains highly condensed on the chromosome axis, where genes are generally not expressed.

4–64 At the final level of condensation each chromatid of a mitotic chromosome is organized into loops of chromatin that emanate from a central axis.

THOUGHT PROBLEMS

4–65 Although mammalian chromosomes have not yet been found to form lampbrush chromosomes in amphibian oocytes, chromosomes from different amphibians form typical lampbrush chromosomes when injected into oocytes in the form of demembranated sperm heads. When sperm heads from *Rana pipiens* (Northern leopard frog), which forms large loops in its own oocyte chromosomes, were injected in *Xenopus laevis* oocytes, the resulting lampbrush chromosomes had the small loops typical of those in *X. laevis* oocytes. Similarly, when sperm heads from *X. laevis* were injected into *Notophthalmus viridescens* (red spotted newt) oocytes, the resulting lampbrush chromosomes had the very large loop structure typical of *N. viridescens*.

Do these heterologous injection experiments support the idea that loop structure is an intrinsic property of a chromosome? Why or why not?

DATA HANDLING

4–66 The characteristic banding patterns of the giant polytene chromosomes of *Drosophila melanogaster* provide a visible map of the genome that has proven an invaluable aid in genetic studies for decades. The molecular basis for the more intense staining of bands relative to interbands remains a puzzle. In principle, bands might stain more darkly because they contain more DNA than interbands due to overreplication, or the amount of DNA may be the same in the bands and interbands, but the DNA stains more prominently in the bands because it is more condensed

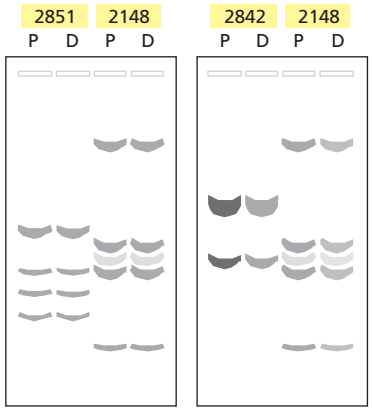


Figure 4–17 Autoradiograph of blot-hybridization analysis of polytene and diploid chromosomes (Problem 4–66). P and D refer to polytene and diploid, respectively. Numbers at the top refer to cloned DNA segments used as probes: 2851 and 2842 are from the 315-kb region under analysis (see Figure 4–18); 2148 is from elsewhere in the genome and was used in all hybridizations to calibrate the amount of DNA added to the gels.

or contains more proteins. These two possibilities—differential replication or differential staining—were distinguished by the experiments described below.

A series of radiolabeled segments spanning 315 kb of a *Drosophila* chromosome were used as hybridization probes to estimate the amount of corresponding DNA present in normal diploid tissues versus DNA from salivary glands, which contain polytene chromosomes. DNA samples from diploid and polytene chromosomes were digested with combinations of restriction enzymes. The fragments were then separated by gel electrophoresis and transferred to nitrocellulose filters for hybridization analysis. In every case the restriction pattern was the same for the DNA from diploid chromosomes and polytene chromosomes, as illustrated for two examples in **Figure 4–17**. The intensities of many specific restriction fragments were measured and expressed as the ratio of the intensity of the fragment from polytene chromosomes to the intensity of the corresponding fragment from diploid chromosomes (**Figure 4–18**).

How do these results distinguish between differential replication and differential staining as the basis for the difference between bands and interbands? Explain your reasoning.

4–67 The typical coiled phone cord provides an everyday example of the phenomenon of supercoiling. Invariably, the cord becomes coiled about itself forming a tangled mess. These coiled coils are supercoils. Dangling the receiver and letting it spin until it stops can remove them. Similarly, supercoils can be reintroduced by twisting the receiver, which of course is how they get there in the first place.

DNA is coiled into a double helix that exhibits the same phenomenon of supercoiling (**Figure 4–19**). A relaxed circular DNA, with 10.5 bp per turn of the helix, will assume a more-or-less circular form when laid onto

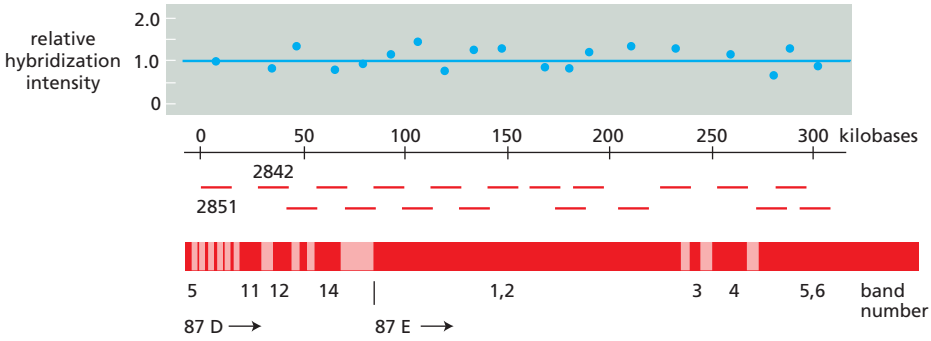


Figure 4–18 Relative amounts of DNA in diploid and polytene chromosomes at different points along the chromosome (Problem 4–66). The chromosomal segment covered by the cloned restriction fragments is shown at the *bottom,* along with the cytological designations for the chromosome regions and bands. The cloned fragments are shown *above* the chromosome, and the positions of 2851 and 2842 are indicated. The ratio of hybridization of each restriction fragment to DNA from polytene chromosomes versus diploid chromosomes is plotted above each fragment.

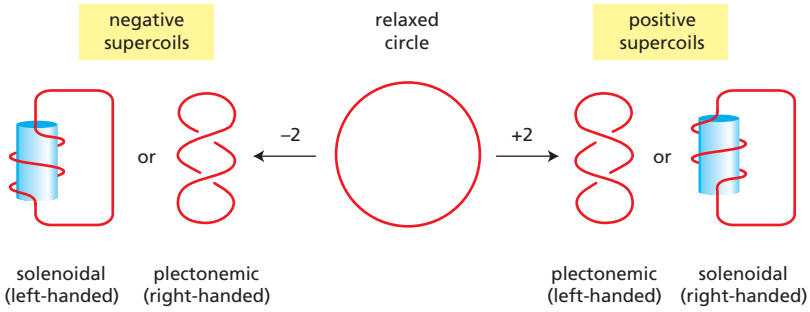


Figure 4–19 Relaxed and supercoiled circular DNA molecules (Problem 4–67). Duplexes of DNA are indicated by single lines. These DNA molecules differ only in the number of times one strand is wound around the other, a quantity known as the linking number. Solenoidal supercoils are shown as wrapped around a cylinder for illustrative purposes.

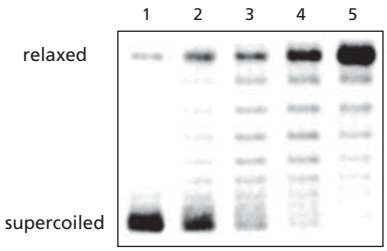


Figure 4–20 Plasmid DNA treated with *E. coli* topoisomerase I for increasing times (Problem 4–67). Plasmid DNA is shown before incubation with *E. coli* topoisomerase I in lane 1 and after increasing times of incubation in lanes 2–5.

a surface. If one strand of the DNA is broken and wound around its partner two extra times (overwound—an increase in linking number of +2) and then rejoined, the molecule will twist on itself, forming two *positive* supercoils. If one strand in a relaxed circular DNA is broken and rejoined with two fewer turns (underwound—a decrease in linking number of –2), the molecule will twist to form two *negative* supercoils. Positive and negative supercoils each can assume two forms termed plectonemic and solenoidal, although plectonemic supercoils are the only ones that are stable in naked DNA. The effect of supercoiling is to preserve the preferred local winding of DNA at 10.5 bp per turn. In cells, the degree of supercoiling of DNA is carefully controlled by special enzymes called topoisomerases that break and rejoin strands of DNA.

Circular plasmid DNA isolated from *E. coli* is highly supercoiled, as is evident when the DNA is separated by electrophoresis on an agarose gel (**Figure 4–20**, lane 1). When incubated for increasing times with *E. coli* topoisomerase I (which breaks and reseals a single DNA strand in negatively supercoiled DNA but not in positively supercoiled DNA), several new bands appear between the supercoiled DNA and the relaxed DNA (lanes 2–5).

A. In the untreated DNA sample isolated from bacteria, why do you suppose a small fraction of the plasmid molecules are relaxed (Figure 4–20, lane 1)?
B. What are the discrete bands between the highly supercoiled and relaxed bands in Figure 4–20 that appear with increasing times of incubation with topoisomerase I? Why do they move at rates intermediate between relaxed and highly supercoiled DNA?
C. Estimate the number of supercoils that were present in the original plasmid molecules.
D. Did the bacterial plasmid originally contain positive or negative supercoils? Explain your answer.

4–68 Imagine that you assemble a single nucleosome on a closed circular, relaxed DNA molecule; that is, a circular duplex DNA with no breaks in either strand and zero supercoiling. Wrapping the DNA molecule around the histone octamer forms solenoidal supercoils, which are compensated for by plectonemic supercoils in another part of the molecule.

Of the four possible arrangements of solenoidal and plectonemic supercoils shown in **Figure 4–21**, which have a net supercoiling of zero? (Since no breaks were introduced into the DNA in the process of forming the nucleosome, it must retain an overall supercoiling of zero.) Indicate the sign of the supercoiling (positive or negative) on the structures you select.

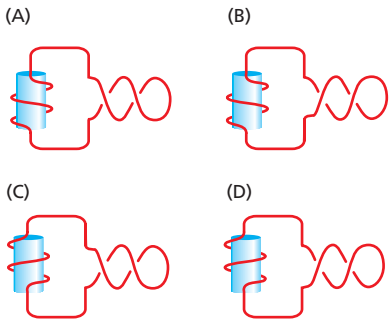


Figure 4–21 Four possible arrangements of circular DNA molecules with two solenoidal and two plectonemic supercoils (Problem 4–68). The position of the nucleosome is indicated by the cylinder.

4–69 Which of the two alternative arrangements of compensating solenoidal and plectonemic supercoils, generated by the formation of a nucleosome (see Problem 4–68), represents the true biological situation? These alternatives were distinguished by incubating the nucleosome-bound DNA with either *E. coli* topoisomerase I, which can remove only negative plectonemic supercoils, or with calf thymus topoisomerase I, which can remove both negative and positive plectonemic supercoils. Histones were removed after the incubation with a topoisomerase, and the presence of supercoils in the naked DNA was assayed by gel electrophoresis (see Figure 4–20, Problem 4–67). (The sign of the plectonemic supercoils in the naked DNA can be determined by subsequent incubation with *E. coli* topoisomerase I, which relaxes negative supercoils but not positive ones.)

It was found that incubation of nucleosomal DNA with *E. coli* topoisomerase I gave DNA molecules with zero supercoils. By contrast, incubation with calf thymus topoisomerase I gave DNA molecules with two negative supercoils. Are the solenoidal supercoils around biological nucleosomes positive (right-handed) or negative (left-handed)? What results would you have expected for the other alternative?

HOW GENOMES EVOLVE

TERMS TO LEARN

copy number variation (CNV)
homologous
pseudogene
purifying selection
single-nucleotide polymorphism (SNP)

DEFINITIONS

Match each definition below with its term from the list above.

4–70 A copy of a functional gene that has become irreversibly inactivated by multiple mutations.

4–71 Long blocks of DNA sequence that differ in the number of times they are present in the genomes of different individuals in a population.

4–72 Evolutionary process that eliminates individuals carrying mutations that interfere with important genetic functions.

4–73 Variation between individuals at a certain nucleotide position in the genome.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

4–74 In a comparison between the DNAs of related organisms such as humans and mice, identifying the conserved DNA sequences facilitates the search for functionally important regions.

4–75 Many human genes so closely resemble their homologs in yeast that the protein-coding portion of the human gene will substitute for the function of the yeast gene in yeast cells.

4–76 The portion of the human genome subjected to purifying selection corresponds to the protein-coding sequences.

4–77 Gene duplication and divergence is thought to have played a critical role in the evolution of increased biological complexity.

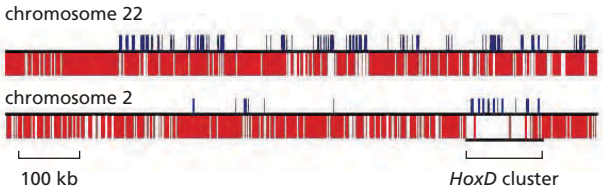


Figure 4–22 Transposable elements and genes in 1-Mb regions of chromosomes 2 and 22 (Problem 4–78). *Blue lines* that project *upward* indicate exons of known genes. *Red lines* that project *downward* indicate transposable elements; they are so numerous that they merge into nearly a solid block outside the *Hox* clusters.

THOUGHT PROBLEMS

4–78 Mobile pieces of DNA—transposable elements—that insert themselves into chromosomes and accumulate during evolution make up more than 40% of the human genome. Transposable elements are inserted more-or-less randomly throughout the human genome. These elements are conspicuously rare at the four homeobox gene clusters, *HoxA*, *HoxB*, *HoxC*, and *HoxD*, as illustrated for *HoxD* in **Figure 4–22**, along with an equivalent region of chromosome 22, which lacks a *Hox* cluster. Each *Hox* cluster is about 100 kb in length and contains 9–11 genes, whose differential expression along the anteroposterior axis of the developing embryo establishes the basic body plan for humans (and for other animals). Why do you suppose that transposable elements are so rare in the *Hox* clusters?

CALCULATIONS

4–79 Nucleotide sequence comparisons are fundamental to our current conception of the tree of life, to our understanding of how mitochondria and chloroplasts were acquired and their subsequent evolution, to the importance and magnitude of horizontal gene transfer, and to the notion that by focusing on a few model organisms we can gain valid insights into all of biology. For these reasons we've designed this problem and the following one to introduce the common methods and assumptions that underlie the art of nucleotide sequence comparison.

A phylogenetic tree represents the history of divergence of species from common ancestors. Construction of such trees from DNA or protein sequences can really only be done with computers: the data sets are enormous and the algorithms are subtle. Nevertheless, some of the fundamental principles of tree construction can be illustrated with a simple example. Consider the first 30 amino acids of the hemoglobin α chains for the five species shown in **Figure 4–23**.

A. In a common approach, known as the distance-matrix method, the first step is to construct a table of all pairwise differences between the sequences. A partially filled-in example is shown in **Table 4–2**. Complete the table by filling in the blanks indicated by question marks.

TABLE 4–2 The difference matrix for the first 30 amino acids of the hemoglobin α chains from five species (Problem 4–79).

	Human	Frog	Chicken	Whale	Fish
Human	0	?	11	8	17
Frog		0	?	17	20
Chicken			0	?	20
Whale				0	?
Fish					0

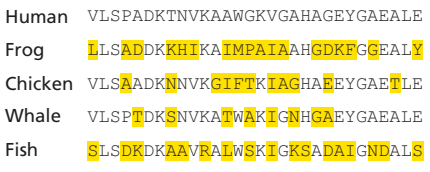


Figure 4–23 Alignment of the first 30 amino acids of the hemoglobin α chains from five species (Problem 4–79). Amino acids are represented by the one-letter code (see Table 8, page 966). Amino acids that differ from the human sequence are highlighted in *yellow.*

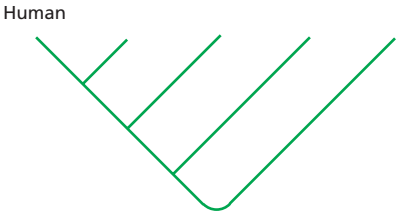


Figure 4–24 A general phylogenetic tree for five species (Problem 4–79).

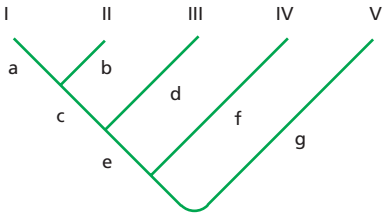


Figure 4–25 A general phylogenetic tree for five species with line segments indicated by *letters* (Problem 4–80).

B. According to the information in the completed Table 4–2, which pair of species is most closely related? What is the assumption that underlies your choice?
C. The information in Table 4–2 can be used to arrange species on the phylogenetic tree shown in **Figure 4–24**. The branching order is determined using a simple kind of cluster analysis. The two most similar species are placed on the adjacent branches at the upper left in Figure 4–24. The species with the fewest average differences relative to this pair is placed on the next branch. In the next step, these three species are combined and the average differences from the remaining species are calculated and used to fill in the next branch, and so on. Use this method to arrange the species on the tree in Figure 4–24.
D. Is the branching order you determined in part C the same as you would get by simply using the number of differences relative to human to place the other species on the tree? Why is the method of cluster analysis superior?

4–80 In the previous question the branching order, or topology, of the tree was established, but actual distances (number of differences) were not assigned to the line segments that make up the tree (**Figure 4–25**). To calculate distances for line segments is, once again, tedious by hand but easy by computer. The following exercise gives a feeling for how such calculations are done.

A. Using the numbers from the completed distance matrix in Table 4–2 and the branching order determined in the previous problem, write down all the equations for the differences between species in terms of the line segments that make up the tree (Figure 4–25). Are there enough equations to solve for the lengths of the seven line segments?
B. One straightforward, not-too-exhausting method for solving these equations is to consider them three at a time; for example,

$$\text{I} \longrightarrow \text{II} = a + b$$
$$\text{I} \longrightarrow \text{III} = a + c + d$$
$$\text{II} \longrightarrow \text{III} = b + c + d$$

There are 10 such three-at-a-time equations for five species. Using the information in the distance-matrix table (see Table 4–2), solve two of these sets of equations—human/whale/chicken and human/whale/frog—for a and b. Are the values for a and b the same in the two solutions?

DATA HANDLING

4–81 The earliest graphical method for comparing nucleotide sequences—the so-called diagon plot—still yields one of the best visual comparisons of sequence relatedness. An example is illustrated in **Figure 4–26**, where the human β-globin gene is compared with the human cDNA for β

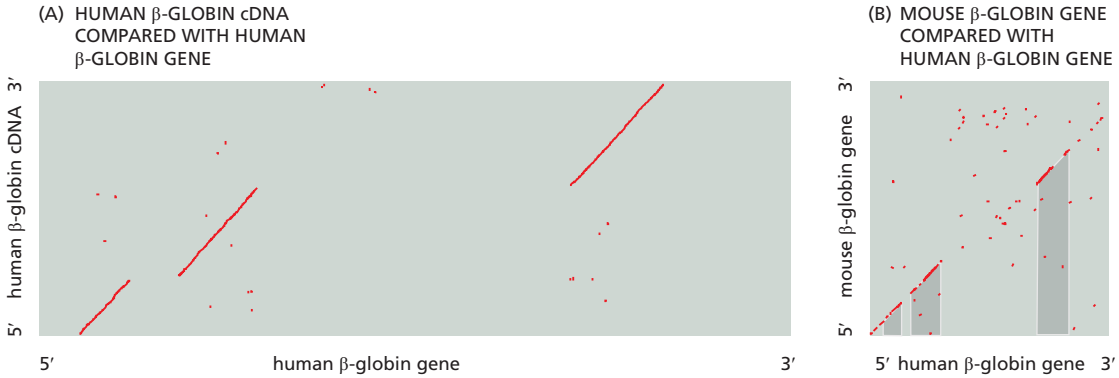


Figure 4–26 Diagon plots (Problem 4–81). (A) Human β-globin cDNA compared with the human β-globin gene. The β-globin cDNA is a complementary DNA copy of the β-globin mRNA. (B) Mouse β-globin gene compared with the human β-globin gene. The positions of the exons in the human β-globin gene are indicated by *shading* in (B). The 5′ and 3′ ends of the sequences are indicated. The human gene sequence is identical in the two plots. To accommodate the short β-globin cDNA sequence (549 nucleotides) and the sequence of the β-globin gene (2052 nucleotides) in similar spaces, while maintaining proportional scales within each plot, the scale of (A) is about three times that of (B).

globin (Figure 4–26A) and with the mouse β-globin gene (Figure 4–26B). (A cDNA is a DNA molecule made as a copy of mRNA and therefore lacking the introns that are present in genomic DNA.) Diagon plots are generated by comparing blocks of sequence, in this case blocks of 11 nucleotides at a time. If 9 or more of the nucleotides match, a dot is placed on the diagram at the coordinates corresponding to the blocks being compared. A comparison of all possible blocks generates diagrams such as the ones shown in Figure 4–26, in which sequence homologies show up as diagonal lines.

A. From the comparison of the human β-globin gene with the human β-globin cDNA (Figure 4–26A), deduce the positions of exons and introns in the β-globin gene.
B. Are the entire exons of the human β-globin gene (indicated by shading in Figure 4–26B) homologous to the mouse β-globin gene? Identify and explain any discrepancies.
C. Is there any homology between the human and mouse β-globin genes that lies outside the exons? If so, identify its location and offer an explanation for its preservation during evolution.
D. Has either of the genes undergone a change of intron length during their evolutionary divergence? How can you tell?

4–82 Your first foray into archaeological DNA studies ended in embarrassment. The dinosaur DNA sequences that you so proudly announced to the world later proved to be derived from contaminating modern human cells—probably your own. Setting your sights slightly lower, you decide to try to amplify residual mitochondrial DNA from a well-preserved Neanderthal skeleton. You also redesign your laboratory to minimize the possibility of stray contamination. You carefully prepare three different samples (A, B, and C) of bone from the femur and perform separate polymerase chain reactions (PCR) on them, one in the laboratory of a foreign collaborator. Sure enough, clear products of the expected size are seen in all three reactions. Cloned products from each PCR are individually sequenced, with the results shown in **Figure 4–27**. The sequence of the corresponding region of mitochondrial DNA from a human is shown at the top. Dots indicate matches to the human sequence; dashes indicate missing DNA.

To determine whether the common sequence differences you observe could be due to normal variation within the human population, you make pairwise comparisons of your consensus (most common) Neanderthal sequence with a large number of individual human sequences. You do the same for individual human sequences versus one another and versus chimpanzee sequences. Your pairwise comparisons are shown in **Figure 4–28**.

A. Have you successfully identified a Neanderthal mitochondrial DNA sequence? Explain your reasoning.

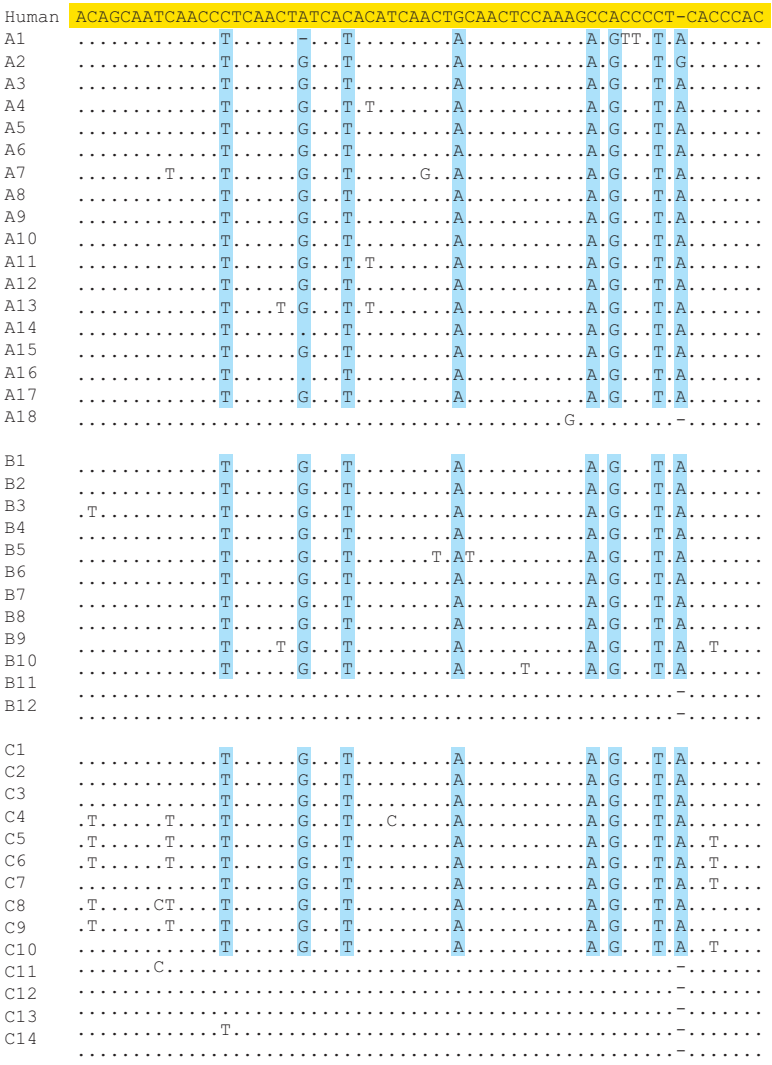


Figure 4–27 Sequences of mitochondrial DNA derived from Neanderthal samples (Problem 4–82). The sequence across the top is a reference human sequence. *Dots* indicate matches to the human sequence; *dashes* indicate missing nucleotides.

B. Did your extensive precautions in handling the sample eliminate human contamination?

C. What is the reason for choosing mitochondrial DNA for archaeological DNA studies? Wouldn't nuclear DNA sequences be more informative?

D. What would you consider to be the most important way to confirm or refute your findings?

4–83 *Alu* sequences are present at six sites in the introns of the human serum albumin and α-fetoprotein genes. (These genes are evolutionary relatives

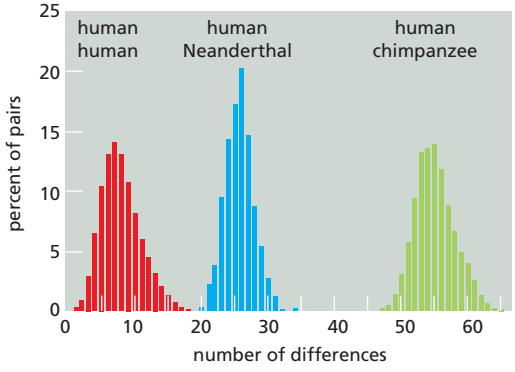


Figure 4–28 Pairwise comparisons of DNA sequences (Problem 4–82). The human–human distribution compared 994 individual, and distinct, human sequences with one another. These 994 sequences represent contemporary human mitochondrial lineages; that is, distinct sequences occurring in one or more individuals. The fraction of pairs with a given number of differences is plotted. The human–Neanderthal comparison is one Neanderthal sequence against the 994 contemporary human sequences. The human–chimpanzee comparison involved 986 contemporary human sequences with 16 contemporary chimpanzee sequences.

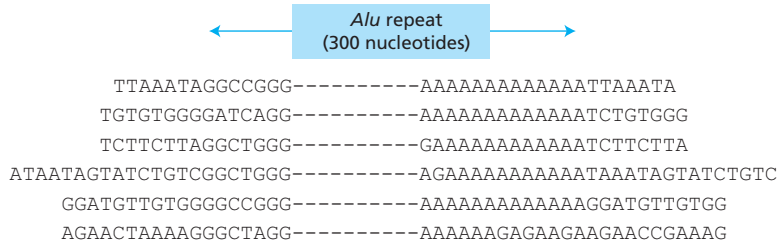


Figure 4–29 Nucleotide sequences of the six *Alu* inserts in the human albumin-gene family (Problem 4–83). *Dashed lines* indicate nucleotides in the internal part of the *Alu* sequences.

that are located side by side in mammalian genomes.) The same pair of genes in the rat contain no *Alu* sequences. The lineages of rats and humans diverged more than 85 million years ago at the time of the mammalian radiation. Does the presence of *Alu* sequences in the human genes and their absence from the corresponding rat genes mean that *Alu* sequences invaded the human genes only recently, or does it mean that the *Alu* sequences have been removed in some way from the rat genes?

To examine this question, you have sequenced all six of the *Alu* sequences in the human albumin-gene family. The sequences around the ends of the inserted *Alu* elements are shown in **Figure 4–29**.

A. *Alu* sequences create duplications of several nucleotides on each side of the target site where they insert. Mark the left and right boundaries of the inserted *Alu* sequences and indicate the nucleotides in the flanking chromosomal DNA that have been altered by mutation.
B. The rate of nucleotide substitution in introns has been measured at about 3×10^{-3} mutations per million years at each site. Assuming the same rate of substitution into the intron sequences that were duplicated by these *Alu* sequences, calculate how long ago the *Alu* sequences inserted into these genes. (Lump all the *Alu* sequences together to make this calculation; that is, treat them as if they inserted at about the same time.)
C. Why are these particular flanking sequences used in the calculation? Why were larger segments of the intron not included? Why were the mutations in the *Alu* sequences themselves not used?
D. Did these *Alu* sequences invade the human genes recently (after the time of the mammalian radiation), or have they been removed from the rat genes?

MEDICAL LINKS

4–84 About 5% of the human genome consists of duplicated segments of chromosomes, many of which are highly homologous, indicating a relatively recent origin. The high degree of homology occasionally allows inappropriate recombination events to occur between the duplications, which can decrease or increase the number of duplicated segments. Such events are responsible for several human diseases, including the red–green color blindness that affects 8% of the male population. The genes for the red and green visual pigments lie near one another on the X chromosome, one in each copy of the duplicated segment. They are 98% identical throughout most of their length, in both exons and introns; however, the genes can be distinguished by the chance presence of an extra RsaI cleavage site in one of the two genes. As a result, digestion with RsaI gives a longer fragment for the *RsaI-A* gene than for the *RsaI-B* gene, and this so-called restriction fragment length polymorphism (RFLP) can be used to track the two genes (**Figure 4–30**).

A. To determine which gene encodes which pigment, several normal, red-blind, and green-blind males were screened using a hybridization probe

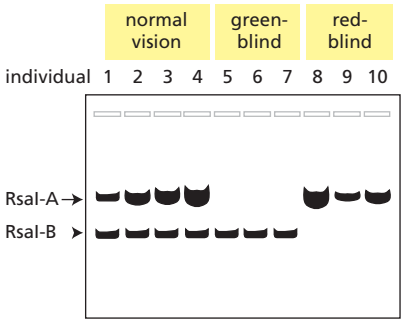


Figure 4–30 RsaI RFLPs in normal, green-blind, and red-blind males (Problem 4–84). RsaI-A refers to the RFLP characteristic of the *RsaI-A* gene; RsaI-B refers to the RFLP characteristic of the *RsaI-B* gene. Individual males are indicated by number.

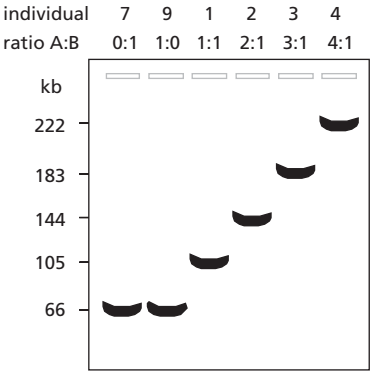


Figure 4–31 NotI digests of DNA from selected normal and color-blind individuals (Problem 4–84). Numbers for individuals correspond to the numbers in Figure 4–30. The ratios of *RsaI-A* genes to *RsaI-B* genes (A:B) were estimated from the intensity of hybridization in Figure 4–30. The sizes of NotI fragments are indicated in kb.

specific for the RsaI RFLP (Figure 4–30). Which gene encodes the red visual pigment, and which encodes the green visual pigment?

B. The intensity of hybridization in normal individuals was constant for the *RsaI-B* gene, but surprisingly variable for the *RsaI-A* gene. This anomaly was investigated by digesting the DNA from selected individuals with NotI, which cleaves once within the *RsaI-A* gene but does not cleave the *RsaI-B* gene. The restriction fragments were separated by pulsed-field gel electrophoresis and hybridized with a probe that recognizes both genes (**Figure 4–31**). What is the basis for the variable intensity of hybridization of *RsaI-A* genes in males with normal color vision? Can your explanation account for the high frequency of color blindness?

C. What is the size of the duplicated chromosomal segment at this site in the human genome?

4–85 There has been a colossal snafu in the maternity ward at your local hospital. Four sets of male twins, born within an hour of each other, were inadvertently shuffled in the excitement occasioned by that unlikely event. You have been called in to set things right. As a first step, you want to get the twins matched up. To that end you analyze a small blood sample from each infant using a hybridization probe that detects polymorphic differences in the numbers of simple sequence repeats such as $(CA)_n$ located in widely scattered regions of the genome. The results are shown in **Figure 4–32**.

A. Which infants are brothers?

B. How will you match brothers to the correct parents?

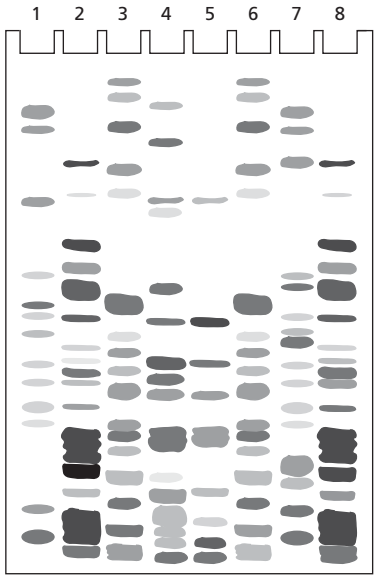


Figure 4–32 DNA fingerprint analysis of shuffled twins (Problem 4–85).

MCAT STYLE

Passage 1 (Questions 4–86 to 4–89)

Important advances in our understanding of epigenetics came from studies of yeast mating. Haploid yeast cells exist in two mating types that are referred to as **a** and **α**. Haploid cells of the opposite mating type can mate, which results in fusion of the two cells to form a diploid cell. Two loci called HML**a** and HMRα contain the key information that controls mating behavior. The genes at these loci are normally repressed, but they can be expressed when they are copied into the MAT locus: if the information from HML**a** is copied into the MAT locus, the cell becomes an **a** cell, and if the information from HMRα is copied, the cell becomes an **α** cell. This system allows cells to easily switch mating types.

4–86 Two short DNA sequences, called the E and I sites, which flank the HML locus (**Figure 4–33**), control expression of the two genes at the HML locus. Deletion of these sites causes the mating-type genes at the

Figure 4–33 The HML locus in yeast (Problem 4–86). The HML locus contains two genes—*a1* and *a2*—both of which are copied into the MAT locus to change the mating type.

HML locus to be expressed. If other, non-mating-type genes are placed between the E and I sites, they become repressed. Which one of the following statements best explains the functions of the E and I sites?

A. They are binding sites for chromatin remodeling complexes that inhibit transcription.
B. They are binding sites for factors that stimulate formation of heterochromatin.
C. They are binding sites for histone chaperones that remove histones from the HML locus.
D. They are telomeric sequences that create a repressed chromatin structure.

4–87 Genetic analyses discovered that mutations in genes called *SIR2*, *SIR3*, and *SIR4* caused expression of genes at the HML locus. Biochemical analysis showed that the SIR proteins form a complex. Which of the following activities might help to explain the roles of the SIR proteins in repression of gene expression?

I. SIR2 is a histone deacetylase
II. SIR3 binds to deacetylated histones
III. SIR4 interacts with the E and I sites

A. I and II
B. I and III
C. II and III
D. I, II, and III

4–88 Which of the following could contribute to repression of gene expression at the HML locus?

I. Binding of barrier proteins adjacent to HML
II. Formation of chromatin loops involving HML
III. Localization of HML near the nuclear envelope

A. I
B. I and II
C. II and III
D. III

4–89 A highly expressed tRNA gene is located just outside the HML. If this gene is deleted, nearby genes that lie beyond the tRNA gene become repressed. Which one of the following hypotheses could explain this observation?

A. A site near the tRNA gene that is included in the deletion acts to position the HML locus in the region near the nuclear envelope.
B. Nucleosomes at the tRNA gene recruit histone chaperones that block the spread of repressive chromatin from the HML locus.
C. Mechanisms that maintain a low density of histones over the highly transcribed tRNA gene act as a barrier to the spread of repressive chromatin.
D. The tRNA gene is covered by heterochromatin that blocks spread of the repressive chromatin from the HML locus.

Passage 2 (Questions 4–90 and 4–91)

Cancer cells proliferate without control, leading to formation of tumors. To investigate the nature of the changes that cause normal cells to become cancer cells, scientists carried out a series of nuclear transfer experiments. They first removed nuclei from mouse oocytes (egg cells), using a micropipette, and then replaced the egg nucleus with a nucleus from one of several different kinds of mouse cancer cells. The reconstituted oocytes were put back into a mouse and allowed to undergo early stages of development. These experiments showed that nuclei from several kinds of cancer cells were able to support early development of a mouse embryo into multiple differentiated cell types, without signs of abnormal proliferation.

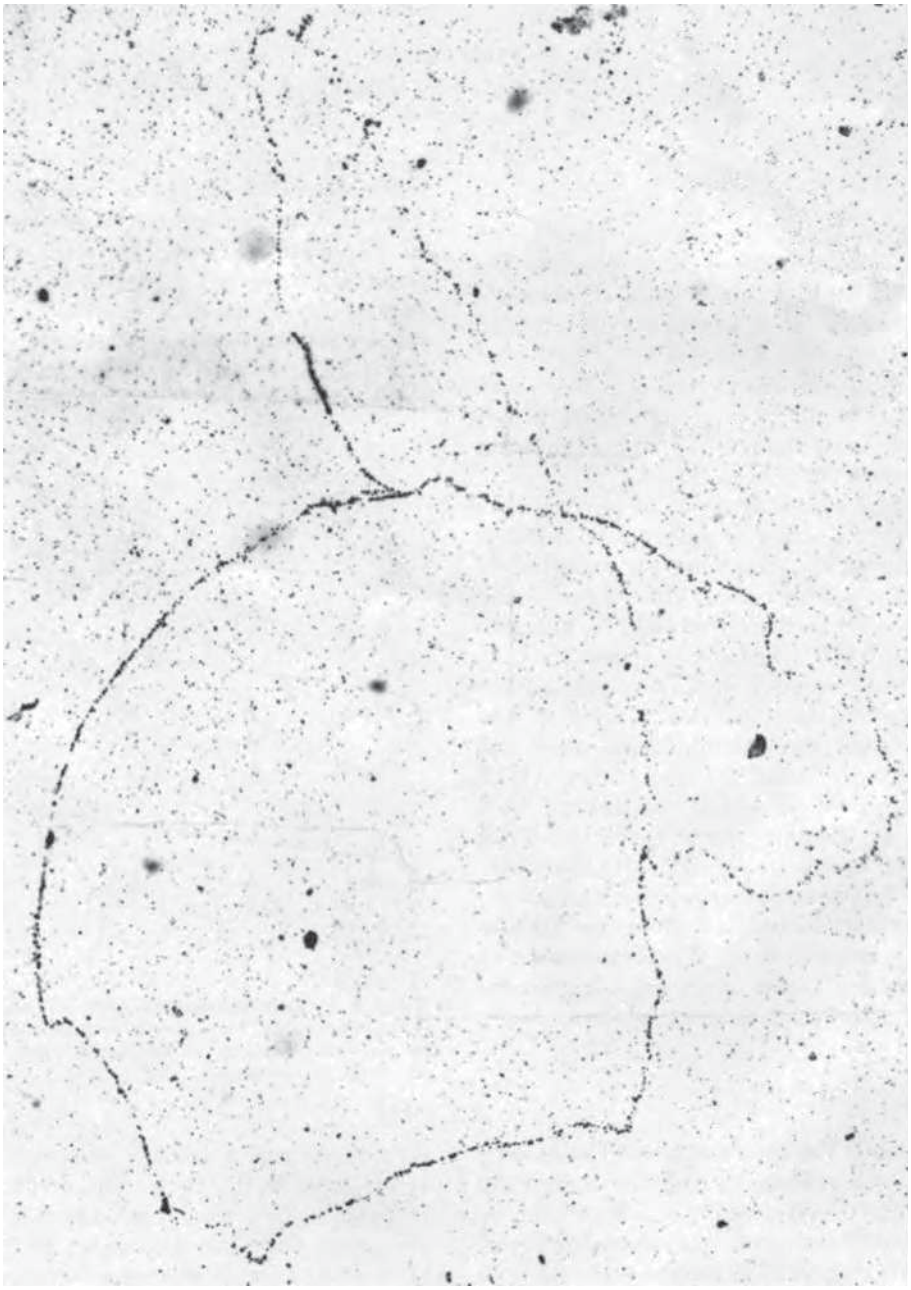
4–90 Which one of the following hypotheses best explains these experimental results?

A. Epigenetic changes at centromeres prevent chromosome segregation, which blocks proliferation of the cancer cells.
B. Epigenetic changes cause the cancer cell chromosomes to become highly condensed, inhibiting expression of cancer-causing genes.
C. Epigenetic changes in gene expression that contribute to cancer are erased when the nucleus is transferred to the mouse oocyte.
D. Mutations that cause cancer are repaired back to the wild-type sequence when the nucleus is transferred to the mouse oocyte.

4–91 In another series of experiments, it was found that inactivation of both copies of the *p16* gene is a key step in the series of events that leads to cancer. In many cancer cells, the *p16* gene is inactivated by mutation or deletion. However, in other cancer cells, the *p16* gene contains no mutations or deletions, yet the p16 protein is not produced. Which of the following could explain the lack of p16 protein in these cancer cells?

I. Formation of euchromatin at the *p16* gene
II. Formation of heterochromatin at the *p16* gene
III. Modification of histone tails at the *p16* gene

A. I
B. II
C. I and III
D. II and III



The Chromosome of *Escherichia coli.* John Cairns published this iconic autoradiograph of the DNA of *E. coli* caught in the act of replication in 1963. The bacteria were labeled with ^{3}H-thymidine for "about two generations" and then lysed with lysozyme in the presence of carrier calf thymus DNA. Most of the chromosomes detected after two months' exposure to film were "more or less tangled" circles about 1.1 to 1.4 mm long. This one, by happy chance, was unbroken and nicely displayed. Cairns assumed, incorrectly as we now know, that replication proceeded in one direction from a fixed point, with a "swivel" at the other Y junction.

DNA Replication, Repair, and Recombination

CHAPTER 5

THE MAINTENANCE OF DNA SEQUENCES

TERMS TO LEARN

germ cell mutation | mutation rate | somatic cell

DEFINITIONS

Match each definition below with its term from the list above.

5–1 A randomly produced, heritable change in the nucleotide sequence of a chromosome.

5–2 Cell type in a diploid organism that carries only one set of chromosomes and is specialized for sexual reproduction. A sperm or an egg.

5–3 Any cell of a plant or animal other than a germ cell or germ-line precursor.

TRUE/FALSE

Decide whether the statement is true or false, and then explain why.

5–4 Both germ-cell DNA stability and somatic-cell DNA stability are essential for the survival of the species.

THOUGHT PROBLEMS

5–5 You infected an *E. coli* culture with a virulent bacterial virus (bacteriophage). Most of the cells lysed, but a few survived: 1×10^{-4} in your sample. You wonder where the resistant bacteria came from. Were they caused by the bacteriophage infection, or did they already exist in the bacterial culture? Earlier, for a different experiment, you had spread a dilute suspension of *E. coli* onto solid medium in a large Petri dish, and, after seeing that about 10^5 colonies were growing up, you made an imprint of the colonies on that plate and transferred it to three other plates—a process known as replica plating—which creates the same pattern of colonies on the three plates. You realize that you can use these plates to distinguish between the two possibilities. You pipette a suspension of the bacteriophage onto each of the three replica plates so the bacteria can be infected. What result do you expect if the bacteriophage cause resistance? What result do you expect if the resistant bacteria preexist?

5–6 The following statement sounds patently false. "The different cells in your body rarely have genomes with the identical nucleotide sequence." Provide an argument for why it might be true.

5–7 Individual organisms that carry harmful mutations tend to be eliminated from a population by natural selection. It is easy to see how deleterious

mutations in bacteria, which have a single copy of each gene, are eliminated by natural selection; the affected bacteria die and the mutation is thereby lost from the population. Eukaryotes, however, have two copies of most genes because they are diploid. It is often the case that an individual with two normal copies of the gene (homozygous, normal) is indistinguishable in phenotype from an individual with one normal copy and one defective copy of the gene (heterozygous). In such cases, natural selection can operate only on an individual with two copies of the defective gene (homozygous, defective). Imagine the situation in which a defective form of the gene is lethal when homozygous, but without effect when heterozygous. Can such a mutation ever be eliminated from the population by natural selection? Why or why not?

CALCULATIONS

5–8 Mutations are introduced into the *E. coli* genome at the rate of about 1 mutation per 10^9 base pairs (bp) per generation. Imagine that you start with a population of 10^6 *E. coli*, none of which carry any mutations in your gene of interest, which is 1000 nucleotides in length and not essential for bacterial growth and survival. In the next generation, after the population doubles in number, what fraction of the cells, on average, would you expect to carry a mutation in your gene? After the population doubles again, what would you expect the frequency of mutants in the population to be? What would the frequency be after a third doubling?

DATA HANDLING

5–9 To determine the reproducibility of mutation frequency measurements, you do the following experiment. You inoculate each of 10 cultures with a single *E. coli* bacterium, allow the cultures to grow until each contains 10^6 cells, and then measure the number of cells in each culture that carry a mutation in your gene of interest. You were so surprised by the initial results that you repeated the experiment to confirm them. Both sets of results display the same extreme variability, as shown in **Table 5–1**. Assuming that the rate of mutation is constant, why do you suppose there is so much variation in the frequencies of mutant cells in different cultures?

TABLE 5–1 Frequencies of mutant cells in multiple cultures (Problem 5–9).

Experiment	Culture (mutant cells/10^6 cells)									
	1	2	3	4	5	6	7	8	9	10
1	4	0	257	1	2	32	0	0	2	1
2	128	0	1	4	0	0	66	5	0	2

DNA REPLICATION MECHANISMS

TERMS TO LEARN

clamp loader
DNA helicase
DNA ligase
DNA polymerase
DNA primase
DNA topoisomerase
lagging strand
leading strand
replication fork
RNA primer
single-strand DNA-binding (SSB) protein
sliding clamp
strand-directed mismatch repair

DEFINITIONS

Match each definition below to its term from the list above.

5–10 Short length of RNA synthesized on the lagging strand during DNA replication and subsequently removed.

5–11 Enzyme that joins two adjacent DNA strands together.

5–12 DNA repair process that replaces incorrect nucleotides inserted during DNA replication.

5–13 Enzyme that opens the DNA helix by separating the single strands.

5–14 A protein complex that encircles the DNA double helix and binds to DNA polymerase, keeping it firmly bound to the DNA while it is moving.

5–15 Enzyme that binds to DNA and reversibly breaks a phosphodiester bond in one or both strands, allowing the DNA to rotate at that point.

5–16 Y-shaped region of a replicating DNA molecule at which the two daughter strands are formed.

5–17 The newly made strand of DNA found at a replication fork that is made in discontinuous segments, which are later joined covalently.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

5–18 When read in the same direction (5′-to-3′), the sequence of nucleotides in a newly synthesized DNA strand is the same as in the parental strand used as the template for its synthesis.

5–19 Each time the genome is replicated, half the newly synthesized DNA is stitched together from Okazaki fragments.

5–20 In *E. coli,* where the replication fork travels at 500 nucleotide pairs per second, the DNA ahead of the fork—in the absence of topoisomerase—would have to rotate at nearly 3000 revolutions per minute.

5–21 Topoisomerase I does not require ATP to break and rejoin DNA strands because the energy of the phosphodiester bond is stored transiently in a phosphotyrosine linkage in the enzyme's active site.

THOUGHT PROBLEMS

5–22 The nucleotide sequence of one DNA strand of a DNA double helix is 5′-GGATTTTTGTCCACAATCA-3′. What is the sequence of the complementary strand?

5–23 The DNA fragment in **Figure 5–1** is double-stranded at each end but single-stranded in the middle. The polarity of the top strand is indicated. Is the phosphate (PO_4^-) shown on the bottom strand at the 5′ end or the 3′ end of the fragment to which it is attached?

5–24 Look carefully at the structures of the molecules in **Figure 5–2**.

A. What would you expect to happen if dideoxycytidine triphosphate (ddCTP) were added to a DNA replication reaction in large excess over

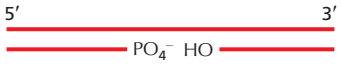


Figure 5–1 A DNA fragment with a single-stranded gap on the bottom strand (Problem 5–23).

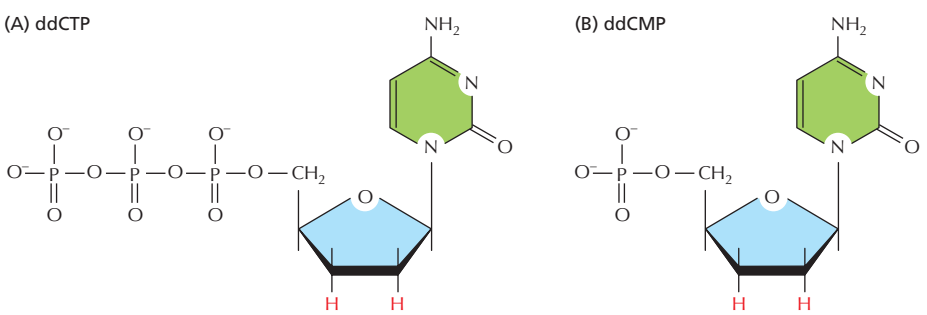


Figure 5–2 Potential replication substrates (Problem 5–24).
(A) Dideoxycytidine triphosphate (ddCTP).
(B) Dideoxycytidine monophosphate (ddCMP).

the concentration of deoxycytidine triphosphate (dCTP)? Would it be incorporated into the DNA? If it were, what would happen after that? Give your reasoning.

B. What would happen if ddCTP were added at 10% of the concentration of dCTP?

C. What effects would you expect if dideoxycytidine monophosphate (ddCMP) were added to a DNA replication reaction in large excess, or at 10% of the concentration of dCTP?

5–25 How would you expect the loss of the 3′-to-5′ proofreading exonuclease activity of DNA polymerase in *E. coli* to affect the fidelity of DNA synthesis? How would its loss affect the rate of DNA synthesis? Explain your reasoning.

5–26 Discuss the following statement: "Primase is a sloppy enzyme that makes many mistakes. Eventually, the RNA primers it makes are replaced with DNA made by a polymerase with higher fidelity. This is wasteful. It would be more energy-efficient if a DNA polymerase made an accurate copy in the first place."

5–27 SSB proteins bind to single-strand DNA at the replication fork and prevent the formation of short hairpin helices that would otherwise impede DNA synthesis. What sorts of sequences in single-strand DNA might be able to form a hairpin helix? Write out an example of a sequence that could form a five-nucleotide hairpin helix, and show the helix.

5–28 Conditional lethal mutations have proven indispensible in genetic and biochemical analyses of complex processes such as DNA replication. Temperature-sensitive (ts) mutations, which are one form of conditional lethal mutation, allow cells to grow at one temperature (for example, 30°C) but not at a higher temperature (for example, 42°C).

A large number of temperature-sensitive replication mutants have been isolated in *E. coli.* These mutant bacteria are defective in DNA replication at 42°C but not at 30°C. If the temperature of the medium is raised from 30°C to 42°C, these mutants stop making DNA in one of two characteristic ways. The "quick-stop" mutants halt DNA synthesis immediately, whereas the "slow-stop" mutants stop DNA synthesis only after many minutes.

A. Predict which of the following proteins, if temperature sensitive, would display a quick-stop phenotype and which would display a slow-stop phenotype. In each case, explain your prediction.
   1. DNA topoisomerase I
   2. A replication initiator protein
   3. Single-strand DNA-binding protein
   4. DNA helicase
   5. DNA primase
   6. DNA ligase

B. Cell-free extracts of the mutants show essentially the same patterns of replication as the intact cells. Extracts from quick-stop mutants halt DNA synthesis immediately at 42°C, whereas extracts from slow-stop mutants do not stop DNA synthesis for several minutes after a shift to 42°C. Suppose extracts from a temperature-sensitive DNA helicase mutant and a temperature-sensitive DNA ligase mutant were mixed together at 42°C. Would you expect the mixture to exhibit a quick-stop phenotype, a slow-stop phenotype, or a nonmutant phenotype?

5–29 DNA repair enzymes preferentially repair mismatched bases on the newly synthesized DNA strand, using the old DNA strand as a template. If mismatches were instead repaired without regard for which strand served as template, would mismatch repair reduce replication errors? Would such a mismatch repair system result in fewer mutations, more

mutations, or the same number of mutations as there would have been without any repair at all? Explain your answers.

5–30 If DNA polymerase requires a perfectly paired primer in order to add the next nucleotide, how is it that any mismatched nucleotides "escape" this requirement and become substrates for mismatch repair enzymes?

5–31 DNA damage can interfere with DNA replication. X-rays, for example, generate highly reactive hydroxyl radicals that can break one or both strands of DNA. Ultraviolet (UV) light commonly generates cyclobutane dimers between adjacent T bases in the same DNA strand, which blocks progression of DNA polymerase. If such damage is not repaired, it can have serious consequences when a replication fork encounters it. See if you can predict the appearance of the replication fork after it encounters a nick or a thymine-dimer block in the templates for the leading and lagging strands. Replication forks just before they encounter the damage are shown in **Figure 5–3**.

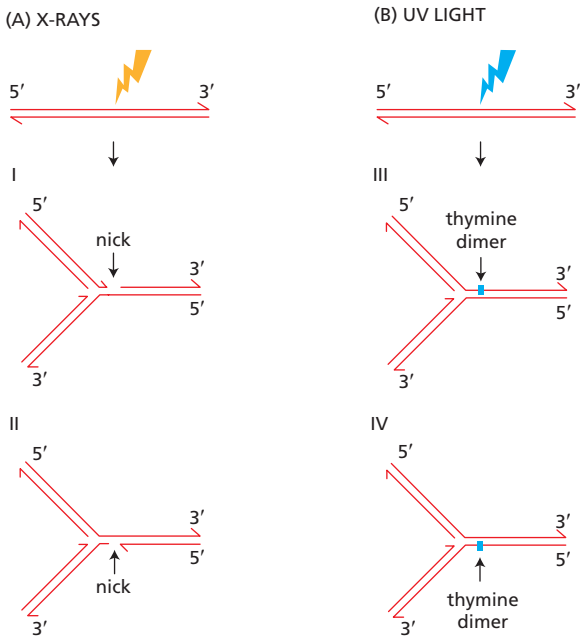


Figure 5–3 Damage to the templates for the leading and lagging strands (Problem 5–31). (A) X-ray-induced nicks. (B) UV-light-induced thymine dimers. Structures I and III have damage in the template for lagging-strand synthesis. Structures II and IV have damage in the template for leading-strand synthesis.

5–32 At the completion of replication of the circular genome of the animal virus SV40, the two daughter circles are interlocked like links in a chain. How do you suppose such interlinked molecules might then separate?

CALCULATIONS

5–33 Like all organisms, bacteriophage T4 encodes an SSB protein that is important for removing secondary structure in the single-strand DNA ahead of the replication fork. The T4 SSB protein is an elongated monomeric protein with a molecular weight of 35,000. It binds tightly to single-strand, but not double-strand, DNA. Binding saturates at a 1:12 weight ratio of DNA to protein. The binding of SSB protein to DNA shows a peculiar property that is illustrated in **Figure 5–4**. In the presence of excess single-strand DNA (10 μg), virtually no binding is detectable at 0.5 μg SSB protein (Figure 5–4A), whereas nearly all the SSB protein is bound to DNA at 7.0 μg (Figure 5–4B).

A. At saturation, what is the ratio of nucleotides of single-strand DNA to molecules of SSB protein? (The average mass of a single nucleotide is 330 daltons.)

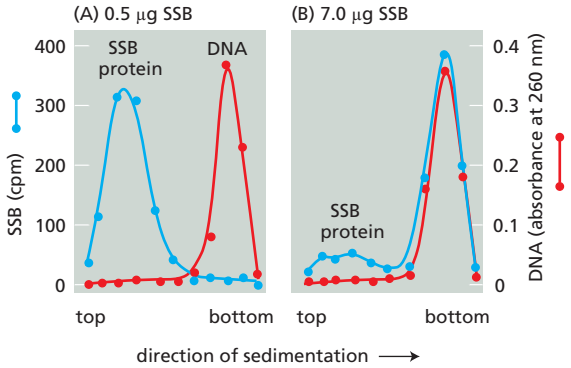


Figure 5–4 Binding of T4 SSB protein to single-strand DNA (Problem 5–33). The binding of (A) 0.5 μg and (B) 7.0 μg SSB protein to DNA was analyzed by centrifugation through sucrose gradients, on which the much more massive DNA sediments more rapidly than protein and is consequently found closer to the bottom of the gradient (cpm stands for counts per minute).

B. When the binding of SSB protein to DNA reaches saturation, are adjacent monomers of SSB protein likely to be in contact? Assume that a monomer of SSB protein extends for 12 nm along the DNA upon binding and that the spacing of bases in single-strand DNA after binding is the same as in double-strand DNA (that is, 10.4 nucleotides per 3.4 nm).

C. Why do you think that the binding of SSB protein to single-strand DNA depends so strongly on the amount of SSB protein?

5–34 Approximately how many high-energy bonds are used to replicate the *E. coli* chromosome? How many molecules of glucose would *E. coli* need to consume to provide enough energy to copy its DNA once? How does this mass of glucose compare with the mass of *E. coli,* which is about 10^{-12} g? (There are 4.6×10^6 base pairs in the *E. coli* genome. Oxidation of one glucose molecule yields about 30 high-energy phosphate bonds. Glucose has a molecular mass of 180 daltons, and there are 6×10^{23} daltons/g.)

DATA HANDLING

5–35 A born skeptic, you plan to confirm for yourself the results of a classic experiment originally performed in the 1960s by Meselson and Stahl. They concluded that each daughter cell inherits only one strand of its mother's DNA. To check their results, you "synchronize" a culture of growing cells, so that virtually all cells begin and then complete DNA synthesis at the same time. You first grow the cells in a medium that contains nutrients highly enriched in heavy isotopes of nitrogen and carbon (^{15}N and ^{13}C in place of the naturally abundant ^{14}N and ^{12}C). Cells growing in this "heavy" medium use the heavy isotopes to build all of their macromolecules, including nucleotides and nucleic acids. You then transfer the cells to a normal, "light" medium containing ^{14}N and ^{12}C nutrients. Finally, you isolate DNA from cells that have grown for different numbers of generations in the light medium and determine the density of their DNA by density-gradient centrifugation. Your data, plotting the amount of DNA isolated versus its density, are shown in **Figure 5–5**. Are these results in agreement with your expectations? Explain the results.

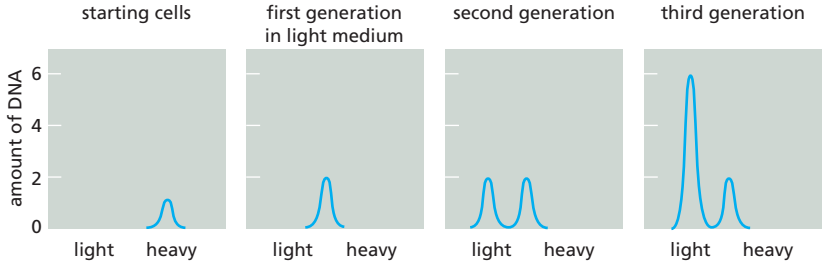


Figure 5–5 Density of DNAs isolated from cells that were grown for different times in "light" medium after initial growth in medium enriched for heavy isotopes of nitrogen and carbon (Problem 5–35). Equal culture volumes were analyzed for each time point. Amount of DNA is in arbitrary units, with the peak amount of DNA in the sample containing starting cells set equal to 1.

5–36 The *dnaB* gene of *E. coli* encodes a helicase (dnaB) that unwinds DNA at the replication fork. Its properties have been studied using artificial substrates like those shown in **Figure 5–6**. In such substrates, dnaB binds preferentially to the longest single-strand region (the largest target) available. The experimental approach is to incubate the substrates under a variety of conditions and then subject a sample to electrophoresis on agarose gels. The short single strand (substrates 1 and 2) or strands (substrate 3) will move slowly if still annealed to the longer DNA strand, but will move much faster if unwound and detached. The migration of these short single strands can be followed selectively by making them radioactive and examining their positions in the gel by autoradiography. The migration of the labeled single strands in the three different substrates is shown in **Figure 5–7**. In the absence of any treatment, the labeled strands move slowly (lanes 4, 8, and 12); when the substrates are heated to 100°C, the labeled strands are detached and migrate more rapidly (lanes 3, 7, and 11).

The results of several experiments are shown in Figure 5–7. Substrate 1, the substrate without tails, was not unwound by dnaB and ATP at 37°C (Figure 5–7, lanes 1 and 2). When either substrate with tails was incubated at 37°C with dnaB and ATP, a significant amount of small fragment was released by unwinding (lanes 6 and 10). For substrate 3, only the 3′ fragment was unwound (lane 10). All unwinding was absolutely dependent on ATP hydrolysis.

Unwinding was considerably enhanced by adding single-strand DNA-binding protein (SSB) (compare Figure 5–7, lanes 5 and 6 and lanes 9 and 10). Interestingly, SSB had to be added about 3 minutes after dnaB; otherwise it inhibited unwinding.

A. Why do you suppose ATP hydrolysis is required for unwinding?
B. In what direction does dnaB move along the long single-strand DNA? Is this direction more consistent with its movement on the template for the leading strand or on the template for the lagging strand at a replication fork?
C. Why do you suppose SSB inhibits unwinding when it is added before dnaB, but stimulates unwinding when added after dnaB?

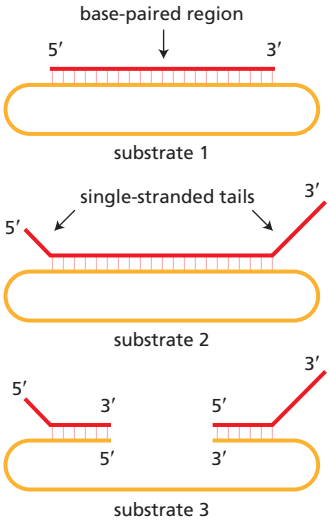


Figure 5–6 Substrates used to test the properties of dnaB (Problem 5–36).

5–37 The different ways in which DNA synthesis occurs on the leading and lagging strands raises the question as to whether synthesis occurs with equal fidelity on the two strands. One clever approach used reversion of specific mutations in the *E. coli LacZ* gene to address this question. *E. coli*

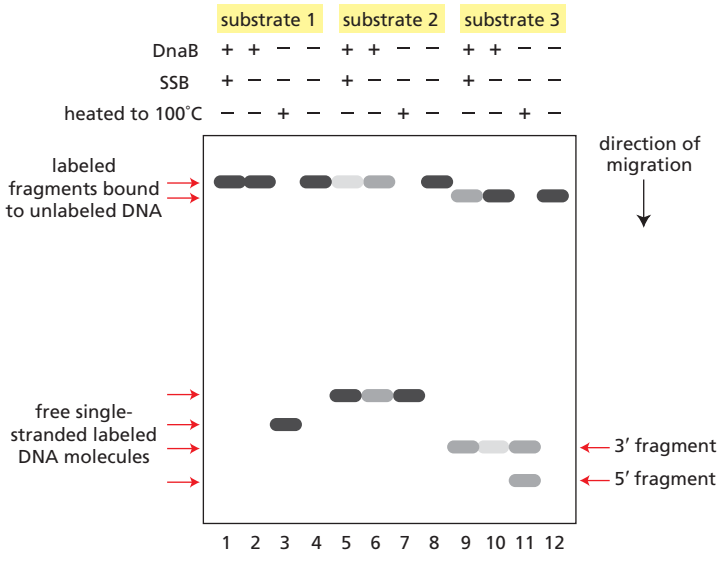


Figure 5–7 Results of several experiments to measure unwinding by dnaB (Problem 5–36). Only the single-strand fragments were radioactively labeled. Their positions are shown by the bands in this schematic diagram. ATP was included with dnaB in all incubations, which were carried out at 37°C.

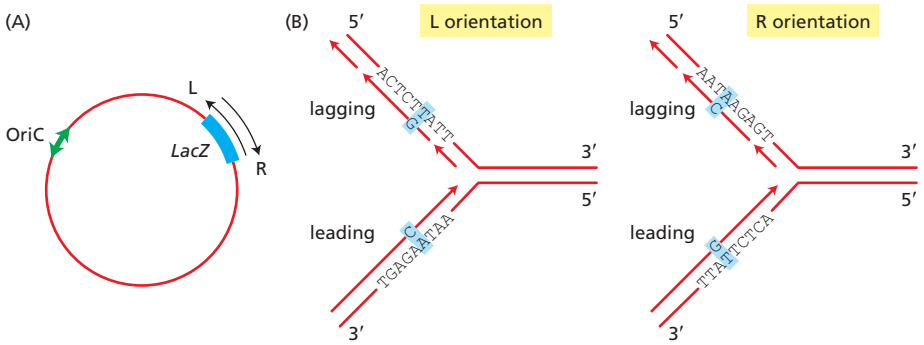


Figure 5–8 Fidelity of synthesis of the leading and lagging strands (Problem 5–37). (A) Site of insertion of *LacZ* into the *E. coli* chromosome. Orientations are denoted R and L. The *arrows* at OriC represent the two forks initiated at that site. (B) Arrangement of sequences relative to the leading and lagging strands in the L and R orientations. The G and C misincorporations that could lead to reversion are indicated.

is a good choice for such a study because the same polymerase (DNA pol III) synthesizes both the leading and the lagging strands.

The *LacZ* CC106 allele can regain its function (revert) by converting the mutant A-T base pair to the normal G-C base pair. This allele was inserted into the *E. coli* chromosome on one side of the normal origin of replication (**Figure 5–8A**). Two *E. coli* strains were isolated: one with the allele in the "L" orientation, and the other with it in the opposite, "R," orientation. As shown in **Figure 5–8B**, misincorporation of G opposite T on one strand, or of C opposite A on the other strand, could lead to reversion. Previous studies had shown that C is very rarely misincorporated opposite A. Thus, the most common source of reversion is from misincorporation of G opposite T.

To eliminate the complicating effects of mismatch repair, the experiments were done in two mutant strains of bacteria. One was defective for mismatch repair, which eliminates it from consideration; the other was defective in the proofreading exonuclease, and introduces so many mismatches that it overwhelms the mismatch-repair machinery.

Accurate frequencies of *LacZ* reversion were measured in the two strains, along with the frequencies of mutation at the *Rif* gene, whose orientation in the chromosome was constant (**Table 5–2**).

A. On which strand, leading or lagging, does DNA synthesis appear to be more accurate? Explain your reasoning.

B. Can you suggest a reason why DNA synthesis might be more accurate on the strand you have chosen?

TABLE 5–2 Frequencies (per 10^6 cells) of revertants of *LacZ* and mutants of *Rif* in strains of *E. coli* deficient for mismatch repair or proofreading (Problem 5–37).

LacZ allele (mutation measured)	*LacZ* orientation	Mismatch-repair deficient		Proofreading deficient	
		Lac⁻ → *Lac*⁺	*Rif*ˢ → *Rif*ʳ	*Lac*⁻ → *Lac*⁺	*Rif*ˢ → *Rif*ʳ
CC106 (AT → GC)	L	0.27	7.0	2.7	82
CC106 (AT → GC)	R	0.51	6.4	6.7	80

THE INITIATION AND COMPLETION OF DNA REPLICATION IN CHROMOSOMES

TERMS TO LEARN

histone chaperone
origin recognition complex (ORC)
replication origin
S phase
telomerase

DEFINITIONS

Match each definition below with its term from the list above.

5–38 Period during a eukaryotic cell cycle in which DNA is synthesized.

5–39 Large multimeric protein structure that is bound to the DNA at origins of replication in eukaryotic chromosomes throughout the cell cycle.

5–40 Special DNA sequence on a bacterial or viral chromosome at which DNA replication begins.

5–41 Enzyme that elongates telomeres, the repetitive nucleotide sequences found at the ends of eukaryotic chromosomes.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

5–42 In a replication bubble, the same parental DNA strand serves as the template strand for leading-strand synthesis in one replication fork and as the template for lagging-strand synthesis in the other fork.

5–43 When bidirectional replication forks from adjacent origins meet, a leading strand always runs into a lagging strand.

5–44 If an origin of replication is deleted from a eukaryotic chromosome, the DNA on either side will ultimately be lost, as well, because it cannot be replicated.

THOUGHT PROBLEMS

5–45 The laboratory you joined is studying the life cycle of an animal virus that uses circular, double-strand DNA as its genome. Your project is to define the location of the origin(s) of replication and to determine whether replication proceeds in one or both directions away from an origin (unidirectional or bidirectional replication). To accomplish your goal, you isolated replicating molecules, cleaved them with a restriction nuclease that cuts the viral genome at only one site to produce a linear molecule from the circle, and examined the resulting molecules in the electron microscope. Some of the molecules you observed are illustrated schematically in **Figure 5–9**. (Note that it is impossible to distinguish the orientation of one DNA molecule from another in the electron microscope.)

You must present your conclusions to the rest of the lab tomorrow. How will you answer the two questions your advisor had posed for you? Is there a single, unique origin of replication or several origins? Is replication unidirectional or bidirectional?

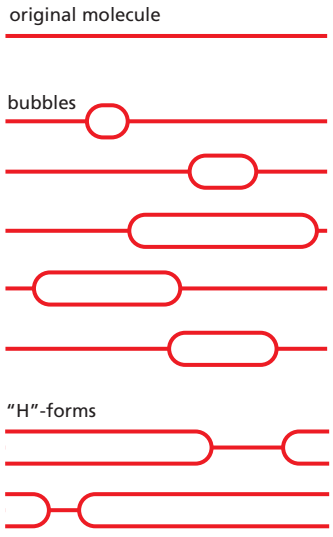


Figure 5–9 Parental and replicating forms of an animal virus (Problem 5–45).

5–46 Which one of the following statements about the newly synthesized strand of a human chromosome is correct?

A. It was synthesized from a single origin solely by continuous DNA synthesis.
B. It was synthesized from a single origin solely by discontinuous DNA synthesis.
C. It was synthesized from a single origin by a mixture of continuous and discontinuous DNA synthesis.
D. It was synthesized from multiple origins solely by continuous DNA synthesis.
E. It was synthesized from multiple origins solely by discontinuous DNA synthesis.
F. It was synthesized from multiple origins by a mixture of continuous and discontinuous DNA synthesis.

G. It was synthesized from multiple origins by either continuous or discontinuous DNA synthesis, depending on which specific daughter chromosome is being examined.

5–47 The mechanism of DNA replication gives rise to the "end-replication problem" for linear chromosomes. Over time, this problem leads to loss of DNA from the ends of chromosomes. In cells such as yeast, loss of nucleotides during replication is balanced by addition of nucleotides by telomerase. In humans, however, telomerase is turned off in most somatic cells early in development, so that chromosomes become shorter with increasing rounds of replication. Consider one round of replication in a human somatic cell. Which one of the following statements correctly describes the status of the two daughter chromosomes relative to the parent chromosome?

A. One daughter chromosome will be shorter at one end; the other daughter chromosome will be normal at both ends.
B. One daughter chromosome will be shorter at both ends; the other daughter chromosome will be normal at both ends.
C. One daughter chromosome will be shorter at both ends; the other daughter chromosome will be shorter at only one end.
D. Both daughter chromosomes will be shorter at one end, which is the same end in the two chromosomes.
E. Both daughter chromosomes will be shorter at one end, which is the opposite end in the two chromosomes.
F. Both daughter chromosomes will be shorter at both ends.

CALCULATIONS

5–48 In the early embryo of *Drosophila*, many replication origins are active so that several can be observed in a single electron micrograph, as shown in **Figure 5–10**.

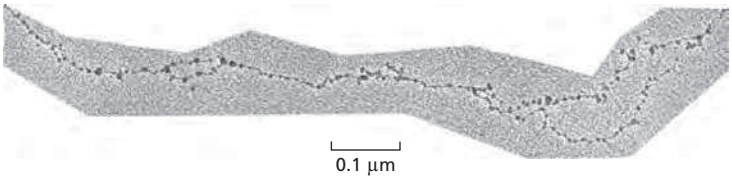


Figure 5–10 Electron micrograph showing four replication bubbles in a chromosome from the early embryo of *Drosophila* (Problem 5–48).

A. Identify the four replication bubbles in Figure 5–10. Indicate the approximate locations of the origins at which each replication bubble was initiated, and label the replication forks 1 through 8 from left to right across the figure.
B. Estimate how long it will take until forks 4 and 5 collide with each other. How long will it take until forks 7 and 8 collide? Each nucleotide in DNA occupies 0.34 nm, and eukaryotic replication forks move at about 50 nucleotides/second. For this problem disregard the nucleosomes evident in Figure 5–10 and assume that the DNA is fully extended.

5–49 Assuming that there were no time constraints on replication of the genome of a human cell, what would be the minimum number of origins that would be required? If replication had to be accomplished in an 8-hour S phase and replication forks moved at 50 nucleotides/second, what would be the minimum number of origins required to replicate the human genome? (Recall that the human genome comprises a total of 6.4×10^9 nucleotides on 46 chromosomes.)

DATA HANDLING

5–50 You are investigating DNA synthesis in tissue-culture cells, using ^{3}H-thymidine to radioactively label the replication forks. By breaking open the

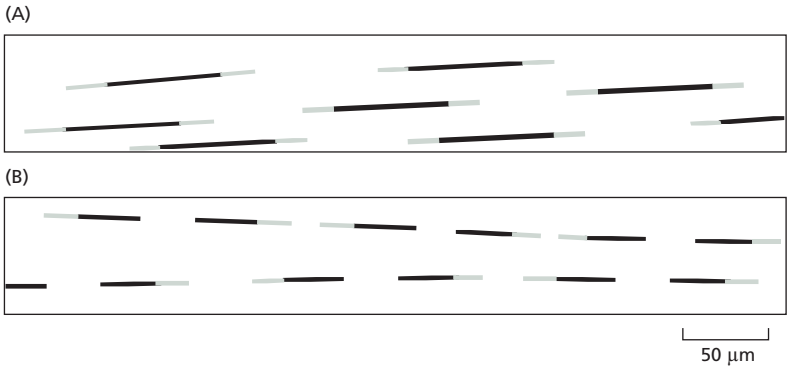


Figure 5–11 Autoradiographic investigation of DNA replication in cultured cells (Problem 5–50). (A) Addition of ^{3}H-labeled thymidine immediately after release from the synchronizing block. (B) Addition of ^{3}H-labeled thymidine 30 minutes after release from the synchronizing block.

cells in a way that allows some of the DNA strands to be stretched out, very long DNA strands can be isolated intact and examined. You overlay the DNA with a photographic emulsion, and expose it for 3 to 6 months, a procedure known as autoradiography. Because the emulsion is sensitive to radioactive emissions, the ^{3}H-labeled DNA shows up as tracks of silver grains. Because the stretching collapses replication bubbles, the daughter duplexes lie side by side and cannot be distinguished from each other.

You pretreat the cells to synchronize them at the beginning of S phase. In the first experiment, you release the synchronizing block and add ^{3}H-thymidine immediately. After 30 minutes, you wash the cells and change the medium so that the total concentration of thymidine is the same as it was, but only one-third of it is radioactive. After an additional 15 minutes, you prepare DNA for autoradiography. The results of this experiment are shown in **Figure 5–11A**. In the second experiment, you release the synchronizing block and then wait 30 minutes before adding ^{3}H-thymidine. After 30 minutes in the presence of ^{3}H-thymidine, you once again change the medium to reduce the concentration of radioactive thymidine and incubate the cells for an additional 15 minutes. The results of the second experiment are shown in **Figure 5–11B**.

A. Explain why, in both experiments, some regions of the tracks are dense with silver grains (dark), whereas others are less dense (light).

B. In the first experiment, each track has a central dark section with light sections at each end. In the second experiment, the dark section of each track has a light section at only one end. Explain the reason for this difference.

C. Estimate the rate of fork movement (µm/min) in these experiments. Do the estimates from the two experiments agree? Can you use this information to gauge how long it would take to replicate the entire genome?

5–51 Segments of DNA that allow plasmids to replicate in yeast are known as autonomous replication sequences (ARSs). They are thought to function as origins of replication. Proving that an ARS is an origin of replication is difficult, however, mainly because it is very hard to obtain enough well-defined replicating DNA to analyze. This problem can be addressed using a two-dimensional gel-electrophoretic analysis that separates DNA molecules by mass in the first dimension and by shape in the second dimension. Because they have branches, replicating molecules migrate more slowly in the second dimension than do linear molecules of equal mass. By cutting replicating molecules with restriction nucleases, it is possible to generate a continuum of different branched forms that together give characteristic patterns on two-dimensional gels (**Figure 5–12**).

You apply this technique to the replication of a plasmid that contains a specific ARS, *Ars1*. To maximize the fraction of plasmid molecules that are replicating, you synchronize a yeast culture and isolate DNA from cells in S phase. You then digest the DNA with BglII or PvuI, which cut the plasmid as indicated in **Figure 5–13A**. You separate the DNA fragments

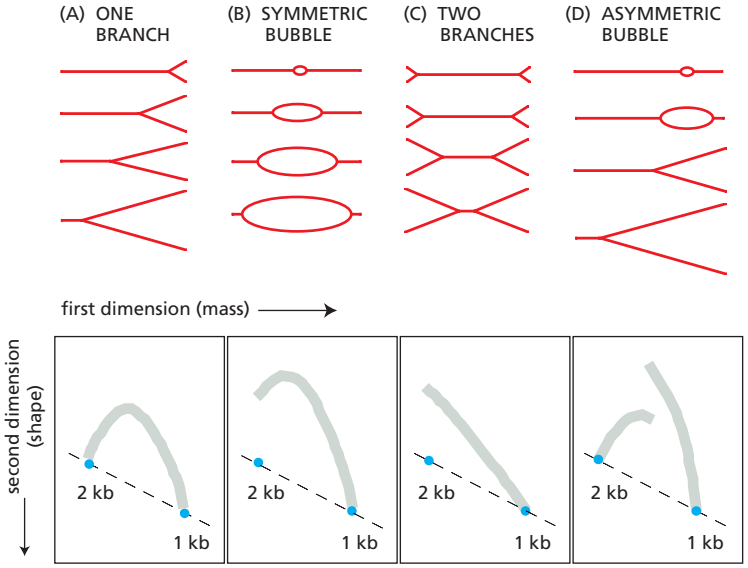


Figure 5–12 Expected patterns on two-dimensional gels for various DNA molecules (Problem 5–51). (A) Molecules with a single branch. (B) Molecules with a symmetrically located replication bubble. (C) Molecules with two branches. (D) Molecules with an asymmetrically located replication bubble. In the upper half of the figure, 1-kb molecules are shown at progressive stages of replication to 2-kb molecules. In the lower half are shown the corresponding gel patterns that would result from a continuum of such intermediates.

by two-dimensional electrophoresis and visualize the plasmid sequences by autoradiography after blot hybridization to radioactive plasmid DNA (**Figure 5–13B**).

A. What is the source of the intense spot of hybridization at the 4.5-kb position in both gels in Figure 5–13B?

B. Do the results of this experiment indicate that *Ars1* is an origin of replication? Explain your answer.

C. There is a gap in the arc of hybridization in the PvuI gel pattern in Figure 5–13B. What is the basis for this discontinuity?

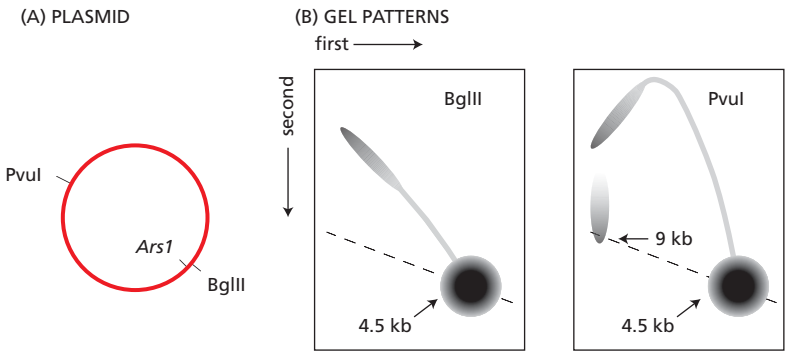


Figure 5–13 Analysis of replication of an *Ars1*-containing plasmid (Problem 5–51). (A) Structure of the *Ars1* plasmid. (B) The two-dimensional gel patterns resulting from cleavage of replicating plasmids with BglII or PvuI.

5–52 The shell of a *Drosophila* egg is made from more than 15 different chorion proteins, which are synthesized at a late stage in egg development by follicle cells surrounding the egg. The various chorion genes are grouped in two clusters, one on chromosome 3 and the other on the X chromosome. In each cluster, the genes are closely spaced with only a few hundred nucleotides separating adjacent genes. During egg development, the number of copies of the chorion genes increases by overreplication of a segment of the surrounding chromosome. The level of amplification around the chorion cluster on chromosome 3 is maximal in the region of the chorion genes, but extends for nearly 50 kb on each side (**Figure 5–14**).

The DNA sequence responsible for amplification of the cluster on chromosome 3 has been narrowed to a 510-nucleotide segment immediately upstream of one of the chorion genes. When this segment is moved to different places in the genome, those new sites are also amplified in

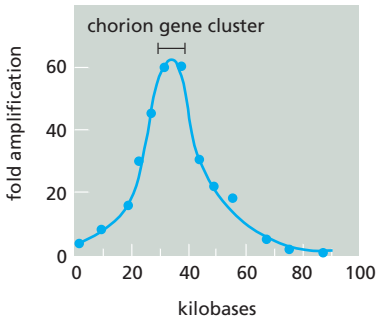


Figure 5–14 Levels of amplification in the region of the chromosome surrounding the chorion gene cluster (Problem 5–52).

follicle cells. No RNA or protein product seems to be synthesized from this amplification-control element.

A. Sketch what you think the DNA from an amplified cluster would look like in the electron microscope.
B. How many rounds of replication would be required to achieve a 60-fold amplification?
C. How do you think the 510-nucleotide amplification-control element promotes the overreplication of the chorion gene cluster?

5–53 In yeast, origin selection is initiated by the origin recognition complex (ORC). ORC is a six-protein DNA-binding complex that recognizes DNA sequences within the yeast origin of replication. One of the components of ORC, Orc1, contains a protein motif (the Walker motif) that is commonly associated with binding and hydrolysis of ATP.

To determine whether binding of ATP, its hydrolysis, or both are required for the recognition of origin DNA by ORC, you carry out the following set of experiments. You first mutate the *Orc1* gene to change an amino acid in the Walker motif of Orc1. You then isolate two versions of ORC: the wild-type form with normal Orc1 and the mutant form with a defective Walker motif. Finally, you measure the binding of these two forms of ORC to origin DNA in the presence of different concentrations of ATP. Binding of the two forms of ORC, as revealed by a DNase I protection assay (DNase footprinting), is shown in **Figure 5–15**. Exactly the same footprints were obtained when the nonhydrolyzable analog, ATPγS, was used in place of ATP.

A. Indicate the location of ORC binding on the origin DNA in Figure 5–15.
B. Is ATP required for ORC to bind to origin DNA? How can you tell?
C. Is ATP hydrolysis required for ORC binding to origin DNA? How can you tell?
D. Is the Walker motif important to the function of ORC? Explain your answer.

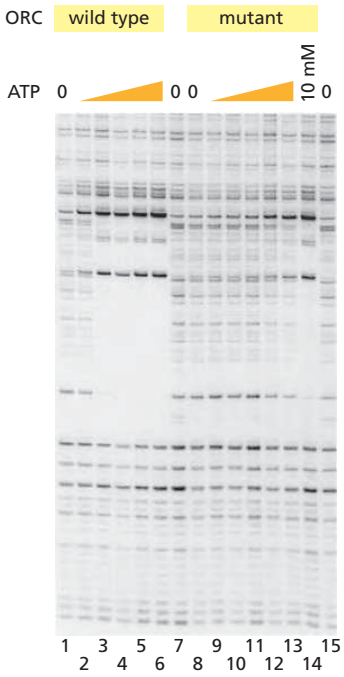


Figure 5–15 DNase footprinting assay to detect ORC binding to origin DNA (Problem 5–53). *Wedges* indicate increasing concentration of ATP in factors of ten, from 10 nM to 100 μM. In the footprinting assay, one strand of the origin-containing DNA was labeled at one end. After ORC was allowed to bind, the complex was treated briefly with DNase I, which breaks the DNA at characteristic places, except where it is protected by ORC. Lanes 7 and 15 omit both ATP and ORC.

5–54 You have developed an assay for assembly of nucleosomes onto DNA in order to define the role of chromosome assembly factor 1 (CAF1). You replicate the circular genome of the SV40 animal virus in a cell-free system in the presence of a labeled nucleotide to tag the replicated molecules, so you can follow them specifically in the assembly assay. After separating the DNA from soluble components in the cell-free replication system, you incubate it with purified CAF1 and a source of histones. You then assay for nucleosome assembly by its effects on the supercoiling status of the circular SV40 genome. Genomes without nucleosomes remain relaxed, whereas genomes with nucleosomes become supercoiled. You separate different supercoiled forms of the DNA by electrophoresis on an agarose gel, which was stained to reveal all forms of DNA and subjected to autoradiography to identify replicated DNA (**Figure 5–16**).

A. Is most of the SV40 DNA replicated or unreplicated? How can you tell?
B. Does CAF1 assemble nucleosomes on replicated DNA, unreplicated DNA, or both? Explain your answer.
C. How do you suppose that CAF1 recognizes the DNA it assembles into nucleosomes?

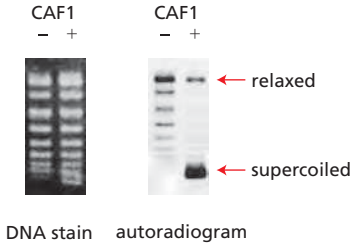


Figure 5–16 CAF1 nucleosome-assembly assay (Problem 5–54). The presence or absence of CAF1 in the assembly assay is indicated by + or –, respectively.

5–55 You have recently purified and partially sequenced a protein from a ciliated protozoan that seems to be the catalytic subunit of telomerase. You then identify the homologous gene in fission yeast, so you can perform genetic studies that are impossible in the protozoan. You make a targeted deletion of one copy of the gene in a diploid strain of the yeast and then induce sporulation to produce haploid organisms. All four spores germinate perfectly, and you are able to grow colonies on nutrient agar plates. Every 3 days, you re-streak colonies onto fresh plates. After four such serial transfers, the descendants of two of the original four spores grow

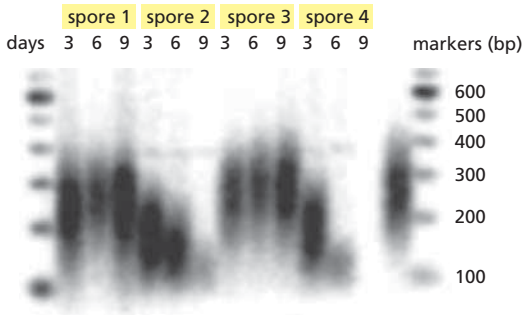


Figure 5–17 Analysis of telomeres from four fission-yeast spores (Problem 5–55). The results from normal diploid yeast are shown on the *right*, adjacent to the markers.

poorly, if at all. You take cells from the 3-, 6-, and 9-day master plates, prepare DNA from them, and cleave the samples at a chromosomal site about 35 nucleotides away from the start of the telomere repeats. You separate the fragments by gel electrophoresis, and hybridize them to a radioactive telomere-specific probe (**Figure 5–17**).

A. What is the average length of telomeres in fission yeast?
B. Do the data support the idea that you have identified yeast telomerase? If so, which spores lack telomerase?
C. Assuming that the generation time of this yeast is about 6 hours when growing on plates, by how much do the chromosomes shorten in each generation in the absence of telomerase?
D. If you were to examine the yeast cells that stop dividing, do you suppose they would be smaller, larger, or about the same size as normal yeast cells?

DNA REPAIR

TERMS TO LEARN

base excision repair
DNA repair
nonhomologous end joining
nucleotide excision repair

DEFINITIONS

Match each definition below with its term from the list above.

5–56 A means for repairing double-strand DNA breaks that links two ends with little regard for sequence homology.

5–57 Collective term for the enzymatic processes that correct deleterious changes affecting the continuity or sequence of a DNA molecule.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

5–58 DNA repair mechanisms all depend on the existence of two copies of the genetic information, one in each of the two homologous chromosomes.

5–59 Spontaneous depurination and the removal of a deaminated C by uracil DNA glycosylase leave identical substrates, which are recognized by AP endonuclease.

5–60 Only the initial steps in DNA repair are catalyzed by enzymes that are unique to the repair process; the later steps are typically catalyzed by enzymes that play more general roles in DNA metabolism.

THOUGHT PROBLEMS

5–61 Discuss the following statement: "The DNA repair enzymes that correct

damage introduced by deamination and depurination must preferentially recognize such defects on newly synthesized DNA strands."

5–62 If you compare the frequency of the sixteen possible dinucleotide sequences in the *E. coli* and human genomes, there are no striking differences except for one dinucleotide, 5′-CG-3′. The frequency of CG dinucleotides in the human genome is significantly lower than in *E. coli* and significantly lower than expected by chance. Why do you suppose that CG dinucleotides are underrepresented in the human genome?

CALCULATIONS

5–63 Ku70 and Ku80, two key proteins used in nonhomologous end joining (NHEJ), form heterodimers that bind to broken DNA ends, helping to align them for joining. How difficult is it for a Ku dimer to find a double-strand break? One way to approach this question is to estimate the average distance between Ku dimers in the nucleus: a break in the DNA will be within half that distance from a Ku dimer. If there are 4×10^5 Ku dimers in a typical nucleus, what is their average separation? Assume that the Ku dimers are randomly distributed, that a Ku dimer can be approximated as a cube, 8 nm on a side, and that the nucleus is 6 μm in diameter. [Volume of a sphere is $(4/3)\pi r^3$; volume of a cube is l^3.]

5–64 With age, somatic cells are thought to accumulate genomic "scars" as a result of the inaccurate repair of double-strand breaks by nonhomologous end joining (NHEJ). Estimates based on the frequency of breaks in primary human fibroblasts suggest that by age 70, each human somatic cell may carry some 2000 NHEJ-induced mutations due to inaccurate repair. If these mutations were distributed randomly around the genome, how many protein-coding genes would you expect to be affected? Would you expect cell function to be compromised? Why or why not? (Assume that 2% of the genome—1.5% protein-coding and 0.5% regulatory—is crucial information.)

DATA HANDLING

5–65 Several genes in *E. coli*, including *UvrA*, *UvrB*, *UvrC*, and *RecA*, are involved in repair of UV damage. Strains of *E. coli* that are defective in any one of these genes are much more sensitive to killing by UV light than are nonmutant (wild-type) cells, as shown for *UvrA* and *RecA* strains in **Figure 5–18A**. Cells mutant for pairs of these genes display a wide range of sensitivity to UV light. The combinations of *Uvr* mutations with

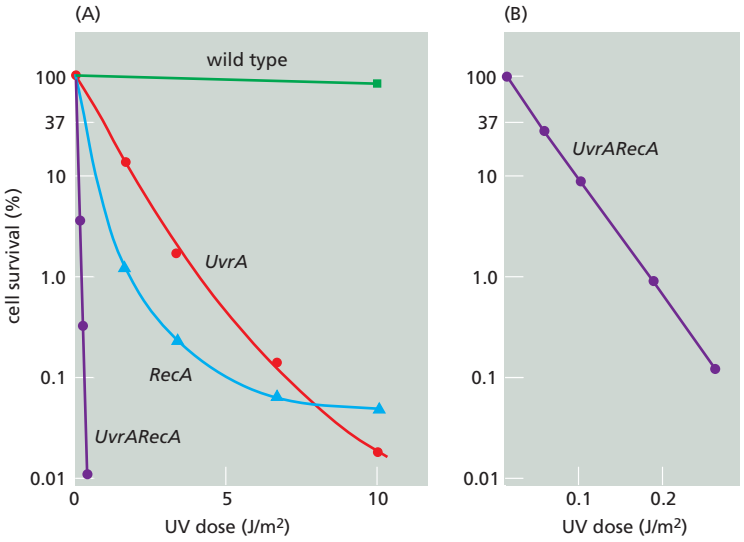


Figure 5–18 Cell survival as a function of UV dose (Problem 5–65). (A) Survival of wild-type cells, a *UvrA* mutant, a *RecA* mutant, and a *UvrARecA* double mutant. (B) An expanded scale for *UvrARecA* survival.

one another show little increase in sensitivity relative to the *Uvr* single mutants. By contrast, the combination of a *RecA* mutation with any of the *Uvr* mutations gives a strain that is exquisitely sensitive to UV light, as shown for *UvrARecA* on an expanded scale in **Figure 5–18B**.

A. Why do combinations of a *RecA* mutation with a *Uvr* mutation give an extremely UV-sensitive strain of bacteria, whereas combinations of mutations in different *Uvr* genes are no more UV-sensitive than the individual mutants?

B. According to the Poisson distribution, when a population of bacteria receives an overall average of one lethal "hit," 37% (which is e^{-1}) will survive because they receive no hits. For the double mutant *UvrARecA*, a UV dose of 0.04 joules/m^2 gives 37% survival (Figure 5–18B). Calculate the number of pyrimidine dimers that constitutes a lethal hit for the *UvrARecA* strain. *E. coli* has 4.6×10^6 base pairs in its genome (assume 50% GC). Exposure of DNA to UV light at 400 joules/m^2 converts 1% of the total pyrimidine pairs (TT, TC, CT, plus CC) to pyrimidine dimers.

5–66 The nature of the mutagenic lesion introduced by the alkylating agent MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) and the mechanism of its removal from DNA were identified in the following experiments. To determine the nature of the mutagenic lesion, untreated bacteria and bacteria that had been exposed to low doses of MNNG were incubated with 50 μg/mL ^{3}H-MNNG for 10 minutes. Bacteria exposed briefly to a low dose of MNNG induce a repair enzyme that removes methyl groups from DNA, allowing these bacteria to survive better and suffer fewer mutations than bacteria that were not pre-treated with MNNG. DNA was isolated from bacteria that were not pre-treated with MNNG and from those that were adapted by exposure to a low concentration of MNNG. The DNAs were hydrolyzed to nucleotides, and the radioactive purines were then analyzed by paper chromatography as shown in **Figure 5–19**.

The mechanism of removal of the mutagenic lesion was investigated by first purifying the enzyme responsible. The kinetics of removal were studied by incubating different amounts of the enzyme (molecular weight 19,000) with DNA containing 0.26 pmol of the mutagenic base, which was radioactively labeled with ^{3}H. At various times, samples were taken, and the DNA was analyzed to determine how much of the mutagenic base remained (**Figure 5–20**). When the experiment was repeated at 5°C instead of 37°C, the initial rates of removal were slower, but exactly the same end points were achieved.

A. Which methylated purine is responsible for the mutagenic action of MNNG?

B. What is peculiar about the kinetics of removal of the methyl group from the mutagenic base? Is this peculiarity due to an unstable enzyme?

C. Calculate the number of methyl groups that are removed by each enzyme molecule. Does this calculation help to explain the peculiar kinetics?

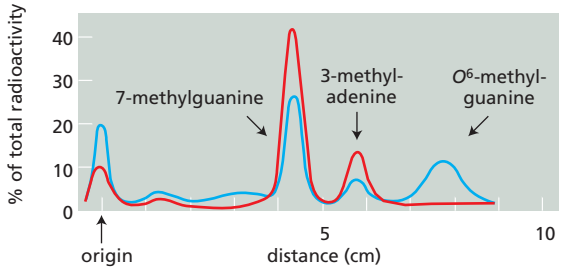


Figure 5–19 Chromatographic separation of labeled methylated purines in the DNA of untreated bacteria and bacteria treated with low doses of MNNG (Problem 5–66). The *red* line indicates methylated purines from bacteria that were not pre-treated with a low dose of MNNG; the *blue* line indicates methylated purines from bacteria that were pre-treated with a low dose of MNNG.

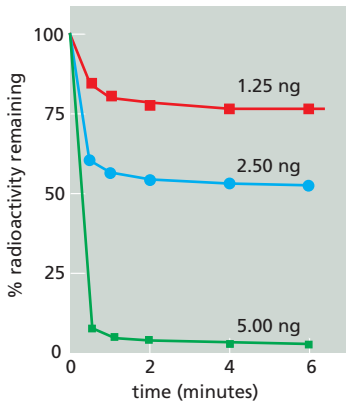


Figure 5–20 Removal of ^{3}H-labeled methyl groups from DNA by the purified enzyme (Problem 5–66). The quantities of purified enzyme are indicated.

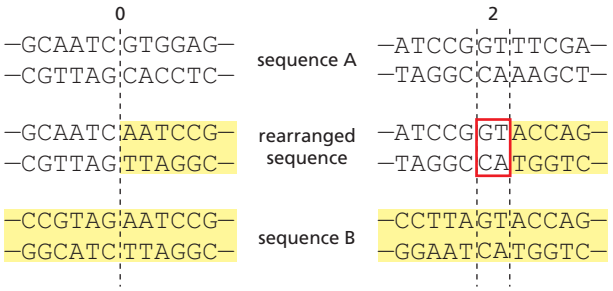


Figure 5–21 Microhomology at rearrangement junctions (Problem 5–67). In the rearrangement junction with two nucleotides of microhomology, the central GT cannot be uniquely assigned to either sequence A or sequence B; it could have come from either.

5–67 Nonhomologous end joining (NHEJ) is responsible for linking DNA sequences that are not homologous to one another. Junctions formed by NHEJ are commonly found at sites of DNA rearrangements, including translocations, inversions, duplications, and deletions. Surveys of large numbers of such junctions have revealed the presence of so-called "microhomology" at the junctions, typically ranging from 0–5 nucleotides. Examples of junctions with 0 and 2 nucleotides of microhomology are shown in **Figure 5–21**.

Are these short homologies relevant to the mechanism of NHEJ, or are they present by chance? One way to decide the issue is to compare the distribution of observed junctional microhomology with the distribution expected by chance. Microhomologies at 110 NHEJ junctions are shown along with the distribution expected by chance in **Table 5–3**. One way to determine whether these two distributions are different is to use the statistical method known as chi-square analysis. The readout of a chi-square analysis is a *P* value, which measures the probability that the observed distribution is the same as the expected distribution. Commonly, a *P* value of less than 0.05 is taken to mean that the two distributions are different. (A *P* value of 0.05 means that there is a 5% chance that the observed distribution is the same as the expected distribution.)

Using chi-square analysis, decide whether the observed distribution in Table 5–3 is the same as or different from the distribution expected by chance. If you don't have a statistics package available, search for "chi square calculator" on the Internet.

TABLE 5–3 Observed distribution of microhomologies at rearrangement junctions along with the distribution expected by chance (Problem 5–67).

	Microhomology					
Distribution	0	1	2	3	4	5
Observed	47	21	14	13	9	6
Expected	62	31	12	4	1	0

The distribution of microhomology expected by chance was calculated on the assumption that the random joining of blunt-ended DNA segments generated the junctions.

MEDICAL LINKS

5–68 Humans with the rare genetic disease xeroderma pigmentosum (XP) are extremely sensitive to sunlight and are prone to developing malignant skin cancers. Defects in any one of eight genes can cause XP. Seven XP genes encode proteins involved in nucleotide excision repair (NER). The eighth gene is associated with the XP variant (XP-V) form of the disease. Cells from XP-V patients are proficient for NER but do not

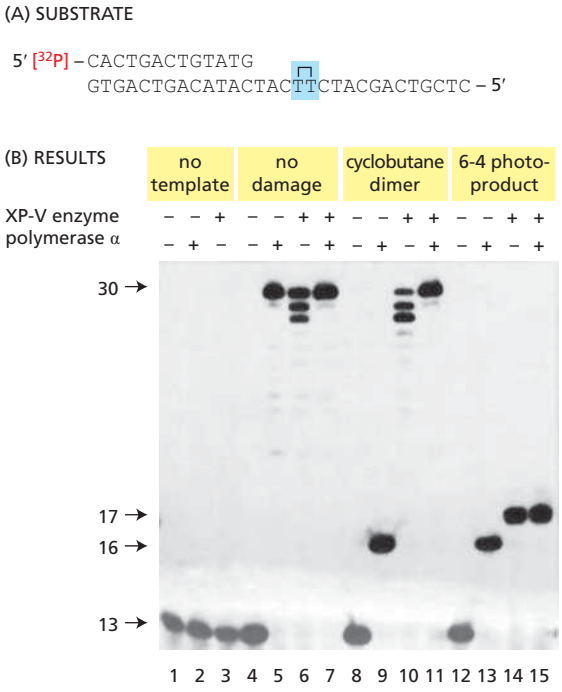


Figure 5–22 Comparison of DNA polymerase α and the XP-V enzyme (Problem 5–68). (A) Substrate for DNA polymerization. The adjacent Ts indicated by the *bracket* can be selectively modified to produce a cyclobutane dimer or a 6-4 photoproduct. (B) Results obtained with DNA polymerase α and the XP-V enzyme on damaged and undamaged templates. Numbers on the *left* indicate lengths of single-strand DNA in nucleotides.

accurately replicate UV-damaged DNA. Using a clever assay, you manage to purify from a normal cell extract the enzyme that is missing from XP-V cells. You test its ability to synthesize DNA from the simple template in **Figure 5–22A**, which contains a TT sequence. This template can be modified to contain either a cyclobutane thymine dimer or the somewhat rarer 6-4 photoproduct (two Ts linked in a different way). You compare the ability of DNA polymerase α (a normal replicative polymerase) and the XP-V enzyme to synthesize DNA from the undamaged template, the template with a cyclobutane dimer, and the template with a 6-4 photoproduct. By labeling the primer (the shorter DNA strand) at its 5′ end and denaturing the reaction products, it is possible to determine whether DNA synthesis has occurred (**Figure 5–22B**).

A. Is the XP-V enzyme a DNA polymerase? Why or why not?
B. How does the XP-V enzyme differ from DNA polymerase α on an undamaged template? On a template with a cyclobutane dimer? On a template with a 6-4 photoproduct?
C. How accurately do you suppose that the XP-V enzyme copies normal DNA? Would you guess it to be error-prone or faithful?
D. If NER is normal in patients with XP-V, why are they sensitive to sunlight and prone to skin cancers?

5–69 Mutagens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and methyl nitrosourea (MNU) are potent DNA-methylating agents and are extremely toxic to cells. Nitrosoguanidines are used in research as mutagens and clinically as drugs in cancer chemotherapy because they preferentially kill cells in the act of replication.

The original experiment that led to the discovery of the alkylation repair system in bacteria was designed to assess the long-term effects of exposure to low doses of MNNG (as in chemotherapy), in contrast to brief exposure to large doses (as in mutagenesis). Bacteria were placed first in a low concentration of MNNG (1 μg/mL) for 1.5 hours and then in fresh medium lacking MNNG. At various times during and after exposure to the low dose of MNNG, samples of the culture were treated with a high concentration of MNNG (100 μg/mL) for 5 minutes and then tested for viability and the frequency of mutants. As shown in **Figure 5–23**,

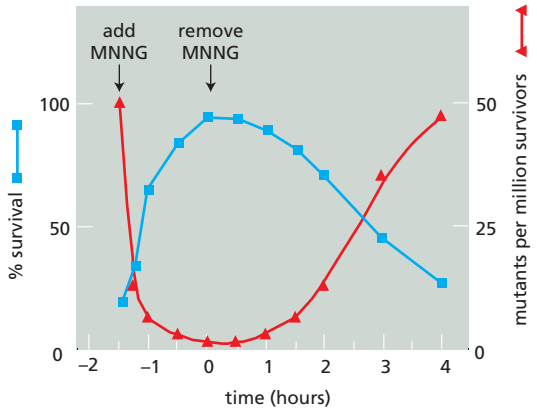


Figure 5–23 Adaptive response of *E. coli* to low doses of MNNG (Problem 5–69). MNNG at 1 μg/mL was present from –1.5 to 0 hours, as indicated by *arrows*. Samples were removed at various times and treated briefly with 100 μg/mL MNNG to assess the number of survivors and the frequency of mutants.

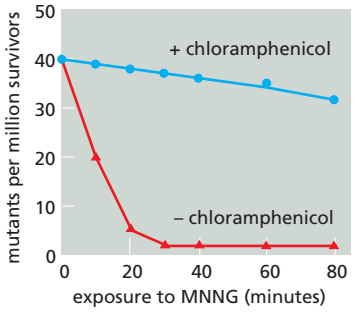


Figure 5–24 Effects of chloramphenicol on the adaptive response to low doses of MNNG (Problem 5–69). After different times of exposure to 1 μg/mL MNNG, samples were removed and treated with 100 μg/mL MNNG to measure susceptibility to mutagenesis.

exposure to low doses of MNNG temporarily increased the number of survivors and decreased the frequency of mutants among the survivors. As shown in **Figure 5–24**, the adaptive response to low doses of MNNG was prevented if chloramphenicol (an inhibitor of protein synthesis) was included in the incubation.

A. Does the adaptive response of *E. coli* to low levels of MNNG require activation of preexisting protein or the synthesis of new protein?
B. Why do you think the adaptive response to a low dose of MNNG is so short-lived?

HOMOLOGOUS RECOMBINATION

TERMS TO LEARN

allele	homologous recombination	Rad51
gene conversion	hybridization	RecA
Holliday junction	loss of heterozygosity	strand exchange

DEFINITIONS

Match each definition below with its term from the list above.

5–70 One of a set of alternative forms of a gene. In a diploid cell, each gene will have two of these, each occupying the same position (locus) on homologous chromosomes.

5–71 Process by which DNA sequence information can be transferred from one DNA helix (which remains unchanged) to another DNA helix whose sequence is altered.

5–72 Experimental process in which two complementary nucleic acid strands form a double helix; a powerful technique for detecting specific nucleotide sequences.

5–73 X-shaped structure observed in DNA undergoing recombination, in which the two DNA molecules are held together at the site of crossing-over, also called a cross-strand exchange.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

5–74 Homologous recombination requires relatively long regions of homologous DNA on both partners in the exchange.

5–75 Gene conversion requires a limited amount of DNA synthesis.

THOUGHT PROBLEMS

5–76 Using **Figure 5–25A** as a guide, draw the products of a crossover recombination event between the homologous regions of the molecules represented in **Figure 5–25B**. In the figure, single lines represent the DNA duplex and arrows represent the targets for homologous recombination.

5–77 Haploid yeast cells that preferentially repair double-strand breaks by homologous recombination are especially sensitive to agents that cause double-strand breaks in DNA. If the breaks occur in the G_1 phase of the cell cycle, most yeast cells die; however, if the breaks occur in the G_2 phase, a much higher fraction of cells survive. Explain these results.

5–78 When plasmid DNA is extracted from *E. coli* and examined by electron microscopy, the majority of molecules are monomeric circles, but there are a variety of other forms, including dimeric and trimeric circles. In addition, about 1% of the molecules appear as figure-8 forms, in which the two loops are equal (**Figure 5–26A**).

You suspect that the figure-8 forms are recombination intermediates in the formation of a dimer from two monomers (or two monomers from a dimer). To rule out the possibility that they might represent twisted dimers or touching monomers, you digest the DNA sample with a restriction nuclease that cuts at a single site in the monomer, and then examine the molecules. After cutting, only two forms are seen: 99% of the DNA molecules are linear monomers, and 1% are χ (chi) forms (**Figure 5–26B**). You note that the χ forms have an interesting property: the two longer arms are the same length, as are the two shorter arms. In addition, the sum of the lengths of a long arm and a short arm is equal to the length of the monomer plasmid. The position of the crossover point, however, is completely random.

Unsure of yourself and feeling you are probably missing some hidden artifact, you show your pictures to a friend. She points out that your observations prove you are looking at recombination intermediates that arose by random pairing at homologous sites.

A. Is your friend correct? What is her reasoning?
B. How would you expect your observations to differ if you repeated the experiments in a strain of *E. coli* that carried a nonfunctional *RecA* gene?
C. How would the χ forms have differed from those you observed if the figure-8 forms had arisen as intermediates in a site-specific recombination between the monomers?
D. What would the χ forms have looked like if the figure-8 forms had arisen as intermediates in a totally random, nonhomologous recombination between the monomers?

5–79 Discuss the following statement: "The Holliday junction contains two distinct pairs of strands (crossing strands and noncrossing strands), which cannot be interconverted without breaking the phosphodiester backbone of at least one strand."

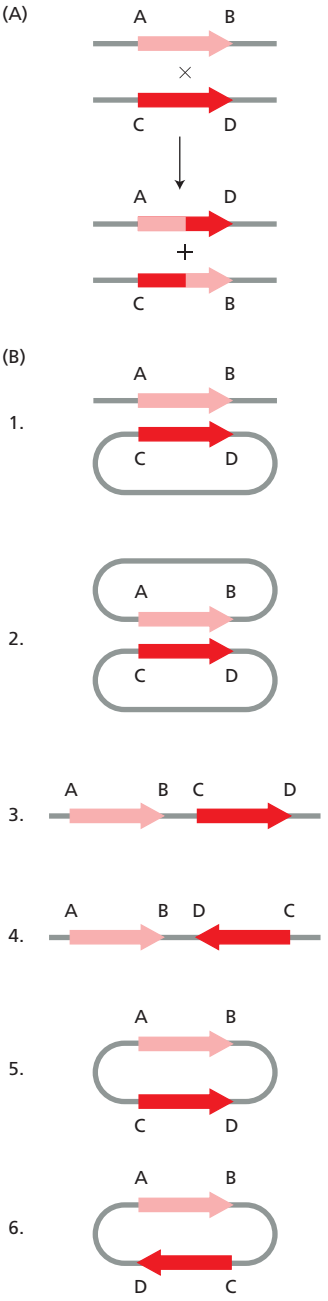


Figure 5–25 A variety of recombination substrates (Problem 5–76).

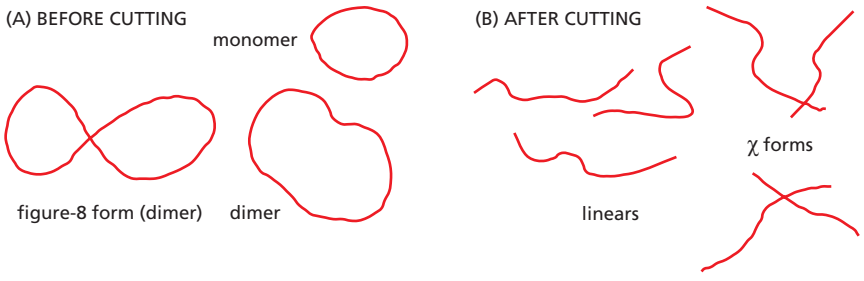


Figure 5–26 Structures of plasmid molecules (Problem 5–78). (A) Before digestion. (B) After digestion with a single-cut restriction nuclease.

5–80 Draw the structure of the double Holliday junction that would result from strand invasion by both ends of the broken duplex into the intact homologous duplex shown in **Figure 5–27**. Label the left end of each strand in the Holliday junction 5′ or 3′ so that the relationship to the parental and recombinant duplexes is clear. Indicate how DNA synthesis would be used to fill in any single-strand gaps in your double Holliday junction.

Figure 5–27 A broken duplex with single-strand tails ready to invade an intact homologous duplex (Problem 5–80).

5–81 In addition to correcting DNA mismatches, the mismatch repair system functions to prevent homologous recombination from taking place between similar but not identical sequences. Why would recombination between similar, but nonidentical sequences pose a problem for human cells?

DATA HANDLING

5–82 In *E. coli*, RecA protein catalyzes both the initial pairing step of recombination and subsequent branch migration. It promotes recombination by binding to single-strand DNA and catalyzing the pairing of such coated single strands to homologous double-strand DNA. One assay for the action of RecA is the formation of double-strand DNA circles from a mixture of double-strand linear molecules and homologous single-strand circles, as illustrated in **Figure 5–28**. This reaction proceeds in two steps: circles pair with linears at an end and then they branch-migrate until a single-strand linear DNA is displaced.

One important question about the RecA reaction is whether branch migration is directional. This question has been studied in the following way. Single-strand circles, which were uniformly labeled with ^{32}P, were mixed with unlabeled double-strand linear molecules in the presence of RecA. As the single-strand DNA pairs with the linear DNA, it becomes sensitive to cutting by restriction nucleases, which do not cut single-strand DNA. By sampling the reaction at various times, digesting the DNA with a restriction nuclease, and separating the labeled fragments by electrophoresis, you obtain the pattern shown in **Figure 5–29A**.

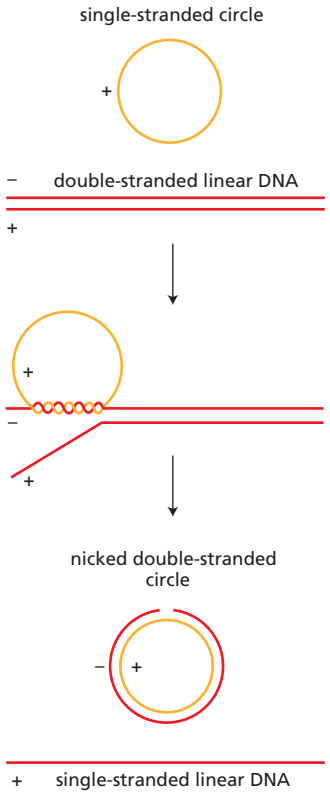


Figure 5–28 Strand assimilation assay for RecA (Problem 5–82). The single-strand (+) circle is complementary to the minus (–) strand and identical to the plus strand of the duplex.

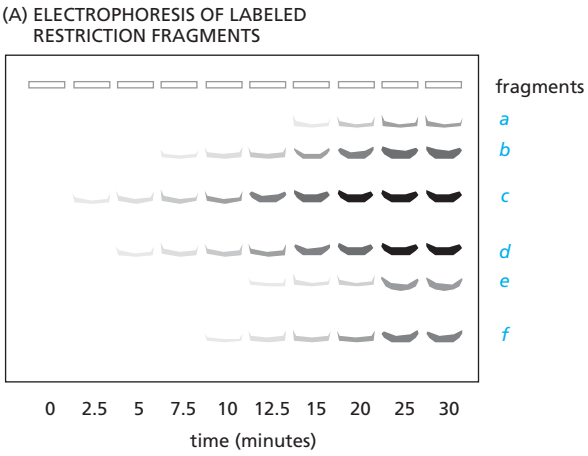


Figure 5–29 Analysis of branch migration catalyzed by RecA (Problem 5–82). (A) Electrophoretic separation of labeled restriction fragments as a function of time of incubation with RecA. (B) Sites of cleavage represented on the single-strand DNA circle. Clockwise around the circle is 5′ to 3′.

A. By comparing the time of appearance of labeled fragments with the restriction map of the circular DNA in **Figure 5–29B**, deduce which end (5′ or 3′) of the minus strand of the linear DNA the circular plus strand pairs with initially. Also deduce the direction of branch migration along the minus strand. (The linear double-strand DNA was cut at the boundary between fragments *a* and *c* on the restriction map.)
B. Estimate the rate of branch migration, given that the length of this DNA is 7 kb.
C. What would you expect to happen if the linear double-strand DNA carried an insertion of 500 nonhomologous nucleotides between restriction fragments *e* and *a*?

5–83 Most eukaryotic cells use two different mechanisms to repair double-strand breaks in DNA: homologous recombination and nonhomologous end joining. The recombination pathway is favored by a homolog of the Rad52 protein, first defined in yeast. Relatives of the protein known as Ku stimulate the end-joining pathway.

You have purified the human Rad52 protein and are studying its properties. When you mix Rad52 with linear DNA that has short (300-nucleotide) single-strand tails and examine the mixture in the electron microscope, you see structures like the one shown in **Figure 5–30A**. (Such structures are much rarer when the linear DNA is blunt-ended.) You then expose Rad52-bound, uniformly radiolabeled DNA to nucleases and measure digestion of DNA by release of soluble fragments. As shown in **Figure 5–30B**, Rad52 protects the DNA from exonuclease digestion but not from digestion by an endonuclease.

A. Where does Rad52 bind on the linear DNA? What features of the DNA are important for Rad52 binding?
B. How does Rad52 protect against digestion by the exonuclease, but not against digestion by the endonuclease?
C. How do the properties of Rad52 revealed by these observations fit into its role in recombinational repair of double-strand breaks?

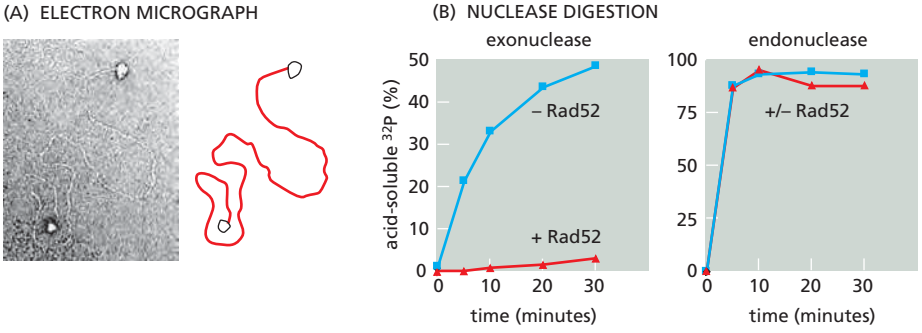


Figure 5–30 Analysis of the role of Rad52 in repair of double-strand breaks (Problem 5–83). (A) Binding of Rad52 to linear DNA. A schematic representation is shown adjacent to the micrograph. (B) Nuclease digestion of DNA in the presence (+) and absence (–) of bound Rad52.

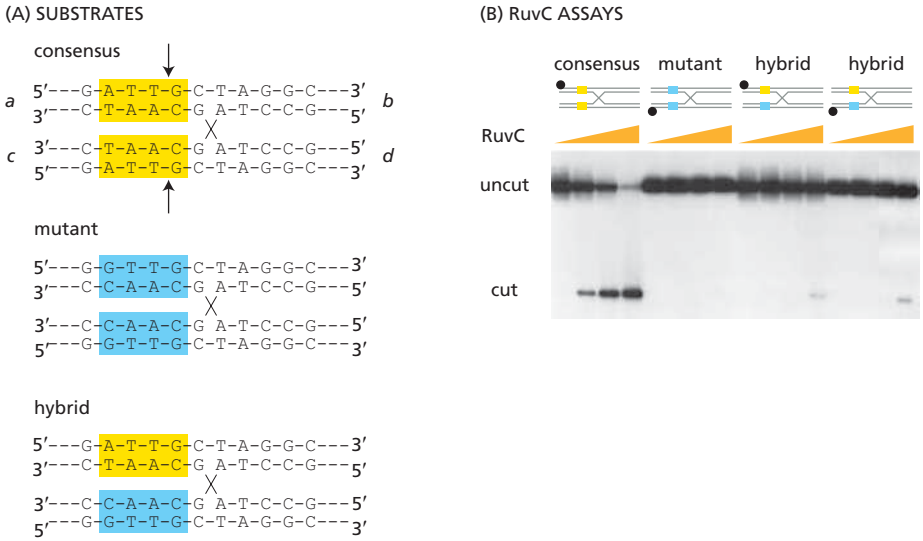


Figure 5–31 Analysis of Holliday junction cleavage (Problem 5–84). (A) Effects of DNA sequence on resolution of Holliday junctions. Cleavable sequences are shown in *yellow* boxes; uncleavable sequences are shown in *blue* boxes. *Arrows* indicate the sites of cleavage in a Holliday junction with consensus sequences. (B) Resolution of Holliday junctions by RuvC. Increasing concentrations of RuvC in each lane are indicated schematically by *triangles.* The locations of the labeled 5′ ends are indicated by *black dots.*

5–84 The mechanism of Holliday junction cleavage by RuvC has been investigated using artificial junctions created by annealing oligonucleotides together (**Figure 5–31A**). Each of the duplexes involved in these junctions has unique sequences at its ends, which allowed the oligonucleotides to anneal to form the indicated Holliday junctions; they also possess a core of 11 nucleotides that are homologous. The Holliday junction can branch-migrate within the core region. A dimer of RuvC binds to Holliday junctions and cleaves a pair of adjacent strands between nucleotides 3 and 4 in the sequences 5′-ATTG, 5′-ATTC, 5′-TTTG, or 5′-TTTC, as shown for 5′-ATTG in Figure 5–31A. These four sequences are represented by the consensus sequence 5′-$^{A}/_{T}TT^{G}/_{C}$.

You want to know whether the two subunits of the RuvC dimer coordinate the cleavages on the two strands or can act independently. To investigate this you make the three Holliday junctions shown in Figure 5–31A: one with two cleavable sequences, one with two uncleavable sequences, and one with one cleavable and one uncleavable sequence. You label the 5′ end of one strand in each, incubate them with RuvC, and analyze cleavage by electrophoresis on denaturing gels, so that the oligonucleotides separate from one another. The results are shown in **Figure 5–31B**.

A. What fraction of all possible four-nucleotide sequences is cleaved by RuvC?
B. Does the requirement for resolution at a limited set of specific sequences seriously restrict the sites in the *E. coli* genome at which Holliday junctions can be resolved?
C. Do the two subunits of the RuvC dimer coordinate their cleavage of the two strands or can they act independently? Explain your reasoning.
D. Draw out the duplex products generated by RuvC resolution of the "consensus" Holliday junction in Figure 5–31A, the one with its ends marked by letters.

TRANSPOSITION AND CONSERVATIVE SITE-SPECIFIC RECOMBINATION

TERMS TO LEARN

conservative site-specific recombination
DNA-only transposon
nonretroviral retrotransposon
phase variation
retroviral-like retrotransposon
retrovirus
reverse transcriptase
transposable element
transposition
transposon

DEFINITIONS

Match each definition below with its term from the list above.

5–85 Length of DNA that moves from a donor site to a target site either by cut-and-paste transposition or by replicative transposition.

5–86 Enzyme that makes a double-strand DNA copy from a single-strand RNA template molecule.

5–87 RNA-containing virus that replicates in a cell by first making a double-strand DNA intermediate.

5–88 Rearrangement of DNA that depends on the breakage and rejoining of two DNA helices at specific sequences on each DNA molecule.

TRUE/FALSE

Consider the following statement and explain your answer.

5–89 When transposable elements move around the genome, they rarely integrate into the middle of a gene because gene disruption—a potentially lethal event to the cell and the transposon—is selected against by evolution.

THOUGHT PROBLEMS

5–90 Cre recombinase is a site-specific enzyme that catalyzes recombination between two LoxP DNA recognition sequences. Cre recombinase pairs two LoxP sites in the same orientation, breaks both duplexes at the same point in each LoxP site, and joins the ends with new partners so that each LoxP site is regenerated, as shown schematically in **Figure 5–32A**. Based on this mechanism, predict the arrangement of sequences that will be generated by Cre-mediated site-specific recombination for each of the two DNAs shown in **Figure 5–32B**.

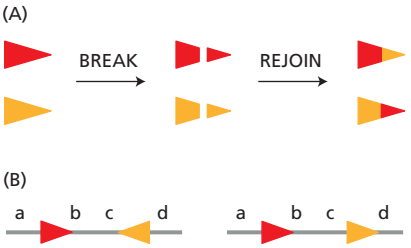


Figure 5–32 Cre recombinase-mediated site-specific recombination (Problem 5–90). (A) Schematic representation of Cre/LoxP site-specific recombination. The LoxP sequences in the DNA are represented by *triangles* that are colored so that the site-specific recombination event can be followed more readily. In reality their DNA sequences are identical. (B) DNA substrates containing two arrangements of LoxP sites.

DATA HANDLING

5–91 You are studying the prokaryotic transposon Tn10 and have just figured out an elegant way to determine whether Tn10 replicates during transposition or moves by a cut-and-paste mechanism. Your idea is based on the key difference between these two mechanisms: both parental strands of the Tn10 move in cut-and-paste transposition, whereas only one parental strand moves in replicative transposition (**Figure 5–33**).

You plan to mark the individual strands by annealing strands from two different Tn10s. Both Tn10s contain a gene for tetracycline resistance and a gene for lactose metabolism (*LacZ*), but in one, the *LacZ* gene is inactivated by a mutation. This difference provides a convenient way to follow the two Tn10s since *LacZ*$^+$ bacterial colonies (when incubated with an appropriate substrate) turn blue, but *LacZ*$^-$ colonies remain white. You denature and reanneal a mixture of the two transposon DNAs, which produces an equal mixture of heteroduplexes and homoduplexes

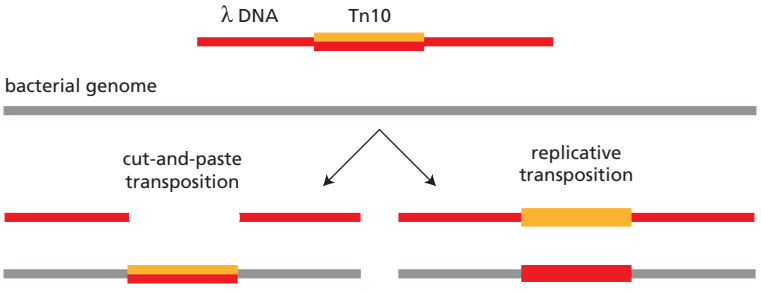


Figure 5–33 Replicative and cut-and-paste transposition of a transposable element (Problem 5–91). The transposable element is shown as a heteroduplex, which is composed of two genetically different strands—one *red* and one *yellow.* During replicative transposition, one strand stays with the donor DNA and one strand is transferred to the bacterial genome. In cut-and-paste transposition, the transposable element is cut out of the donor DNA and transferred entirely to the bacterial genome.

(**Figure 5–34**). You introduce them into *LacZ*⁻ bacteria, and spread the bacteria onto Petri dishes that contain tetracycline and the color-generating substrate.

Once inside a bacterium, the transposon will move (at very low frequency) into the bacterial genome, where it confers tetracycline resistance on the bacterium. The rare bacterium that gains a Tn10 survives the selective conditions and forms a colony. When you score a large number of such colonies, you find that roughly 25% are white, 25% are blue, and 50% are mixed, with one blue sector and one white sector.

A. Explain the source of each kind of bacterial colony and decide whether the results support a replicative or a cut-and-paste mechanism for Tn10 transposition.

B. You performed these experiments using a recipient strain of bacteria that was incapable of repairing mismatches in DNA. How would you expect the results to differ if you used a bacterial strain that could repair mismatches?

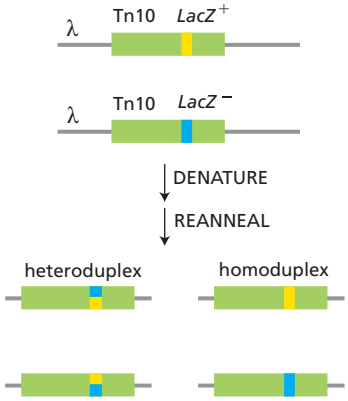


Figure 5–34 Formation of a mixture of heteroduplexes and homoduplexes by denaturing and reannealing two different Tn10 genomes (Problem 5–91). The Tn10 DNA is shown as a *box*. The sequence differences between the two Tn10s are indicated by the *blue* and *yellow* segments.

5–92 The Ty elements of the yeast *Saccharomyces cerevisiae* move to new locations in the genome by transposition through an RNA intermediate. Normally, the Ty-encoded reverse transcriptase is expressed at such a low level that transposition is very rare. To study the transposition process, you engineer a cloned version of the Ty element so that the gene for reverse transcriptase is linked to the galactose control elements. You also "mark" the element with a segment of bacterial DNA so that you can detect it specifically and thus distinguish it from other Ty elements in the genome. As a target gene to detect transposition, you use a defective histidine (*His*⁻) gene whose expression is dependent on the insertion of a Ty element near its 5′ end. You show that yeast cells carrying a plasmid with your modified Ty element generate *His*⁺ colonies at a frequency of 5×10^{-8} when grown on glucose. When the same cells are grown on galactose, the frequency of *His*⁺ colonies is 10^{-6}, a 20-fold increase.

You notice that cultures of cells with the Ty-bearing plasmid grow normally on glucose but very slowly on galactose. To investigate this phenomenon, you isolate individual colonies that arise under three different conditions: *His*⁻ colonies grown in the presence of glucose, *His*⁻ colonies grown in the presence of galactose, and *His*⁺ colonies grown in the presence of galactose. You eliminate the plasmid from each colony, isolate DNA from each culture, and analyze it by gel electrophoresis and blot hybridization, using the bacterial marker DNA as a probe. Your results are shown in **Figure 5–35**.

A. Why does transposition occur so much more frequently in cells grown on galactose than it does in cells grown on glucose?

B. As shown in Figure 5–35, *His*⁻ cells isolated after growth on galactose have about the same number of marked Ty elements in their chromosomes as *His*⁺ cells that were isolated after growth on galactose. If transposition is independent of histidine selection, why is the frequency of Ty-induced *His*⁺ colonies so low (10^{-6})?

C. Why do you think it is that cells with the Ty-bearing plasmid grow so slowly on galactose?

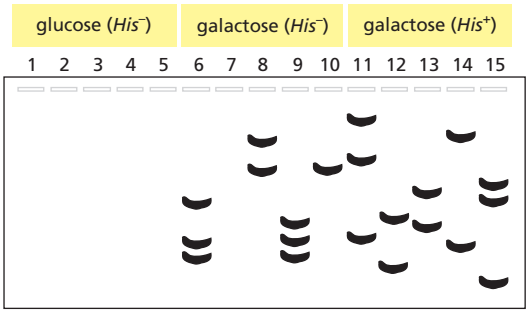


Figure 5–35 Analysis of cells that harbored a plasmid carrying a modified Ty element (Problem 5–92). Cells were initially grown on glucose or galactose as a carbon source. *His*⁻ cells were grown in the presence of histidine; *His*⁺ cells were grown in its absence. Bands indicate restriction fragments that hybridized to the marker DNA originally present in the Ty element carried by the plasmid.

MCAT STYLE

Passage 1 (Questions 5–93 to 5–95)

Taq DNA polymerase, which comes from the thermotolerant bacterium *Thermus aquaticus*, is used extensively for the polymerase chain reaction (PCR) because it remains functional at the high temperatures required for PCR. In a typical PCR, a DNA sample is heated to separate the two strands; two short single strands of DNA, called primers, are annealed to their complementary sequences in the DNA on either side of the segment to be amplified; and Taq polymerase then synthesizes DNA starting from the primers. After synthesis has occurred, the reaction is heated to high temperatures to separate the DNA strands, and then cooled to begin another round of primer-directed DNA synthesis. The cycle is repeated many times, leading to amplification of the DNA between the primers.

5–93 One problem with Taq polymerase is that it incorporates the wrong base approximately once every 8000 bases, a frequency of mistakes that is far higher than the error rates for DNA polymerases that carry out DNA replication in cells. Which one of the following statements best explains the high error rate of Taq polymerase in PCR?

A. Taq polymerase cannot make Okazaki fragments efficiently.
B. Taq polymerase lacks a 3′-to-5′ proofreading exonuclease.
C. Taq polymerase often falls off the DNA, interrupting synthesis.
D. Taq polymerase synthesizes DNA in the 3′-to-5′ direction.

5–94 Another problem with Taq polymerase is that it is only capable of synthesizing relatively small pieces of DNA, with an upper limit of around 4000 bases. Which one of the following is a likely explanation for the inability of Taq polymerase to synthesize long pieces of DNA?

A. Taq polymerase can make only relatively short Okazaki fragments.
B. Taq polymerase cannot remove mismatched bases from the 3′ end.
C. Taq polymerase lacks the helicase required for strand separation.
D. Taq polymerase reactions lack the primase needed for new primers.

5–95 Imagine that you have used PCR to amplify a specific region of DNA about 4000 bases long. You then ligate the PCR products into a plasmid vector and transform it into bacteria. One of the transformed bacteria receives a plasmid that includes a PCR product with two separate mismatched bases resulting from Taq polymerase errors the last time the DNA was copied. You grow this bacterium into a colony and isolate the population of plasmids derived from the single transformed plasmid. Assuming that the mismatches were resolved by the cell's mismatch repair machinery prior to replication of the plasmid, what are the chances that both mismatches were converted to mutations?

A. 100% chance
B. 50% chance
C. 25% chance
D. 0% chance

Passage 2 (Question 5–96)

The first DNA polymerase to be discovered was purified from *E. coli* and called DNA polymerase I. It synthesized DNA at a rate of about 10–20 bases per second, but could only synthesize about 25–30 bases before falling off the template DNA. In addition to its polymerase activity, it also possessed both 5′-to-3′ and 3′-to-5′ exonuclease activities, which could act on DNA or RNA hybridized to a DNA template. Another DNA polymerase—DNA polymerase III—was discovered later. It could synthesize much longer stretches of DNA at a rate of about a thousand bases per second, and had only a 3′-to-5′ exonuclease activity.

5–96 Based on this information, which one of the following functions best describes the role of DNA polymerase I in bacterial cells?

A. Modifies Okazaki fragments for joining into intact strands.
B. Repairs any DNA that is damaged during transcription.
C. Synthesizes most of the cellular DNA during replication.
D. Synthesizes RNA primers to initiate Okazaki fragments.

Reading the Genome.
The complete sequence of the human genome printed out occupies 7 shelves of 16 volumes, each containing hundreds of pages like the one shown here, with almost 10 million characters in each volume printed in 4-point courier type without any index. Try to see if you can find the gene in this sequence, which comes from human chromosome 11, open on the shelf in the photograph that was taken at the opening of the Wellcome Museum, London, in July 2007.

How Cells Read the Genome: From DNA to Protein

CHAPTER 6

IN THIS CHAPTER

FROM DNA TO RNA

TERMS TO LEARN

consensus nucleotide sequence
DNA supercoiling
DNA transcription
exon
exosome
general transcription factor
intron
mRNA (messenger RNA)
noncoding RNA
nuclear pore complex
promoter
RNA polymerase
RNA splicing
rRNA gene
snoRNA (small nucleolar RNA)
snRNA (small nuclear RNA)
spliceosome
TATA box
terminator

DEFINITIONS

Match the definition below with its term from the list above.

6–1 Helps to position the RNA polymerase correctly at the promoter, to aid in pulling apart the two strands of DNA to allow transcription to begin, and to release RNA polymerase from the promoter into the elongation mode once transcription has begun.

6–2 Small RNA molecules that are complexed with proteins to form the ribonucleoprotein particles involved in RNA splicing.

6–3 Nucleotide sequence in DNA to which RNA polymerase binds to begin transcription.

6–4 A large protein complex containing multiple 3′-to-5′ RNA exonucleases that degrade improperly processed mRNAs, introns, and other RNA debris retained in the nucleus.

6–5 The enzyme that carries out transcription.

6–6 RNA molecule that specifies the amino acid sequence of a protein.

6–7 Process in which intron sequences are excised from RNA transcripts in the nucleus during the formation of messenger and other RNAs.

6–8 Signal in bacterial DNA that halts transcription.

6–9 Segment of a eukaryotic gene consisting of a sequence of nucleotides that will be represented in mRNA or other functional RNAs.

6–10 Large multiprotein structure forming a channel through the nuclear envelope that allows selected molecules to move between nucleus and cytoplasm.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

6–11 The consequences of errors in transcription are less severe than those of errors in DNA replication.

6–12 The σ subunit is a permanent component of the RNA polymerase holoenzyme from *E. coli*, allowing it to initiate at appropriate promoters in the bacterial genome.

6–13 Eukaryotic mRNA molecules carry 3′ ribosyl OH groups at both their 3′ and 5′ ends.

6–14 Since introns are largely genetic "junk," they do not have to be removed precisely from the primary transcript during RNA splicing.

6–15 RNA polymerase II generates the end of a pre-mRNA transcript when it ceases transcription and releases the transcript; a poly-A tail is then quickly added to the free 3′ end.

THOUGHT PROBLEMS

6–16 Consider the expression "central dogma," which refers to the proposition that genetic information flows from DNA to RNA to protein. Is the word "dogma" appropriate in this scientific context?

6–17 In the electron micrograph in **Figure 6–1**, are the RNA polymerase molecules moving from right to left or from left to right? How can you tell? Why do the RNA transcripts appear so much shorter than the length of DNA that encodes them?

6–18 Match the following list of RNAs with their functions.

A. mRNA	1. blocks translation of selected mRNAs
B. rRNA	2. modification and processing of rRNA
C. snoRNA	3. protects germ line from transposable elements
D. snRNA	4. components of ribosome
E. tRNA	5. splicing of RNA transcripts
F. piRNA	6. directs degradation of selected mRNAs
G. miRNA	7. codes for proteins
H. siRNA	8. adaptor for protein synthesis

6–19 An RNA polymerase is transcribing a segment of DNA that contains the sequence

```
5′-GTAACGGATG-3′
3′-CATTGCCTAC-5′
```

If the polymerase transcribes this sequence from left to right, what will the sequence of the RNA be? What will the RNA sequence be if the polymerase moves right to left?

6–20 What are the roles of general transcription factors in RNA polymerase II-mediated transcription, and why are they referred to as "general"?

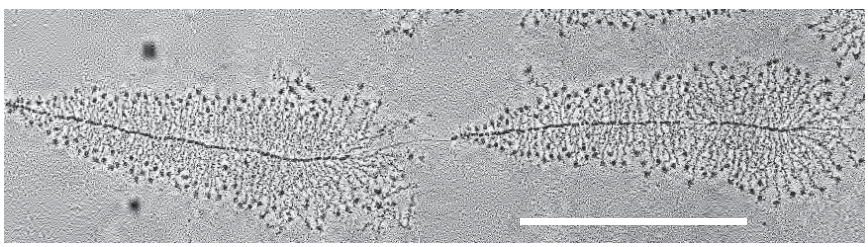


Figure 6–1 Transcription of two adjacent rRNA genes (Problem 6–17). The scale bar is 1 μm. (Courtesy of Ulrich Scheer.)

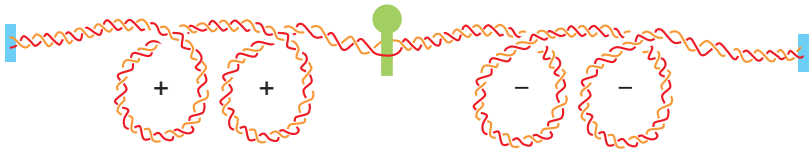


Figure 6–2 Supercoils around a moving RNA polymerase (Problem 6–21).

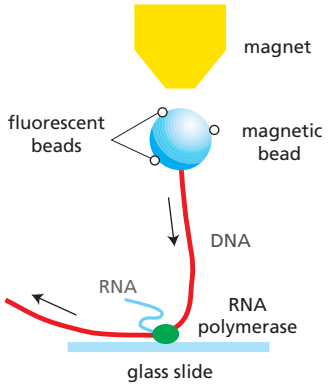


Figure 6–3 System for measuring the rotation of DNA caused by RNA polymerase (Problem 6–22). The magnet holds the bead upright (but doesn't interfere with its rotation), and the attached tiny fluorescent beads allow the direction of motion to be visualized under the microscope. RNA polymerase is held in place by attachment to the glass slide.

6–21 In which direction along the template must the RNA polymerase in Figure 6–2 be moving to have generated the supercoiled structures that are shown? Would you expect supercoils to be generated if the RNA polymerase were free to rotate about the axis of the DNA as it progressed along the template?

6–22 You have attached an RNA polymerase molecule to a glass slide and have allowed it to initiate transcription on a template DNA that is tethered to a magnetic bead as shown in Figure 6–3. If the DNA with its attached magnetic bead moves relative to the RNA polymerase as indicated in the figure, in which direction will the bead rotate?

6–23 Why doesn't transcription cause a hopeless tangle? If the RNA polymerase does not revolve around the DNA as it moves, it will induce two DNA supercoils—one in front and one behind—for every 10 nucleotides it transcribes. If, instead, RNA polymerase revolves around the DNA—avoiding DNA supercoiling—then it will coil the RNA around the DNA duplex, once for every 10 nucleotides it transcribes. Thus, for any reasonable-size gene, the act of transcription should result in hundreds of coils or supercoils...and that's for every single RNA polymerase! So why doesn't transcription lead to a complete snarl?

6–24 You are studying a DNA virus that makes a set of abundant proteins late in its infectious cycle. An mRNA for one of these proteins maps to a DNA restriction fragment from the middle of the linear genome. To determine the precise location of this mRNA you anneal it with the purified restriction fragment under conditions where only DNA–RNA hybrid duplexes are stable and DNA strands do not reanneal. When you examine the reannealed DNA–RNA duplexes by electron microscopy, you see structures such as that in Figure 6–4. Why are there single-stranded tails at the ends of the DNA–RNA duplex region?

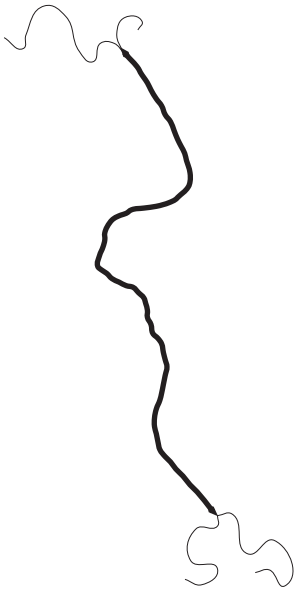


Figure 6–4 DNA–RNA hybrid between an mRNA and a DNA restriction fragment from adenovirus (Problem 6–24).

6–25 Smilin is a (hypothetical) protein that causes people to be happy. It is inactive in many chronically unhappy people. The mRNA isolated from a number of different unhappy individuals in the same family was found to lack an internal stretch of 173 nucleotides that are present in the Smilin mRNA isolated from a control group of generally happy people. The DNA sequences of the Smilin genes from the happy and unhappy persons were determined and compared. They differed by just one nucleotide change—and no nucleotides were deleted. Moreover, the change was found in an intron.

A. Can you hypothesize a molecular mechanism by which a single nucleotide change in a gene could cause the observed *internal* deletion in the mRNA?
B. What consequences for the Smilin protein would result from removing a 173-nucleotide-long internal stretch from its mRNA? Assume that the 173 nucleotides are deleted from the coding region of the Smilin mRNA.
C. What can you say about the molecular basis of unhappiness in this family?

6–26 The human α-tropomyosin gene is alternatively spliced to produce different forms of α-tropomyosin mRNA in different cell types (Figure 6–5).

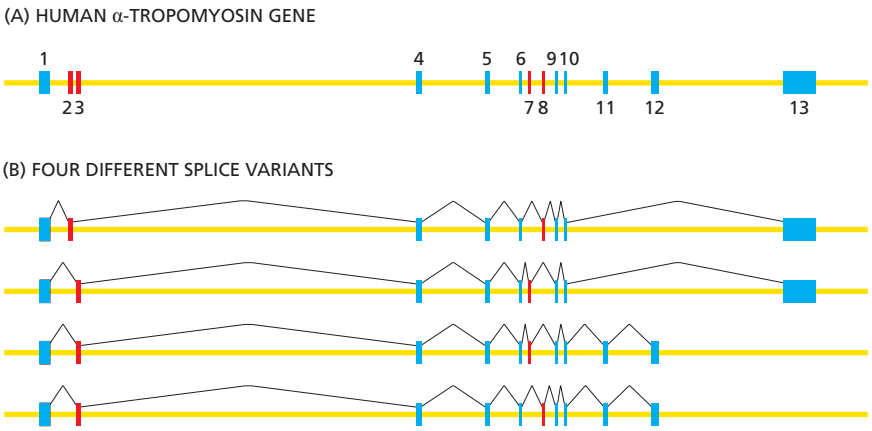


Figure 6–5 Alternatively spliced mRNAs from the human α-tropomyosin gene (Problem 6–26). (A) Exons in the human α-tropomyosin gene. The locations and relative sizes of exons are shown by *blue* and *red rectangles*, with alternative exons in *red*. (B) Splicing patterns for four α-tropomyosin mRNAs. Splicing is indicated by *lines* connecting the exons that are included in the mRNA.

For all forms of the mRNA, the protein sequences encoded by exon 1 are the same, as are the protein sequences encoded by exon 10. Exons 2 and 3 are alternative exons used in different mRNAs, as are exons 7 and 8. Which of the following statements about exons 2 and 3 is the most accurate? Is that statement also the most accurate one for exons 7 and 8? Explain your answers.

A. Exons 2 and 3 must have the same number of nucleotides.
B. Exons 2 and 3 must each contain an integral number of codons (that is, the number of nucleotides divided by 3 must be an integer).
C. Exons 2 and 3 must each contain a number of nucleotides that when divided by 3 leaves the same remainder (that is, 0, 1, or 2).

6–27 You have printed out a set of DNA sequences around the intron/exon boundaries for genes in the β-globin family, and have taken the thick file to the country to study for the weekend. When you look at the printout, you discover to your annoyance that there's no indication of where in the gene you are. You know that the sequences in **Figure 6–6** come from one of the exon/intron or intron/exon boundaries and that the boundaries lie on the dotted line, but you don't know the order of the intron and exon. You know that introns begin with the dinucleotide sequence GT and end with AG, but you realize that these particular sequences would fit *either* as the start *or* the finish of an intron.

If you cannot decide which side is the intron, you will have to cut your weekend short and return to the city (or find a neighbor with Internet access). In desperation, you consider the problem from an evolutionary perspective. You know that introns evolve faster (suffering more nucleotide changes) than exons because they are not constrained by function. Does this perspective allow you to identify the intron, or will you have to pack your bags?

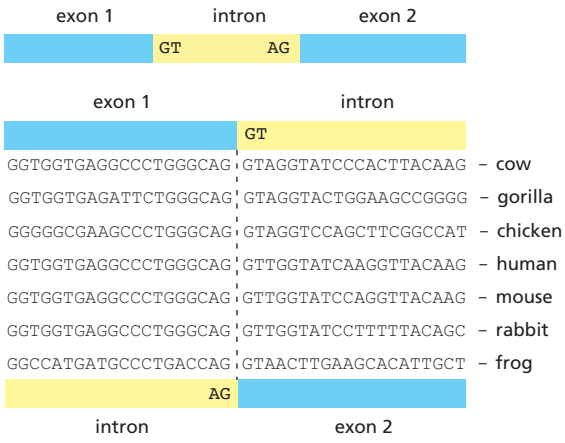
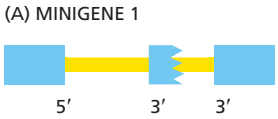


Figure 6–6 Aligned DNA sequences from the β-globin genes in different species (Problem 6–27). As indicated by the gene structures shown *above* and *below*, the DNA sequences could come from the boundary of exon 1 with the intron, or from the boundary of the intron with exon 2.



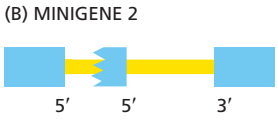


Figure 6–7 A test for intron scanning during RNA splicing (Problem 6–28). (A) Minigene with two 3′ splice sites. (B) Minigene with two 5′ splice sites. *Boxes* represent complete (*rectangles*) or partial (*ragged edge*) exons; 5′ and 3′ splice junctions are indicated.

6–28 Many eukaryotic genes contain a large number of exons. Correct splicing of such genes requires that neighboring exons be ligated to one another; if they are not, exons will be left out. Since 5′ splice sites look alike, as do 3′ splice sites, it is remarkable that skipping an exon occurs so rarely. Some mechanism must keep track of neighboring exons and ensure that they are brought together.

One early proposal suggested that the splicing machinery bound to a splice site at one end of an intron and scanned through the intron to find the splice site at the other end. Such a scanning mechanism would guarantee that an exon was never skipped. This hypothesis was tested with two minigenes: one with a duplicated 5′ splice site and the other with a duplicated 3′ splice site (**Figure 6–7**). These minigenes were transfected into cells and their RNA products were analyzed to see which 5′ and 3′ splice sites were selected during splicing.

A. Draw a diagram of the products you expect from each minigene if the splicing machinery binds to a 5′ splice site and scans toward a 3′ splice site. Diagram the expected products if the splicing machinery scans in the opposite direction.

B. When the RNA products from the transfected minigenes were analyzed, it was found that each minigene generated a mixture of the two possible 5′-to-3′ splice products. Based on these results, would you conclude that neighboring exons are brought together by intron scanning? Why or why not?

6–29 What does "export ready" mRNA mean, and what distinguishes an "export ready" mRNA from a bit of excised intron that needs to be degraded?

6–30 The nucleolus disappears at each mitosis and then reappears during G_1 of the next cell cycle. How is this reversible process thought to be accomplished?

CALCULATIONS

6–31 You have established a transcription assay in which transcripts initiate at a specific adenovirus promoter in a plasmid (see Problem 6–35). Each transcript is 400 nucleotides long and has an overall composition of C_2AU. These transcripts accumulate linearly for about an hour and then reach a plateau. Your assay conditions use a 25 μL reaction volume containing 16 μg/mL of DNA template (the plasmid, which is 3.5 kb in length) with all other components in excess. From the specific activity of the ^{32}P-CTP and the total radioactivity in the transcripts, you calculate that at the plateau 2.4 pmol of CMP was incorporated. (The mass of a nucleotide pair is 660 daltons.)

A. How many transcripts are produced per reaction?

B. How many templates are present in each reaction?

C. How many transcripts are made per template in the reaction?

DATA HANDLING

6–32 If RNA polymerase proofreads its product in a manner analogous to DNA polymerase, it must slow its rate of nucleotide incorporation after adding an incorrect base to the end of the growing RNA chain. This delay will allow time for removal of the mismatched nucleotide before the next one is added.

TABLE 6–1 Rate of incorporation of correct and incorrect nucleotides (Problem 6–32).

Incorporated nucleotide (bold)	V_{max} (units/sec)
CU**A**	0.20
CU**G**	0.0015
CUA**C**	0.17
CUG**C**	0.036

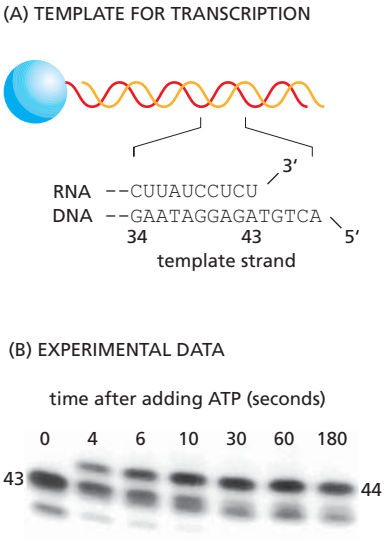


Figure 6–8 Determination of kinetic parameters for RNA polymerase (Problem 6–32). (A) DNA template attached to an agarose bead. (B) Rate of incorporation of the correct A nucleotide in the nascent RNA chain. (The bands in adjacent lanes form a curve because of an artifact of gel electrophoresis.)

To investigate this possibility, scientists devised a clever technique to measure the rate of nucleotide incorporation by RNA polymerase at a defined point in a DNA template. The template contained a promoter and was covalently attached to agarose beads, so that it (and the attached RNA polymerase) could be removed from solution and washed, and then reincubated in a new mixture of nucleotides (**Figure 6–8A**). By using a series of solutions containing just one or a couple of nucleotides, the RNA polymerase was "walked" along the template to the site shown in Figure 6–8A. At that point, the RNA polymerase and template were resuspended in a solution containing one nucleotide, and the rate of incorporation of that nucleotide was measured as shown in **Figure 6–8B**. The rates of incorporation of A and G at position +44 in the RNA strand are shown in **Table 6–1**. In addition, the incorporation of the following C at position +45 was measured after A or G was incorporated at position +44 (Table 6–1).

A. Imagine that the RNA polymerase was stopped so that the last nucleotide in the RNA chain was the C at position +34. Describe how you might "walk" the polymerase from there to position +43 to do these experiments.

B. Is the correct nucleotide preferred over the incorrect nucleotide? If so, by what factor is it preferred? Does the presence of an incorrect nucleotide influence the rate of addition of the next nucleotide? Explain your answer.

6–33 In **Figure 6–9**, the sequences of 13 promoters recognized by the σ^{70} factor of RNA polymerase have been aligned. Deduce the consensus sequences for the –10 and –35 regions of these promoters.

6–34 Deletion analysis of protein-binding sequences in a promoter can be difficult to interpret because altered spacing between elements can critically affect their function. The "linker-scanning" method eliminates this potential difficulty by replacing 10-nucleotide segments throughout the promoter with oligonucleotide linkers. A classic paper described this method in an analysis of the promoter for the thymidine kinase (*Tk*)

Figure 6–9 Sequences recognized by σ^{70} factor (Problem 6–33). Different σ factors are designated by their molecular masses; σ^{70} has a mass of 70 kilodaltons. *Dashes* represent spaces that have been added to maximize alignment of sequences in the –10 and –35 regions.

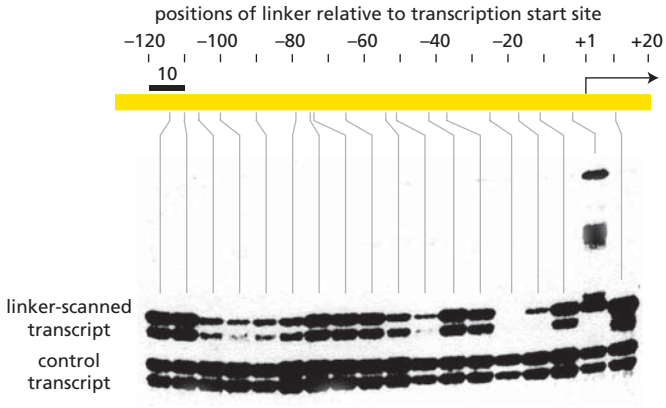


Figure 6–10 Linker-scanning analysis of the *Tk* promoter (Problem 6–34). Transcripts from linker-scanned plasmids and control plasmids were analyzed by primer extension, using a radiolabeled primer corresponding to sequences about 80 nucleotides from the 5′ end of the transcript. These primers were extended to the 5′ ends of the transcripts and the products were displayed on a denaturing polyacrylamide gel. Two bands are present for both the control and linker-scanned transcripts because of inefficient extension to the very end of the transcript. The position of each linker is indicated at the *center* of the segment it replaced. A 10-base-pair bar—the length of the replacements—is shown at the *top left* of the figure.

gene. Plasmids in which 10-nucleotide segments had been replaced with linkers were injected into *Xenopus laevis* oocytes along with control plasmids to measure injection efficiency. Results of these injections are shown in **Figure 6–10**.

A. Estimate from these experiments the locations of sequences that are critical for promoter function, and rank their relative importance.

B. Which, if any, of these elements do you suppose corresponds to the TATA box?

6–35 Purification of a transcription factor typically requires a rapid assay to prevent inactivation of the factor before it can be identified. One key technical advance was to use a promoter linked to a 400-nucleotide DNA sequence that contained no C nucleotides. When GTP is omitted from the assay mixture (but CTP, UTP, and ATP are included), the only long RNA transcript is made from the synthetic DNA sequence because all other transcripts terminate when a G is required. This set-up allows a rapid assay of specific transcription simply by measuring the incorporation of a radioactive nucleotide into the long transcript.

To test this idea, two plasmids were constructed that carried the synthetic sequence: one with a promoter from adenovirus (pML1), the other without a promoter (pC1) (**Figure 6–11A**). Each plasmid was mixed with pure RNA polymerase II, transcription factors, UTP, ATP, and ^{32}P-CTP. In addition, various combinations of GTP, RNAse T1 (which cleaves RNA adjacent to each G nucleotide), and 3′ *O*-methyl GTP (which terminates transcription whenever G is incorporated) were added. The products were measured by gel electrophoresis, with the results shown in **Figure 6–11B**.

A. Why is the 400-nucleotide transcript absent from lane 4 but present in lanes 2, 6, and 8?

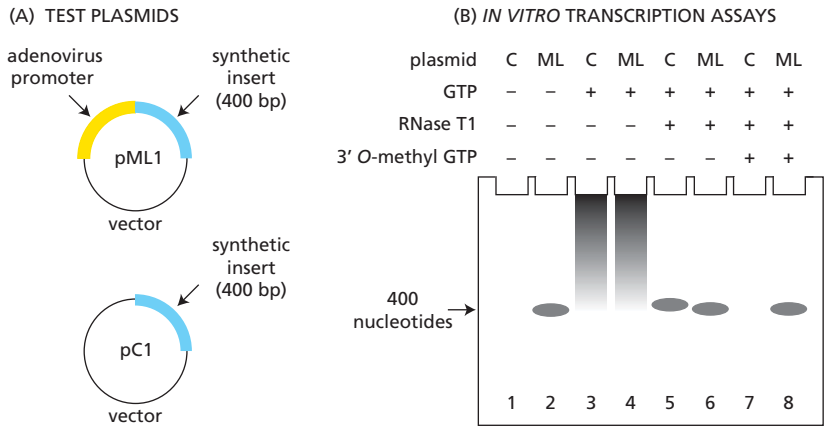


Figure 6–11 Characterization of transcription using a template without C nucleotides (Problem 6–35). (A) Structures of test plasmids. (B) Results of transcription assays under various conditions. All reactions contain RNA polymerase II, transcription factors, UTP, ATP, and ^{32}P-CTP. Other components are listed *above* each lane. C is plasmid pC1; ML is plasmid pML1.

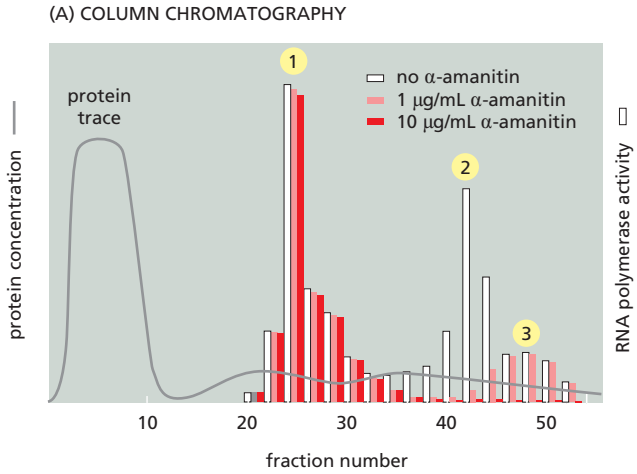


Figure 6–12 Characterization of RNA polymerase activities (Problem 6–36). (A) Peaks of RNA polymerase activity after column chromatography. Activities were measured in the presence of various concentrations of α-amanitin. (B) The *Amanita phalloides* mushroom. (Courtesy of Fred Stevens.)

B. Can you guess the source of the synthesis in lane 3 when the promoterless pC1 plasmid is used?
C. Why is a transcript of about 400 nucleotides present in lane 5 but not in lane 7?
D. The goal in developing this ingenious assay was to aid the purification of transcription factors; however, that process will begin with crude cell extracts, which will contain GTP. How do you suppose you might assay specific transcription in crude extracts?

6–36 When RNA polymerases were first being characterized in eukaryotes, three peaks of polymerizing activity (1, 2, and 3 in **Figure 6–12A**) were commonly obtained by fractionating cell extracts on chromatography columns. It was unclear whether these peaks corresponded to different RNA polymerases or just to different forms of one polymerase. Incubating the three polymerase fractions in the presence of 1 μg/mL or 10 μg/mL α-amanitin (from *Amanita phalloides*, the world's deadliest mushroom; **Figure 6–12B**) gave the results shown in Figure 6–12A. Do these results argue for different RNA polymerases or different forms of the same RNA polymerase? Explain your answer.

6–37 The large subunit of eukaryotic RNA polymerase II in yeast has a CTD (C-terminal domain) that comprises 27 near-perfect repeats of the sequence YSPTSPS. If the normal RNA polymerase II gene is replaced with one that encodes a CTD with only 11 repeats, the cells are viable at 30°C but are unable to grow at 12°C. This cold sensitivity allows suppressors to be selected for growth at 12°C. Some of these suppressors proved to be dominant mutations in the previously unknown gene *Srb2*.

Extracts prepared from yeast that are lacking the *Srb2* gene cannot transcribe added DNA templates, but they can be activated for transcription by the addition of Srb2 protein. To test the role of Srb2 in transcription, plasmid DNAs with either a short or a long G-free sequence downstream of a promoter (**Figure 6–13A**) were incubated separately in the presence or absence of a *limiting* amount of Srb2 [and in the absence of added nucleotide triphosphates (NTPs) so that transcription could not begin]. The reactions were then mixed and transcription was initiated at various times afterward by adding a mixture of all four NTPs that included ^{32}P-CTP (**Figure 6–13B**). After a brief incubation (so that transcription did not have time to reinitiate) the products were displayed by gel electrophoresis. Whichever template was preincubated with Srb2 was the one that was transcribed (**Figure 6–13C**). By contrast, if an *excess* of Srb2 was mixed with one template during the preincubation, transcription was observed from both templates after mixing.

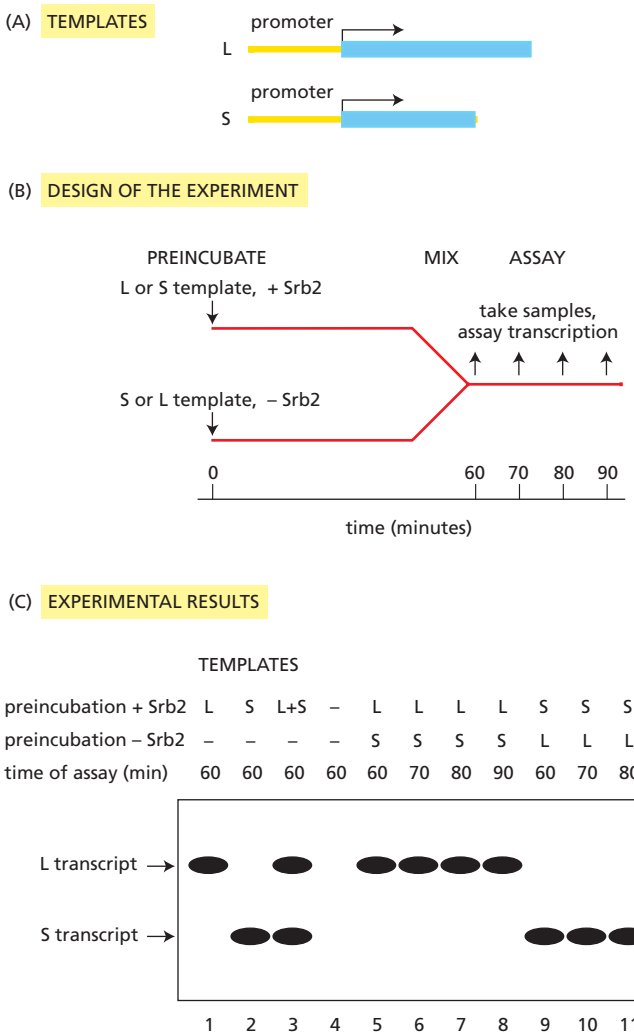


Figure 6–13 Experiments to test the role of Srb2 in transcription (Problem 6–37). (A) Short (S) and long (L) G-free templates. (B) Experimental design. (C) Experimental results. The transcripts from the S and L templates were visualized by autoradiography.

A. Did Srb2 show a preference for either template when the preincubation was carried out with the individual templates or the mixture (Figure 6–13C, lanes 1 to 3)?
B. Do the results indicate that Srb2 acts stoichiometrically or catalytically? How so?
C. Is Srb2 part of the complex of proteins that forms on the template before transcription is initiated, or does it act after transcription has begun? How can you tell?
D. What do you think happens during the preincubation that so strongly favors transcription from the template that was included in the preincubation?
E. Do these results indicate that Srb2 binds to the CTD of RNA polymerase II?

6–38 Detailed features of the active site of RNA polymerase have been probed using modified RNA nucleotides that can react with nearby targets. In one study, an analog of U carrying a reactive chemical group (**Figure 6–14A**) was incorporated at one of two positions in a radiolabeled RNA and then "walked" to specific locations relative to the 3′ end of the RNA chain using a technique like that described in Problem 6–32 (**Figure 6–14B**). When the analog was then activated, it reacted almost exclusively either with the adenine it was paired with in the DNA template strand or with the polymerase (**Figure 6–14C**). In both series of experiments, the pattern

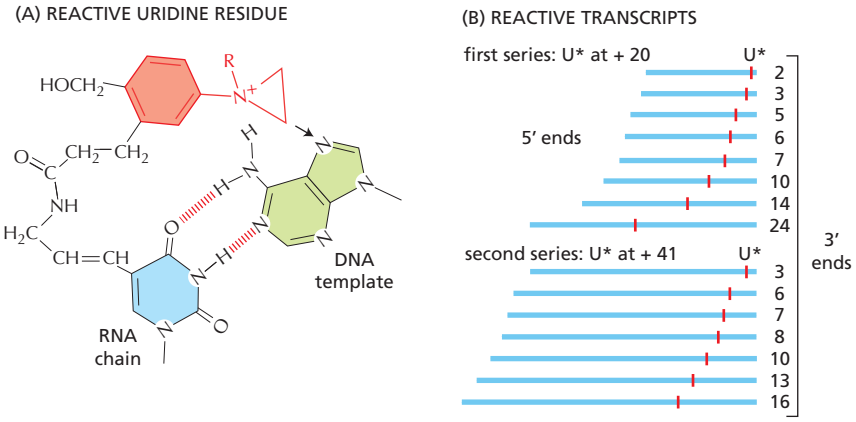


Figure 6–14 Probing the active site of RNA polymerase (Problem 6–38). (A) The reactivity of a U analog with adenine in the template DNA. The *arrow* indicates the site of attack. (B) Locations of the U analog in the RNA chain. *Numbers* refer to the number of nucleotides from the reactive U to the 3′ end. (C) The pattern of reaction of the reactive U with DNA and protein.

of reactivity with DNA and protein varied depending on the position of the reactive analog relative to the 3′ end of the RNA. Why do you suppose that the activated U reacts so strongly with DNA up to a point and then abruptly begins to react more strongly with protein?

6–39 The intron–exon structure of eukaryotic genes came as a shock. In the early, skeptical days, the most convincing demonstration was visual, as shown, for example, by the electron micrograph in **Figure 6–15A**. This image was obtained by hybridizing ovalbumin mRNA to a long segment of DNA that contained the gene. To those used to looking at single- and double-stranded nucleic acids in the electron microscope, the structure was clear: a set of single-stranded tails and loops emanating from a central duplex segment whose ends corresponded to the ends of the mRNA (**Figure 6–15B**). To the extent possible, describe the intron–exon structure of this gene.

6–40 The interaction of U1 snRNP with the sequences at the 5′ ends of introns is usually shown to involve pairing between the nucleotides in the pre-mRNA and those in the RNA component of the U1 snRNP, as illustrated in **Figure 6–16A**. But given the relatively small number of bases involved,

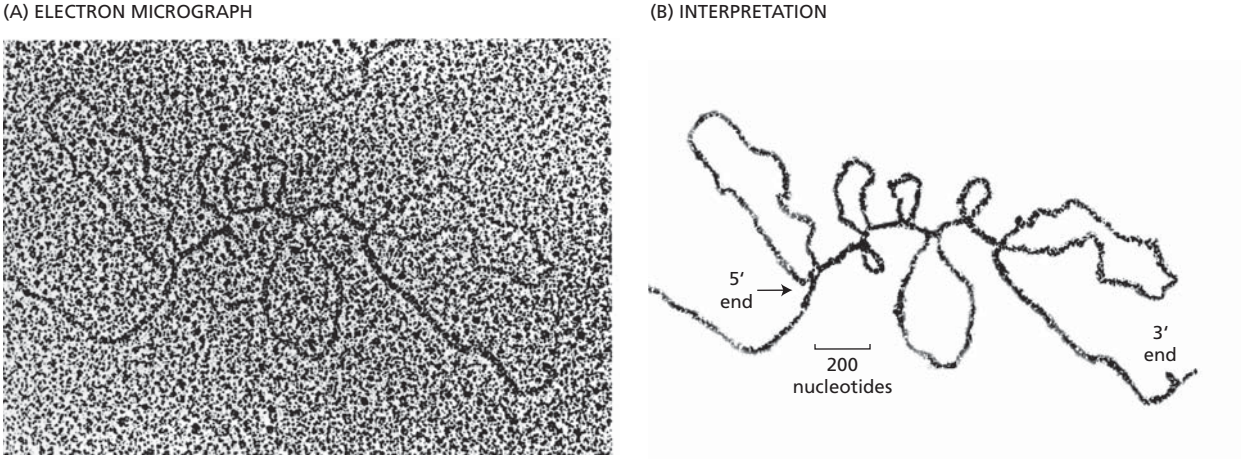


Figure 6–15 Hybrid between ovalbumin mRNA and a DNA segment containing its gene (Problem 6–39). (A) An electron micrograph. (B) The hybrid with background eliminated. Each of the loops is formed by single-stranded DNA emanating from a central RNA–DNA duplex.

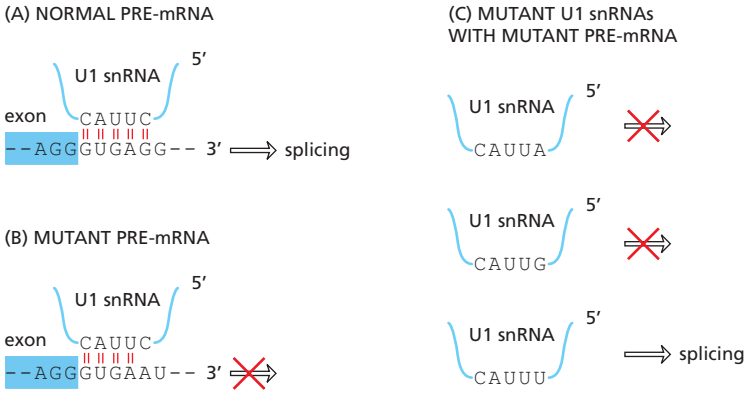


Figure 6–16 Characterization of the pairing of U1 snRNP with pre-mRNA (Problem 6–40). (A) Interaction of U1 snRNP with a normal pre-mRNA. (B) Interaction of U1 snRNP with a mutant pre-mRNA. (C) Mutant U1 snRNAs.

that arrangement is hardly convincing. A series of experiments tested the hypothesis that base-pairing was critical to the function of U1 snRNP in splicing. As shown in **Figure 6–16B**, a mutant pre-mRNA was generated that could not be spliced. Several mutant U1 snRNAs were then tested for their ability to promote splicing of the mutant pre-mRNA, with the results shown in **Figure 6–16C**. Do these experiments argue that base-pairing is critical to the role of U1 snRNP in splicing? If so, how?

6–41 The AAUAAA sequence just 5′ of the polyadenylation site is a critical signal for polyadenylation, as has been verified in many ways. One elegant confirmation used chemical modification to interfere with specific protein interactions. RNA molecules containing the signal sequence were radiolabeled at one end and then treated with diethylpyrocarbonate, which can modify A and G nucleotides, rendering the RNA sensitive to breakage by subsequent aniline treatment. The experimental conditions employed were such that the RNA molecules contained about one modification each. They were then cleaved with aniline. The resulting series of fragments have lengths that correspond to the positions of As and Gs in the RNA (**Figure 6–17**, lane 1).

To define critical A and G nucleotides, the modified, but still intact, RNAs were incubated with an extract capable of cleavage and polyadenylation. The RNAs were then separated into those that had acquired a poly-A tail (poly-A^+) and those that had not (poly-A^-). These two fractions were treated with aniline and the fragments were analyzed by gel electrophoresis (Figure 6–17, lanes 2 and 3). In a second reaction, EDTA was added to the extract along with the modified RNAs; this does not affect RNA cleavage but prevents addition of the poly-A tail. These cleaved RNAs were isolated, treated with aniline, and examined by electrophoresis (lane 4).

A. At which end were the starting RNA molecules labeled?
B. Explain why the bands corresponding to the AAUAAA signal (bracket in Figure 6–17) are missing from the poly-A^+ RNA and the cleaved RNA.
C. Explain why the band at the arrow (the normal nucleotide to which poly A is added) is missing from the poly-A^+ RNA but is present in the cleaved RNA.
D. Which A and G nucleotides are important for cleavage, and which A and G nucleotides are important for addition of the poly-A tail?
E. What additional information might be obtained by labeling the RNA molecules at the other end?

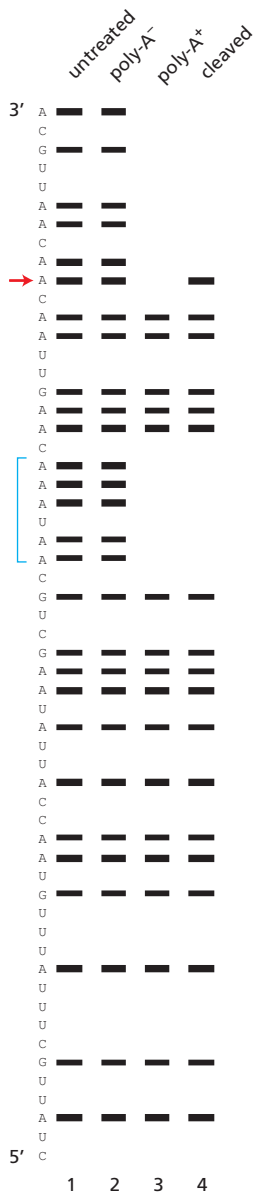


Figure 6–17 Autoradiographic analysis of experiments to define the purines important in RNA cleavage and polyadenylation (Problem 6–41). The sequence of the precursor RNA is shown at the *left* with the 5′ end at the *bottom* and the 3′ end at the *top*. All RNAs were modified by reaction with diethylpyrocarbonate. RNA that was not treated with extract (untreated) is shown in lane 1. RNA that was treated with extract but was not polyadenylated (poly-A^-) is shown in lane 2. RNA that was polyadenylated (poly-A^+) in the extract is shown in lane 3. RNA that was cleaved but not polyadenylated (cleaved) is shown in lane 4. Shorter RNA fragments run faster; that is, they travel farther toward the bottom of the gel during electrophoresis.

Figure 6–18 Processing of histone precursor RNAs (Problem 6–42). (A) Nucleotide sequences of histone precursor RNAs. *Horizontal arrows* indicate the inverted repeat sequences capable of forming a stem-loop structure in the precursor, the *vertical arrow* indicates the site of cleavage, and the *red shading* shows the position of the conserved region. (B) DNA oligonucleotides used in the experiments. (C) Sequence of human U7 snRNA. The trimethylated cap, m_3G, is characteristic of "U" RNAs. N refers to nucleotides whose identity was unknown.

6–42 Unlike most mRNAs, histone mRNAs do not have poly-A tails. They are processed from a longer precursor by cleavage just 3′ of a stem-loop structure in a reaction that depends on a conserved sequence near the cleavage site (**Figure 6–18A**). A classic paper defined the role of U7 snRNP in cleavage of the histone precursor RNA.

When nuclear extracts from human cells were treated with a nuclease to digest RNA, they lost the ability to cleave the histone precursor. Adding back a crude preparation of snRNPs restored the activity. The extract could also be inactivated by treatment with RNAse H (which cleaves RNA in an RNA–DNA hybrid) in the presence of DNA oligonucleotides containing the conserved sequence—the suspected site of snRNP interaction in the histone precursor (**Figure 6–18B**). Somewhat surprisingly, the mouse and mammalian consensus oligonucleotides completely blocked histone processing, but the human oligonucleotide had no effect. The two inhibitory oligonucleotides also caused the disappearance of a 63-nucleotide snRNA, which was then partially sequenced and identified as U7 snRNA (**Figure 6–18C**).

A. Explain the design of the oligonucleotide experiment. What were these scientists trying to accomplish by incubating the extract with a DNA oligonucleotide in the presence of RNAse H?

B. Since a human extract was used, do you find it surprising that the human oligonucleotide did not inhibit processing, whereas the mouse and consensus oligonucleotides did? Can you offer an explanation for this result?

6–43 In eukaryotes, two distinct classes of snoRNAs, which are characterized by conserved sequence motifs termed boxes, are responsible for 2′-*O*-methylation and pseudouridylation. Box C/D snoRNAs direct 2′-*O*-ribose methylation, and box H/ACA snoRNAs direct pseudouridylation of target RNAs. Recently a novel snoRNA, called U85, was shown to contain both box elements (**Figure 6–19**). You want to know how these box elements participate in the function of U85 snoRNA. Examination of potential substrate RNAs revealed that U85 snoRNA could potentially pair with a region of the U5 spliceosomal snRNA that carries two 2′-*O*-methylated nucleotides, U41 and C45, and two pseudouridines, ψ43 and ψ46 (**Figure 6–20A**).

To determine whether these U5 snRNA sequences were true substrates for modification by U85 snoRNA, the relevant segment of U5 was inserted into a region of the U2 snRNA gene to create a distinctive U2–U5 hybrid molecule that could be readily followed. In addition, a second hybrid molecule, U2–U5m, was constructed to contain a mutant segment of U5, and a mutant U85 snoRNA, U85m, was generated with compensating changes in the guide sequences adjacent to the box H and box D regions, so that it could pair with U2–U5m (**Figure 6–20B**). When expression vectors for U2–U5, U2–U5m, and U85m were transfected into cells, the encoded RNAs were shown to accumulate normally. 2′-*O*-methylation and pseudouridylation in the critical region in the

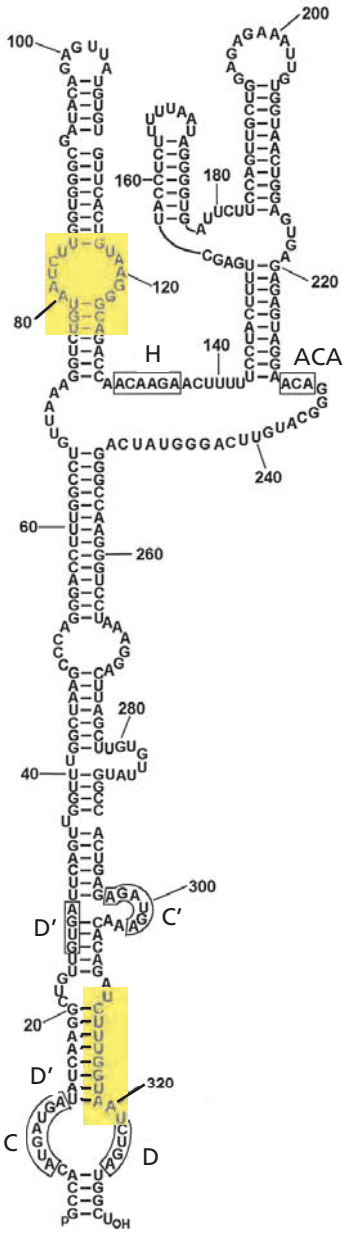


Figure 6–19 Proposed secondary structure of the human U85 snoRNA (Problem 6–43). Box elements are indicated by *boxes*. Regions that are thought to be important for targeting 2′-*O*-methylation and pseudouridylation are highlighted in *yellow*.

TABLE 6–2 Modification of potential target nucleotides in various transfections (Problem 6–43).

		Transfected molecules			Modifications[b]			
					Pseudouridine		2′-*O*-Methyl	
	U85[a]	U85m	U2–U5	U2–U5m	U43	U46	U41	C45
1	+		+		–	ψ	m	m
2	+			+	–	–	m	–
3	+	+		+	–	ψ	m	m

[a]Endogenous U85 is present in all cells.
[b]Nucleotides that have been converted to pseudouridine are indicated by ψ; nucleotides that have been 2′-*O*-methylated are indicated by an m.

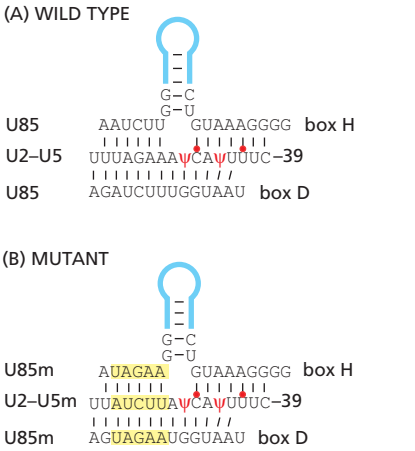


Figure 6–20 Analysis of potential RNA targets for modification by U85 snoRNA (Problem 6–43). (A) Potential pairing between the guide sequences in U85 snoRNA and a segment of U5 in U2–U5 snRNA. Pairing with the guide sequence adjacent to box H, which typically directs pseudouridylation, is shown *above* U2–U5. Pairing with the guide sequence adjacent to box D, which typically directs 2′-*O*-methylation, is shown *below* U2–U5. Only one pairing could occur at a time. Pseudouridines are indicated with ψ, and sites of 2′-*O*-methylation are indicated with *dots*. (B) Potential pairing between the mutant guide sequences in U85m snoRNA and the mutant segment of U5 in U2–U5m.

U2–U5 and U2–U5m molecules were detected by sequencing, as summarized in **Table 6–2**.

What are the expectations of these experiments if bases in U5 snRNA serve as bona fide targets for U85 snoRNA-dependent modification? Which, if any, of the naturally modified nucleotides in U5—methylated riboses at U41 and C45, and the pseudouridines ψ43 and ψ46—are dependent on U85 snoRNA? Explain your reasoning.

MEDICAL LINKS

6–44 The trypanosome, which is the microorganism that causes sleeping sickness, can vary its surface glycoprotein coat and thus evade the immune defenses of its host. The promoter for the variable surface glycoprotein (VSG) gene proved difficult to locate, but was mapped by measuring the sensitivity of the transcript to ultraviolet (UV) irradiation. Since RNA polymerases cannot transcribe through pyrimidine dimers (the major damage produced by UV irradiation), the sensitivity of transcription to UV irradiation is a measure of the distance between the start of transcription (the promoter) and the point at which transcription is assayed (*Vsg* gene).

Transcription through rRNA genes was used to calibrate the system. The 5S RNA transcription unit is just over 100 nucleotides long, whereas the 18S, 5.8S, and 28S rRNAs are part of a single transcription unit that is about 8 kb in length (**Figure 6–21A**). Trypanosomes were exposed to increasing doses of UV irradiation; their nuclei were then isolated and incubated with ^{32}P-NTPs to radioactively label the RNA. RNA isolated from the nuclei was hybridized to DNA probes corresponding to the 5S RNA gene and various parts of the rRNA gene (**Figure 6–21B**). Plots of the logarithm of the counts in each spot against the UV dose gave straight lines (**Figure 6–21C**), with slopes that were proportional to the distance from the hybridization probe to the promoter.

When the experiment was done with a probe from the beginning of the *Vsg* gene, transcription was found to be inactivated about seven times faster for the *Vsg* gene than for probe 4 from the ribosomal transcription unit.

A. Why does RNA transcription increase in sensitivity to UV irradiation with increasing distance from the promoter?
B. Roughly how far is the *Vsg* gene from its promoter? What assumption do you have to make in order to estimate this distance?
C. Transcription through another gene, located about 10 kb upstream of the *Vsg* gene, was about 20% less sensitive to UV irradiation than transcription

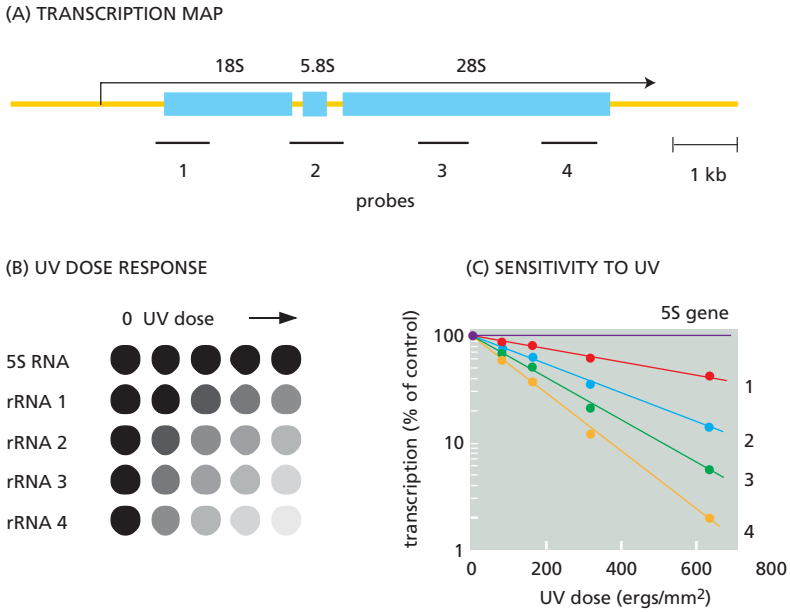


Figure 6–21 UV mapping of the promoter in trypanosomes (Problem 6–44). (A) Structure of the ribosomal RNA transcription unit. The positions of the hybridization probes along with a scale marker are shown. The transcription unit begins at the left end of the *arrow.* (B) A dot blot of transcripts from the 5S RNA and rRNA genes. The dot blot is done by placing an excess of the DNA probe in spots on a filter paper and then hybridizing radiolabeled RNA to it. (C) Sensitivities of the transcription units to increasing doses of UV irradiation.

through the *Vsg* gene. Is this measurement consistent with the possibility that these two genes are transcribed from the same promoter? Explain your reasoning.

FROM RNA TO PROTEIN

TERMS TO LEARN

aminoacyl-tRNA synthetase
anticodon
codon
eukaryotic initiation factor (eIF)
genetic code
induced fit
initiator tRNA
kinetic proofreading
molecular chaperone
nonsense-mediated mRNA decay
proteasome
reading frame
ribosome
ribozyme
rRNA (ribosomal RNA)
translation
tRNA (transfer RNA)

DEFINITIONS

Match the definition below with its term from the list above.

6–45 Large protein complex in the cytosol and nucleus with proteolytic activity that is responsible for degrading the proteins marked for destruction.

6–46 Set of rules specifying the correspondence between nucleotide triplets in DNA or RNA and amino acids in proteins.

6–47 Special tRNA that carries methionine and is used to begin translation.

6–48 Sequence of three nucleotides in a tRNA that is complementary to a three-nucleotide sequence in an mRNA molecule.

6–49 RNA molecule with catalytic activity.

6–50 Surveillance system in eukaryotes that eliminates defective mRNAs before they can be translated into protein.

6–51 The three-nucleotide phase in which nucleotides in an mRNA are translated into amino acids in a protein.

6–52 Enzyme that attaches the correct amino acid to a tRNA molecule to form the activated intermediate used in protein synthesis.

6–53 Protein that helps other proteins fold correctly.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

6–54 Wobble pairing occurs between the first position in the codon and the third position in the anticodon.

6–55 During protein synthesis, the thermodynamics of base-pairing between tRNAs and mRNAs sets the upper limit for the accuracy with which protein molecules are made.

THOUGHT PROBLEMS

6–56 For the RNA sequence below, indicate the amino acids that are encoded in the three reading frames. If you were told that this segment of RNA was in the middle of an mRNA that encoded a large protein, would you know which reading frame was used? How so? (The genetic code is shown in Table 7 on page 966.)

AGUCUAGGCACUGA

6–57 After treating cells with a chemical mutagen, you isolate two mutants. One carries alanine and the other carries methionine at a site in the protein that normally contains valine (**Figure 6–22**). After treating these two mutants again with the mutagen, you isolate mutants from each that now carry threonine at the site of the original valine (Figure 6–22). Assuming that all mutations involve single-nucleotide changes, deduce the codons that are used for valine, methionine, threonine, and alanine at the affected site. Would you expect to be able to isolate a valine-to-threonine mutant in one step?

6–58 The genetic code was deciphered in part by experiments in which polynucleotides of repeating sequences were used as mRNAs to direct protein synthesis in cell-free extracts. In the test tube, artificial conditions were used that allowed ribosomes to start protein synthesis anywhere on an RNA molecule, without the need for a translation start codon, as required in a living cell. What polypeptides would you expect to be synthesized if the following polynucleotides were used as templates in such a cell-free extract?

A. UUUUUUUUUUUU...
B. AUAUAUAUAUAU...
C. AUCAUCAUCAUC...

6–59 In *B. licheniformis*, a few amino acids are removed from the C-terminus of the β-lactamase enzyme after it is synthesized. The sequence of the original C-terminus can be deduced by comparing it to a mutant in which the reading frame is shifted by the insertion or deletion of a nucleotide and the mutant β-lactamase escapes cleavage. The amino acid sequences of the purified wild-type enzyme and the frameshift mutant from amino acid 263 to the C-terminal end are given below.

					↓ site of protein cleavage						
wild type:	N	M	N	G	K						
frameshift mutant:	N	M	I	W	Q	I	C	V	M	K	D

A. What was the mutational event that gave rise to the frameshift mutant?
B. Deduce the number of amino acids in the synthesized form of the wild-type enzyme and, as far as possible, the sequence of the deleted C-terminus.

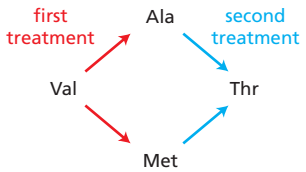


Figure 6–22 Two rounds of mutagenesis and the altered amino acids at a single position in a protein (Problem 6–57).

6–60 Which of the following mutational changes would you predict to be the most deleterious to gene function? Explain your answers.

1. Insertion of a single nucleotide near the end of the coding sequence.
2. Removal of a single nucleotide near the beginning of the coding sequence.
3. Deletion of three consecutive nucleotides in the middle of the coding sequence.
4. Deletion of four consecutive nucleotides in the middle of the coding sequence.
5. Substitution of one nucleotide for another in the middle of the coding sequence.

6–61 Consider the properties of two hypothetical genetic codes constructed with the four common nucleotides: A, G, C, and T.

A. Imagine that one genetic code is constructed so that pairs of nucleotides are used as codons. How many different amino acids could such a code specify?

B. Imagine that the other genetic code is a triplet code; that is, it uses three nucleotides to specify each amino acid. In this code, the amino acid specified by each codon depends only on the composition of the codon—not the sequence. Thus, for example, CCA, CAC, and ACC, which all have the composition C_2A, would encode the same amino acid. How many different amino acids could such a code specify?

C. Would you expect the genetic codes in A and B to lead to difficulties in the process of translation, using mechanisms analogous to those used in translating the standard genetic code?

6–62 One remarkable feature of the genetic code is that amino acids with similar chemical properties often have similar codons. Codons with U or C as the second nucleotide, for example, tend to specify hydrophobic amino acids. Can you suggest a possible explanation for this phenomenon in terms of the early evolution of the protein-synthesis machinery?

6–63 The rules for wobble base-pairing in bacteria and eukaryotes are shown in **Table 6–3**. On the left side of the table, the rules are expressed as a wobble codon base and its recognition by possible anticodon bases. [The anticodon base I (inosine) is a common modification in tRNAs; it is generated by deamination of A.] Reformulate these rules as particular anticodon bases and their recognition by possible codon bases, as suggested by the partial information on the right side of the table.

TABLE 6–3 Rules for wobble base-pairing between codon and anticodon (Problem 6–63).

	Wobble codon base	Possible anticodon base		Wobble anticodon base	Possible codon base
Bacteria	U C A G	A, G, or I G or I U or I C or U	Bacteria	U C A G I	
Eukaryotes	U C A G	G or I G or I U C	Eukaryotes	U C A G I	

6–64 Given the wobble rules for codon–anticodon pairing in bacteria, the minimum number of different tRNAs that would be required to recognize all 61 codons is 31. What is the minimum number of different tRNAs that is consistent with the wobble rules used in eukaryotes (see Table 6–3)?

6–65 A mutation in a bacterial gene generates a UGA stop codon in the middle of the mRNA coding for the protein product. A second mutation in the cell leads to a single nucleotide change in a tRNA that allows the correct translation of the protein; that is, the second mutation "suppresses" the defect caused by the first. The altered tRNA translates the UGA codon as tryptophan. What nucleotide change has probably occurred in the mutant tRNA molecule? What consequences would the presence of such a mutant tRNA have for the translation of the normal genes in this cell?

6–66 In a clever experiment performed in 1962, a cysteine that was already attached to $tRNA^{Cys}$ was chemically converted to an alanine. These alanyl-$tRNA^{Cys}$ molecules were then added to a cell-free translation system from which the normal cysteinyl-$tRNA^{Cys}$ molecules had been removed. When the resulting protein was analyzed, it was found that alanine had been inserted at every point in the protein chain where cysteine was supposed to be. Discuss what this experiment tells you about the role of aminoacyl-tRNA synthetases during the normal translation of the genetic code.

6–67 The charging of a tRNA with an amino acid occurs according to the reaction

$$\text{amino acid} + \text{tRNA} + \text{ATP} \longrightarrow \text{aminoacyl-tRNA} + \text{AMP} + PP_i$$

where PP_i is pyrophosphate, the linked phosphates that were cleaved from ATP to generate AMP. In the aminoacyl-tRNA, the amino acid and tRNA are linked by a high-energy bond. Thus, a large portion of the energy derived from the hydrolysis of ATP is stored in this bond and is available to drive peptide bond formation at the later stages of protein synthesis. The free-energy change ($\Delta G°$) for the charging reaction shown above is close to zero, so that attachment of the amino acid to tRNA would not be expected to be dramatically favored. Can you suggest a further step that could help drive the charging reaction to completion?

6–68 The protein you are studying contains five leucines and consists of a single polypeptide chain. One leucine is C-terminal and another is N-terminal. In a suspension of cells, the average time required to synthesize this polypeptide is 8 minutes. At time zero, radioactive leucine is added to five different suspensions of cells that are *already* in the process of synthesizing the protein. You isolate the complete protein from individual suspensions at 2, 4, 6, 8, and 80 minutes. (Any incomplete polypeptide chains are eliminated at this step.) The proteins are then analyzed for N-terminal and total radioactive leucine. With increasing time of exposure of the cells to the radioactive leucine, the ratio of N-terminal radioactivity to total radioactivity in the isolated protein should:

A. Increase to a final value of 0.2.
B. Remain constant at a value of 0.2.
C. Decrease to a final value of 0.2.
D. An answer cannot be determined from this information.

6–69 It is commonly reported that 30–50% of a cell's energy budget is spent on protein synthesis. How do you suppose such a measurement might be made?

6–70 One strand of a section of DNA isolated from *E. coli* reads

5′-GTAGCCTACCCATAGG-3′

A. Suppose that an mRNA were transcribed using the complement of this DNA strand as the template. What would the sequence of the mRNA in this region be?
B. How many different peptides could potentially be made from this sequence of RNA? Would the same peptides be made if the other strand of the DNA served as the template for transcription?
C. What peptide would be made if translation started exactly at the 5′ end of the mRNA in part A? When $tRNA^{Ala}$ leaves the ribosome, what tRNA will be bound next? When the amino group of alanine forms a peptide bond, which bonds, if any, are broken, and what happens to $tRNA^{Ala}$?

6–71 Polycistronic mRNAs are common in prokaryotes but extremely rare in eukaryotes. Describe the key differences in protein synthesis that underlie this observation.

6–72 Prokaryotes and eukaryotes both protect against the dangers of translating broken mRNAs. What dangers do partial mRNAs pose for the cell?

6–73 The antibiotic edeine inhibits protein synthesis but has no effect on either DNA synthesis or RNA synthesis. When added to a reticulocyte lysate, edeine stops protein synthesis after a short lag, as shown in **Figure 6–23**. By contrast, cycloheximide stops protein synthesis immediately (Figure 6–23). Analysis of the edeine-inhibited lysate by density-gradient centrifugation showed that no polyribosomes remained at the time protein synthesis had stopped. Instead, all the globin mRNA accumulated in an abnormal 40S peak, which contained equimolar amounts of the small ribosomal subunit and initiator tRNA.
A. What step in protein synthesis does edeine inhibit?
B. Why is there a lag between addition of edeine and cessation of protein synthesis? What determines the length of the lag?
C. Would you expect the polyribosomes to disappear if you added cycloheximide at the same time as edeine?

6–74 In a reticulocyte lysate, the polynucleotide 5′-AUGUUUUUUUUU directs the synthesis of Met–Phe–Phe–Phe. In the presence of farsomycin, a new antibiotic perfected by Fluhardy Pharmaceuticals, this polymer directs synthesis of Met–Phe only. From this information, which of the following deductions could you make about farsomycin?
A. It prevents formation of the 80S initiation complex, which contains the initiator tRNA and both ribosomal subunits.
B. It inhibits binding of aminoacyl-tRNAs to the A site in the ribosome.
C. It inactivates peptidyl transferase activity of the large ribosomal subunit.
D. It blocks translocation of peptidyl-tRNA from the A site to the P site of the ribosome.
E. It interferes with chain termination and release of the peptide.

6–75 Both hsp60-like and hsp70 molecular chaperones share an affinity for exposed hydrophobic patches on proteins, using them as indicators of incomplete folding. Why do you suppose hydrophobic patches serve as critical signals for the folding status of a protein?

6–76 Most proteins require molecular chaperones to assist in their correct folding. How do you suppose the chaperones themselves manage to fold correctly?

6–77 Your advisor, the brilliant bioinformatician, has a high regard for your intellect and industry. She suggests that you write a computer program that will identify the exons of protein-encoding genes directly from the sequence of the human genome. In preparation for that task, you decide to write down a list of the features that might distinguish coding sequences from intronic DNA and sequences outside of genes. What features would you list?

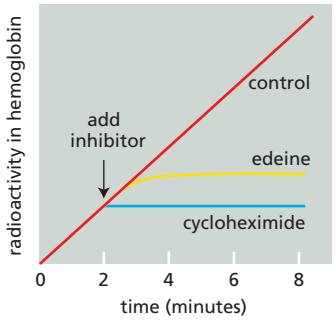


Figure 6–23 Effects of the inhibitors edeine and cycloheximide on protein synthesis in reticulocyte lysates (Problem 6–73).

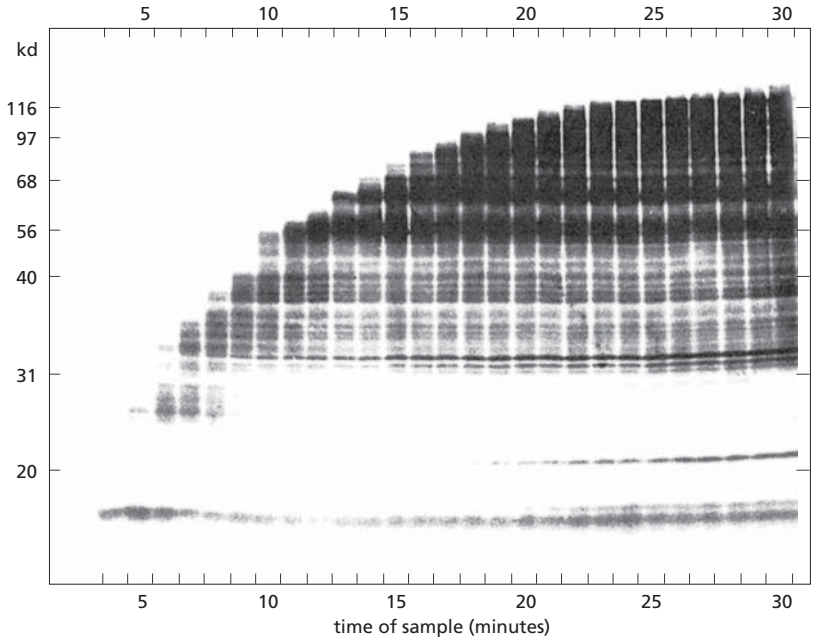


Figure 6–24 Time course of synthesis of a TMV protein in a rabbit-reticulocyte lysate (Problem 6–78). No radioactivity was detected during the first 3 minutes because the short chains ran off the bottom of the gel. SDS denatures proteins so that they run approximately according to their molecular masses. A scale of molecular masses in kilodaltons is shown on the *left*.

CALCULATIONS

6–78 Rates of peptide-chain growth can be estimated from data such as those shown in **Figure 6–24**. In this experiment, a tobacco mosaic virus (TMV) mRNA, which encodes a 116,000 dalton protein, was translated in a rabbit-reticulocyte lysate in the presence of ^{35}S-methionine. Samples were removed at one-minute intervals and subjected to electrophoresis on SDS-polyacrylamide gels. The separated translation products were visualized by autoradiography. As is apparent in Figure 6–24, the largest detectable polypeptides get larger with time, until the full-length protein appears at about 25 minutes.

A. Is the rate of synthesis linear with time? One simple way to answer this question is to determine the molecular mass of the largest peptide in each sample, as determined by reference to the standards shown on the left in Figure 6–24, and then plot each of these masses against the time at which the relevant sample was taken.

B. What is the rate of protein synthesis (in amino acids/minute) in this experiment? Assume the average molecular mass of an amino acid is 110 daltons.

C. Why does the autoradiograph have so many bands in it rather than just a few bands that get larger as time passes; that is, why does the experiment produce the "actual" result (**Figure 6–25A**) rather than the "theoretical" result (**Figure 6–25B**)? Can you think of a way to manipulate the experimental conditions to produce the theoretical result?

6–79 The average molecular weight of proteins encoded in the human genome is about 50,000. A few proteins are very much larger than this average. For example, the protein called titin, which is made by muscle cells, has a molecular weight of 3,000,000.

A. Estimate how long it will take a muscle cell to translate an mRNA coding for an average protein and one coding for titin. The average molecular mass of amino acids is about 110 daltons. Assume that the translation rate is two amino acids per second.

B. If the nucleotides in the coding portion of the mRNA constitute 5% of the total that are transcribed, how long will it take a muscle cell to transcribe a gene for an average protein versus the titin gene. Assume that the transcription rate is 20 nucleotides per second.

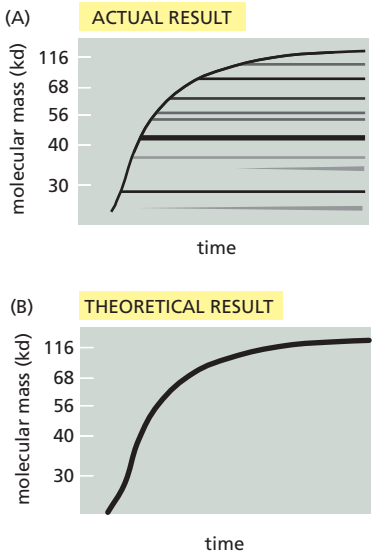


Figure 6–25 Potential outcomes of experiments on rates of protein synthesis (Problem 6–78).

6–80 Protein synthesis consumes four high-energy phosphate bonds per added amino acid. Transcription consumes two high-energy phosphate bonds per added nucleotide. Calculate how many protein molecules will have been made from an individual mRNA at the point when the energy cost of translation is equal to the energy cost of transcription. Assume that the nucleotides in the coding portion of the mRNA constitute 5% of the total that are transcribed.

6–81 The overall accuracy of protein synthesis is difficult to measure because mistakes are very rare. One ingenious approach used flagellin (molecular weight 40,000). Flagellin is the sole protein in bacterial flagella and thus easy to purify. Because flagellin contains no cysteine, it allows for sensitive detection of cysteine that has been misincorporated into the protein.

To generate radioactive cysteine, bacteria were grown in the presence of $^{35}SO_4^{2-}$ (specific activity 5.0×10^3 cpm/pmol) for exactly one generation with excess unlabeled methionine in the growth medium (to prevent the incorporation of ^{35}S label into methionine). Flagellin was purified and assayed: 8 μg of flagellin was found to contain 300 cpm of ^{35}S radioactivity.

A. Of the flagellin molecules that were synthesized during the labeling period, what fraction contained cysteine? Assume that the mass of flagellin doubles during the labeling period and that the specific activity of cysteine in flagellin is equal to the specific activity of the $^{35}SO_4^{2-}$ used to label the cells.
B. In flagellin, cysteine is misincorporated at the arginine codons CGU and CGC. In terms of anticodon–codon interaction, what mistake is made during the misincorporation of cysteine for arginine?
C. Given that there are 18 arginines in flagellin, and assuming that all arginine codons are equally represented, what is the frequency of misreading of each sensitive (CGU and CGC) arginine codon?
D. Assuming that the error frequency per codon calculated above applies to all amino acid codons equally, calculate the percentage of molecules that are correctly synthesized for proteins 100, 1000, and 10,000 amino acids in length. The probability of synthesizing a correct protein is $P = (1 - E)^n$, where E is the error frequency and n is the number of amino acids added.

DATA HANDLING

6–82 Many of the errors in protein synthesis occur because tRNA synthetases have difficulty discriminating between related amino acids. For example, isoleucyl-tRNA synthetase (IleRS) normally activates isoleucine.

$$\text{IleRS} + \text{Ile} + \text{ATP} \longrightarrow \text{IleRS(Ile-AMP)} + \text{PP}_i$$

At a frequency about 1/180 of the correct activation, IleRS misactivates valine.

$$\text{IleRS} + \text{Val} + \text{ATP} \longrightarrow \text{IleRS(Val-AMP)} + \text{PP}_i$$

Protein synthesis is more accurate than this frequency might suggest because the synthetase subsequently edits out most of its mistakes, in a reaction that depends on the presence of $tRNA^{Ile.}$

$$\text{IleRS(Val-AMP)} + tRNA^{Ile} \longrightarrow \text{IleRS} + \text{Val} + \text{AMP} + tRNA^{Ile} \quad \text{(EDITING)}$$

The $tRNA^{Ile}$ is, of course, also required for proper aminoacylation (charging) by isoleucine to make Ile-$tRNA^{Ile}$.

$$\text{IleRS(Ile-AMP)} + tRNA^{Ile} \longrightarrow \text{Ile-}tRNA^{Ile} + \text{IleRS} + \text{AMP} \quad \text{(CHARGING)}$$

Are the parts of $tRNA^{Ile}$ that are required for isoleucine charging the same as those that are required for valine editing?

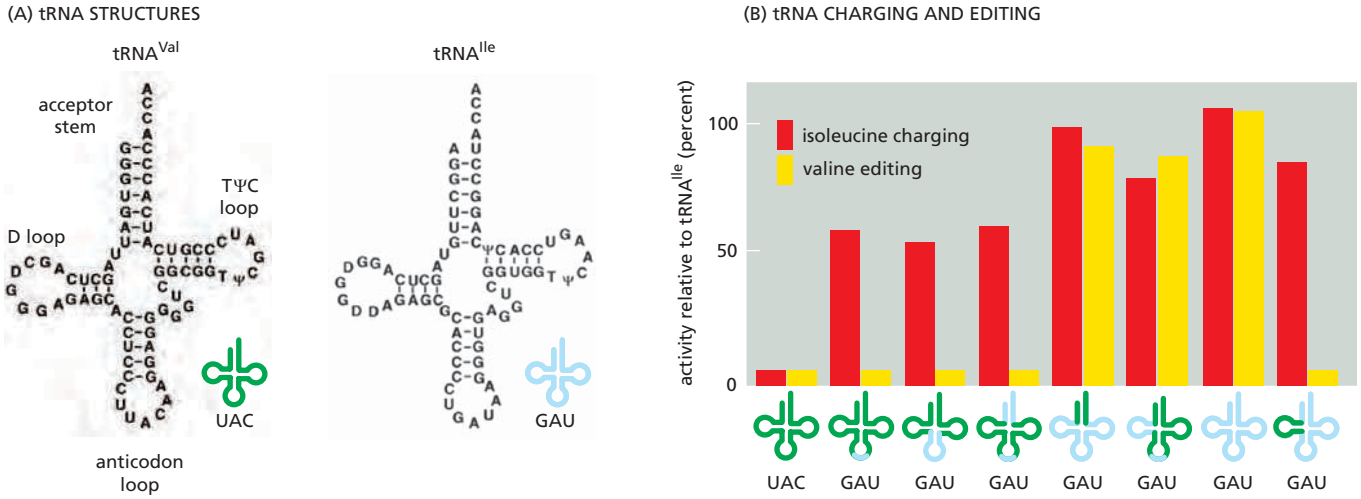


Figure 6–26 Portions of tRNA^{Ile} responsible for charging and editing (Problem 6–82). (A) tRNA^{Val} and tRNA^{Ile}. (B) Isoleucine charging and valine editing. Chimeric tRNAs composed of bits from tRNA^{Val} *(green)* and tRNA^{Ile} *(blue)* are shown *below* the results for isoleucine charging *(red bars)* and valine editing *(yellow bars)*.

One approach to this question is to make changes in the tRNA^{Ile} to see whether the two activities track with one another. Rather than change the sequence nucleotide by nucleotide, blocks of sequence changes were made, using tRNA^{Val} as a donor. tRNA^{Val} by itself does not stimulate isoleucine charging or valine editing by IleRS. Changing its anticodon from 5′-CAU to 5′-GAU, however, allows it to be charged fairly efficiently by IleRS. A variety of chimeric tRNAs were made by combining bits of tRNA^{Ile} and tRNA^{Val}. The ability of each chimera to stimulate isoleucine charging and valine editing was then tested, as shown in **Figure 6–26**.

A. What (at a minimum) must be inserted into tRNA^{Val} to permit isoleucine charging by IleRS?
B. What (at a minimum) must be inserted into tRNA^{Val} to permit valine editing by IleRS?
C. Does IleRS recognize the same features of tRNA^{Ile} when it catalyzes isoleucine charging that it does when it carries out valine editing? Explain your answer.

6–83 Consider the following experiment on the coordinated synthesis of the α and β chains of hemoglobin. Rabbit reticulocytes were labeled with ^{3}H-lysine for 10 minutes, which is very long relative to the time required for the synthesis of a single globin chain. The ribosomes, with attached nascent globin chains, were then isolated by centrifugation to give a preparation free of soluble (finished) globin chains. The nascent globin chains were digested with trypsin, which gives peptides ending in C-terminal lysine or arginine. The peptides were then separated by high-performance liquid chromatography (HPLC), and their radioactivity was measured. A plot of the radioactivity in each peptide versus the position of the lysines in the chains (numbered from the N-terminus) is shown in **Figure 6–27**.

A. Do these data allow you to decide which end of the globin chain (N- or C-terminus) is synthesized first? How so?
B. In what ratio are the two globin chains produced? Can you estimate the relative numbers of α- and β-globin mRNA molecules from these data?
C. How long does a protein chain stay attached to the ribosome once the termination codon has been reached?
D. It was once suggested that heme is added to nascent globin chains during their synthesis and, furthermore, that ribosomes must wait for the

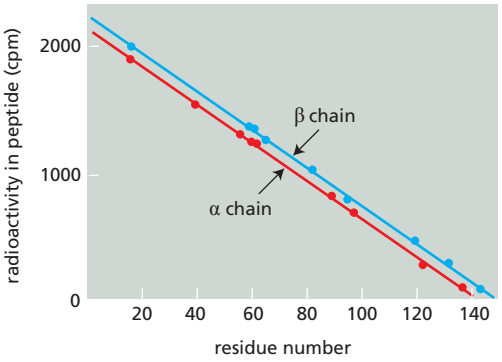


Figure 6–27 Synthesis of α- and β-globin chains (Problem 6–83).

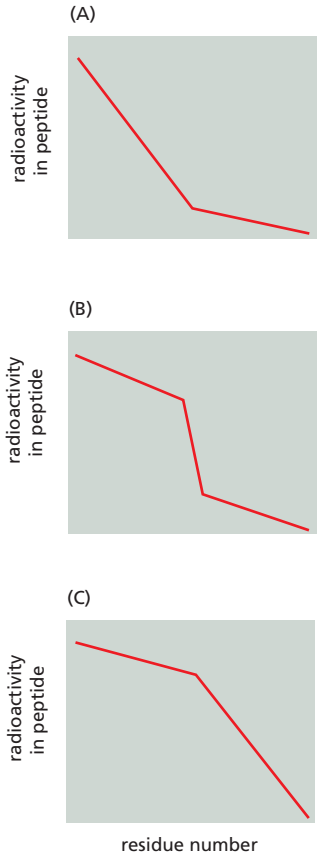


Figure 6–28 Hypothetical curves for globin synthesis with a roadblock to ribosome movement at the midpoint of the mRNA (Problem 6–83). These schematic diagrams are analogous to the graph in Figure 6–27.

insertion of heme before they can proceed. The straight lines in Figure 6–27 indicate that ribosomes do not pause significantly, and heme is now thought to be added after synthesis. From among the graphs shown in **Figure 6–28**, choose the one that would have resulted if there were a significant roadblock to ribosome movement halfway down the globin mRNA.

6–84 Termination codons in bacteria are decoded by one of two proteins. Release factor 1 (RF1) recognizes UAG and UAA, whereas RF2 recognizes UGA and UAA. For RF2, a comparison of the nucleotide sequence of the gene with the amino acid sequence of the protein revealed a startling surprise, which is contained within the sequences shown below the gene in **Figure 6–29**. Sequences of the gene and protein were checked carefully to rule out any artifacts.

A. What is the surprise?

B. What hypothesis concerning the regulation of RF2 expression is suggested by this observation?

6–85 You are studying protein synthesis in *Tetrahymena*, which is a unicellular ciliate. You have good news and bad news. The good news is that you have the first bit of protein and nucleic acid sequence data for the C-terminus of a *Tetrahymena* protein, as shown below:

```
 I   M   Y   K   Q   V   A   Q   T   Q   L   *
AUU AUG UAU AAG UAG GUC GCA UAA ACA CAA UUA UGA GAC UUA
```

The bad news is that you have been unable to translate a preparation of *Tetrahymena* mRNA in a reticulocyte lysate, which is a standard system for analyzing protein synthesis *in vitro*. The mRNA preparation looks good by all criteria, but the translation products are mostly small polypeptides (**Figure 6–30**, lane 1).

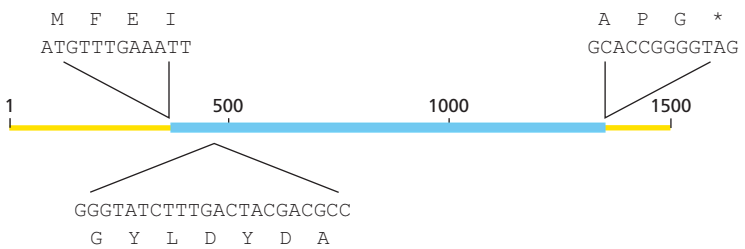


Figure 6–29 Schematic representation of the gene for RF2 (Problem 6–84). The coding sequence is shown as a *blue line*, with sequences at the start and finish shown for reference.

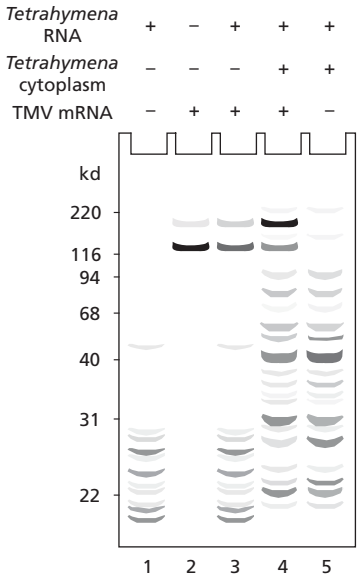


Figure 6–30 Translation of TMV and *Tetrahymena* mRNA in a reticulocyte lysate in the presence and absence of various components from *Tetrahymena* (Problem 6–85). The molecular masses of marker proteins are indicated in kilodaltons on the *left*.

To figure out what is wrong, you do a number of control experiments using a pure mRNA from tobacco mosaic virus (TMV) that encodes a 116 kd protein. TMV mRNA alone is translated just fine in the *in vitro* system, giving a major band at 116 kd—the expected product—and a very minor band about 50 kd larger (Figure 6–30, lane 2). When *Tetrahymena* RNA is added, there is a decrease in the smaller of the two bands and a significant increase in the larger one (lane 3). When some *Tetrahymena* cytoplasm (minus the ribosomes) is added, the TMV mRNA now gives mostly the higher molecular mass product (lane 4); furthermore, much to your delight, the previously inactive *Tetrahymena* mRNA now appears to be translated (lane 4). You confirm this by leaving out the TMV mRNA (lane 5).

A. What is unusual about the sequence data for the *Tetrahymena* protein?
B. How do you think the more minor of the two higher molecular mass bands is produced from pure TMV mRNA in the reticulocyte lysate (lane 2)?
C. Explain the basis for the shift in proportions of the major and minor TMV proteins upon addition of *Tetrahymena* RNA alone and in combination with *Tetrahymena* cytoplasm. What *Tetrahymena* components are likely to be required for the efficient translation of *Tetrahymena* mRNA?
D. Comment on the evolutionary implications of your results.

6–86 Hsp70 molecular chaperones are thought to bind to hydrophobic regions of nascent polypeptides on ribosomes. This binding was difficult to demonstrate for dnaK, which is one of the two major hsp70 chaperones in *E. coli*. In one approach, nascent proteins were labeled with a 15-second pulse of ^{35}S-methionine, isolated in the absence of ATP, and then incubated with antibodies against dnaK. A collection of labeled proteins was precipitated as shown in **Figure 6–31A**, lane 1. The proteins were not precipitated if they were treated beforehand with the strong detergent SDS (lane 2), or if they were isolated from a mutant strain missing dnaK (*dnaK*-deletion, lane 3). If labeled *dnaK*-deletion cells were mixed with unlabeled wild-type cells before the proteins were isolated, dnaK antibodies did not precipitate labeled proteins (lane 4). Finally, if unlabeled methionine was added in excess after the pulse of ^{35}S-methionine, the labeled proteins disappeared with time (**Figure 6–31B**).

A. Do the series of control experiments in Figure 6–31A, lanes 2 to 4, argue that dnaK is bound to the labeled proteins in a meaningful way (as opposed to a random aggregation, for example)?

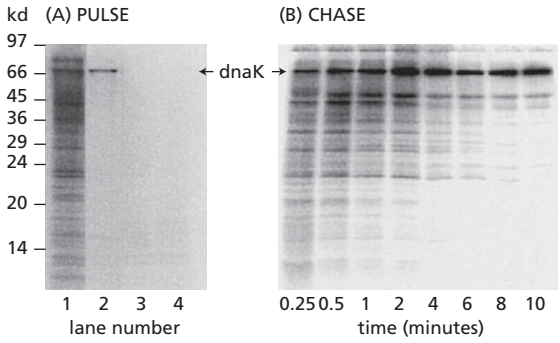


Figure 6–31 Association of dnaK with nascent proteins (Problem 6–86). (A) Pulse-labeled proteins immunoprecipitated by antibodies to dnaK. Wild-type bacteria (lane 1); SDS-treated wild-type bacteria (lane 2); *dnaK*-deletion strain (lane 3); and mixture of labeled *dnaK*-deletion strain and unlabeled wild-type strain (lane 4). Size markers are indicated on the *left* and the position of dnaK is indicated on the *right*. (B) Pulse-chase experiment. Wild-type bacteria labeled for 15 seconds with ^{35}S-methionine were then incubated for varying times in the presence of an excess of unlabeled methionine before immunoprecipitation by antibodies against dnaK.

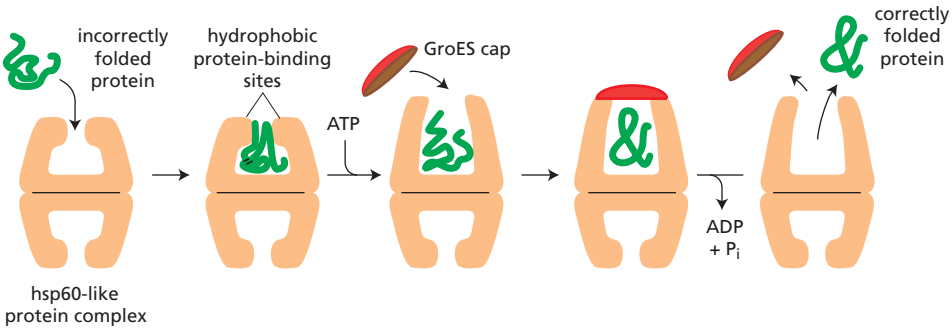


Figure 6–32 Protein refolding by the bacterial GroEL chaperone (Problem 6–87). A misfolded protein is initially captured by hydrophobic interactions along one rim of the barrel. The subsequent binding of ATP plus the GroES cap increases the size of the cavity and confines the protein in the enclosed space, where it has a new opportunity to fold. After about 15 seconds, ATP hydrolysis ejects the protein, whether folded or not, and the cycle repeats.

B. When ATP was present during the isolation of the proteins, antibodies against dnaK did not precipitate any proteins. How do you suppose ATP might interfere with precipitation of labeled proteins?
C. Why do you suppose that the labeled proteins disappeared with time in the presence of excess unlabeled methionine?
D. Do these experiments show that dnaK binds to proteins as they are being synthesized on ribosomes? Why or why not?

6–87 Hsp60-like molecular chaperones provide a large central cavity in which misfolded proteins can attempt to refold. Two models, which are not mutually exclusive, can be considered for the role of the hsp60-like chaperones in the refolding process. They might act passively to provide an isolation chamber that aids protein folding by preventing aggregation with other proteins. Alternatively, hsp60-like chaperones might actively unfold misfolded proteins to remove stable, but incorrect, intermediate structures that block proper folding. The involvement of ATP binding and hydrolysis and the associated conformational changes of the hsp60-like chaperones could be used in favor of either model.

In bacteria, the hsp60-like chaperone GroEL binds to a misfolded protein, then binds ATP and the GroES cap, and after about 15 seconds hydrolyzes the ATP and ejects the protein (**Figure 6–32**). To distinguish between a passive and an active role for GroEL in refolding, you label a protein by denaturing it in tritiated water, ^3H_2O. When the denaturant is removed and the protein is transferred to normal water, ^1H_2O, most of the radioactivity is lost within 10 minutes, but a stable core of 12 tritium atoms exchanges on a much longer time scale—a behavior typical of amide hydrogen atoms involved in stable hydrogen bonds. Disruption of these bonds would allow their exchange within a few milliseconds.

You prepare the radioactive substrate and mix it immediately with a slight molar excess of GroEL, and then wait 10 minutes for the rapidly exchanging tritium atoms to be lost. The addition of GroES or ATP alone has no effect on the exchange, as shown by the upper curve in **Figure 6–33**. The addition of GroES and ATP together causes a rapid loss of tritium (Figure 6–33, lower curve). Addition of GroES plus ADP has no

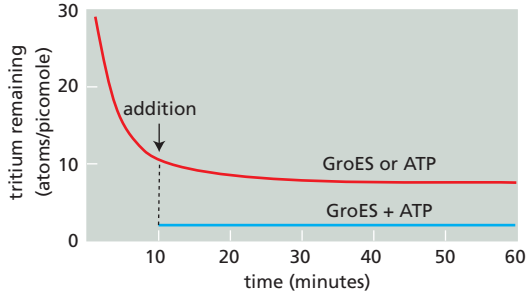


Figure 6–33 Effects of chaperone components on rates of tritium exchange (Problem 6–87).

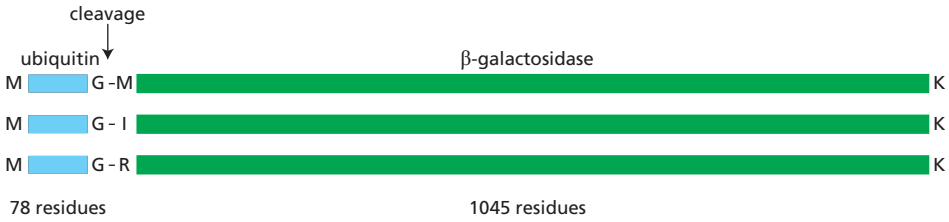


Figure 6–34 Fusion proteins encoded by three yeast plasmids (Problem 6–88). Upon expression in yeast, the fusion proteins are cleaved at the peptide bonds indicated by the *arrow.* The β-galactosidase molecules liberated by cleavage differ only at their N-termini.

effect, but GroES plus AMPPNP, a nonhydrolyzable analog of ATP, promotes a rapid exchange that is indistinguishable from GroES plus ATP.

A. After the addition of components to the complex of tritiated protein and GroEL, it took a minimum of 45 seconds to separate the protein from the freed tritium label. Did the exchange of tritium occur within one cycle of binding and ejection by GroEL, which takes about 15 seconds, or might it have required more than one cycle? Explain your answer.
B. Do the results support a passive isolation-chamber model, or an active-unfolding model, for GroEL action? Explain your reasoning.

6–88 The life-spans of proteins are appropriate to their *in vivo* tasks: structural proteins tend to be long-lived; regulatory proteins are usually short-lived. In eukaryotic cells, life-spans are strongly influenced by the N-terminal amino acid. The first experiments that revealed these effects used hybrid proteins consisting of ubiquitin fused to β-galactosidase, as shown in **Figure 6–34**. When plasmids encoding these proteins were introduced into yeast, the hybrid proteins were synthesized but the ubiquitin was cleaved off exactly at the junction with β-galactosidase, generating proteins with different N-termini (Figure 6–34).

To measure the half-lives of these β-galactosidase molecules, yeast cells containing the plasmids were grown for several generations in the presence of a radioactive amino acid. Protein synthesis was then blocked with the inhibitor cycloheximide (CHX). The rate of degradation of β-galactosidase was determined by removing samples from the cultures at various times, purifying β-galactosidase, and measuring the amount of associated radioactivity after SDS-gel electrophoresis. The results at the 5-minute time point are shown in **Figure 6–35A**, and a graph depicting results for all time points is shown in **Figure 6–35B**.

A. Using recombinant DNA techniques, it would have been straightforward to generate a series of plasmids in which the first codon in the β-galactosidase gene was changed. Why do you suppose this more direct approach was not tried?
B. Estimate the half-life (time at which half the material has been degraded) of each of the three species of β-galactosidase.

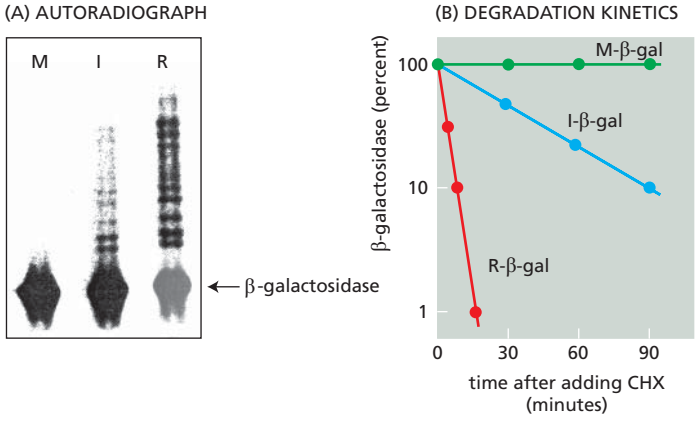


Figure 6–35 Analysis of half-lives of proteins with different N-termini (Problem 6–88). (A) Electrophoretic separation of radioactive β-galactosidase. Antibodies directed against β-galactosidase were used to precipitate the protein. The bands *above* β-galactosidase carry one or more ubiquitin molecules that were attached as a prelude to proteasomal degradation. The N-terminal amino acids are indicated *above* the lanes. (B) Disappearance of β-galactosidases (β-gal) with time after termination of protein synthesis by the addition of cycloheximide (CHX). The level of β-galactosidase is expressed as a percentage of that present immediately after protein synthesis was blocked.

THE RNA WORLD AND THE ORIGINS OF LIFE

TERM TO LEARN
RNA world

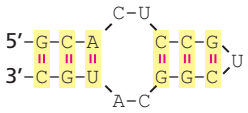


Figure 6–36 An RNA hairpin with a symmetric internal loop (Problem 6–94).

DEFINITIONS

Match the definition below with its term from the list above.

6–89 A hypothetical state of evolution that existed on Earth before modern cells arose, in which RNA both stored genetic information and catalyzed chemical reactions in primitive cells.

TRUE/FALSE

Decide whether the statement is true or false, and then explain why.

6–90 Protein enzymes are thought to greatly outnumber ribozymes in modern cells because they can catalyze a much greater variety of reactions and all of them have faster rates than any ribozyme.

THOUGHT PROBLEMS

6–91 What is so special about RNA that it is hypothesized to be an evolutionary precursor to DNA and protein?

6–92 Discuss the following statement: "During the evolution of life on Earth, RNA has been demoted from its glorious position as the first replicating catalyst. Its role now is as a mere messenger in the information flow from DNA to protein."

6–93 Imagine a warm pond on the primordial Earth. Chance processes have just assembled a single copy of an RNA molecule with a catalytic site that can carry out RNA replication. This RNA molecule folds into a structure that is capable of linking nucleotides according to instructions in an RNA template. Given an adequate supply of nucleotides, will this single RNA molecule be able to use itself as a template to catalyze its own replication? Why or why not?

6–94 If an RNA molecule could form a hairpin with a symmetric internal loop, as shown in **Figure 6–36**, could the complement of this RNA form a similar structure? If so, would there be any regions of the two structures that are identical? Which ones?

6–95 What is it about DNA that makes it a better material than RNA for the storage of genetic information?

6–96 An RNA molecule with the ability to catalyze RNA replication—the linkage of RNA nucleotides according to the information in an RNA template—would have been a key ribozyme in the RNA world. Your advisor wants to evolve such an RNA replicase *in vitro* by selection and amplification of rare functional molecules from a pool of random RNA sequences. The difficulty has been to devise a strategy that models a reasonable first step and that can be fit into an *in vitro* selection scheme. When you arrived in the lab today, you found the scheme shown in **Figures 6–37** and **6–38** on your desk with a note from your advisor that he wants to talk with you about it tomorrow when he returns from an out-of-town trip. He's even left you some questions to "focus" the discussion.

A. As shown in Figure 6–37, the activity being selected for is the attachment of an oligonucleotide "tag" to the end of the catalytic RNA molecule. How is this reaction an analog of nucleotide addition to the end of a primer on a template RNA?

B. Why is it important in the selection scheme in Figure 6–38 that the "tag" RNA be linked to the same RNA that catalyzed its attachment?

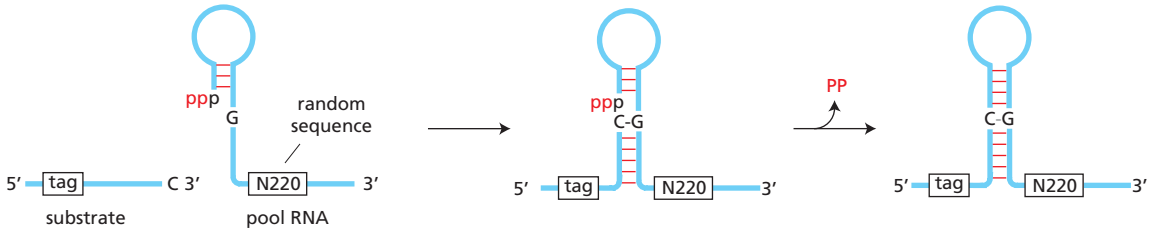


Figure 6–37 Ribozyme-catalyzed reaction selected for in this scheme for *in vitro* evolution (Problem 6–96). The random sequence in the pool RNA is 220 nucleotides in length (N220). The 3′ end of the substrate oligonucleotide is complementary to the constant 5′ end of the pool RNA molecules, so that it can pair with the pool RNAs as shown.

C. Why does the starting pool of RNA molecules have constant regions at each end and a random segment in the middle? What specific roles do these segments play in the overall scheme?
D. How is a catalytic RNA molecule selected from the pool and specifically amplified?
E. Why do you suppose it is necessary to repeat the cycles of selection and amplification? Why not simply purify the ribozymes at the end of the first cycle?

CALCULATIONS

6–97 Curses! Your advisor has sent you an email with still more questions about his selection scheme for evolving an RNA replicase (see Figures 6–37 and 6–38).

A. He thinks you will be able to generate about a milligram of RNA to begin the selection. How many molecules will be present in this amount of RNA? (Assume that an RNA nucleotide has a mass of 330 daltons and that the RNA molecules are 300 nucleotides in length.)
B. How many different molecules are possible if the central 220-nucleotide segment is completely random? What fraction of all possible molecules will be present in your 1 mg sample?
C. If the ligation reaction could only be catalyzed by a single, *unique* 50-nucleotide RNA sequence, what do you suppose your chances of success would be? What does the general success of such selection schemes imply about the range of RNA molecules that are capable of catalysis?

DATA HANDLING

6–98 Once you and your advisor had ironed out the details of the selection scheme, the work went fairly quickly. You have now carried out 10 rounds of selection with the results shown in **Table 6–4**. You have cloned and sequenced 15 individual RNA molecules from pool 10: no two are the

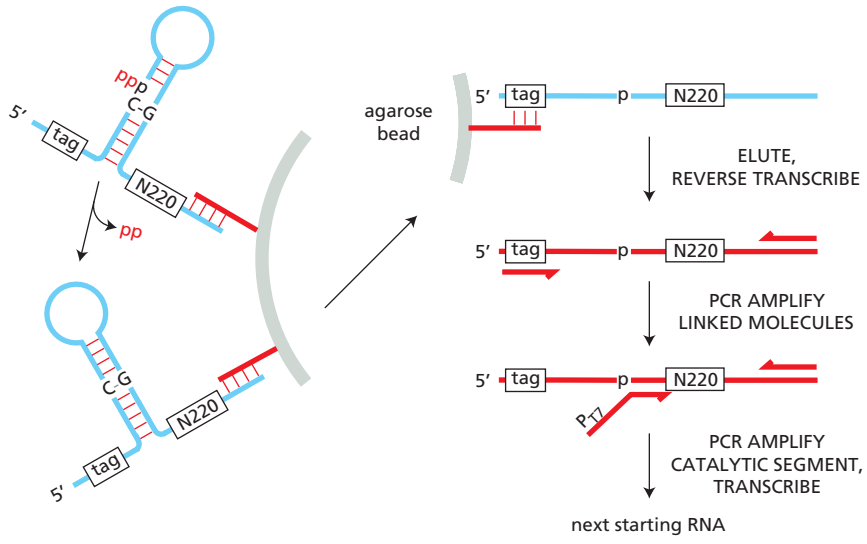


Figure 6–38 One round in the cyclic selection scheme to amplify individual ribozymes from a random pool (Problem 6–96). Each pool RNA is linked at one end to the substrate RNA molecule and at the other end to a complementary DNA oligonucleotide attached to an agarose bead for ease of manipulation. On the *right,* the hairpin is not shown for simplicity; the "p" indicates the site of linkage between the substrate and the presumptive catalytic RNA. In the final PCR amplification, the noncomplementary portion of the oligonucleotide carries the promoter for T7 RNA polymerase (P_{T7}), which allows the final DNA product to be transcribed back into RNA.

TABLE 6–4 Summary of rounds of selection for a ribozyme with RNA ligation activity (Problem 6–98).

Round	Error-prone PCR	Ligation conditions		Ligation rate (per hour)
		$MgCl_2$ (mM)	Time (hours)	
0				0.000003
1	No	60	16	<0.000004
2	No	60	16	0.0008
3	No	60	16	0.0094
4	No	60	16	0.027
5	Yes	60	0.50	0.16
6	Yes	60	0.17	0.40
7	Yes	60	0.02	0.86
8	No	4	0.12	3.2
9	No	2.5	0.17	4.5
10	No	2.5	0.17	8.0

same, although 11 of the 15 have very similar sequences. Your advisor is excited by your results and has asked you to give the next departmental seminar. You know from your conversations with other students that you will need to prepare careful explanations for the questions listed below.

A. Why did you use error-prone PCR, which can introduce mutations, in some of the rounds of amplification?
B. Why did you reduce the time and Mg^{2+} concentration—both changes increase the difficulty of ligation—in successive rounds of selection?
C. How much of an improvement in ligation rate have you found in your 10 rounds of selection and amplification?
D. Why is there still such diversity among the RNA molecules after 10 rounds of selection and amplification?

MCAT STYLE

Passage 1 (Questions 6–99 to 6–102)

Congenital heart defects are present in 1–2% of newborns and cause a significant number of stillbirths. They are also a major cause of heart disease in adults. Many congenital heart defects are due to mutations in genes for transcription factors that control expression of genes that are required for normal heart development. For example, over 30 different mutations have been found in the gene for transcription factor Tbx5 in patients with congenital heart defects. To carry out its functions, Tbx5 must bind to another transcription factor called Nkx2-5, which also plays a role in heart development and is mutated in many individuals with congenital heart defects. Imagine that you can isolate developing cardiac cells with homozygous Tbx5 mutations and study the molecular defects caused by the mutations.

6–99 In one mutant cell line, you find that the normal, mature Tbx5 mRNA is present at its usual levels, but that full-length Tbx5 protein cannot be detected. What kind of mutation most likely accounts for this phenotype?

A. A mutation that blocks binding of Tbx5 to Nkx2-5
B. A mutation that causes a splicing defect
C. A mutation that disrupts the normal folding of Tbx5
D. A mutation that introduces a premature stop codon

6–100 In another mutant cell line, you find that levels of the mature, properly spliced Tbx5 mRNA are greatly reduced. What kind of mutation could account for this phenotype?

I. A short deletion in the coding region that preserves the reading frame
II. A short deletion in the coding region that disrupts the reading frame
III. A mutation in the promotor region of the Tbx5 gene

A. I
B. I and II
C. I and III
D. II and III

6–101 In another mutant cell line, you find that full-length Tbx5 mRNA and protein are present at normal levels, yet individuals carrying this mutation have severe defects in heart development. What kind of mutation could cause this phenotype?

A. A mutation that blocks association of the Tbx5 mRNA with the ribosome
B. A mutation that blocks export of the Tbx5 mRNA from the nucleus
C. A mutation that causes a splicing defect
D. A mutation that disrupts binding of Tbx5 with Nkx2-5

6–102 Which one of the following is a plausible mechanism by which Tbx5 could control initiation of transcription?

A. Activation of transcription elongation factors
B. Recruitment of RNA polymerase II to promoters
C. Recruitment of splicing factors to RNA polymerase II
D. Regulation of 5′ cap formation

Passage 2 (Questions 6–103 to 6–105)

A key discovery in cell biology came from studying autoimmune diseases. Sera from patients with autoimmune diseases were found to contain antibodies that bind to cellular proteins. To characterize these proteins further, the antibodies were used to purify the proteins they recognized. In one case, it was found that the antibodies bound to small nuclear ribonucleoprotein complexes or snRNPs (pronounced "snurps"). The RNAs within each complex were snRNAs (small nuclear RNAs). The functions of these snRNPs were initially mysterious, but it was eventually found that they are the key functional components of the spliceosome.

6–103 Which one of the following would have provided key evidence that snRNPs are involved in the splicing of pre-mRNA?

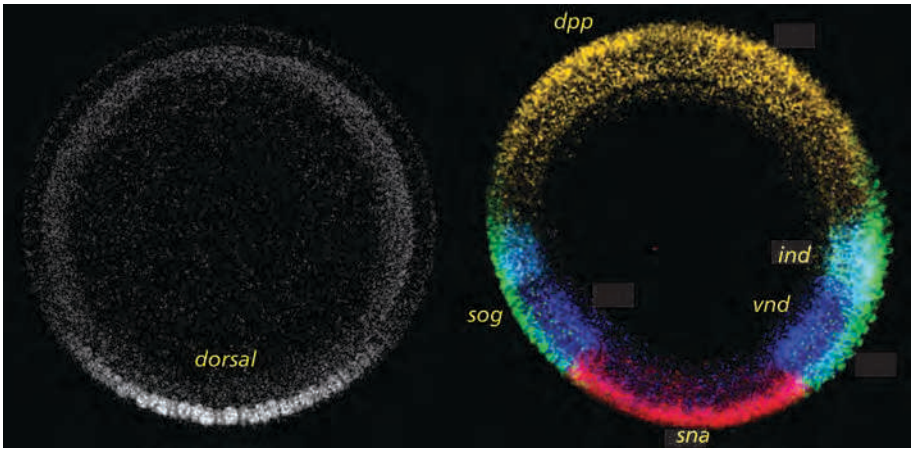
A. Association of snRNPs with ribosomes
B. Base-pairing between snRNAs and pre-mRNA splice sites
C. Introns within snRNAs
D. Lariat structures within snRNAs

6–104 Autoimmune antibodies from patients were used to determine the localization of snRNPs in the cell. Which of the following best describes where snRNP particles would be found within the cell?

A. In the cytoplasm, near ribosomes
B. In the cytoplasm, near the nucleus
C. In the nucleus, near RNA polymerase II
D. In the nucleus, near RNA polymerase III

6–105 Which one of the following proteins might be expected to be found in a snRNP?

A. A DNA helicase
B. An ATPase
C. An enhancer protein
D. A site-specific ribonuclease



Gene Regulation by the Dorsal Gradient in the *Drosophila* Embryo.
This stunning image shows cross sections through a *Drosophila* embryo at about the 14th nuclear division cycle after fertilization. The image on the left shows the gradient of Dorsal protein as revealed by a fluorescent antibody and, on the right, the resulting pattern of expression of a number of genes detected by *in situ* hybridization with fluorescent nucleic acid probes. At least 50 genes are controlled by Dorsal, some activated, others repressed.
Key to gene names: *dpp*, decapentaplegic; *ind*, intermediate neuroblasts defective; *sog,* short gastrulation; *sna*, snail; *vnd*, ventral neuroblasts defective.

CHAPTER 7

Control of Gene Expression

AN OVERVIEW OF GENE CONTROL

TERMS TO LEARN

mRNA degradation control
protein activity control
RNA localization control
RNA processing control
RNA transport control
transcriptional control
translational control

DEFINITIONS

Match each definition below with its term from the list above.

7–1 Regulates which RNAs are exported from the nucleus.

7–2 Regulates when and how often a given gene sequence is made into RNA.

7–3 Regulates which mRNA molecules are selectively destabilized in the cytoplasm.

7–4 Regulates which mRNAs are selected to be used for protein synthesis by ribosomes.

7–5 Regulates the splicing and modification of RNA transcripts.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–6 When the nucleus of a fully differentiated carrot cell is injected into a frog egg whose nucleus has been removed, the injected donor nucleus is capable of programming the recipient egg to produce a normal carrot.

7–7 The differences in the patterns of proteins produced in different specialized cell types are accurately reflected in the patterns of expressed mRNAs.

THOUGHT PROBLEMS

7–8 A small portion of a two-dimensional display of proteins from human brain is shown in **Figure 7–1**. These proteins were separated on the basis of size in one dimension and electrical charge (isoelectric point) in the other. Not all protein spots on such displays are products of different genes; some represent modified forms of a protein that migrate to different positions. Pick out a couple of sets of spots that could represent proteins that differ by the number of phosphates they carry. Explain the basis for your selection.

7–9 In principle, a eukaryotic cell can regulate gene expression at any step in the pathway from DNA to the active protein (**Figure 7–2**).

IN THIS CHAPTER

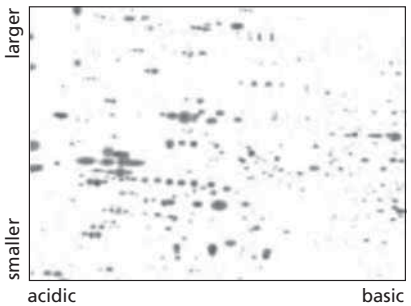


Figure 7–1 Two-dimensional separation of proteins from the human brain (Problem 7–8). The proteins were displayed using two-dimensional gel electrophoresis. Only a small portion of the protein spectrum is shown.

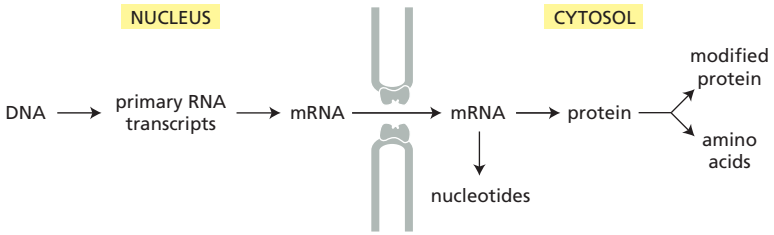


Figure 7–2 Six steps at which the pathway for eukaryotic gene expression can be controlled (Problem 7–9).

A. Place the types of control listed below at appropriate places on the diagram in Figure 7–2.
   1. mRNA degradation control
   2. protein activity control
   3. RNA processing control
   4. RNA transport and localization control
   5. transcriptional control
   6. translational control

B. Which of the types of control listed above are unlikely to be used in bacteria?

DATA HANDLING

7–10 In the original cloning of sheep from somatic cells, the success rate was very low. For example, only one lamb (Dolly) was born from 277 zygotes that were reconstructed using nuclei derived from breast cells and enucleated, unfertilized eggs. Other experiments using nuclei from embryonic or fetal lamb cells had a higher success rate, albeit still a low one. Given the rarity of successful events, it was critical to eliminate inadvertent mating—of either the oocyte donor or the surrogate mother—as the source of newborn lambs. To determine whether the cloned animals were derived from the donor nuclei, the researchers analyzed DNA microsatellites (short, repetitive DNA sequences) at four loci in surrogate mothers and donor cells (**Figure 7–3**). These loci were chosen because many different lengths are present in sheep populations.

A. Do the results in Figure 7–3 argue that the lambs were derived from the transplanted nuclei, or from an inadvertent mating? Explain your answer.

B. What would the results have looked like for the alternative you did not choose in question A?

7–11 One of the rare examples of developmentally programmed genome rearrangements in mammals occurs during the generation of antibody diversity in the immune system. In B cells, which produce antibodies, the variable (V) and constant (C) segments of immunoglobulin genes are brought close together by deletion of a long segment of DNA that lies

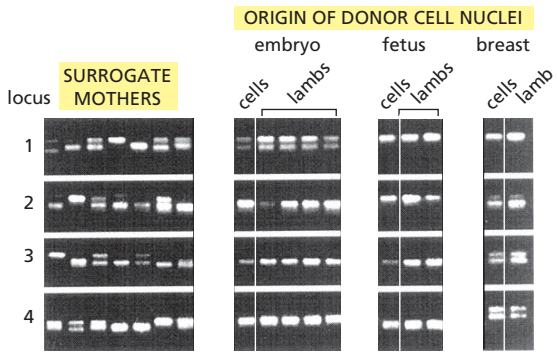


Figure 7–3 Microsatellite analysis of seven surrogate mothers, the three different nuclear donor cell types, and the seven lambs that were born (Problem 7–10). Four polymorphic loci were used in the analysis. The surrogate mothers are arranged *left* to *right* in the same order as the lambs they gave birth to. Nuclear donor cells were derived from embryo, fetus, or breast. At each of the four polymorphic loci, flanking polymerase chain reaction (PCR) primers were used to amplify DNA that included a particular microsatellite. Microsatellites with different numbers of repeats give rise to different length PCR products.

between them. Digestion of unrearranged germ-line DNA with a restriction nuclease that cuts in DNA flanking the V and C segments generates two bands on a Southern blot when hybridized to radioactive probes specific for the V and the C segments (**Figure 7–4**). Would you expect the V- and C-segment probes to hybridize to the same or different DNA fragments after digestion of B cell DNA with the same restriction nuclease? Sketch a possible pattern of hybridization to B cell DNA that is consistent with your expectations. Explain the basis for your pattern of hybridization. (Without a lot more information you cannot predict the exact pattern, so focus on the general features of the pattern.)

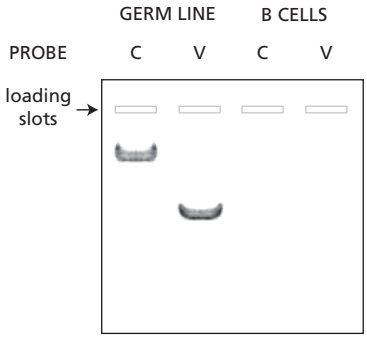


Figure 7–4 Southern blot of DNAs from the germ line and B cells (Problem 7–11). Germ-line DNA and B cell DNA were digested with the same restriction nuclease. Only the hybridization to germ-line DNA is shown.

MEDICAL LINKS

7–12 Comparisons of the patterns of mRNA levels across different human cell types show that the level of expression of almost every active gene is different. The patterns of mRNA abundance are so characteristic of cell type that they can be used to determine the tissue of origin of cancer cells, even though the cells may have metastasized to different parts of the body. By definition, however, cancer cells are different from their noncancerous precursor cells. How do you suppose then that patterns of mRNA expression might be used to determine the tissue source of a human cancer?

CONTROL OF TRANSCRIPTION BY SEQUENCE-SPECIFIC DNA-BINDING PROTEINS

TERMS TO LEARN

cis-regulatory sequence
transcription regulator

DEFINITIONS

Match each definition below with its term from the list above.

7–13 A protein that binds to a specific DNA sequence and influences the rate at which a gene is transcribed.

7–14 A short segment of DNA, 5–10 nucleotide pairs in length, in the neighborhood of the promoter, that serves as a binding site for a protein that modulates gene expression.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–15 Because the individual contacts are weak, the interactions between regulatory proteins and DNA are among the weakest in biology.

7–16 In terms of the way it interacts with DNA, the helix–loop–helix motif is more closely related to the leucine zipper motif than it is to the helix–turn–helix motif.

THOUGHT PROBLEMS

7–17 **Figure 7–5** shows a short stretch of a DNA helix displayed as a space-filling model. Indicate the major and minor grooves and provide a length scale. Is it possible to tell the polarity of each of the strands in this figure?

7–18 Explain how DNA-binding proteins can make sequence-specific contacts to a double-stranded DNA molecule without breaking the hydrogen bonds that hold the bases together. Indicate how, by making such

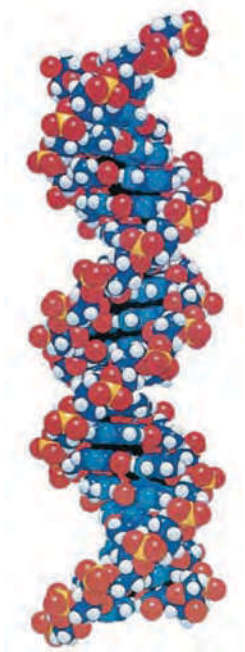


Figure 7–5 A space-filling model of a DNA duplex (Problem 7–17).

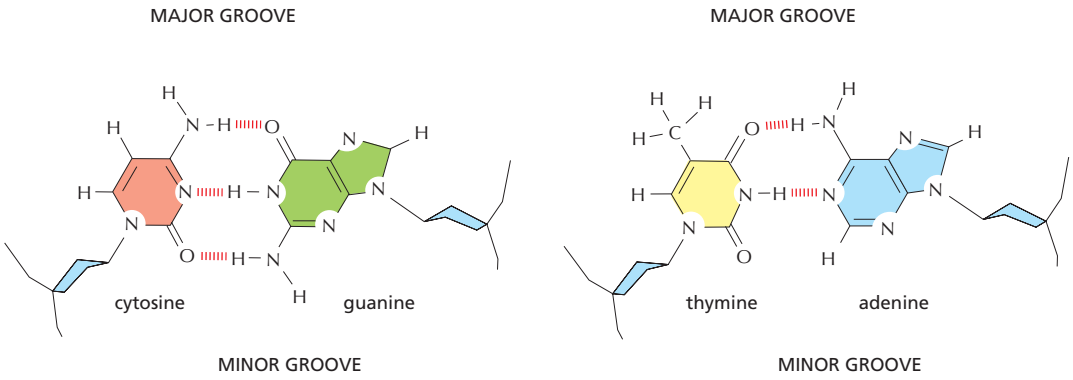


Figure 7–6 C-G and T-A base pairs (Problem 7–18).

contacts, a protein can distinguish a C-G from a T-A base pair. Use **Figure 7–6** to indicate what sorts of noncovalent bonds (hydrogen bonds, electrostatic attractions, or hydrophobic interactions) could be used to discriminate between C-G and T-A. (You do not need to specify any particular amino acid on the protein.)

7–19 What are the two fundamental components of a genetic switch?

7–20 The nucleus of a eukaryotic cell is much larger than a bacterium, and it contains much more DNA. As a consequence, a transcription regulator in a eukaryotic cell must be able to select its specific binding site from among many more unrelated sequences than does a transcription regulator in a bacterium. Does this present any special problems for eukaryotic gene regulation?

Consider the following situation. Assume that the eukaryotic nucleus and the bacterial cell each have a single copy of the same DNA binding site. In addition, assume that the nucleus is 500 times the volume of the bacterium, and has 500 times as much DNA. If the concentration of the transcription regulator that binds the site were the same in the nucleus and in the bacterium, would the regulator occupy its binding site equally as well in the eukaryotic nucleus as it does in the bacterium? Explain your answer.

7–21 One type of zinc finger motif consists of an α helix and a β sheet held together by a zinc ion (**Figure 7–7**). When this motif binds to DNA, the α helix is positioned in the major groove, where it makes specific contacts with the bases. This type of zinc finger is often found in a cluster with additional zinc fingers, an arrangement that allows strong and specific DNA–protein interactions to be built up through a repeating basic structural unit. Why do you suppose this motif is thought to enjoy a particular advantage over other DNA-binding motifs when the strength and specificity of the DNA–protein interaction needs to be adjusted during evolution?

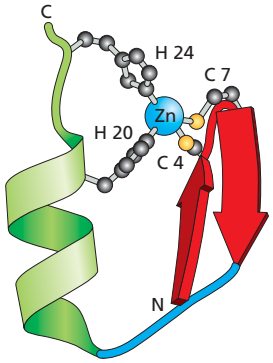


Figure 7–7 Zinc finger DNA-binding motif (Problem 7–21). The zinc ion interacts with Cys (C) and His (H) side chains so that the α helix is held tightly to one end of the β sheet.

7–22 Many transcription regulators form dimers of identical or slightly different subunits on the DNA. Suggest two advantages of dimerization.

7–23 Genetic analyses in bacteria in the 1950s provided the first evidence for the existence of transcription regulators. The lambda repressor, one such regulator, is encoded by the bacterial virus, bacteriophage lambda. The lambda repressor binds as a dimer to critical sites on the bacteriophage lambda genome to keep the lytic genes turned off, which allows the bacteriophage lambda genome to be maintained as a silent resident in the bacterial genome. Each molecule of the repressor consists of an N-terminal DNA-binding domain and a C-terminal dimerization domain (**Figure 7–8**). Upon induction (for example, by irradiation with ultraviolet light), the genes for lytic growth are expressed, bacteriophage lambda

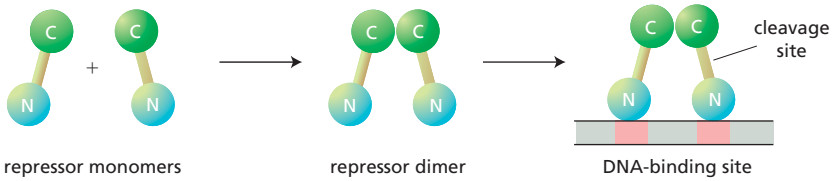


Figure 7–8 Domains of the lambda repressor and the binding of repressor dimers to DNA (Problem 7–23).

progeny are produced, and the bacterial cell lyses to release the viral progeny. Induction is initiated by cleavage of the lambda repressor at a site between the DNA-binding domain and the dimerization domain. In the absence of bound repressor, RNA polymerase initiates transcription of the lytic genes, triggering lytic growth. Given that the number (concentration) of DNA-binding domains is unchanged by cleavage of the repressor, why do you suppose its cleavage results in its removal from the DNA?

7–24 The differentiation of muscle cells from the somites of the developing embryo is controlled by myogenin, a member of the MyoD family of helix–loop–helix transcription regulators. Myogenin functions as a heterodimer with another member of the MyoD family of helix–loop–helix proteins (**Figure 7–9A**). The activity of myogenin must be carefully controlled lest it trigger premature expression of the muscle program of cell differentiation. The *myogenin* gene is turned on in advance of the time when it is needed, but myogenin is prevented from functioning by phosphorylation of its DNA-binding domain and by its tight binding to Id, a helix–loop–helix protein that lacks a DNA-binding domain (**Figure 7–9B**). Explain how phosphorylation of the DNA-binding domain and dimerization with Id might act to keep myogenin nonfunctional.

CALCULATIONS

7–25 One method for determining the DNA sites bound by a transcription regulator is to mix random sequences of DNA with the regulator, separate the bound sequences from the starting mixture, amplify the bound sequences by PCR, and then repeat this binding-release-amplification cycle until a consensus sequence emerges. But is it reasonable to expect that all possible consensus-site binding sequences will actually be present in the starting sample of oligonucleotides?

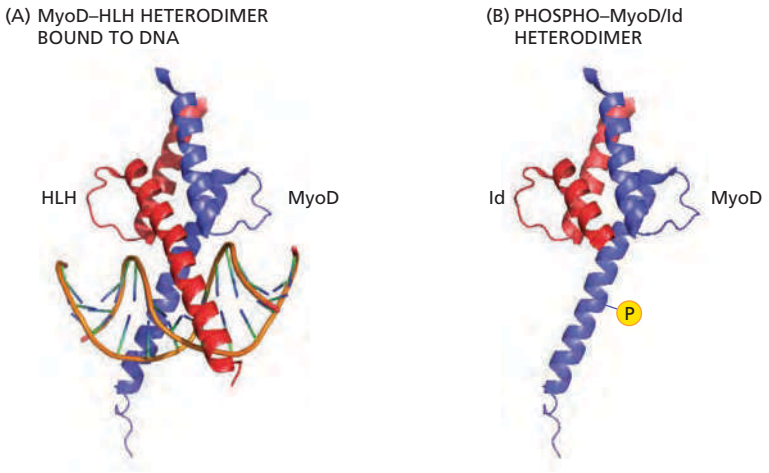


Figure 7–9 The transcription regulator myogenin (Problem 7–24). (A) Myogenin as part of a heterodimer bound to DNA. HLH stands for helix–loop–helix. (B) An inactive form of phosphorylated myogenin bound to Id.

TABLE 7–1 Nucleotide sequences of selected and amplified DNAs (Problem 7–25).

GAATTCGCCTCGAGCACATCATTGCCCATATATGGCACGACAGGATCC
GAATTCGCCTCTTCTAATGCCCATATATGGACTTGCTCGACAGGATCC
GGATCCTGTCGGTCCTTTATGCCCATATATGGTCATTGAGGCGAATTC
GAATTCGCCTCATGCCCATATATGGCAATAGGTGTTTCGACAGGATCC
GAATTCGCCTCTATGCCCATATAAGGCGCCACTACCCCGACAGGATCC
GAATTCGCCTCGTTCCCAGTATGCCCATATATGGACACGACAGGATCC
GGATCCTGTCGACACCATGCCCATATTTGGTATGCTCGAGGCGAATTC
GAATTCGCCTCATTTATGAACATGCCCTTATAAGGACCGACAGGATCC
GAATTCGCCTCTAATACTGCAATGCCCAAATAAGGAGCGACAGGATCC
GAATTCGCCTCATGCCCAAATATGGTCATCACCTACACGACAGGATCC

Underlined sequences correspond to PCR primer sites. The sequences have been ordered so that the binding sites are all oriented in the same direction (to make it easier to pick out the consensus sequence by eye).

Consider the following specific example. You wish to test all possible consensus sequences that are 14 nucleotides long. You synthesize a population of oligonucleotides that carry a central 26-nucleotide-long random sequence, flanked on either side by 25-nucleotide-long defined sequences to serve as primer sites for PCR amplification. You convert the single-stranded oligonucleotides to double-stranded ones, using one of the PCR primers. You begin the first cycle of binding with a 0.4 ng sample of the synthesized population of double-stranded oligonucleotides.

A. How many double-stranded oligonucleotides, 76-nucleotide pairs long, are present in the 0.4 ng sample with which you started the experiment? (The average mass of a nucleotide pair is 660 daltons.)
B. Assuming that the starting population of oligonucleotides was truly random in the 26 central nucleotides, would you expect to find all possible 14-base-pair-long sequences represented in the starting sample?
C. The sequences of 10 individual clones resulting from this procedure are shown in **Table 7–1**. What is the consensus sequence to which the transcription regulator binds?

DATA HANDLING

7–26 When Jacob and Wollman tried to check the genetic linkage between the *Gal* gene and an integrated bacteriophage lambda genome (termed a lambda prophage), they discovered a surprising phenomenon they referred to as "erotic induction" (which was later called zygotic induction for publication). In a bacterial mating used to determine genetic linkage, a portion of the chromosome is transferred via a narrow tube from the donor bacterium to the recipient. Jacob and Wollman found that if the donated chromosome carried a lambda prophage, but the recipient cell did not, lambda growth was induced in the recipient cell, which then lysed, producing lambda phage. If the recipient cell carried the lambda prophage, however, no lysis was observed. A summary of results from all their matings is shown in **Figure 7–10**.

A. Are these results consistent with the notion that a transcription regulator encoded by the prophage normally represses the bacteriophage's lytic genes. Why or why not?

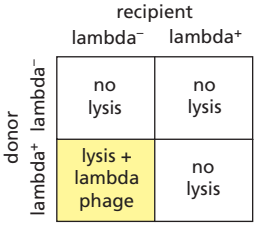


Figure 7–10 Results of matings between bacteria with and without lambda prophages (Problem 7–26). Lambda⁻ indicates the absence of a prophage; lambda⁺ indicates its presence.

B. Suppose that the prophage prevented lytic growth by expressing a transcription regulator that turned on a gene for an anti-lysis protein. Would the results of the matings have been the same or different? Explain your answer.

7–27 Transcription regulators often find their specific sites much faster than would be anticipated by simple three-dimensional diffusion. The *Lac* repressor, for example, associates with the *Lac* operator—its DNA binding site—more than 100 times faster than expected for three-dimensional diffusion. Clearly, the repressor must find the operator by mechanisms that reduce the dimensionality or volume of the search in order to hasten target acquisition.

Several techniques have been used to investigate this problem. One of the most elegant used fluorescent RNA polymerase molecules that could be followed individually. An array of DNA molecules was aligned in parallel by an electrophoretic technique and anchored to a glass slide. Fluorescent RNA polymerase molecules were then allowed to flow across them at an oblique angle (**Figure 7–11A**). Traces of individual RNA polymerases showed that about half flowed in the same direction as the bulk and about half deviated from the bulk flow in a characteristic manner (**Figure 7–11B**). If the RNA polymerase molecules were first incubated with short DNA fragments containing a strong promoter, all the traces followed the bulk flow.

A. Offer an explanation for why some RNA polymerase molecules deviated from the bulk flow as shown in Figure 7–11B. Why did incubation with short DNA fragments containing a strong promoter eliminate traces that deviated from the bulk flow?

B. Do these results suggest an explanation for how transcription regulators manage to find their sites faster than expected by diffusion?

C. Based on your explanation, would you expect a transcription regulator to find its target site faster in a population of short DNA molecules or in a population of long DNA molecules? Assume that the concentration of DNA is the same in both populations and that both populations have the same number of target sites.

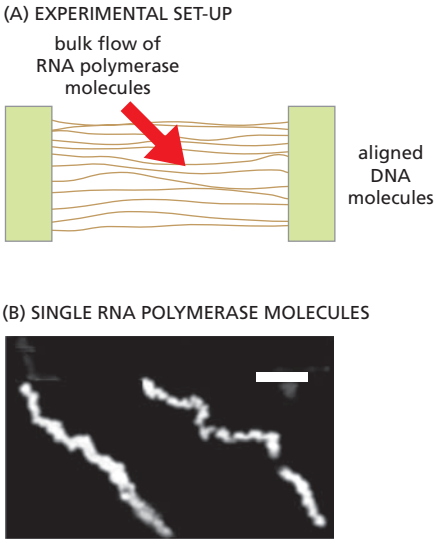


Figure 7–11 Interactions of individual RNA polymerase molecules with DNA (Problem 7–27). (A) Experimental set-up. DNA molecules are aligned and anchored to a glass slide at their ends, and highly fluorescent RNA polymerase molecules are allowed to flow across them. (B) Traces of two individual RNA polymerase molecules. The one on the *left* has traveled with the bulk flow, and the one on the *right* has deviated from it. The scale bar is 10 μm.

7–28 The binding of a transcription regulator to a DNA sequence can cause the DNA to bend to make appropriate contacts with groups on the surface of the protein. Such protein-induced DNA bending can be readily detected by the way the protein–DNA complexes migrate through polyacrylamide gels. The rate of migration of bent DNA depends on the average distance between its ends as it gyrates in solution: the more bent the DNA, the closer together the ends are on average, and the more slowly it migrates. If there are two sites of bending, the end-to-end distance depends on whether the bends are in the same (*cis*) or opposite (*trans*) directions (**Figure 7–12A**).

You have shown that the catabolite activator protein (CAP) causes DNA to bend by more than 90° when it binds to its regulatory site. You wish to know the details of the bent structure. Specifically, is the DNA at the center of the CAP-binding site bent so that the minor groove of the DNA helix is on the inside, or is it bent so that the major groove is on the inside? To answer this, you prepare two kinds of constructs, as shown in **Figure 7–12B**. In one, you place two CAP-binding sequences on either side of a central site into which you insert a series of DNA segments that vary from 10 to 20 nucleotide pairs in length. In the other, you flank the insertion site with one CAP-binding sequence and one $(A_5N_5)_4$ sequence, which is known to bend with the major groove on the inside. You measure the migration of the CAP-bound constructs relative to the corresponding CAP-bound DNA with no insert. You then plot the relative migration versus the number of nucleotide pairs between the centers of bending (**Figure 7–12C and D**).

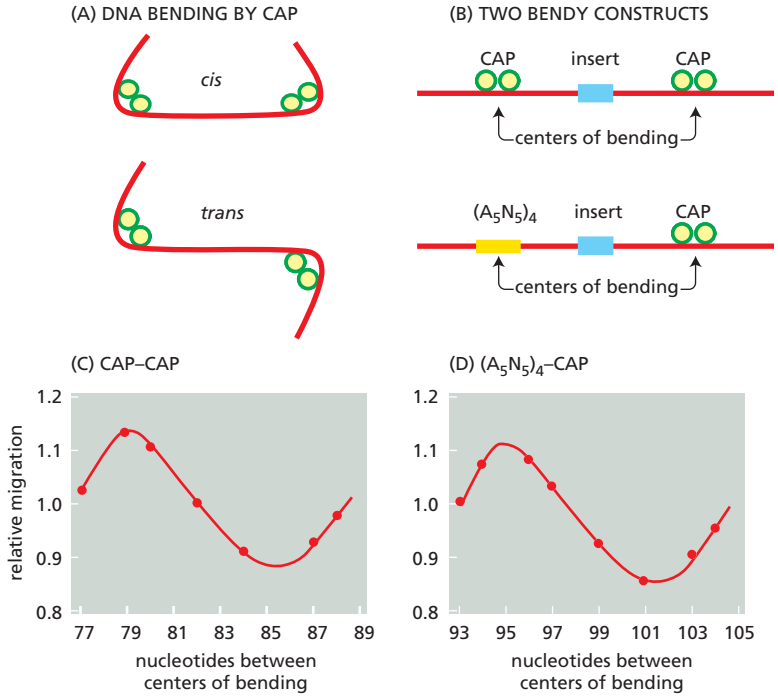


Figure 7–12 Bending of DNA by CAP binding (Problem 7–28). (A) The *cis* and *trans* configurations of a pair of bends. (B) Two constructs used to investigate DNA bending by CAP binding. (C) Relative migration as a function of the number of nucleotides between the centers of bending in the CAP–CAP construct. (D) Relative migration as a function of the number of nucleotides between the centers of bending in the $(A_5N_5)_4$–CAP construct.

A. Assuming that there are 10.6 nucleotides per turn of the DNA helix, estimate the number of turns that separate the centers of bending of the two CAP-binding sites at the point of minimum relative migration. How many helical turns separate the centers of bending at the point of maximum relative migration?
B. Is the relationship between the relative migration and the separation of the centers of bending of the CAP sites what you would expect, assuming that the *cis* configuration migrates slower than the *trans* configuration (Figure 7–12C)? Explain your answer.
C. How many helical turns separate the centers of bending at the point of minimum migration of the construct with one CAP site and one $(A_5N_5)_4$ site (Figure 7–12D)?
D. Which groove of the helix faces the inside of the bend at the center of bending of the CAP site?

7–29 The *Fos* and *Jun* oncogenes encode proteins that form a heterodimeric transcription regulator. Leucine zipper domains in each protein mediate their dimerization through coiled-coil interactions. Dimerization juxtaposes the DNA-binding domains of each protein, positioning them for interaction with regulatory sites in DNA. The dynamics of Fos–Jun interaction in the presence and absence of AP-1 DNA (the sequence to which the Fos–Jun heterodimer binds) were investigated by fluorescence resonance energy transfer (FRET), which is well suited for the rapid measurements that are necessary in such studies.

Fos was tagged with fluorescein (Fos–F) and Jun was tagged with rhodamine (Jun–R), as shown in **Figure 7–13A**. Fluorescein absorbs light at 490 nm and emits light at 530 nm, whereas rhodamine absorbs light at 530 nm and emits light at 603 nm. When Fos–F and Jun–R are brought into close proximity through heterodimerization, light energy absorbed by fluorescein at 490 nm is efficiently transferred to rhodamine through nonradiative energy transfer and emitted by rhodamine at 603 nm. Dimerization thus decreases fluorescence by fluorescein at 530 nm and increases fluorescence by rhodamine at 603 nm, as shown in **Figure 7–13B**. In the presence of AP-1 DNA, FRET is even more efficient

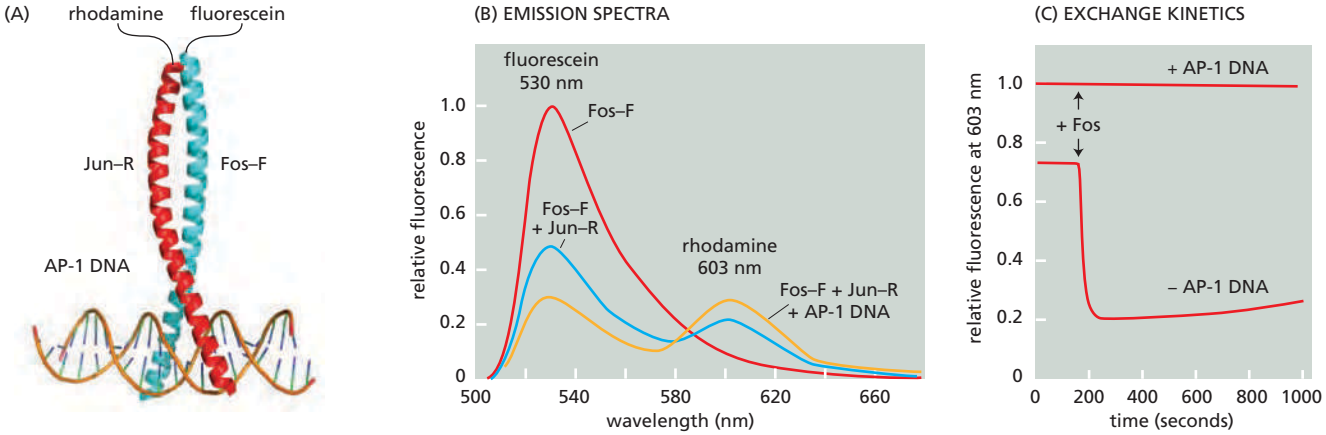


Figure 7–13 Dynamics of Fos–Jun heterodimerization in the presence and absence of AP-1 DNA (Problem 7–29). (A) Arrangement of fluorophores in Fos–F and Jun–R. In the dimer, the excited fluorescein on Fos transfers energy to the rhodamine on Jun. (B) Emission spectra of Fos–F alone, Fos–F with Jun–R, and Fos–F with Jun–R and AP-1 DNA. The emission spectra were recorded between 500 and 700 nm after excitation at 490 nm. (C) Analysis of the exchange of Fos–F and Jun–R in the presence and absence of AP-1 DNA. Rhodamine fluorescence at 603 nm was followed over time after excitation of fluorescein at 490 nm. A 10-fold excess of unlabeled Fos was added at the time indicated by the *arrow*.

(Figure 7–13B), indicating that binding to the DNA brings the two fluorophores into even closer proximity.

FRET was also used to measure the ability of Fos–Jun dimers to exchange subunits with monomers in solution in the presence and absence of AP-1 DNA (**Figure 7–13C**). Fos–F and Jun–R were preincubated in the absence of DNA to allow free heterodimers to form, or in its presence to allow DNA-bound heterodimers to form. A 10-fold excess of Fos (without fluorescein) was then added to both solutions, and rhodamine fluorescence at 603 nm was followed after excitation at 490 nm (Figure 7–13C).

A. Do free heterodimers exchange subunits with added unlabeled Fos? Do heterodimers bound to DNA exchange subunits? How can you tell? Explain any significant differences in the behavior of free and DNA-bound heterodimers.

B. In most cells, there are many distinct leucine zipper transcription regulators, several of which can interact to form a variety of heterodimers. If the results in Figure 7–13C were typical of the leucine zipper proteins, what would they imply about the leucine zipper heterodimers in cells?

7–30 The binding of a protein to DNA can protect it from chemical cleavage, which is the basis for the technique known as DNA footprinting. You have used DNA footprinting to determine the DNA binding site of a transcription regulator after labeling one strand. To check your results, you repeat the experiment after labeling the other strand of the duplex. You find that the footprints are slightly offset from one another relative to the sequence of the DNA (**Figure 7–14**). If the protein binds to the same duplex in both cases, how can the footprints on the two strands be different?

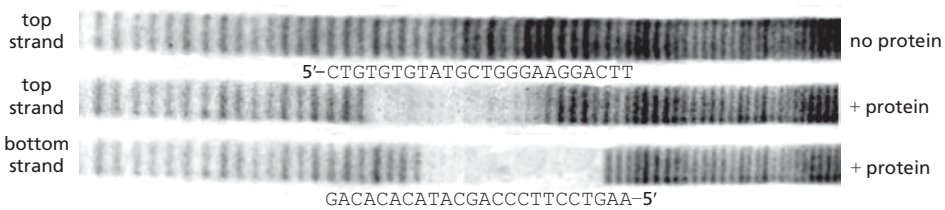


Figure 7–14 DNA footprints (Problem 7–30). The sequences of the DNA from the top and bottom strands around the footprint are shown. The 5′ ends of the top or bottom strands were labeled with ^{32}P. Chemical cleavage was used to introduce DNA breaks, which are fairly randomly distributed, as shown by the roughly equal intensities of the individual bands. Each band corresponds to a fragment of DNA that differs from those on either side by one nucleotide.

TRANSCRIPTION REGULATORS AS GENE SWITCHES

TERMS TO LEARN

cis-regulatory sequence
gene
gene control region
promoter

DEFINITIONS

Match each definition below with its term from the list above.

7–31 Nucleotide sequence in DNA to which RNA polymerase binds to begin transcription.

7–32 The whole expanse of DNA involved in regulating and initiating transcription of a gene.

7–33 The segment of DNA that is transcribed into RNA.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–34 Many transcription regulators in eukaryotes can act even when they are bound to DNA thousands of nucleotide pairs away from the promoter they influence.

7–35 The close-packed arrangement of bacterial genes and genetic switches may have developed from more extended forms of switches in response to the evolutionary pressure to maintain a small genome.

THOUGHT PROBLEMS

7–36 The genes encoding the enzymes for arginine biosynthesis are located at several positions around the genome of *E. coli*. The ArgR transcription regulator coordinates their expression. The activity of ArgR is modulated by arginine. Upon binding arginine, ArgR dramatically changes its affinity for the *cis*-regulatory sequences in the promoters of the genes for the arginine biosynthetic enzymes. Given that ArgR is a transcription repressor, would you expect that ArgR would bind more tightly, or less tightly, to the regulatory sequences when arginine is abundant? If ArgR functioned instead as a transcription activator, would you expect the binding of arginine to increase, or to decrease, its affinity for its regulatory sequences? Explain your answers.

7–37 Bacterial cells can take up the amino acid tryptophan from their surroundings, or, if the external supply is insufficient, they can synthesize tryptophan from small molecules in the cell. The tryptophan repressor inhibits transcription of the genes in the tryptophan operon, which encodes the tryptophan biosynthetic enzymes. Upon binding tryptophan, the tryptophan repressor binds to a site in the promoter of the operon.

A. Why is tryptophan-dependent binding to the operon a useful property for the tryptophan repressor?
B. What would you expect to happen to the regulation of the tryptophan biosynthetic enzymes in cells that express a mutant form of the tryptophan repressor that (i) cannot bind to DNA or (ii) binds to DNA even when no tryptophan is bound to it?
C. What would happen in scenarios (i) and (ii) if the cell produced normal tryptophan repressor from a second, unmutated copy of the gene?

7–38 In **Figure 7–15**, the bacterial activator protein CAP and the *Lac* repressor have been placed in the four possible combinations on their binding

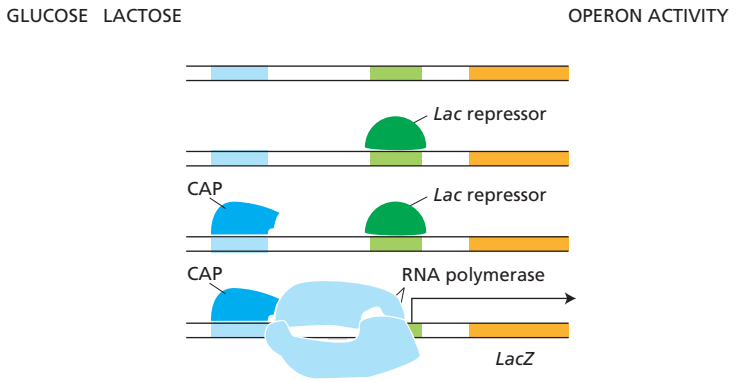


Figure 7–15 Arrangement of binding sites and the four possible combinations of transcription regulators on the promoter for the *Lac* operon (Problem 7–38).

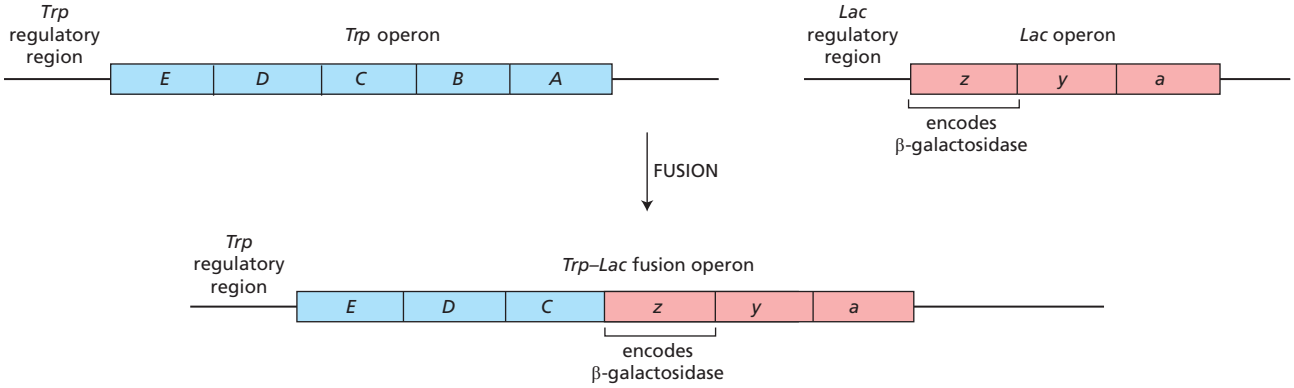
sites in the promoter for the *Lac* operon. Each combination of transcription regulators corresponds to the expected binding in a particular mixture of glucose and lactose. For each of the four combinations, indicate on the left-hand side of the figure which sugars are present and, on the right-hand side, whether the operon is expected to be turned on or off.

7–39 Imagine that you have created a fusion between the *Trp* operon, which encodes the enzymes for tryptophan biosynthesis, and the *Lac* operon, which encodes the enzymes necessary for lactose utilization (**Figure 7–16**). Under which set of conditions (A–F below) will β-galactosidase be expressed in the strain that carries the fused operon?

A. Only when lactose and glucose are both absent.
B. Only when lactose and glucose are both present.
C. Only when lactose is absent and glucose is present.
D. Only when lactose is present and glucose is absent.
E. Only when tryptophan is absent.
F. Only when tryptophan is present.

7–40 When *cis*-regulatory sequences were initially found to influence activity at distant promoters, two principal models were invoked to explain this action at a distance. In the "DNA looping" model, direct interactions between transcription regulators bound at *cis*-regulatory sequences and the distant promoters were proposed to stimulate RNA polymerase. In the "scanning" model, RNA polymerase (or a transcription regulator) was proposed to bind at the regulatory sequence and then slide along the DNA until it reached the promoter. These two models were distinguished using a *cis*-regulatory sequence on one piece of DNA and the *β-globin* gene with its promoter on a separate piece of DNA (**Figure 7–17**). The *β-globin* gene was not expressed from the mixture of pieces. However, when the two segments of DNA were joined via a protein linker, the *β-globin* gene was expressed.

Figure 7–16 Separated (normal) and fused *Trp* and *Lac* operons (Problem 7–39).



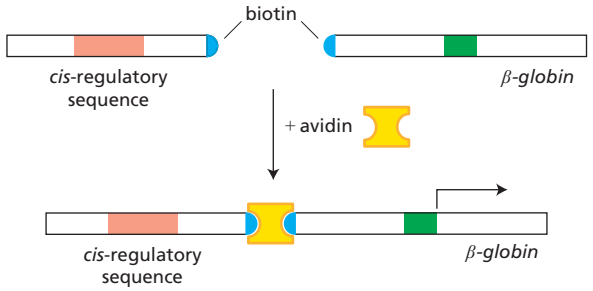


Figure 7–17 Stimulation of *β-globin* gene expression by a *cis*-regulatory sequence linked via a protein bridge (Problem 7–40). Each DNA molecule carries biotin attached to one end, as shown. In the presence of the protein avidin, the two molecules are linked together and transcription occurs, as shown by the *arrow* above the *β-globin* gene.

How does this experiment distinguish between the DNA looping model and the scanning model? Explain your answer.

7–41 Some transcription regulators bind to DNA and cause the double helix to bend at a sharp angle. Such "bending proteins" can affect the initiation of transcription without directly contacting any other protein. Can you devise a plausible explanation for how such proteins might work to modulate transcription? Draw a diagram that illustrates your explanation.

7–42 The yeast Gal4 transcription activator comprises two domains: a DNA-binding domain and an activation domain. The DNA-binding domain allows Gal4 to bind to appropriate DNA sequences located near genes that are required for metabolism of the sugar galactose. The activation domain binds to components of the transcriptional machinery (including RNA polymerase), attracting them to the promoter, so the regulated genes can be turned on. In the absence of Gal4, the galactose genes cannot be turned on. When Gal4 is expressed normally, the genes can be maximally activated. When Gal4 is massively overexpressed, however, the galactose genes are turned off. Why do you suppose that too much Gal4 squelches expression of the galactose genes?

7–43 How are histone modification enzymes and chromatin remodeling complexes recruited to unmodified chromatin, and how are they thought to aid in the activation of transcription from previously silent genes?

7–44 How is it that protein–protein interactions that are too weak to cause proteins to assemble in solution can nevertheless allow the same proteins to assemble into complexes on DNA?

7–45 Consider the following argument: "If the expression of every gene depends on a set of transcription regulators, then the expression of these transcription regulators must also depend on the expression of other transcription regulators, and their expression must depend on the expression of still other transcription regulators, and so on. Cells would therefore need an infinite number of genes, most of which would code for transcription regulators." How does the cell get by without having to achieve the impossible?

DATA HANDLING

7–46 *E. coli* proliferates faster on the monosaccharide glucose than it does on the disaccharide lactose for two reasons: (1) lactose is taken up more slowly than glucose and (2) lactose must be hydrolyzed to glucose and galactose (by β-galactosidase) before it can be further metabolized.

When *E. coli* is grown on a medium containing a mixture of glucose and lactose, it proliferates with complex kinetics (**Figure 7–18**, squares). The bacteria proliferate faster at the beginning than at the end, and there is a lag between these two phases when they virtually stop dividing. Assays of the concentrations of the two sugars in the medium show

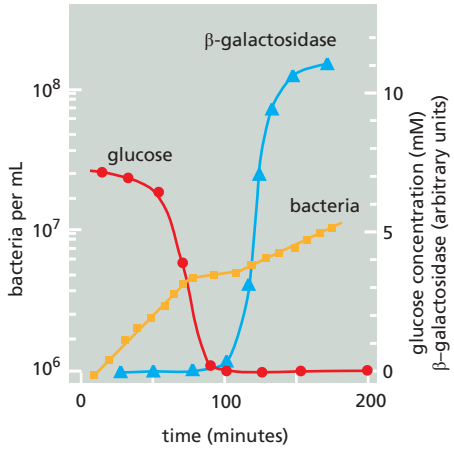


Figure 7–18 Proliferation of *E. coli* on a mixture of glucose and lactose (Problem 7–46).

that glucose falls to very low levels after a few cell doublings (Figure 7–18, circles), but lactose remains high until near the end of the experimental time course (not shown). Although the concentration of lactose is high throughout most of the experiment, β-galactosidase, which is regulated as part of the *Lac* operon, is not induced until more than 100 minutes have passed (Figure 7–18, triangles).

A. Explain the kinetics of bacterial proliferation during the experiment. Account for the rapid initial rate, the slower final rate, and the delay in the middle of the experiment.

B. Explain why the *Lac* operon is not induced by lactose during the rapid initial phase of bacterial proliferation.

7–47 Transcription of the bacterial gene encoding the enzyme glutamine synthetase is regulated by the availability of nitrogen in the cell. The key transcription regulator is the NtrC protein, which stimulates transcription when it is phosphorylated. Transcription of the gene for glutamine synthetase can be achieved *in vitro* by adding RNA polymerase, a special sigma factor, and phosphorylated NtrC. Although NtrC binds regardless of its phosphorylation state and RNA polymerase binds strongly to the promoter in the absence of NtrC, transcription is absolutely dependent on phosphorylated NtrC.

Activation of transcription by NtrC was characterized using three different templates: the normal gene with five regulatory sequences, a gene with all of the NtrC-binding sites deleted, and a gene with three NtrC-binding sites at its 3′ end (**Figure 7–19**). For half-maximal rates of transcription, the normal gene (Figure 7–19A) required 5 nM NtrC, the gene with 3′ NtrC-binding sites (Figure 7–19C) required 10 nM NtrC, and the gene without NtrC-binding sites (Figure 7–19B) required 50 nM NtrC.

A. If RNA polymerase can bind to the promoter of the glutamine synthetase gene in the absence of NtrC, why do you suppose NtrC is needed to activate transcription?

B. If NtrC can bind to its binding sites regardless of its state of phosphorylation, why do you suppose phosphorylation is necessary for transcription?

C. If NtrC can activate transcription even when its binding sites are absent, what role do the binding sites play?

7–48 Coactivators do not bind to DNA, but instead serve as bridging molecules that link transcription activators to general transcription factors at the promoter. The SAGA complex in yeast is a large multiprotein complex that is required for transcription of many genes. SAGA contains a variety of proteins, including a histone acetyl transferase and a subset of TATA-binding protein associated factors (TAFs). If SAGA functions as a

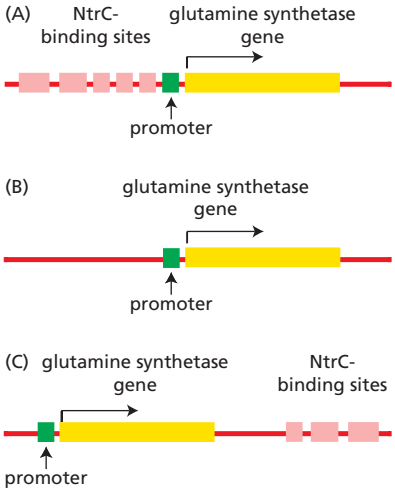


Figure 7–19 Three templates for studying the role of NtrC in transcription of the glutamine synthetase gene (Problem 7–47). (A) The normal gene with intact upstream regulatory sequences. (B) A gene with all of the NtrC-binding sites deleted. (C) A gene with three NtrC-binding sites at the 3′ end of the gene.

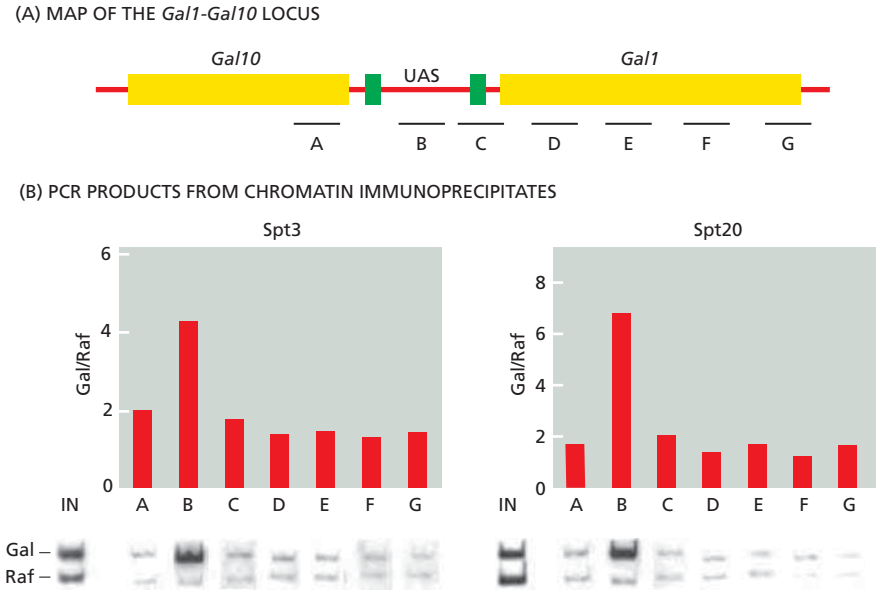


Figure 7–20 Analysis of SAGA association with the *Gal* promoter (Problem 7–48). (A) Organization of the *Gal10* and *Gal1* genes. A common UAS serves both promoters, which are indicated by *green boxes*. The positions of the PCR fragments that were used for quantification are shown by A–G below. (B) Results of chromatin immunoprecipitation using antibodies against Spt3 or Spt20. The strains were grown in medium containing raffinose (Raf) or galactose (Gal), their chromatin was fragmented and immunoprecipitated, and the DNA was amplified by quantitative PCR. The PCR products from strains grown on raffinose were loaded onto the gels and run for a few minutes before the PCR products from strains grown on galactose, so that the products could be compared within the same lanes. Finally, the amounts of PCR products were measured, normalized to the input chromatin before immunoprecipitation (IN), and the ratio (Gal/Raf) was calculated. About 50- to 100-fold less input chromatin (IN) was loaded per lane than immunoprecipitated chromatin, reflecting the inefficiency of chromatin immunoprecipitation.

coactivator, it should be physically present at the promoters it regulates under appropriate conditions.

To test this idea, you focus on the regulation of the *Gal1* and *Gal10* genes by the Gal4 activator, which binds to *cis*-regulatory sequences (termed UAS) adjacent to their promoters (**Figure 7–20A**, green boxes). When cells are grown on galactose, the *Gal1* and *Gal10* genes are transcribed; when the cells are grown on the sugar raffinose, the genes are not transcribed.

To determine whether the SAGA complex associates with Gal4 on the chromosome, you use the technique of chromatin immunoprecipitation. You shear the chromatin to small pieces and precipitate those that are bound to the SAGA complex, using antibodies against two components of the complex—Spt3 and Spt20. You strip the precipitated chromatin of protein and analyze the DNA by quantitative PCR to measure the amounts of specific DNA segments in the precipitates. The ratio of precipitated DNA sequences from galactose-grown cells to those from raffinose-grown cells (Gal/Raf) is shown for several segments around the galactose promoter in **Figure 7–20B**.

A. Which of the tested fragments would you have expected to be enriched if SAGA behaved as a coactivator? Explain your answer.

B. Does SAGA meet the criteria for a coactivator? Is it physically present at the promoter under appropriate conditions?

7–49 Genes expressed at the yeast mating type (*Mat*) locus on chromosome III determine the mating type of a haploid yeast cell. Identical mating-type genes also exist at two other loci—*Hml* and *Hmr*—on the same chromosome (**Figure 7–21A**). At these two silent loci, the mating-type genes are not expressed, even though all the signals for expression are present. Each silent mating-type locus is bracketed by two short DNA sequences, designated E and I, which bind a variety of proteins and serve to establish and maintain repression of genes within each locus.

To investigate the function of these elements, you insert the *Ura3* gene at different locations between E and I and outside the *Hml* locus, as shown in **Figure 7–21B**. You then test the growth of these strains in complete medium, in the absence of uracil (–uracil), and in the presence of 5-fluoroorotic acid (+FOA) (Figure 7–21B). These growth conditions

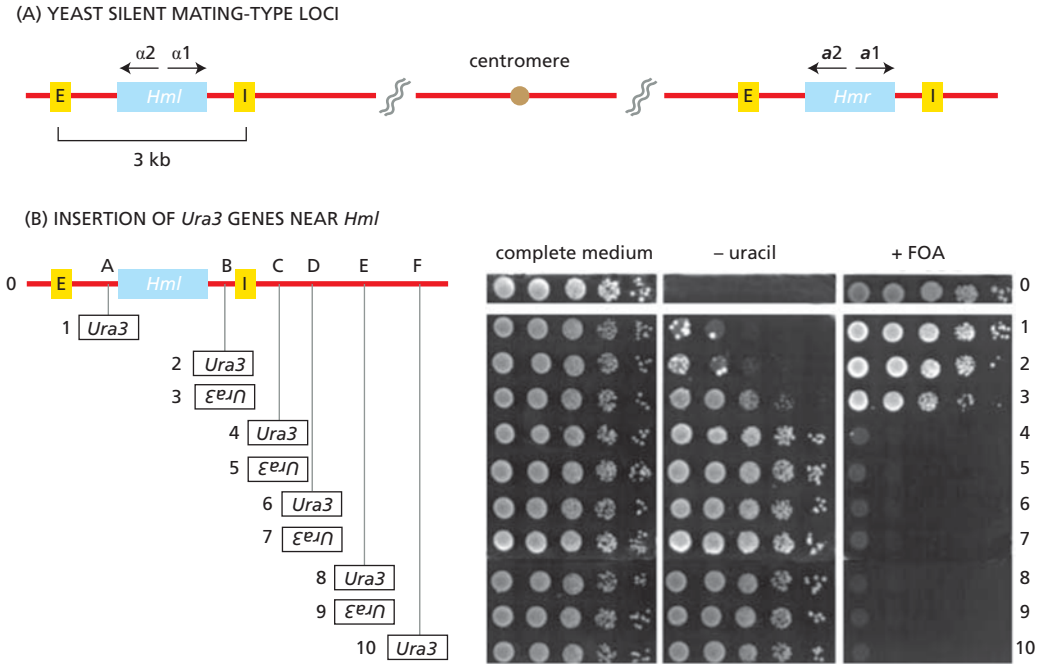


Figure 7–21 Effects of E and I elements at *Hml* on the expression of the *Ura3* gene inserted nearby (Problem 7–49). (A) Arrangement of E and I elements around *Hml* and *Hmr*. The mating-type genes are shown as α1, α2, ***a***1, and ***a***2. (B) Locations of the *Ura3* gene inserted around the *Hml* locus. Orientations of the *Ura3* gene are indicated by the direction of lettering. Growth phenotypes of each strain are indicated in the panel on the *right*. Strain 0 is the parental strain with no *Ura3* gene; strains 1 to 10 have the *Ura3* gene inserted as indicated. Serial dilutions of each strain were spotted on agar plates (with the highest concentration on the *left*). The agar contained complete medium, complete medium minus uracil (–uracil), or complete medium plus FOA (+FOA). *White areas* indicate growth.

test for the activity of the *Ura3* gene: in complete medium, expression of the Ura3 protein is irrelevant; in the absence of uracil, expression of Ura3 is required for growth; and in the presence of FOA, expression of Ura3 is lethal to the cell.

Do these control elements specifically repress the mating-type genes, or do they act like insulators that control the expression of any gene placed between them?

MOLECULAR GENETIC MECHANISMS THAT CREATE AND MAINTAIN SPECIALIZED CELL TYPES

TERMS TO LEARN

cell memory induced pluripotent stem (iPS) cell

DEFINITIONS

Match each definition below with its term from the list above.

7–50 Cell derived from a fibroblast, by artificial expression of specific transcription regulators, that looks and behaves like an embryonic stem cell.

7–51 The property that allows a proliferating cell to maintain its identity through subsequent cell divisions.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–52 In most organisms, extracellular molecules that bind to receptors on the cell surface communicate the positional cues that allow cells in the embryo to differentiate into appropriate cell types.

7–53 The fibroblasts that are converted to muscle cells by expression of the transcription regulator MyoD have probably already accumulated a number of transcription regulators that can cooperate with MyoD to switch on muscle-specific genes.

7–54 Once cells have differentiated to their final specialized forms, they never again alter expression of their genes.

THOUGHT PROBLEMS

7–55 Bacteriophage lambda can replicate as a prophage or lytically. In the prophage state, the viral DNA is integrated into the bacterial chromosome and is copied once per cell division. In the lytic state, the viral DNA is released from the chromosome and replicates many times. This viral DNA then produces viral coat proteins that enclose the replicated viral genomes to form many new virus particles, which are released when the bacterial cell bursts.

These two states are controlled by the transcription regulators cI and Cro, which are encoded by the virus. In the prophage state, cI is expressed; in the lytic state, Cro is expressed. In addition to regulating the expression of other genes, cI is a repressor of transcription of the gene that encodes Cro, and Cro is a repressor of the gene that encodes cI (**Figure 7–22**).

When bacteria containing a lambda prophage are briefly irradiated with ultraviolet (UV) light, cI protein is degraded.

A. What will happen next?
B. Will the change in question A be reversed when the UV light is switched off?
C. How is this mechanism beneficial to the virus?

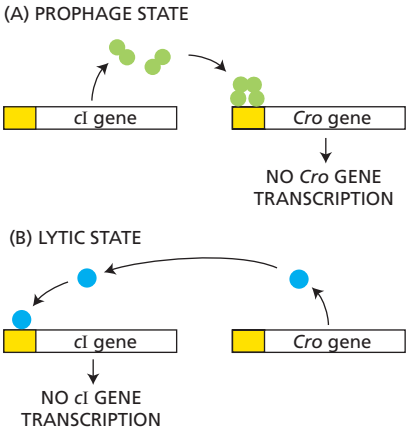


Figure 7–22 Regulation of bacteriophage lambda replication by cI and Cro (Problem 7–55). (A) The prophage state. (B) The lytic state.

7–56 Imagine the two situations shown in **Figure 7–23**. In cell 1, a transient signal induces the synthesis of protein A, which is a transcription activator that turns on many genes including its own. In cell 2, a transient signal induces the synthesis of protein R, which is a transcription repressor that turns off many genes including its own. In which, if either, of these situations will the descendants of the original cell "remember" that the progenitor cell had experienced the transient signal? Explain your reasoning.

7–57 **Figure 7–24** shows a simple scheme by which three transcription regulators might be used during development to create eight different cell types. How many cell types could you create, using the same rules, with four different transcription regulators? MyoD is a transcription regulator that can trigger the entire program of muscle differentiation when expressed in fibroblasts. How might you accomodate this observation into the scheme shown in Figure 7–24?

DATA HANDLING

7–58 The protein encoded by the *Even-skipped* (*Eve*) gene of *Drosophila* is a transcription regulator required for proper segmentation in the middle

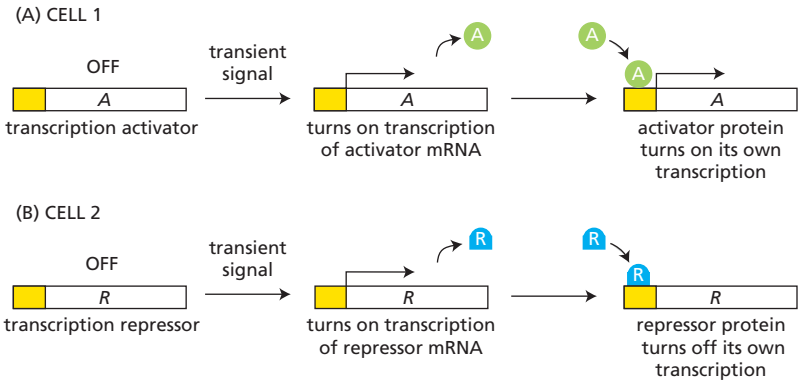


Figure 7–23 Gene regulatory circuits and cell memory (Problem 7–56). (A) Induction of synthesis of transcription activator A by a transient signal. (B) Induction of synthesis of transcription repressor R by a transient signal.

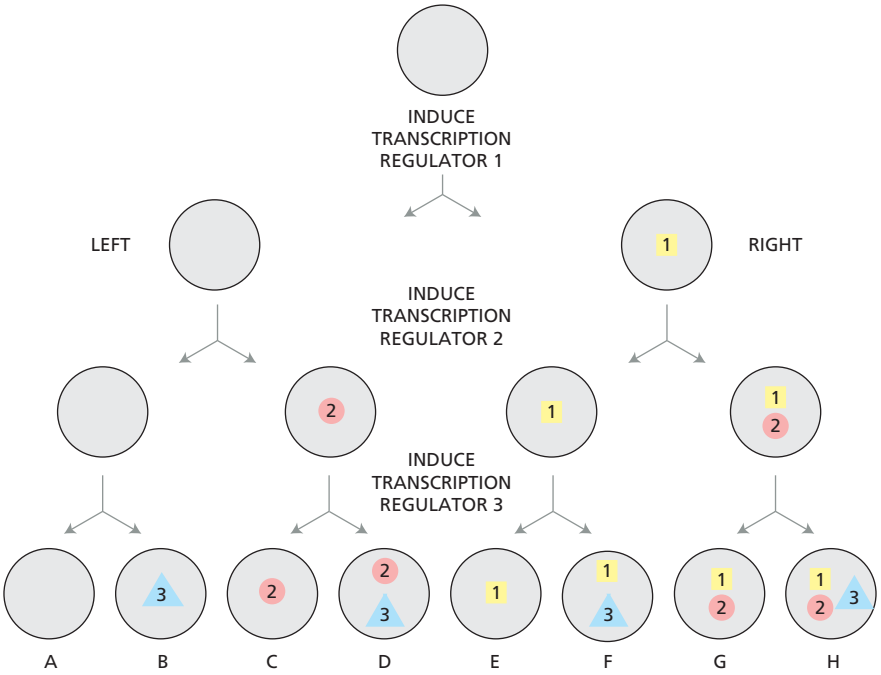


Figure 7–24 An illustration of combinatorial gene control for development (Problem 7–57). In this simple, idealized scheme, a "decision" to make a new transcription regulator *(numbered symbols)* is made after each cell division. In this scheme, the daughter cell on the *right* is induced to make the new transcription regulator. Each transcription regulator is assumed to continue to be expressed after it is induced, thereby allowing different combinations of regulatory proteins to be built up. In this example, eight different cell types have been created.

of the body. It first appears about 2 hours after fertilization at a uniform level in all the embryonic nuclei. Not long after that, it forms a pattern of seven stripes across the embryo. Each stripe is under the control of a separate module in the *Eve* promoter, which provides binding sites for both repressors and activators of *Eve* transcription. In the case of stripe 2, there are multiple, overlapping binding sites for activators and repressors (**Figure 7–25**). Two transcription activators, Hunchback (Hb) and Bicoid (Bcd), and two transcription repressors, Giant (Gt) and Krüppel (Kr), are required to give the normal pattern. The binding sites for these proteins have been mapped onto the 670-nucleotide segment shown in Figure 7–25; deletion of this upstream segment abolishes *Eve* expression in stripe 2. The patterns of expression of Hunchback, Bicoid, Giant, and Krüppel in the embryo are shown in **Figure 7–26**. It seems that *Eve* expression in stripe 2 occurs only in the region of the embryo that expresses both transcription activators, but neither transcription repressor: a simple enough rule.

To check if this rule is correct, you construct a *β-galactosidase* reporter gene driven by a 5 kb upstream segment from the *Eve* promoter. (This segment also includes the controlling elements for stripes 3 and 7.) In addition to the normal upstream element, you make three mutant versions in which several of the binding sites in the *Eve* stripe 2 control segment have been deleted.

Construct 1. Deletion of all the Krüppel-binding sites
Construct 2. Deletion of all the Giant-binding sites
Construct 3. Deletion of two Bicoid-binding sites (indicated by Δ in Figure 7–25)

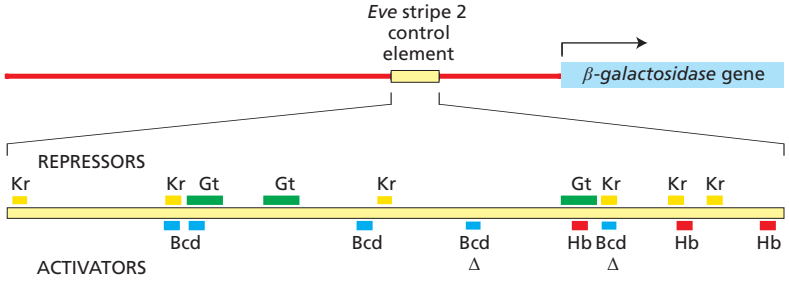


Figure 7–25 Binding sites for transcription repressors and activators of *Eve* stripe 2 (Problem 7–58). The *Eve* stripe 2 control segment, with additional DNA upstream of the *Eve* promoter, was fused to the *β-galactosidase* gene, whose product can be readily visualized in the embryo.

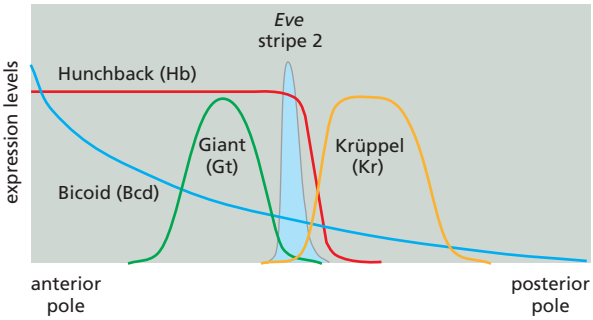


Figure 7–26 Expression of transcription repressors and activators of *Eve* stripe 2 in the *Drosophila* embryo (Problem 7–58).

You make flies containing these novel genetic constructs integrated into their chromosomes and determine the patterns of β-galactosidase expression in their embryos, which are shown in **Figure 7–27**.

A. Match the mutant embryos to the mutant constructs.
B. You began these experiments to test the simple rule that *Eve* expression in stripe 2 occurs in the embryo where the two transcription activators are present and the two transcription repressors are absent. Do the results with the mutant embryos confirm this rule? Explain your answer.
C. Offer a plausible explanation for why there is no expression of β-galactosidase at the anterior pole of mutant embryo D in Figure 7–27.
D. In the *Eve* stripe 2 control segment, the binding sites for the two transcription activators do not overlap, nor do the binding sites for the two transcription repressors; however, it is often the case that binding sites for activators overlap binding sites for repressors. What does this overlap suggest about the mode of genetic control of *Eve* in stripe 2, and what might be the consequences for stripe morphology?

7–59 Hormone receptors for glucocorticoids, upon hormone binding, become transcription regulators that activate a specific set of responsive genes. The DNA- and hormone-binding sites occupy distinct regions of the C-terminal half of the glucocorticoid receptor. Hormone binding could generate a functional DNA-binding protein either by altering receptor conformation to create a DNA-binding domain or by altering the conformation to uncover a preexisting DNA-binding domain.

These possibilities were investigated by comparing the activities of a series of C-terminal deletions (**Figure 7–28**). Segments of the cDNA that encode N-terminal portions of the receptor were expressed in cells, and their ability to activate a chloramphenicol acetyl transferase (*Cat*) gene—linked to a glucocorticoid-responsive *cis*-regulatory sequence—was measured. As shown in Figure 7–28, the full-length receptor (with its C-terminus at position 0) displayed CAT activity only in the presence of the glucocorticoid dexamethasone. Most of the mutant receptors failed to activate CAT expression in the presence or absence of dexamethasone. In contrast, the four mutant receptors lacking 190, 200, 239, and 270 C-terminal amino acids activated CAT expression in the presence and absence of dexamethasone.

How do these experiments distinguish between the proposed models for hormone-dependent conversion of the normal receptor to a DNA-binding form? Does hormone binding create a DNA-binding site or does it uncover a preexisting one?

7–60 One of the key transcription regulators produced by the yeast mating-type locus is a repressor protein known as α2. In haploid cells of the α mating type, α2 is essential for turning off a set of genes that are specific for the

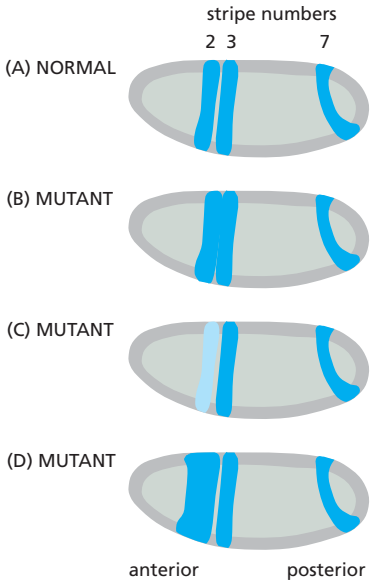


Figure 7–27 Embryonic expression of β-galactosidase from constructs with normal or mutated *Eve* stripe 2 control elements (Problem 7–58).

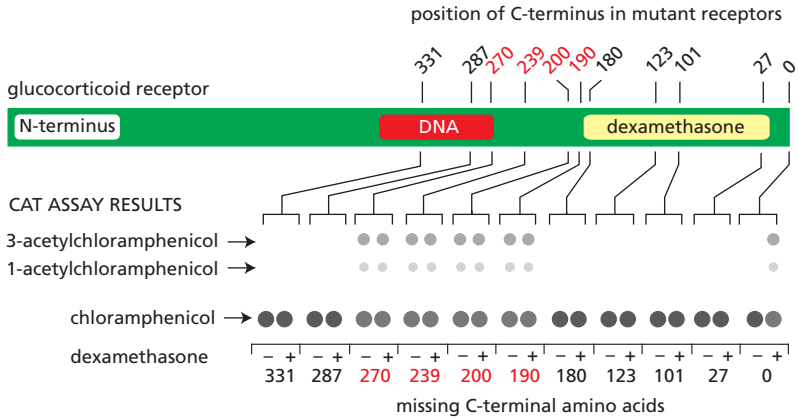


Figure 7–28 Effect of C-terminal deletions on the activity of the glucocorticoid receptor (Problem 7–59). The schematic diagram at the *top* illustrates the position of the DNA-binding site (DNA) and the glucocorticoid-binding site (dexamethasone) in the receptor, as well as the positions of the C-terminal deletions. The *lower* diagram shows the results of a standard CAT assay. The *lowest* spot is unreacted chloramphenicol; the *upper* spots show the attachment of acetyl groups to one or the other of two positions on chloramphenicol. The presence (+) or absence (–) of dexamethasone is indicated *below* appropriate lanes.

a mating type. In **a**/α diploid cells, the α2 repressor collaborates with the product of the **a**1 gene to turn off a set of haploid-specific genes in addition to the **a** mating-type-specific genes. Two distinct but related types of conserved DNA sequences are found upstream of these two sets of regulated genes: one in front of the **a**-specific genes and the other in front of the haploid-specific genes. Given the relatedness of these upstream sequences, it is most likely that α2 binds to both; however, its binding properties must be modified in some way by the **a**1 protein before it can recognize the haploid-specific sequence. You wish to understand the nature of this modification. Does **a**1 catalyze covalent modification of α2, or does it modify α2 by binding to it stoichiometrically?

To study these questions, you perform three types of experiment. In the first, you measure the binding of **a**1 and α2, alone and together, to the two kinds of upstream regulatory DNA sites. As shown in **Figure 7–29**, **a**1 alone does not bind radiolabeled DNA fragments that contain either regulatory site (Figure 7–29, lane 2), whereas α2 binds to **a**-specific fragments, but not to haploid-specific fragments (lane 3). The mixture of **a**1 and α2 binds to **a**-specific *and* haploid-specific fragments (lane 4).

In the second set of experiments, you add a vast excess of unlabeled DNA containing the **a**-specific sequence, along with the mixture of **a**1 and α2 proteins. Under these conditions, the haploid-specific fragment is still bound (Figure 7–29, lane 5). Similarly, if you add an excess of unlabeled haploid-specific DNA, the **a**-specific fragment is still bound (lane 6).

In the third set of experiments, you vary the ratio of **a**1 relative to α2. When α2 is in excess, binding to the haploid-specific fragment is decreased (Figure 7–29, lane 7); when **a**1 is in excess, binding to the **a**-specific fragment is decreased (lane 8).

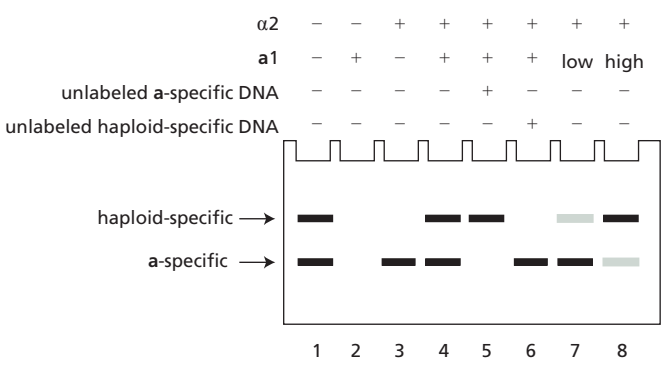


Figure 7–29 Binding of transcription regulators to fragments of DNA containing the **a**-specific or haploid-specific *cis*-regulatory sequences (Problem 7–60). Various combinations of regulators were incubated with a mixture of **a**-specific and haploid-specific radioactive DNA fragments (shown in lane 1). At the end of the incubation, the samples were precipitated with antibody against the proteins, and the DNA fragments in the precipitate were run on the gel. The gel was then placed against x-ray film to expose the positions of the radioactive DNA fragments.

A. In the presence of **a**1, is α2 present in two forms with different binding specificities, or in one form that can bind to both regulatory sequences? How do your experiments distinguish between these alternatives?
B. An α2 repressor with a small deletion in its DNA-binding domain does not bind to DNA fragments containing the haploid-specific sequence. When this mutant protein is expressed in a diploid cell along with normal α2 and **a**1 proteins, the haploid-specific genes are turned on. (These genes are normally off in a diploid.) In the light of this result and your other experiments, do you consider it more likely that **a**1 catalyzes a covalent modification of α2, or that **a**1 modifies α2 by binding to it to form an **a**1–α2 complex?

MECHANISMS THAT REINFORCE CELL MEMORY IN PLANTS AND ANIMALS

TERMS TO LEARN

CG island
DNA methylase
DNA methylation
epigenetic inheritance
genomic imprinting
monoallelic gene expression
X-inactivation
X-inactivation center (XIC)

DEFINITIONS

Match each definition below with its term from the list above.

7–61 Transcriptional silencing of gene expression on one of the two X chromosomes in the somatic cells of female mammals.

7–62 Addition of a $-CH_3$ group to cytosines in CG sequences in vertebrate DNA.

7–63 Heritable difference in the phenotype of a cell or an organism that does not result from changes in the nucleotide sequence of the DNA.

7–64 Expression from only one of the two copies of a gene in the diploid genome.

7–65 Situation in which one copy of a gene is either expressed or not expressed in the embryo, depending on which parent it is inherited from.

7–66 Long region of DNA with a much greater than average density of CG sequences, which usually remain unmethylated.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–67 Because CG sequences in one strand are paired with GC sequences in the other strand, two different methyl transferases are required to place methyl groups on the two strands.

7–68 CG islands are thought to have arisen during evolution because they were associated with portions of the genome that remained unmethylated in the germ line.

7–69 In most differentiated tissues, daughter cells retain a memory of gene expression patterns that were present in the parent cell through mechanisms that do not involve changes in the sequence of their genomic DNA.

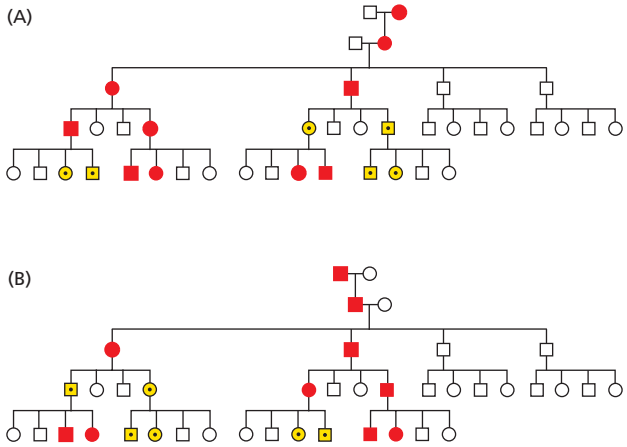


Figure 7–30 Pedigrees reflecting maternal and paternal imprinting (Problem 7–71). In one pedigree, the gene is paternally imprinted; in the other, it is maternally imprinted. In generations 3 and 4, only one of the two parents in the indicated matings is shown; the other parent is a normal individual from outside this pedigree. Affected individuals are represented by *red circles* for females and *red squares* for males. *Dotted yellow symbols* indicate individuals that carry the deletion but do not display the phenotype.

THOUGHT PROBLEMS

7–70 Maintenance methyl transferase, *de novo* DNA methyl transferases, and demethylating enzymes play crucial roles in the changes in methylation patterns during development. Starting with the unfertilized egg, describe in a general way how these enzymes bring about the observed changes in genomic DNA methylation.

7–71 Examine the two pedigrees shown in **Figure 7–30**. One results from deletion of a maternally imprinted autosomal gene. The other pedigree results from deletion of a paternally imprinted autosomal gene. In both pedigrees, affected individuals (*red* symbols) are heterozygous for the deletion. These individuals are affected because one copy of the chromosome carries an imprinted, inactive gene, while the other carries a deletion of the gene. Dotted *yellow* symbols indicate individuals that carry the deleted locus, but do not display the mutant phenotype. Which pedigree is based on paternal imprinting and which on maternal imprinting? Explain your answer.

7–72 Imprinting occurs only in mammals, and why it should exist at all is a mystery. One idea is that it represents an evolutionary end point in a tug of war between the sexes. In most mammalian species, a female can mate with multiple males, generating multiple embryos with different fathers. If one father could cause more rapid growth of his embryo, it would prosper at the expense of the other embryos. While this would be good for the father's genes (in an evolutionary sense), it would drain the resources of the mother, potentially putting her life at risk (not good for her genes). Thus, it is in the mother's interest to counter these paternal effects with maternal changes that limit the growth of the embryo.

Based on this scenario, decide whether the *Igf2* gene, whose product—insulin-like growth factor-2—is required for prenatal growth, is more likely to be imprinted in the male or in the female.

DATA HANDLING

7–73 You are studying the role of DNA methylation in the control of gene expression, using the human *γ-globin* gene as a test system (**Figure 7–31**). Globin mRNA can be detected when this gene is integrated into the genome of mouse fibroblasts, even though it is expressed at much lower levels than in red blood cells. If the gene is methylated at all 27 of its CG sites before integration, its expression is blocked completely. You are using this system to decide whether a single critical methylation site is sufficient to determine globin expression.

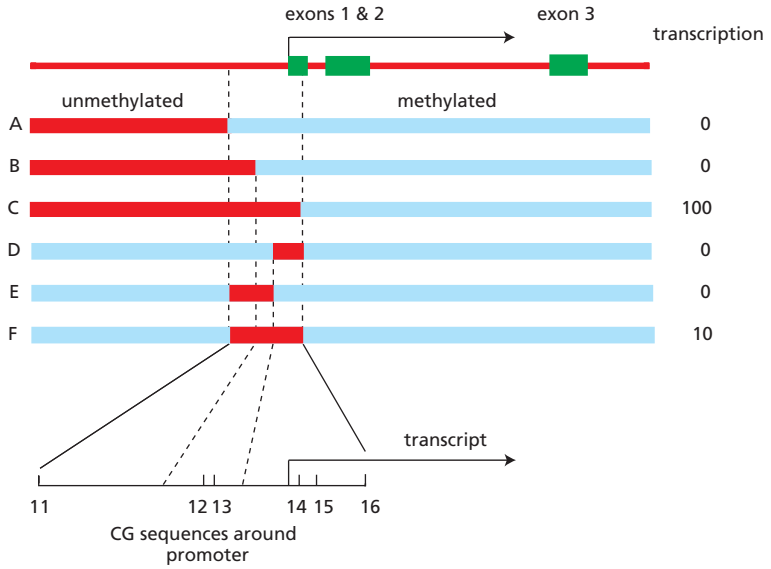


Figure 7–31 Effects of methylation on transcription of the γ-*globin* gene (Problem 7–73). The methylated segments of the gene are shown in *blue*; unmethylated segments are shown in *red*. The six CG sites around the promoter are shown in more detail *below* the constructs. The level of expression of γ-globin RNA from each construct is shown on the *right* as a percentage of the expression from a fully unmethylated construct.

You create several different γ-globin constructs that are unmethylated in specific regions of the gene. These constructs are illustrated in Figure 7–31, with the methylated regions shown in *blue*. The arrangement of six methylation sites around the promoter is shown below the constructs. Sites 11, 12, and 13 are unmethylated in construct E; sites 14, 15, and 16 are unmethylated in construct D; and all six sites are unmethylated in construct F. You integrate these constructs in mouse fibroblasts, grow cell lines containing individual constructs, verify their methylation patterns, and measure γ-globin RNA synthesis relative to cell lines containing the fully unmethylated construct (Figure 7–31).

Does the γ-globin transcription associated with the cell lines containing the constructs (Figure 7–31) indicate that a single critical site of methylation determines whether the gene is expressed? How can you tell?

7–74 In female mammals, one X chromosome in each cell is chosen at random for inactivation early in development. X-inactivation, which involves more than 1000 genes in humans, is crucial for equalizing expression of X-chromosome genes in males and females. A critical clue to the mechanism of X-inactivation came from the isolation of a large number of cDNAs for genes on the human X chromosome. Their expression patterns were characterized in cells from normal males and females, in cells from individuals with abnormal numbers of X chromosomes, and in rodent:human hybrid cell lines that retained either one inactive human X chromosome (X_i) or one active human X chromosome (X_a). Among all these cDNAs, there were three patterns of expression, as illustrated by cDNAs A, B, and C in **Figure 7–32**.

A. For each pattern of expression, decide whether the gene is expressed from the active X, the inactive X, or both. Which pattern do you expect to be the most common? Which pattern is the most surprising?

B. From the results with cells from abnormal individuals, formulate a rule as to how many chromosomes are inactivated, and how many remain active during X-inactivation.

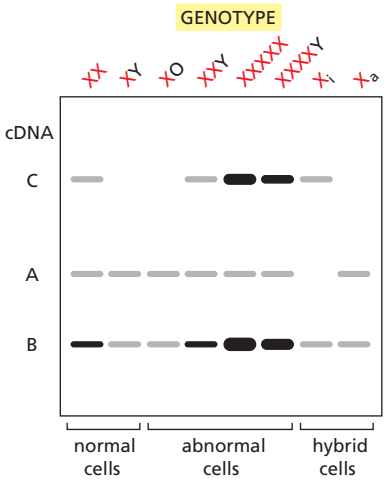


Figure 7–32 Northern analysis of gene expression from cells with different numbers and types of X chromosomes (Problem 7–74). RNA from cells was run out on gels, blotted onto nitrocellulose, probed with a mixture of radioactive cDNAs A, B, and C, and visualized by autoradiography. The positions of the RNA bands that correspond to genes A, B, and C were determined in a separate experiment.

POST-TRANSCRIPTIONAL CONTROLS

TERMS TO LEARN

alternative RNA splicing
internal ribosome entry site (IRES)
post-transcriptional control
regulated nuclear transport
RNA editing

DEFINITIONS

Match each definition below with its term from the list above.

7–75 Production of a functional RNA by insertion or alteration of individual nucleotides in an RNA transcript after it has been synthesized.

7–76 A way to generate different proteins from the same gene by combining different segments of the initial RNA transcript to make distinct mRNAs.

7–77 General term for a regulatory event that occurs after RNA polymerase has bound to the gene's promoter and begun RNA synthesis.

7–78 A sequence in the interior of an mRNA that folds into structures that bind translation initiation proteins.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–79 If the site of transcript cleavage and poly-A addition for a particular RNA in one cell is downstream of the site used for cleavage and poly-A addition for the same RNA in a second cell, the protein produced from the longer polyadenylated RNA will necessarily contain additional amino acids at its C-terminus.

7–80 In one extreme case, a single gene in *Drosophila*—the *Dscam* gene—has the potential to produce more than 38,000 different proteins by alternative splicing; thus, the complexity of this one gene rivals the complexity of the whole human genome.

THOUGHT PROBLEMS

7–81 RNA polymerase II commonly terminates transcription of the HIV (the human AIDS virus) genome a few hundred nucleotides after it begins, unless helped along by a virus-encoded protein called Tat, which binds to a specific hairpin structure in the nascent viral RNA. Tat then recruits a collection of proteins, including the protein kinase Cdk9, which phosphorylates RNA polymerase, enhancing its ability to continue transcription. Flavopiridol is the most potent inhibitor of Cdk9 yet discovered; it blocks Cdk9-mediated phosphorylation. Would you expect flavopiridol to interfere with HIV transcription? Why or why not?

7–82 Several distinct mechanisms for mRNA localization have been discovered. They all require specific sequences in the mRNA itself, usually in the 3′ untranslated region (UTR). Briefly outline three mechanisms by which cellular mRNAs might become localized in the cell.

7–83 Regulation of ferritin translation is controlled by the interaction between a hairpin structure in the mRNA, termed an iron-response element (IRE), and the iron-response protein (IRP) that binds to it. When the IRE is bound by the IRP, translation is inhibited. The location of the IRE in the mRNA is critical for its function. To work properly, it must be positioned near the 5′ end of the mRNA. If it is moved more than 60 nucleotides downstream of the cap structure, the IRE no longer inhibits translation.

Why do you suppose there is such a critical position-dependence for regulation of translation by IRE and IRP?

7–84 Although essential for the cell, iron is also potentially toxic. It is maintained at optimal levels in mammalian cells by the actions of three proteins. Transferrin binds to extracellular iron ions and delivers them to cells; the transferrin receptor binds iron-loaded transferrin and brings it into the cell; and ferritin binds iron (up to 4500 atoms in the internal cavity in each complex) to provide an intracellular storage site.

The regulation of the transferrin receptor, like that of ferritin (see Problem 7–83), is accomplished by IREs and IRPs, and, in both cases, iron binding to IRPs prevents their binding to IREs. Nevertheless, the mechanism of transferrin-receptor regulation is quite different from that of ferritin. The transferrin receptor mRNA contains five IREs that are all located in the 3′ untranslated region of the mRNA (instead of at the 5′ end, as in ferritin mRNA). In addition, binding of IRPs to these IREs increases the translation of transferrin receptor (as opposed to a decrease for ferritin).

A. Does this opposite regulation of ferritin and transferrin receptor in response to iron levels make biological sense? Consider the consequences of high and low iron levels.

B. In the presence of iron, the transferrin receptor mRNA is rapidly degraded; in the absence of iron, it is stable. Can you suggest a mechanism for how iron levels might be linked to the stability of transferrin receptor mRNA?

7–85 *Vg1* mRNA encodes a member of the TGFβ family of growth factors, which is important for mesoderm induction. In *Xenopus*, *Vg1* mRNA is localized to the vegetal pole of the oocyte. Translation of *Vg1* mRNA does not occur until a late stage, after localization is complete. Analysis of the 3′ untranslated region identified two elements: a localization element and a UA-rich translation control element. Translational regulation was shown to be independent of the poly-A tail. By contrast, translational repression was abolished when an internal ribosome entry site (IRES) sequence was inserted into the mRNA upstream of the coding sequence. How do you suppose translation of *Vg1* mRNA is controlled?

DATA HANDLING

7–86 A repeated hexanucleotide element, TGCATG, has been shown to regulate the tissue-specific splicing of the fibronectin alternative exon EIIIB. You wonder if it might also regulate alternative splicing in the calcitonin/CGRP gene (**Figure 7–33**). In thyroid cells, an mRNA containing exons 1, 2, 3, and 4 encodes calcitonin. In neuronal cells, an mRNA containing exons 1, 2, 3, 5, and 6 encodes calcitonin gene-related peptide (CGRP).

When you examine the calcitonin/CGRP gene you find five copies of a related repeat, GCATG, within 500 nucleotides of the exon-4 splice site (Figure 7–33). To analyze their potential role in alternative splicing, you make several calcitonin/CGRP constructs in which the repeats have been altered singly or in combination. You transfect the constructs into HeLa cells, which normally give the calcitonin splicing pattern, and into F9 cells, which normally give the CGRP splicing pattern. You find that no single mutation alters the pattern seen with wild type (not shown); however, various combinations of the mutations have dramatic effects (Figure 7–33).

A. Why do you suppose the most dramatic changes were seen in HeLa cells rather than in F9 cells?

B. Is there a particular combination of GCATG repeats that is critical for proper calcitonin mRNA production in HeLa cells? If so, what is it?

7–87 In humans, two closely related forms of apolipoprotein B (ApoB) are found in blood as constituents of the plasma lipoproteins. ApoB48,

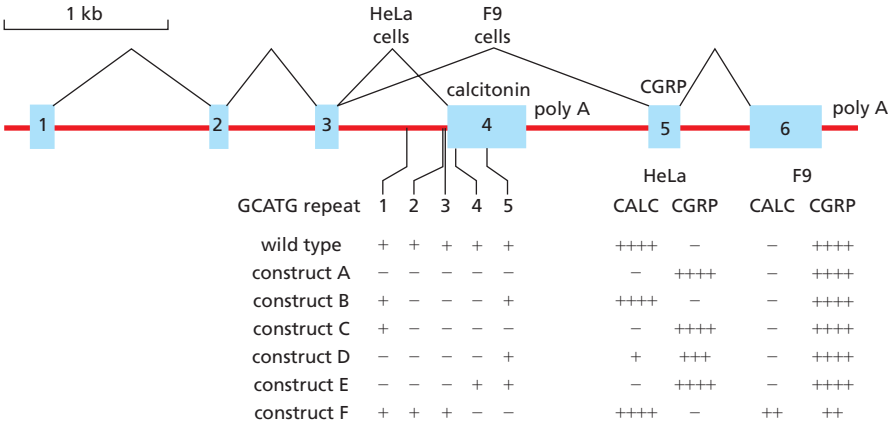


Figure 7–33 Effects of mutations in GCATG repeats on alternative splicing of calcitonin/CGRP pre-mRNA (Problem 7–86). In HeLa cells, exon 3 is spliced to exon 4 to make calcitonin mRNA; in F9 cells, exon 3 is spliced to exon 5 to make CGRP mRNA. The positions of the GCATG repeats around exon 4 are indicated. In the various constructs, the presence of a GCATG repeat is indicated by a *plus*, and its absence by a *minus*. Production of calcitonin- (CALC) and CGRP-specific spliced RNA is indicated relative to wild type as follows: ++++ = 80–100%, +++ = 60–80%, ++ = 40–60%, + = 20–40%, and – = 0–20%.

which has a molecular mass of 48 kilodaltons, is synthesized by the intestine and is a key component of chylomicrons, the large lipoprotein particles responsible for delivery of dietary triglycerides to adipose tissue for storage. ApoB100, which has a molecular mass of 100 kilodaltons, is synthesized in the liver for formation of the much smaller, very-low-density lipoprotein particles used in the distribution of triglycerides to meet energy needs. A classic set of studies defined the surprising relationship between these two proteins.

Sequences of cloned cDNA copies of the mRNAs from these two tissues revealed a single difference: cDNAs from intestinal cells had a T, as part of a stop codon, at a point where the cDNAs from liver cells had a C, as part of a glutamine codon (**Figure 7–34**). To verify the differences in the mRNAs and to search for corresponding differences in the genome, RNA and DNA were isolated from intestinal and liver cells and then subjected to PCR amplification, using oligonucleotides that flanked the region of interest. The amplified DNA segments from the four samples were tested for the presence of the T or C by hybridization to oligonucleotides containing either the liver cDNA sequence (oligo-Q) or the intestinal cDNA sequence (oligo-STOP). The results are shown in **Table 7–2**.

Are the two forms of ApoB produced by transcriptional control from two different genes, by a processing control of the RNA transcript from a single gene, or by differential cleavage of the protein product from a single gene? Explain your reasoning.

7–88 The level of *β-tubulin* gene expression is established in cells by an unusual regulatory pathway, in which the intracellular concentration of free tubulin dimers (composed of one α-tubulin and one β-tubulin subunit) regulates the rate of new β-tubulin synthesis. The initial evidence for such an autoregulatory pathway came from studies with drugs that cause assembly or disassembly of all cellular tubulin. For example, treatment of cells with colchicine, which causes microtubule depolymerization into

TABLE 7–2 Hybridization of specific oligonucleotides to the amplified segments from liver and intestine RNA and DNA (Problem 7–87).

	RNA		DNA	
	Intestine	Liver	Intestine	Liver
Oligo-Q	+	–	+	+
Oligo-STOP	–	+	–	–

Hybridization is indicated by +; absence of hybridization is indicated by –.

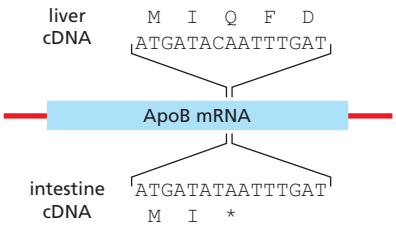


Figure 7–34 Location of the sequence difference in cDNA clones from ApoB RNA isolated from liver and intestine (Problem 7–87). The encoded amino acid sequences, in the one-letter code, are shown aligned with the cDNA sequences. The *asterisk* indicates a stop codon.

	M	R	E	I	regulation
--ATGAGGGAAATC--					+
-----T----------					–
-----C----------					+
------C---------					–
-------A--------					+
--------C-------					–
---------G------					–
----------G-----					+
-----TA---------					–
-----C-C--------					+
-----G--A-------					–
-----G---T------					–
-----C----G-----					+

Figure 7–35 Effects of mutations on the regulation of β-tubulin mRNA stability (Problem 7–88). The wild-type sequence for the first 12 nucleotides of the coding portion of the gene is shown at the *top*, and the first four amino acids beginning with methionine (M) are indicated *above* the codons. The nucleotide changes in the 12 mutants are shown *below*; only the altered nucleotides are indicated. Regulation of mRNA stability is shown on the *right*: + indicates wild-type response to changes in intracellular tubulin concentration and – indicates no response to changes. *Vertical lines* mark the position of the first nucleotide in each codon.

tubulin dimers, represses β-tubulin synthesis 10-fold. Autoregulation of β-tubulin synthesis by tubulin dimers is accomplished at the level of β-tubulin mRNA stability. The first 12 nucleotides of the coding portion of the mRNA were found to contain the site responsible for this autoregulatory control.

Since the critical segment of the mRNA involves a coding region, it is not clear whether the regulation of mRNA stability results from the interaction of tubulin dimers with the RNA or with the nascent protein. Either interaction might plausibly trigger a nuclease that would destroy the mRNA.

These two possibilities were tested by mutagenizing the regulatory region on a cloned version of the gene. The mutant genes were then expressed in cells, and the stability of their mRNAs was assayed in the presence of excess free tubulin dimers. The results from a dozen mutants that affect a short region of the mRNA are shown in **Figure 7–35**.

Does the regulation of β-tubulin mRNA stability result from an interaction with the RNA or from an interaction with the encoded protein? Explain your reasoning.

7–89 In nematodes, the choice between spermatogenesis and oogenesis in the hermaphrodite germ line depends on translational regulation of the *Tra2* and *Fem3* genes, as shown in **Figure 7–36**. When they are expressed, *Tra2* promotes oogenesis and *Fem3* promotes spermatogenesis. Translation of each gene's mRNA is regulated by the binding of proteins to elements within their 3′ untranslated regions. In each case, the protein-bound mRNA, although stable, is not efficiently translated and has a short poly-A tail. In each case, the mRNA in its unbound form is translationally active and has a long poly-A tail. How do you suppose that the lengths of the poly-A tails might affect the efficiency of translation of these mRNAs?

7–90 To investigate the molecular mechanism by which the *Tra2* regulatory elements (TGEs) control translation, you inject *Tra2* mRNAs into *Xenopus* oocytes and follow their fate. You have shown that an oocyte protein binds to the TGEs and inhibits translation of injected *Tra2* mRNA. Now you wish to determine how mRNAs with bound proteins come to have short poly-A tails. To investigate this question, you prepare two kinds

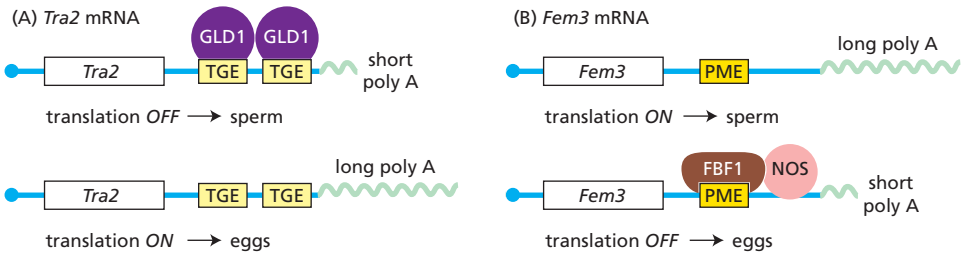


Figure 7–36 Translational control of the choice between spermatogenesis and oogenesis (Problem 7–89). (A) *Tra2* mRNA. (B) *Fem3* mRNA. Control elements within the 3′ untranslated regions of the two genes are shown, along with the specific proteins that bind to those elements.

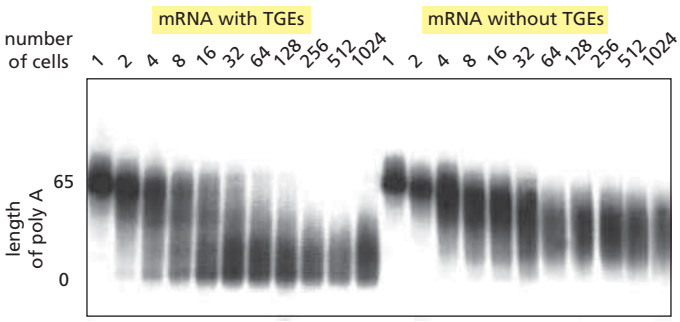
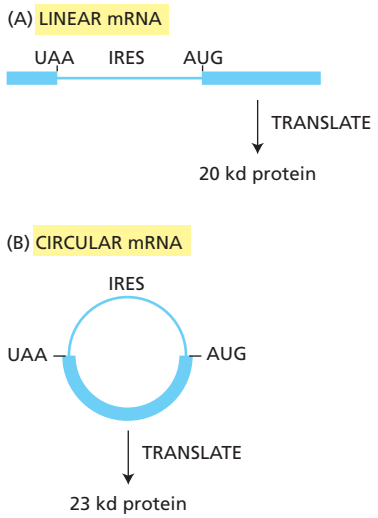


Figure 7–37 Injection of radiolabeled mRNAs with and without TGEs into *Xenopus* one-cell embryos (Problem 7–90). The lengths of the poly-A tails are indicated on the *left*. Cell number refers to the number of cells in the embryos when they were harvested.

of radiolabeled *Tra2* mRNA: one with the normal pair of TGE elements, and one with those elements deleted (see Figure 7–36A). Each RNA has a tail of 65 A nucleotides. You inject these mRNAs into one-cell *Xenopus* embryos and then re-isolate the mRNAs at various cell stages thereafter. The re-isolated radiolabeled RNA was analyzed by gel electrophoresis and autoradiography (**Figure 7–37**).

A. Do the TGEs alter the overall stability of the mRNAs; that is, are the mRNAs destroyed more rapidly in the presence or absence of the TGEs? How can you tell?
B. Do the TGEs influence the length of the poly-A tails? How can you tell?

7–91 You are skeptical that IRESs really allow direct binding of the eukaryotic translation machinery to the interior of an mRNA. As a critical test of this notion, you prepare a set of linear and circular RNA molecules, with and without IRESs (**Figure 7–38A and B**). You translate these various RNAs in rabbit reticulocyte lysates and display the translation products by sodium dodecyl sulfate (SDS) gel electrophoresis (**Figure 7–38C**). Do these results support or refute the idea that IRESs allow ribosomes to initiate translation of mRNAs in a cap-independent fashion? Explain your answer.



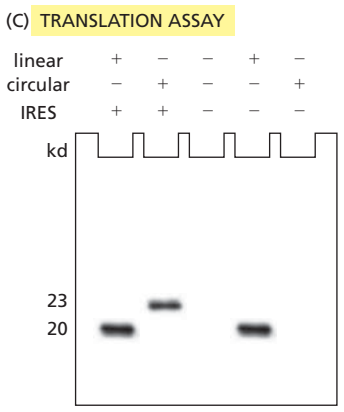


Figure 7–38 Analysis of effects of IRESs on translation (Problem 7–91). (A) Linear mRNA that contains an IRES. The structure of the mRNA without the IRES was the same. (B) Circular mRNA that contains an IRES. The structure of the mRNA without the IRES was the same. Circular RNAs were prepared by ligating the ends together; they were purified from the linear starting molecules by gel electrophoresis. (C) Display of translation products from various species of mRNA.

REGULATION OF GENE EXPRESSION BY NONCODING RNAs

TERMS TO LEARN

CRISPR
crRNAs
long noncoding RNA (lncRNA)
microRNA (miRNA)
piRNA (piwi-interacting RNA)
RNA interference (RNAi)
small interfering RNA (siRNA)

DEFINITIONS

Match each definition below with its term from the list above.

7–92 Natural defense mechanism in many organisms that is directed against foreign RNA molecules, especially those that occur in double-stranded form.

7–93 A class of short noncoding RNAs that regulate gene expression; roughly one-third of human genes are thought to be regulated in this way.

7–94 Small RNAs that transcriptionally silence intact transposon genes and destroy any mRNA produced by them.

7–95 A defense mechanism in bacteria that allows them to destroy viral invaders they have seen before.

7–96 An RNA longer than 200 nucleotides that does not encode a protein.

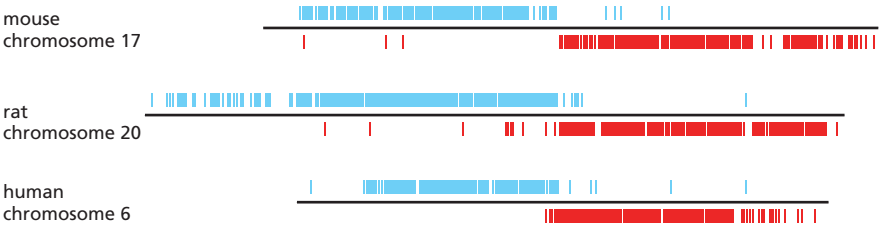


Figure 7–39 Conservation of piRNA clusters among mammalian species (Problem 7–101). The figure is a schematic depiction of the distribution of piRNAs cloned from a conserved region of the mouse, rat, and human genomes. Segments that are transcribed from the top strand of the DNA are shown in *blue*; segments transcribed from the bottom strand are shown in *red*. The three genomes are aligned according to this pattern of transcription.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

7–97 Because siRNAs are so widespread among species, they are believed to be the most ancient form of RNA interference, with miRNAs being a later refinement.

7–98 Although the functions of lncRNAs are still mysterious, it is now clear that they do not function as scaffolds for binding groups of proteins.

7–99 piRNAs and crRNAs serve analogous functions; they defend against foreign invaders.

THOUGHT PROBLEMS

7–100 List and briefly discuss three features that make miRNAs especially useful regulators of gene expression.

7–101 piRNAs have several general characteristics: they are roughly 29–30 nucleotides in length, they have a strong bias for U in the first nucleotide, they bind to the Piwi class of Argonaute proteins, they are found mostly in germ cells, and they are clustered in conserved regions of the mouse, rat, and human genomes (**Figure 7–39**). These characteristics suggest an important function, but the role of not a single piRNA has yet been defined. Why do you suppose that many biologists are convinced that piRNAs serve a critical function in organisms?

7–102 If you insert a *β-galactosidase* gene lacking its own transcription control region into a cluster of piRNA genes in *Drosophila*, you find that β-galactosidase expression from a normal copy elsewhere in the genome is strongly inhibited in the fly's germ cells. If the inactive *β-galactosidase* gene is inserted outside the piRNA gene cluster, the normal gene is properly expressed. What do you suppose is the basis for this observation? How would you test your hypothesis?

DATA HANDLING

7–103 miRNAs were discovered in nematode worms, but it is still unclear whether miRNAs reduce protein expression by causing rapid mRNA degradation or by interfering with translation. The *Let7* miRNA, for example, recognizes sites in the 3′ end of the *Daf12* mRNA, reducing Daf12 protein synthesis. To investigate how *Let7* miRNA controls protein synthesis from *Daf12* mRNA, you make extracts of wild-type larvae at a stage when *Let7* miRNA levels are highest, and also make extracts of mutant larvae that do not make *Let7* miRNA at the same stage. Analysis of the polyribosomes (or polysomes) in normal and mutant larvae revealed no significant difference, nor was there any difference in the distribution of a control mRNA from the *Act1* gene (**Figure 7–40A**). By contrast, the distribution of *Daf12* mRNA changed significantly in the presence of *Let7* miRNA (**Figure 7–40B**).

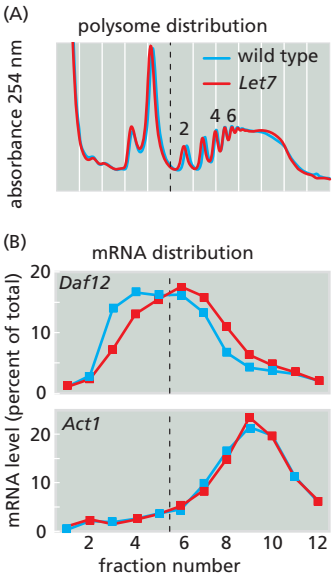


Figure 7–40 Polysome distributions of mRNAs in larval extracts with or without *Let7* miRNA (Problem 7–103). (A) Polysome distribution. mRNAs with attached ribosomes (polyribosomes) were isolated from wild-type and *Let7* mutant larvae and separated by centrifugation through a sucrose gradient. The distributions of all mRNAs in normal (wild type; *blue*) and *Let7* mutant larvae *(red)* are shown in the *top* panel. The monoribosomes are shown to the *left* of the *dashed line* and the polyribosomes are shown to the *right*, with a few individual peaks numbered to indicate how many ribosomes are present in each polysome peak. (B) mRNA distribution. The polysome distribution was divided into 12 fractions, as indicated by the *gray lines.* The RNA was extracted from each fraction and the amounts of *Daf12* and *Act1* mRNAs in each fraction were quantified. The distributions of *Daf12* mRNA and *Act1* mRNA in normal and *Let7* mutant strains are shown *below*.

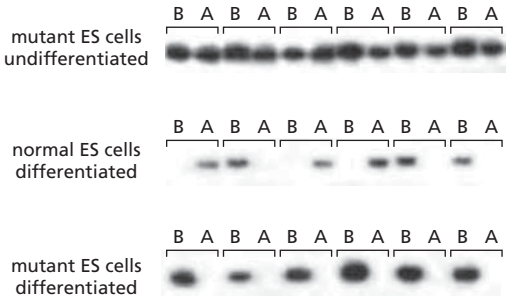


Figure 7–41 Analysis of expression of X-chromosome-specific alleles in undifferentiated and differentiated ES cells (Problem 7–104). Analysis of the A and B alleles from individual cells is indicated by the *brackets*.

A. Does *Let7* miRNA cause degradation of *Daf12* mRNA? How can you tell?
B. How does *Let7* miRNA alter the distribution of *Daf12* mRNA? Is *Daf12* mRNA in smaller or larger polysomes in the presence of *Let7* miRNA?
C. Propose a mechanism by which *Let7* miRNA reduces synthesis of Daf12 protein.

7–104 To determine whether the *Xist* gene, which encodes the *Xist* lncRNA, is required for X-inactivation in mice, scientists deleted the *Xist* gene on one X chromosome. Using female embryonic stem (ES) cells in which genes on the two X chromosomes could be distinguished due to polymorphisms, they followed X-inactivation during differentiation of ES cells in culture. ES cells normally maintain both X chromosomes in the active state; however, when they are induced to differentiate, they randomly inactivate one.

The scientists considered three hypotheses. (1) ES cells mutant for one *Xist* gene would fail to register the presence of two X chromosomes and thus fail to undergo X-inactivation. (2) The *Xist* knockout would prevent X-inactivation of the targeted X chromosome, thus predisposing the normal X chromosome to preferential X-inactivation. (3) The mutation would have no effect on X-inactivation at all.

Using allele-specific (X-chromosome-specific) oligonucleotide probes, they were able to determine which allele of an X-chromosome gene was expressed in individual cells. As shown in **Figure 7–41**, they examined mutant ES cells that were undifferentiated, and mutant and nonmutant ES cells that had undergone differentiation. Only a few cells were examined in Figure 7–41, but analysis of many more confirmed the patterns shown. The A allele marks the X chromosome whose *Xist* gene is intact in the mutant ES cells; the B allele marks the X chromosome from which the *Xist* gene was deleted.

Which of the three hypotheses do these results support? Explain your reasoning.

7–105 Using strand-specific probes of transcription in the X-inactivation center, a second gene was found to be transcribed in the opposite direction to *Xist* and was named *Tsix* to indicate its antisense orientation. The *Tsix* transcript, like the *Xist* transcript, has no significant open reading frame and is thought to be a functional lncRNA. As shown in **Figure 7–42**, the *Tsix* transcript extends all the way across the *Xist* gene. To determine whether *Tsix* plays a role in counting X chromosomes, in choosing which one to inactivate, or in silencing the inactive X, female embryonic stem (ES) cells were generated in which the promoter for *Tsix* had been deleted from one X chromosome. When these ES cells were allowed to

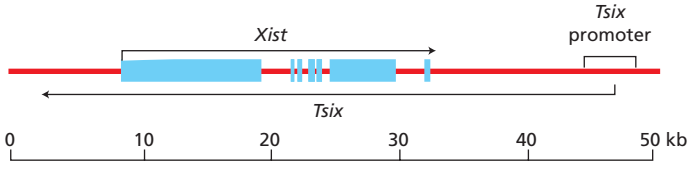


Figure 7–42 Arrangement of *Xist* and *Tsix* transcripts in the X-inactivation center (Problem 7–105). *Boxes* indicate the exons of *Xist*; exons for *Tsix* are not shown. The promoter deletion in the *Tsix* mutant is indicated.

differentiate into females, it was found that the X chromosome with the *Tsix* deletion was always inactivated.

A. Is *Tsix* important for the counting, choice, or silencing of X chromosomes? Explain your answer.
B. Before X-inactivation, *Tsix* is expressed from both alleles, as is *Xist*. At the onset of X-inactivation, *Tsix* expression becomes confined to the future active X, whereas *Xist* expression is restricted to the future inactive X. Can you suggest some possible ways that *Tsix* might regulate *Xist*?

MCAT STYLE

Passage 1 (Questions 7–106 to 7–108)

Embryonic stem cells differentiate into diverse cell types once they receive the appropriate signals. Until then they are kept in an undifferentiated state by the action of three transcription regulators called Oct4, Sox2, and Nanog. An important target of Oct4 and Sox2 is the *FGF4* gene. Analysis of the mechanism by which Oct4 and Sox2 promote transcription of *FGF4* provided early clues to how they work. By analyzing deletions of the DNA regions around the *FGF4* gene, scientists identified a DNA element that was required for the ability of Oct4 and Sox2 to promote transcription of *FGF4*. The element was located beyond the coding sequence at the 3′ end of the gene. Analysis of the DNA element revealed that it contained binding sites for Oct4 and Sox2 that were separated by three nucleotides. When the element was mutated to increase the spacing between the binding sites by 3 or more nucleotides, Oct4 and Sox2 could no longer stimulate transcription of *FGF4*.

The level of expression of Oct4 has strong effects on stem cell differentiation. When expressed at normal levels, Oct4 helps maintain the undifferentiated state. Overexpression of Oct4 by as little as 1.5-fold, however, causes stem cells to differentiate into mesoderm.

7–106 What term best describes the DNA element that is required for Oct4 and Sox2 to promote *FGF4* transcription?

A. Enhancer
B. Mediator
C. Promoter
D. TATA box

7–107 Which hypothesis best explains the observation that increasing the spacing between the Oct4 and Sox2 binding sites prevented stimulation of *FGF4* transcription?

A. Oct4 and Sox2 block the spread of repressive chromatin into the 3′ end of the *FGF4* gene.
B. Oct4 and Sox2 must be properly positioned to interact with the RNA polymerase II complex.
C. Precise positioning of the binding sites promotes cooperative binding of Oct4 and Sox2.
D. The position of Oct4 and Sox2 influences the spacing of nucleosomes over the *FGF4* gene.

7–108 Which one of the following actions best explains how a minor change in Oct4 levels could lead to a big change in the differentiation state of cells?

A. Chromatin modification
B. Cooperative binding
C. Dimerization
D. Negative feedback

Passage 2 (Questions 7–109 to 7–111)

Treatment of mouse fibroblast cells with 5-azacytidine causes some of the cells to differentiate into muscle cells. A search for proteins that are responsible for

this differentiation identified a transcription regulator called MyoD. Transfection of cells with a vector that expresses MyoD from an inducible promoter caused efficient differentiation of fibroblast cells into muscle cells when expression of MyoD was turned on. Moreover, when expression of MyoD from the vector was later turned off, the cells remained differentiated as muscle cells. MyoD controls the transcription of numerous genes. Some of these genes are turned on early in the differentiation process, whereas others are turned on late. Interestingly, MyoD binds to the promoters of the late genes immediately after MyoD expression is first turned on, yet expression of these genes does not occur until later.

7–109 Which of the following possible effects of 5-azacytidine would best account for its ability to cause fibroblasts to differentiate into muscle cells?

A. Activation of Mediator complex
B. Inhibition of a DNA demethylase
C. Inhibition of a specific riboswitch
D. Inhibition of histone modification

7–110 Which one of the following mechanisms best explains how cells remain differentiated as muscle cells, even after the original pulse of MyoD expression has been turned off?

A. Cooperative binding
B. Negative feedback
C. Positive feedback
D. Synergistic transcriptional activation

7–111 Which one of the regulatory circuits in **Figure 7–43** best explains all of the observations regarding the role of MyoD in induction of muscle cell differentiation?

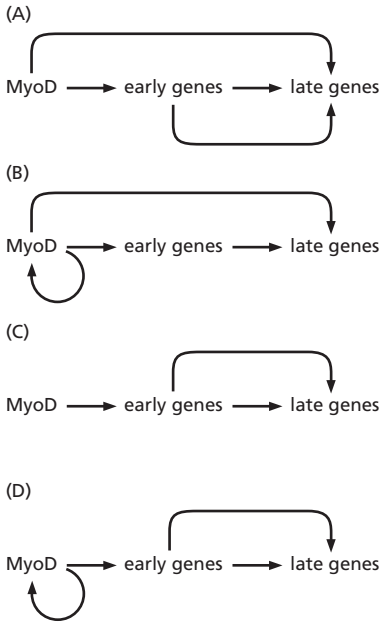
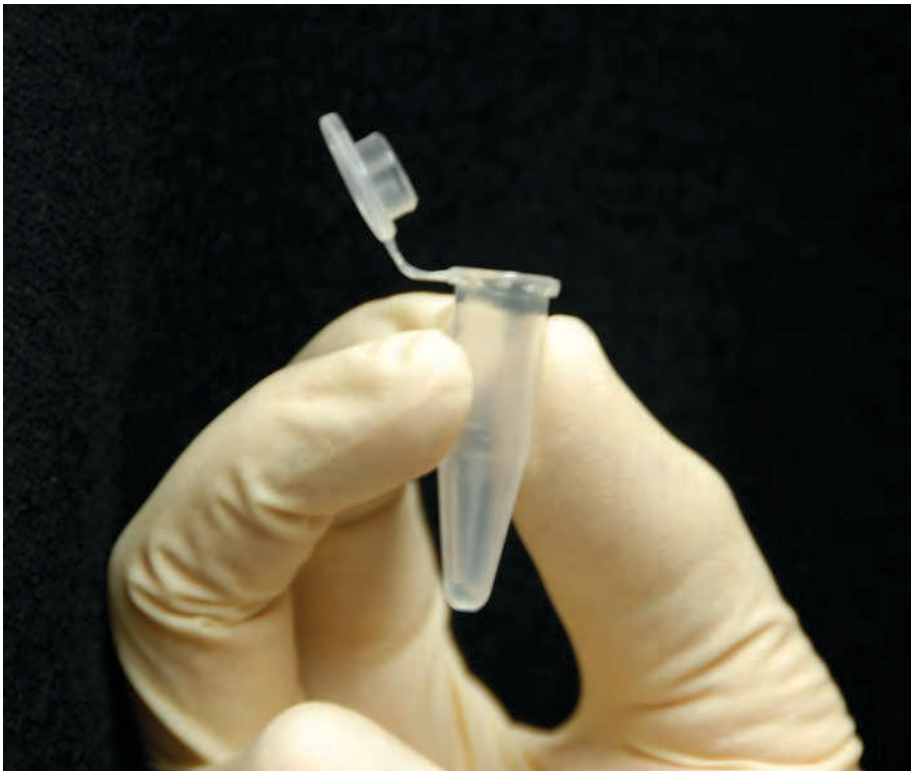


Figure 7–43 Four potential MyoD regulatory circuits (Problem 7–111).



A 1.5 mL Eppendorf Tube.
An Eppendorf tube containing 10 microliters of solution. Most molecular biology reactions are carried out in these tiny plastic tubes, which were invented in 1961 at the Eppendorf hospital laboratories in the suburbs of Hamburg, Germany, as part of a system for handling very small volumes of clinical samples. These tubes are inert, robust, and inexpensive.

Analyzing Cells, Molecules, and Systems

CHAPTER 8

IN THIS CHAPTER

ISOLATING CELLS AND GROWING THEM IN CULTURE

TERMS TO LEARN

hybridoma monoclonal antibody

DEFINITIONS

Match each definition below with its term from the list above.

8–1 Antibody secreted by a hybridoma cell line.

8–2 Cell line used in the production of monoclonal antibodies; obtained by fusing antibody-secreting B cells with cells of a lymphocyte tumor.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

8–3 Laser-capture microdissection permits isolation of individual cells from a sample of tissue.

8–4 Because a monoclonal antibody recognizes a specific antigenic site (epitope), it binds only to the specific protein against which it was made.

THOUGHT PROBLEMS

8–5 A common step in the isolation of cells from a sample of animal tissue is to treat it with trypsin, collagenase, and EDTA. Why is such a treatment necessary, and what does each component accomplish? Why doesn't this treatment kill the cells?

8–6 Isolation of cells from tissues, fluorescence-activated cell sorting, and laser-capture microdissection are just a few of the ways for generating homogeneous cell populations. Why do you suppose it is important to have a homogeneous cell population for many experiments?

8–7 Distinguish among the terms "primary culture," "secondary culture," and "cell line."

8–8 Consider the following two statements. "The most important advantage of the hybridoma technique is that monoclonal antibodies can be made against molecules that constitute only a minor component of a complex mixture." "The most important advantage of the hybridoma technique is that antibodies that may be present as only minor components in conventional antiserum can be produced in quantity in pure form as monoclonal antibodies." Are these two statements equivalent? Why or why not?

8–9 Do you suppose it would be possible to raise an antibody against another antibody? Explain your answer.

CALCULATIONS

8–10 You want to isolate rare cells that are present in a population at a frequency of 1 in 10^5 cells, and you need 10 of those cells to do an experiment. If your fluorescence-activated cell sorter can sort cells at the rate of 1000 per second, how long would it take to collect enough rare cells for your experiment?

DATA HANDLING

8–11 Panels of human–rodent cell hybrids that retain one or a few human chromosomes, parts of human chromosomes, or radiation-induced fragments of human chromosomes have proven enormously useful in mapping genes to defined locations. Now that the human genome has been sequenced, it is a trivial matter to know a gene's location if you have a bit of sequence from the gene. Nevertheless, there are many instances in which such panels of cells are still invaluable; for example, when you know a phenotype, but not the identity of the gene responsible for it, as in the following case.

You wish to map the location of the receptor for feline leukemia virus type C (FeLV-C), which infects human cells but not rodent cells. Using a panel of hybrid cells carrying whole chromosomes, you have shown that FeLV-C infects only those hybrids carrying human chromosome 1. Using a second panel carrying portions of chromosome 1, you show that FeLV-C infects several of the hybrid cell lines. The segments of chromosome 1 that are present in the infectable hybrid cell lines are shown in **Figure 8–1**. Where on chromosome 1 is the gene for the FeLV-C receptor located?

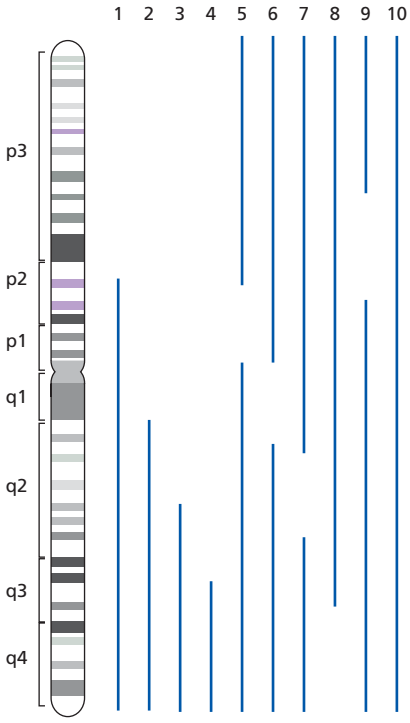


Figure 8–1 Mapping the gene for the FeLV-C receptor using human–rodent hybrid cell lines that carry portions of human chromosome 1 (Problem 8–11). The hybrid cell lines that could be infected by FeLV-C are shown, with the portions of human chromosome 1 retained in the individual hybrid cell lines indicated as *blue lines*. The p and q designations refer to a standard convention (the Paris nomenclature) for describing chromosome positions. Short-arm locations are labeled p (*petit*) and long arms q (*queue*). Each chromosome arm is divided into regions labeled p1, p2, p3, q1, q2, q3, etc., counting outward from the centromere. Regions are delimited by specific landmarks, which are distinct morphological features, including the centromere and certain prominent bands. Regions are divided into bands labeled p11 (one-one, not eleven), p12, etc. Sub-bands are designated p11.1, p11.2, etc., and sub-sub bands are designated p11.11, p11.12, etc. In all cases, the numbers increase from the centromere toward the telomere.

PURIFYING PROTEINS

TERMS TO LEARN

column chromatography
fusion protein
high-performance liquid chromatography (HPLC)
purified cell-free system

DEFINITIONS

Match each definition below with its term from the list above.

8–12 General term for purification technique in which a mixture of proteins is passed through a cylinder containing a porous solid matrix.

8–13 Type of chromatography that uses columns packed with special chromatography resins composed of tiny spheres that attain a high degree of resolution, even at very fast flow rates.

8–14 Artificial product generated by linking the coding sequences for two different proteins, or protein segments, and expressing the hybrid gene in cells.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

8–15 It is possible to pellet hemoglobin by centrifugation at sufficiently high speed.

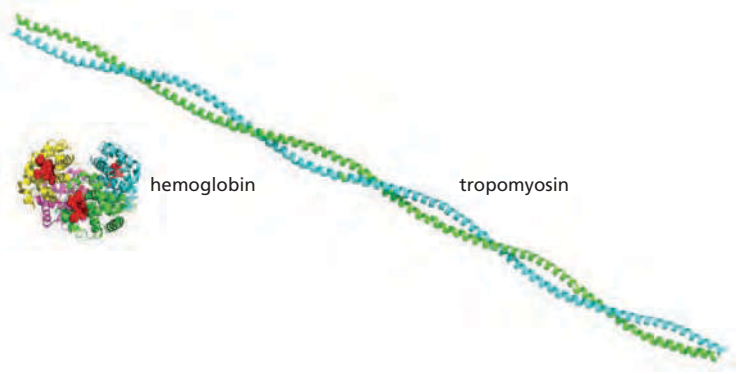


Figure 8–2 Backbone models of tropomyosin and hemoglobin (Problem 8–19).

8–16 If the beads used in gel-filtration chromatography had pores of a uniform size, proteins would either be excluded from the pores or included in them, but would not be further fractionated.

THOUGHT PROBLEMS

8–17 Describe how you would use preparative centrifugation to purify mitochondria from a cell homogenate.

8–18 Distinguish between velocity sedimentation and equilibrium sedimentation. For what general purpose is each technique used? Which do you suppose might be best suited for separating two proteins of different size?

8–19 Tropomyosin, at 93 kd, sediments at 2.6S, whereas the 65-kd protein, hemoglobin, sediments at 4.3S. (The sedimentation coefficient S is a linear measure of the rate of sedimentation: both increase or decrease in parallel.) These proteins are drawn to scale in **Figure 8–2**. How is it that the bigger protein sediments more slowly than the smaller one? Can you think of an analogy from everyday experience that might help you with this problem?

8–20 Distinguish among ion-exchange chromatography, hydrophobic chromatography, gel-filtration chromatography, and affinity chromatography in terms of the column material and the basis for separation of a mixture of proteins.

CALCULATIONS

8–21 The purification of a protein usually requires multiple steps and often involves several types of column chromatography. A key component of any purification is an assay for the desired protein. The assay can be a band on a gel, a structure in the electron microscope, the ability to bind to another molecule, or enzyme activity. The purification of an enzyme is particularly instructive because the assay allows one to quantify the extent of purification at each step. Consider the purification of the enzyme shown in **Table 8–1**. The total volume, total protein, and total enzyme activity are shown at each step.

A. For each step in the purification procedure, calculate the specific activity of the enzyme (units of activity per mg of protein). How can you tell that purification has occurred at each step?
B. Which of the purification steps was most effective? Which was least effective?
C. If you were to carry the purification through additional steps, how would the specific activity change? How could you tell from specific activity measurements that the enzyme was pure? How might you check on that conclusion?

TABLE 8–1 Purification of an enzyme (Problem 8–21).

Procedure	Total volume (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude extract	2000	15,000	150,000	
2. Ammonium sulfate precipitation	320	4000	140,000	
3. Ion-exchange chromatography	100	550	125,000	
4. Gel-filtration chromatography	85	120	105,000	
5. Affinity chromatography	8	5	75,000	

D. If the enzyme is pure at the end of the purification scheme in Table 8–1, what proportion of the protein in the starting cell does it represent?

DATA HANDLING

8–22 In the classic paper that demonstrated the semiconservative replication of DNA, Meselson and Stahl began by showing that DNA itself will form a band when subjected to equilibrium sedimentation. They mixed randomly fragmented *E. coli* DNA with a solution of CsCl so that the final solution had a density of 1.71 g/mL. As shown in Figure 8–3, with increasing length of centrifugation at 70,000 times gravity, the DNA, which was initially dispersed throughout the centrifuge tube, became concentrated over time into a discrete band in the middle.

A. Describe what is happening with time and explain why the DNA forms a discrete band.

B. What is the buoyant density of the DNA? (The density of the solution at which DNA "floats" at equilibrium defines the "buoyant density" of the DNA.)

C. Even if the DNA were centrifuged for twice as long—or even longer—the width of the band remains about what is shown at the bottom of Figure 8–3. Why doesn't the band become even more compressed? Suggest some possible reasons to explain the thickness of the DNA band at equilibrium.

8–23 The result of gel-filtration chromatography of six roughly spherical proteins is shown in **Figure 8–4**. The identities of the proteins, their molecular masses, and their elution volumes are indicated in **Table 8–2**. (The elution volume identifies when each protein came off the column.)

TABLE 8–2 Proteins separated by gel-filtration chromatography (Problem 8–23).

Protein	Molecular mass (kd)	Molecular mass (log)	Elution volume (mL)
Ribonuclease A	13	4.11	250
Chymotrypsinogen	25	4.40	228
Ovalbumin	43	4.63	199
Bovine serum albumin	67	4.83	176
Aldolase	158	5.20	146
Catalase	232	5.37	123

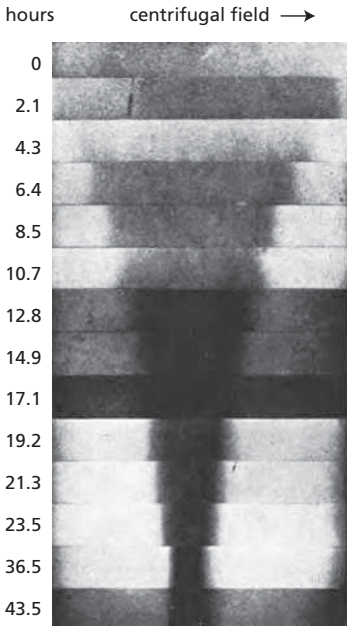


Figure 8–3 Ultraviolet (UV) absorption photographs showing successive stages in the banding of *E. coli* DNA (Problem 8–22). DNA, which absorbs UV light, shows up as *dark regions* in the photographs. The bottom of the centrifuge tube is on the *right*.

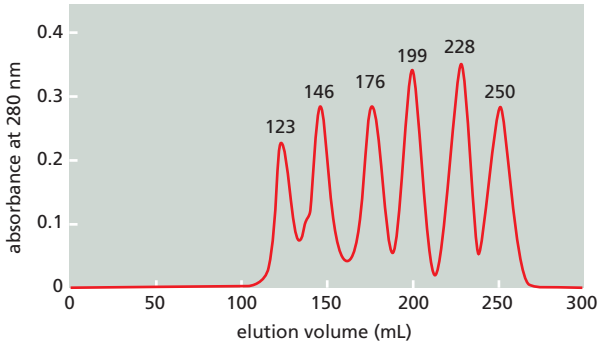


Figure 8–4 Elution profile for proteins fractionated by gel-filtration chromatography (Problem 8–23). The absorbance at 280 nm is a measure of protein concentration. Each of the peaks is identified by its elution volume.

A. Why do the smaller proteins come off the column later than the larger proteins?
B. Plot molecular mass versus elution volume. Now plot the log of the molecular mass versus the elution volume. Which plot gives a straight line? What do you suppose is the basis for that result?

8–24 In preliminary studies, you've determined that your partially purified protein is stable (retains activity) between pH 5.0 and pH 7.5. On either side of that pH range, the protein is no longer active. Your advisor now wants you to do a quick experiment to determine conditions for ion-exchange chromatography. He has left instructions for you. First, you're supposed to mix a bit of the crude preparation with a small amount of the ion-exchange resin DEAE-Sepharose™ in a series of buffer solutions that have a pH between 5.0 and 7.5. Next, you are to pellet the resin and assay the supernatant for the presence of your protein. Finally, he tells you to use this information to pick the proper pH to do the ion-exchange chromatography. You have completed the first two steps and have obtained the results shown in **Figure 8–5**. But you are a little uncertain as to how to use the information to pick the pH for the chromatography.

A. At which end of the pH range is the charge on your protein more positive and at which end is it more negative? [Over this pH range, the positively charged amine groups on the DEAE-Sepharose beads (Figure 8–5A) are unaffected.]
B. For the chromatography, should you pick a pH at which the protein binds to the beads (pH 6.5 to 7.5) or a pH where it does not bind (pH 5.0 to 6.0)? Explain your choice.
C. Should you pick a pH close to the boundary (that is, pH 6.0 or 6.5) or far away from the boundary (that is, pH 5.0 or pH 7.5)? Explain your reasoning.
D. How will you carry out ion-exchange chromatography of your protein? What are the various steps you will use to accomplish the separation of your protein from others via ion-exchange chromatography?

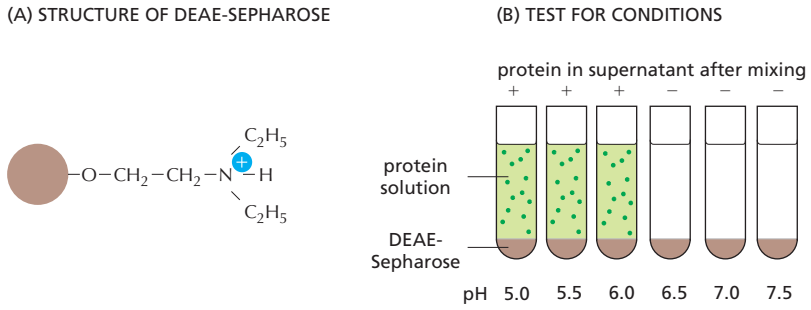


Figure 8–5 Preliminary test to determine conditions for ion-exchange chromatography (Problem 8–24). (A) Structure of the charged amine groups attached to Sepharose beads. (B) Results of mixing your protein with DEAE-Sepharose beads. Samples of the protein were mixed with DEAE-Sepharose beads in buffers at a range of pH values, and then the mixtures were centrifuged to pellet the beads. The presence of the protein in the supernatant is indicated by a +; its absence is indicated by a –.

ANALYZING PROTEINS

TERMS TO LEARN

chemical biology
nuclear magnetic resonance (NMR) spectroscopy
SDS polyacrylamide-gel electrophoresis (SDS-PAGE)
two-dimensional gel electrophoresis
Western blotting (immunoblotting)
x-ray crystallography

DEFINITIONS

Match each definition below with its term from the list above.

8–25 Analysis of the release of electromagnetic radiation by atomic nuclei in a magnetic field, due to flipping of the orientation of their magnetic dipole moments.

8–26 Technique for protein separation in which the protein mixture is run first in one direction and then in a direction at right angles to the first.

8–27 Technique in which a protein mixture is separated by running it through a gel containing a detergent that binds to and unfolds the proteins.

8–28 The main technique that has been used to discover the three-dimensional structure of molecules, including proteins, at atomic resolution.

8–29 Technique by which proteins are separated by electrophoresis, immobilized on a paper sheet, and then analyzed, usually by means of a labeled antibody.

TRUE/FALSE

Decide whether the statement is true or false, and then explain why.

8–30 Given the inexorable march of technology, it seems inevitable that the sensitivity of detection of molecules will ultimately be pushed beyond the yoctomole level (10^{-24} mole).

THOUGHT PROBLEMS

8–31 How is it that smaller molecules move through a gel-filtration column more slowly than larger molecules, whereas in SDS polyacrylamide-gel electrophoresis (SDS-PAGE) the opposite is true: larger molecules move more slowly than small molecules?

8–32 You are set to run your first SDS polyacrylamide-gel electrophoresis. You have boiled your samples of protein in SDS in the presence of mercaptoethanol and loaded them into the wells of a polyacrylamide gel. You are now ready to attach the electrodes. Uh oh, does the positive electrode (the anode) go at the top of the gel, where you loaded your proteins, or at the bottom of the gel?

8–33 You hate the smell of mercaptoethanol. Since disulfide bonds in intracellular proteins are very rare (see Problem 3–37), you have convinced yourself that it is not necessary to treat a cytoplasmic homogenate with mercaptoethanol prior to SDS-PAGE. You heat a sample of your homogenate in SDS and subject it to electrophoresis. Much to your surprise, your gel looks horrible; it is an ugly smear! You show your result to a fellow student with a background in chemistry, and she suggests that you treat your sample with *N*-ethylmaleimide (NEM), which reacts with free sulfhydryls. You run another sample of your homogenate after treating it with NEM and SDS. Now the gel looks perfect!

If the vast majority of intracellular proteins don't have disulfide bonds—and they don't—why didn't your original scheme work? And how does treatment with NEM correct the problem?

8–34 For separation of proteins by two-dimensional polyacrylamide-gel electrophoresis, what are the two types of electrophoresis that are used in each dimension? Do you suppose it makes any difference which electrophoretic method is applied first? Why or why not?

8–35 Discuss the following statement: "With the ever-expanding databases of protein sequences and structures, it will soon be possible to input an amino acid sequence of an unknown protein and, by analogy to known proteins, determine its structure and function. Thus, it will not be long before biochemists are put out of work."

8–36 Hybridoma technology allows one to generate monoclonal antibodies to virtually any protein. Why is it, then, that genetically tagging proteins with epitopes is such a commonly used technique, especially since an epitope tag has the potential to interfere with the function of the protein?

8–37 Specific activity refers to the amount of radioactivity per unit amount of substance, most commonly in biology expressed on a molar basis, for example, as Ci/mmol. [One curie (Ci), which is the standard unit of radioactive decay, corresponds to 2.22×10^{12} disintegrations per minute (dpm).] If you examine **Table 8–3**, you will see that there seems to be an inverse relationship between maximum specific activity and half-life. Do you suppose this is just a coincidence or is there an underlying reason? Explain your answer.

8–38 You just developed an autoradiograph after a two-week exposure. You had incubated your protein with a cell-cycle kinase in the presence of ^{32}P-ATP in hopes of demonstrating that it was indeed a substrate for the kinase. You see the hint of a band on the gel at the right position, but it is just too faint to be convincing. You show your result to your advisor and tell him that you've put the blot against a fresh sheet of film, which you plan to expose for a longer period of time. He gives you a sideways look and tells you to do the experiment over again and use more radioactivity. What's wrong with your plan to reexpose the blot for a longer time?

CALCULATIONS

8–39 How many copies of a protein need to be present in a cell in order for it to be visible as a band on an SDS gel? Assume that you can load 100 μg of cell extract onto a gel and that you can detect 10 ng in a single band by silver staining. The concentration of protein in cells is about 200 mg/mL, and a typical mammalian cell has a volume of about 1000 μm^3 and a typical bacterium a volume of about 1 μm^3. Given these parameters,

TABLE 8–3 Radioactive isotopes and some of their properties (Problem 8–37).

Radioactive isotope	Emission	Half-life	Maximum specific activity (Ci/mmol)
^{14}C	β particle	5730 years	0.062
^{3}H	β particle	12.3 years	29
^{35}S	β particle	87.4 days	1490
^{32}P	β particle	14.3 days	9120

calculate the number of copies of a 120-kd protein that would need to be present in a mammalian cell and in a bacterium in order to give a detectable band on a gel. You might try an order-of-magnitude guess before you make the calculations.

8–40 How many molecules of your labeled protein are required for detection by autoradiography? You added 1 µL containing 10 µCi of γ-^{32}P-ATP (a negligible amount of ATP) to 9 µL of cell extract that had an ATP concentration of 1 mM. You incubated the mixture to allow transfer of phosphate to proteins in the extract. You then subjected 1 µL of the mixture to SDS-PAGE, dried the gel, and placed it against a sheet of x-ray film. After an overnight exposure you saw a barely detectable band in the location of your protein. You know from previous experience that a protein labeled at 1 count per minute per band (1 cpm equals 1 disintegration per minute, dpm, for ^{32}P) will form such a band after an overnight exposure. How many molecules of labeled protein are in the band, if you assume 1 phosphate per molecule? (Some useful conversion factors for radioactivity are shown in Table 3 on page 964.)

8–41 You want to know the sensitivity for detection of immunoblotting (Western blotting), using an enzyme-linked second antibody to detect the antibody directed against your protein (**Figure 8–6A**). You are using the mouse monoclonal antibody 4G10, which is specific for phosphotyrosine residues, to detect phosphorylated proteins. You first phosphorylate the myelin basic protein *in vitro* using a tyrosine protein kinase that adds one phosphate per molecule. You then prepare a dilution series of the phosphorylated protein and subject the samples to SDS-PAGE. Next, the protein is transferred (blotted) onto a nitrocellulose filter, incubated with the 4G10 antibody, and washed to remove unbound antibody. The blot is then incubated with a second goat anti-mouse antibody that carries horseradish peroxidase (HRP) conjugated to it, and any excess unbound antibody is again washed away. You place the blot in a thin plastic bag, add reagents that chemiluminesce when they react with HRP (Figure 8–6A), and place the bag against a sheet of x-ray film. When the film is developed, you see the picture shown in **Figure 8–6B**.

A. Given the amounts of phosphorylated myelin basic protein indicated in each lane in Figure 8–6B, calculate the detection limit of this method in terms of molecules of protein per band.

B. Assuming that you were using monoclonal antibodies to detect proteins, would you expect that the detection limit would depend on the molecular mass of the protein? Why or why not?

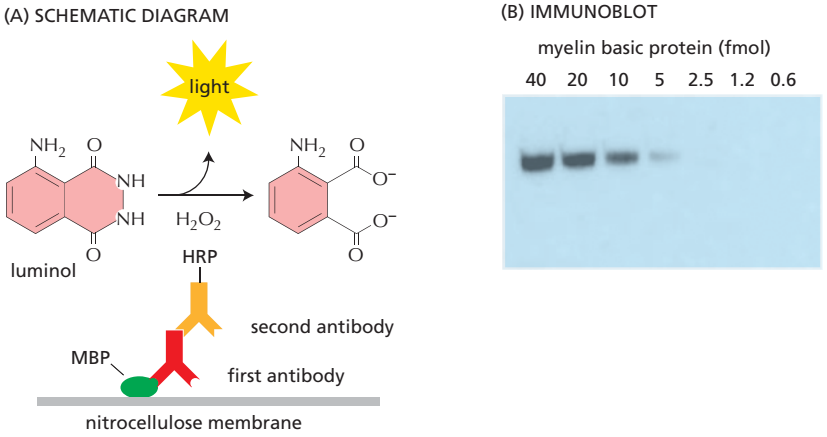


Figure 8–6 Sensitivity of detection of immunoblotting (Problem 8–41). (A) Schematic diagram of the experiment. MBP stands for myelin basic protein. In the presence of hydrogen peroxide, horseradish peroxidase (HRP) converts luminol to a chemiluminescent molecule that emits light, which is detected by exposure of an x-ray film. (B) Exposed film of an immunoblot. The number of femtomoles of myelin basic protein in each band is indicated.

DATA HANDLING

8–42 **Figure 8–7** shows an autoradiograph of an SDS-PAGE separation of radiolabeled proteins in a cell-free extract of sea urchin eggs. Alongside are shown a set of radiolabeled marker proteins of defined molecular mass. Two bands that contain known proteins—the small subunit of ribonucleotide reductase and cyclin B—are indicated.

A. Do the standard set of proteins migrate at a rate that is inversely proportional to their molecular masses? That is to say, would you expect a protein of 35 kd, for example, to migrate twice as far down the gel as a protein of 70 kd? Do you suppose a plot of log molecular mass versus migration would give a more linear relationship?

B. How would you use the standard set of proteins to estimate the molecular masses of ribonucleotide reductase and cyclin B? What would you estimate the molecular masses of these two proteins to be?

C. The sequences of the genes for these two proteins give molecular masses of 44 kd for ribonucleotide reductase and 46 kd for cyclin B. Can you offer some possible reasons why the SDS-PAGE estimate for the molecular mass of cyclin B is so far off?

8–43 You have isolated the proteins from two adjacent spots after two-dimensional polyacrylamide-gel electrophoresis and digested them with trypsin. When the masses of the peptides were measured by MALDI-TOF mass spectrometry, the peptides from the two proteins were found to be identical except for one (**Figure 8–8**). For this peptide, the mass-to-charge (m/z) values differed by 80, a value that does not correspond to a difference in amino acid sequence. (For example, glutamic acid instead of valine at one position would give an m/z difference of around 30.) Can you suggest a possible difference between the two peptides that might account for the observed m/z difference?

8–44 You have raised four different monoclonal antibodies to *Xenopus* Orc1, which is a component of the DNA replication origin recognition complex (ORC) found in eukaryotes. You want to use the antibodies to immunopurify other members of ORC. To decide which of your monoclonal antibodies—TK1, TK15, TK37, or TK47—is best suited for this purpose, you covalently attach them to beads, incubate them with a *Xenopus* egg extract, spin the beads down and wash them carefully, and then solubilize the bound proteins with SDS. You use SDS-PAGE to separate the solubilized proteins and stain them, as shown in **Figure 8–9**.

A. From these results, which bands do you think arise from proteins that are present in ORC?

B. Why do you suppose the various monoclonal antibodies give such different results?

C. Which antibody do you think is the best one to use in future studies of this kind? Why?

D. How might you determine which band on this gel is Orc1?

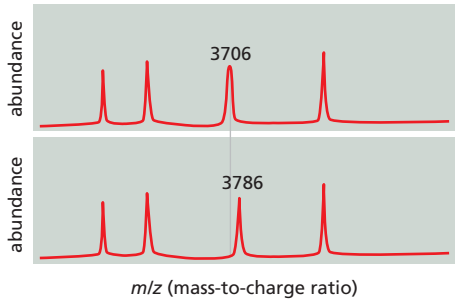


Figure 8–8 Masses of peptides measured by MALDI-TOF mass spectrometry (Problem 8–43). Only the numbered peaks differ between the two protein samples.

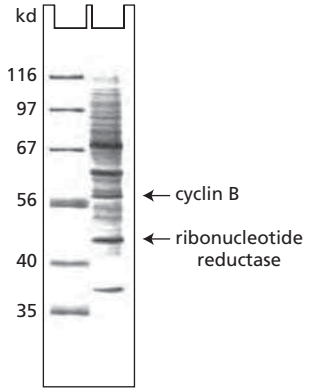


Figure 8–7 Autoradiograph of radiolabeled proteins separated by SDS-PAGE (Problem 8–42). A set of radiolabeled marker proteins with known molecular masses is shown in the *left-hand* lane, along with their molecular masses in kilodaltons. Radiolabeled proteins from a sea urchin egg extract are shown in the *right-hand* lane. *Arrows* mark the bands that correspond to cyclin B and the small subunit of ribonucleotide reductase.

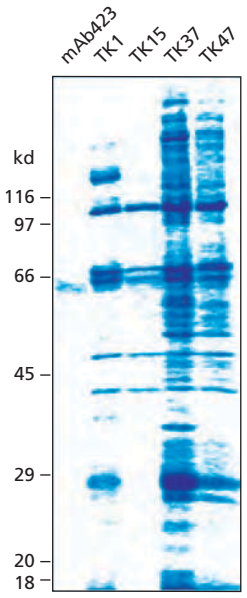


Figure 8–9 Immunoaffinity purification of *Xenopus* ORC (Problem 8–44). The monoclonal antibody mAb423 is specific for an antigen not found in *Xenopus* extracts and thus serves as a control. The positions of marker proteins are shown at the *left* with their masses indicated in kilodaltons.

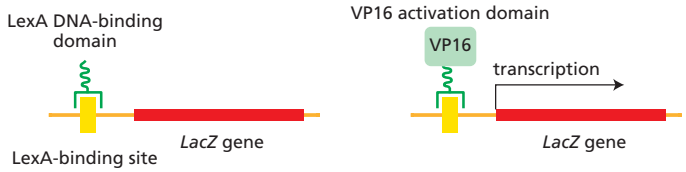


Figure 8–10 Activation of transcription by a hybrid transcription regulator (Problem 8–45).

8–45 The yeast two-hybrid system relies on the cell's own mechanisms to reveal protein–protein interactions. This method takes advantage of the modular nature of many transcription regulators, which have one domain that binds to DNA and another domain that activates transcription. Domains can be interchanged by recombinant DNA methods, allowing hybrid transcription regulators to be constructed. Thus, the DNA-binding domain of the *E. coli* LexA repressor can be combined with the powerful VP16 activation domain from herpesvirus to activate transcription of genes downstream of a LexA DNA binding site (**Figure 8–10**).

If the two domains of the transcription regulator can be brought into proximity by protein–protein interactions, they will activate transcription. This is the key feature of the two-hybrid system. Thus, if one member of an interacting pair of proteins is fused to the DNA-binding domain of LexA (to form the "bait") and the other is fused to the VP16 activation domain (to form the "prey"), transcription will be activated when the two hybrid proteins interact inside a yeast cell. It is possible to design powerful screens for protein–protein interactions, if the gene whose transcription is turned on is essential for growth or can give rise to a colored product.

To check out the ability of the system to find proteins with which Ras interacts, hybrid genes were constructed that contained the LexA DNA-binding domain, one fused to Ras (LexA–Ras) and the other fused to nuclear lamin (LexA–lamin). A second pair of constructs contained the VP16 activation domain alone (VP16) or fused to the adenylyl cyclase gene (VP16–CYR). Adenylyl cyclase is known to interact with Ras and serves as a positive control; nuclear lamins do not interact with Ras and serve as a negative control. These plasmid constructs were introduced into a strain of yeast containing copies of the *His3* gene and the *LacZ* gene, both with LexA-binding sites positioned immediately upstream. Individual transformed colonies were tested for the ability to grow on a plate lacking histidine, which requires expression of the *His3* gene. In addition, they were tested for ability to form blue colonies (as compared to the normal white colonies) when grown in the presence of an appropriate substrate (XGAL) for β-galactosidase. The set-up for the experiment is outlined in **Table 8–4**.

TABLE 8–4 Experiments to test the two-hybrid system (Problem 8–45).

Plasmid constructs		Growth on plates lacking histidine	Color on plates with XGAL
Bait	Prey		
lexA–Ras			
LexA–lamin			
	VP16		
	VP16–CYR		
LexA–Ras	VP16		
LexA–Ras	VP16–CYR		
LexA–lamin	VP16		
LexA–lamin	VP16–CYR		

A. Fill in Table 8–4 with your expectations. Use a plus sign to indicate growth on plates lacking histidine and a minus sign to indicate no growth. Write "blue" or "white" to indicate the color of colonies grown in the presence of XGAL.
B. For any combinations of bait and prey in the table that you expect to confer growth in the absence of histidine and to form blue colonies with XGAL, sketch the structure of the active transcription regulator on the *LacZ* gene.
C. If you want two proteins to be expressed in a single polypeptide chain, what must you be careful to do when you fuse the two genes together?

ANALYZING AND MANIPULATING DNA

TERMS TO LEARN

bacterial artificial chromosome (BAC)
cDNA clone
cDNA library
deep RNA sequencing (RNA-seq)
dideoxy sequencing (Sanger sequencing)
DNA cloning
DNA library
genome annotation
genomic library
hybridization
open reading frame (ORF)
plasmid vector
polymerase chain reaction (PCR)
recombinant DNA technology
restriction nuclease

DEFINITIONS

Match each definition below with its term from the list above.

8–46 Small, circular DNA molecule that replicates independently of the genome and can be used for DNA cloning.

8–47 A collection of clones that contain a variety of DNA segments from the genome of an organism.

8–48 The process of marking out all the genes in a genome and ascribing a biological function to each.

8–49 One of a large number of enzymes that can cleave a DNA molecule at any site where a specific short sequence of nucleotides occurs.

8–50 A DNA clone of a DNA copy of an mRNA molecule.

8–51 Technique for generating multiple copies of specific regions of DNA by the use of sequence-specific primers and multiple cycles of DNA synthesis.

8–52 The sequencing of the entire repertoire of RNA from a cell or tissue.

8–53 Prokaryotic cloning vector that can accommodate large pieces of DNA up to 1 million base pairs.

8–54 The process whereby two complementary nucleic acid strands form a double helix.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

8–55 Bacteria that make a specific restriction nuclease for defense against viruses have evolved in such a way that their own genome does not contain the recognition sequence for that nuclease.

8–56 Pulsed-field gel electrophoresis uses a strong electric field to separate very long DNA molecules, stretching them out so that they travel end-first through the gel at a rate that depends on their length.

8–57 By far the most important advantage of cDNA clones over genomic clones is that they can contain the complete coding sequence of a gene.

8–58 If each cycle of PCR doubles the amount of DNA synthesized in the previous cycle, then 10 cycles will give a 10^3-fold amplification, 20 cycles will give a 10^6-fold amplification, and 30 cycles will give a 10^9-fold amplification.

THOUGHT PROBLEMS

8–59 **Figure 8–11** shows a picture of DNA fragments that have been separated by gel electrophoresis and then stained by ethidium bromide, a molecule that fluoresces intensely under long-wavelength UV light when it is bound to DNA. Such gels are a standard way of detecting the products of cleavage by restriction nucleases. For the DNA fragment shown in **Figure 8–12**, decide whether it will be cut by the restriction nucleases EcoRI (5′-GAATTC), AluI (5′-AGCT), and PstI (5′-CTGCAG). For those that cut the DNA, how many products will be produced?

```
5′–AAGAATTGCGGAATTCGAGCTTAAGGGCCGCGCCGAAGCTTTAAA–3′
3′–TTCTTAACGCCTTAAGCTCGAATTCCCGGCGCGGCTTCGAAATTT–5′
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Figure 8–12 A segment of double-stranded DNA (Problem 8–59).

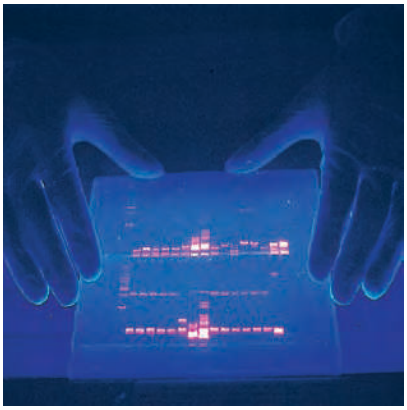


Figure 8–11 DNA fragments separated by gel electrophoresis and stained with ethidium bromide (Problem 8–59). Each of the *orange bands* on the gel represents a site where fragments of DNA have migrated during electrophoresis. Ethidium intercalates between base pairs in double-stranded DNA. Removal of ethidium from the aqueous environment and fixing its orientation in the nonpolar environment of DNA enhance its fluorescence dramatically. When irradiated with long-wavelength UV light, it fluoresces a bright orange.

8–60 The restriction nucleases BamHI and PstI cut their recognition sequences as shown in **Figure 8–13**.

A. Indicate the 5′ and 3′ ends of the cut DNA molecules.
B. How would the ends be modified if you incubated the cut molecules with DNA polymerase in the presence of all four dNTPs?
C. After the reaction in part B, could you still join the BamHI ends together by incubation with T4 DNA ligase? Could you still join the PstI ends together? (T4 DNA ligase will join blunt ends together as well as cohesive ends.)
D. Will joining of the ends in part C regenerate the BamHI site? Will it regenerate the PstI site?

8–61 The restriction nuclease EcoRI recognizes the sequence 5′-GAATTC and cleaves between the G and A to leave 5′ protruding single strands (like BamHI, see Figure 8–13A). PstI, on the other hand, recognizes the sequence 5′-CTGCAG and cleaves between the A and G to leave 3′ protruding single strands (see Figure 8–13B). These two recognition sites are displayed on the helical representations of DNA in **Figure 8–14**.

A. For each restriction site, indicate the position of cleavage on each strand of the DNA.

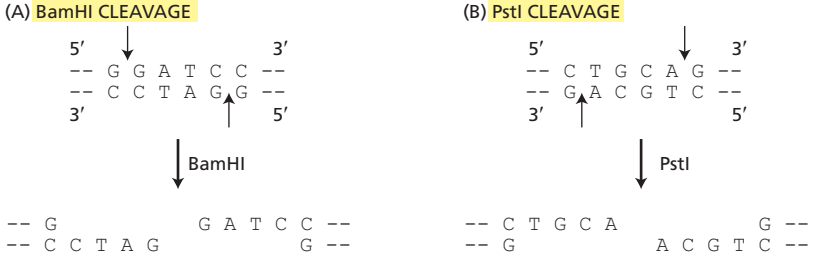


Figure 8–13 Restriction nuclease cleavage of DNA (Problem 8–60). (A) BamHI cleavage. (B) PstI cleavage. Only the nucleotides that form the recognition sites are shown.

B. From the positions of the cleavage sites, decide for each restriction nuclease whether you expect it to approach the recognition site from the major-groove side or from the minor-groove side.

8–62 Which, if any, of the restriction nucleases listed in **Table 8–5** will *definitely* cleave a segment of cDNA that encodes the peptide KIGPACF? (See Tables 7 and 8, page 966, for the genetic code.)

8–63 You wish to make a restriction map of a 3.0-kb BamHI restriction fragment. You digest three samples of the fragment with EcoRI, HpaII, and a mixture of EcoRI and HpaII. You then separate the fragments by gel electrophoresis and visualize the DNA bands by staining with ethidium bromide (**Figure 8–15**). From these results, draw a restriction map that shows the relative positions of the EcoRI and HpaII recognition sites and the distances in kilobases (kb) between them.

8–64 If you add DNA to wells at the top of a gel, should you place the positive electrode (anode) at the top or at the bottom of the gel? Explain your choice.

8–65 You want to clone a DNA fragment that has KpnI ends into a vector that has BamHI ends. The problem is that BamHI and KpnI ends are not compatible: BamHI leaves a 5′ overhang and KpnI leaves a 3′ overhang (**Figure 8–16**). A friend suggests that you try to link them with an oligonucleotide "splint" as shown in Figure 8–16. It is not immediately clear to you that such a scheme will work because ligation requires an adjacent 5′ phosphate and 3′ hydroxyl. Although molecules that are cleaved with restriction nucleases have appropriate ends, oligonucleotides are typically synthesized with hydroxyl groups at both ends. Also, although the junction shown in Figure 8–16 is BamHI–KpnI, the other junction is KpnI–BamHI, and you are skeptical that the same oligonucleotide could splint both junctions.

A. Draw a picture of the KpnI–BamHI junction and the oligonucleotide splint that would be needed. Is this oligonucleotide the same or different from the one shown in Figure 8–16?

B. Draw a picture of the molecule after treatment with DNA ligase. Indicate which if any of the nicks will be ligated.

C. Will your friend's scheme work?

8–66 How would a DNA sequencing reaction be affected if the ratio of dideoxynucleoside triphosphates (ddNTPs) to deoxynucleoside triphosphates (dNTPs) were increased? What would the consequences be if the ratio were decreased?

8–67 DNA sequencing of your own two β-globin genes (one from each of your two copies of chromosome 11) reveals a mutation in one of the genes.

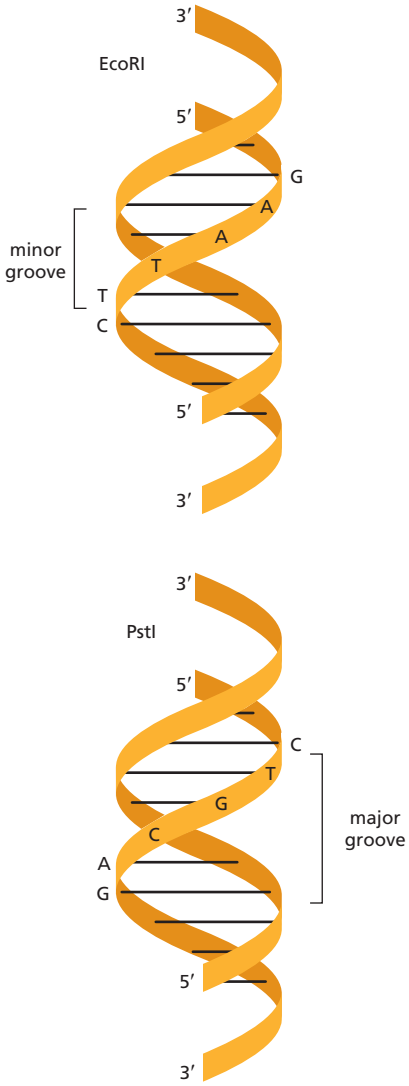


Figure 8–14 Restriction sites on helical DNA (Problem 8–61).

TABLE 8–5 A set of restriction nucleases and their recognition sequences (Problem 8–62).

Restriction nuclease	Recognition sequence
AluI	AGCT
Sau96I	GGNCC
HindIII	AAGCTT
N stands for any nucleotide.	

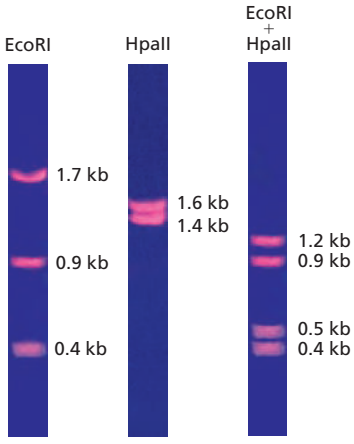


Figure 8–15 Sizes of DNA bands produced by digestion of a 3.0-kb fragment by EcoRI, HpaII, and a mixture of the two (Problem 8–63). Sizes of the fragments are shown in kilobases.

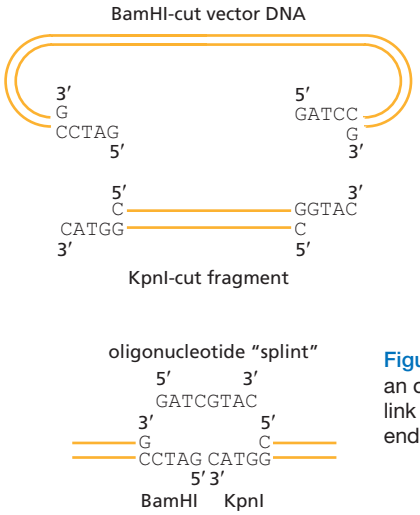


Figure 8–16 Scheme to use an oligonucleotide "splint" to link incompatible restriction ends (Problem 8–65).

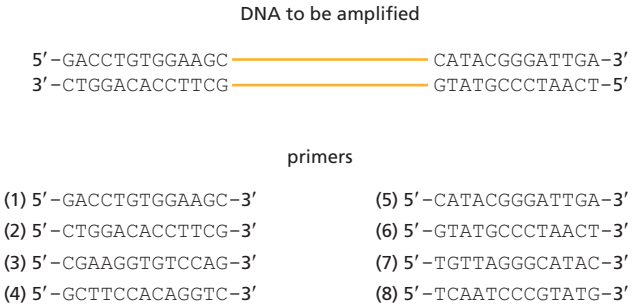


Figure 8–17 DNA to be amplified and potential PCR primers (Problem 8–68).

Given this information alone, how much should you worry about being a carrier of an inherited disease that could be passed on to your children? What other information would you like to have to assess your risk?

8–68 You want to amplify the DNA between the two stretches of sequence shown in **Figure 8–17**. Of the listed primers, choose the pair that will allow you to amplify the DNA by PCR.

8–69 In the very first round of PCR using genomic DNA, the DNA primers prime synthesis that terminates only when the cycle ends (or when a random end of DNA is encountered). Yet, by the end of 20 to 30 cycles—a typical amplification—the only visible product is defined precisely by the ends of the DNA primers (**Figure 8–18**). In what cycle is a double-stranded fragment of the correct size first generated?

8–70 You want to express a rare human protein in bacteria so you can make large quantities of it. To aid in its purification, you decide to add a stretch of six histidines to the N-terminus or the C-terminus of the protein. Such histidine-tagged proteins bind tightly to Ni^{2+} columns but can be readily eluted with a solution of EDTA or imidazole. This procedure allows an enormous purification in one step.

The nucleotide sequence that encodes your protein is shown in **Figure 8–19**. Design a pair of PCR primers, each with 18 nucleotides of homology to the gene, that will amplify the coding sequence and add an initiation codon followed by six histidine codons to the N-terminus. Design a pair of primers that will add six histidine codons followed by a stop codon to the C-terminus.

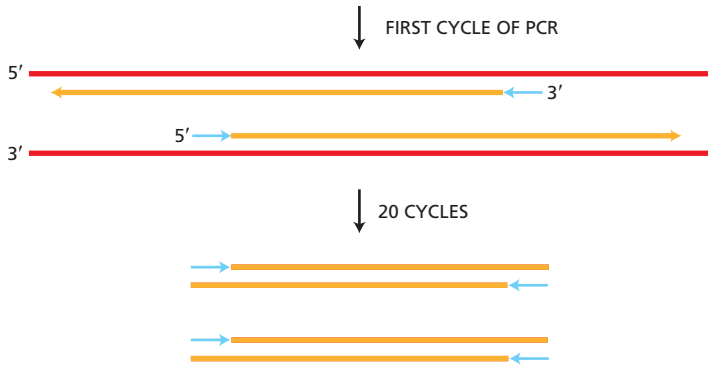


Figure 8–18 Products of PCR after 1 and 20 cycles (Problem 8–69).

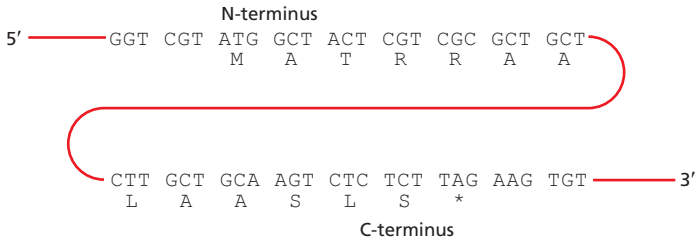


Figure 8–19 Nucleotide sequence around the N- and C-termini of the protein you want to modify (Problem 8–70). The encoded amino acid sequence is indicated *below* each codon using the one-letter code. The *asterisk* (*) indicates the stop codon. Only the top strand of the double-stranded DNA is shown.

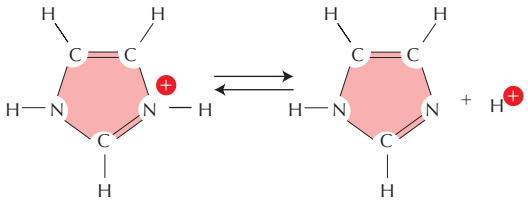


Figure 8–20 Structure of imidazole (Problem 8–71).

8–71 You have now cloned in an expression vector both versions of the histidine-tagged protein you created in Problem 8–70. Neither construct expresses particularly strongly in bacteria, but the product is soluble. You pass the crude extract over a Ni^{2+} affinity column, which binds histidine-tagged proteins specifically. After washing the column extensively, you elute your protein from the column using a solution containing imidazole (**Figure 8–20**), which releases your protein.

When you subject the eluted protein to electrophoresis and stain the gel for protein, you are pleased to find bands in the eluate that are not present when control bacteria are treated similarly. But you are puzzled to see that the construct tagged at the N-terminus gives a ladder of shorter proteins below the full-length protein, whereas the C-terminally tagged construct yields exclusively the full-length protein. The amount of full-length protein is about the same for each construct.

A. Why does a solution of imidazole release a histidine-tagged protein from the Ni^{2+} column?

B. Offer an explanation for the difference in the products generated by the two constructs.

8–72 An example of a dideoxy sequencing gel is shown in **Figure 8–21**. Try reading it. As read from the bottom of the gel to the top, the sequence corresponds to the mRNA for a protein. Can you find the open reading frame in this sequence? What protein does it code for?

CALCULATIONS

8–73 The restriction nuclease Sau3A recognizes the sequence 5′-GATC and cleaves on the 5′ side (to the left) of the G. (Since the top and bottom strands of most restriction sites read the same in the 5′-to-3′ direction, only one strand of the site need be shown.) The single-stranded ends produced by Sau3A cleavage are identical to those produced by BamHI cleavage (see Figure 8–13), allowing the two types of ends to be joined together by incubation with DNA ligase. (You may find it helpful to draw out the product of this ligation to convince yourself that it is true.)

A. What fraction of BamHI sites (5′-GGATCC) can be cut with Sau3A? What fraction of Sau3A sites can be cut with BamHI?

B. If two BamHI ends are ligated together, the resulting site can be cleaved again by BamHI. The same is true for two Sau3A ends. Suppose you ligate a Sau3A end to a BamHI end. Can the hybrid site be cut with Sau3A? Can it be cut with BamHI?

C. What do you suppose is the average size of DNA fragments produced by digestion of chromosomal DNA with Sau3A? What's the average size with BamHI?

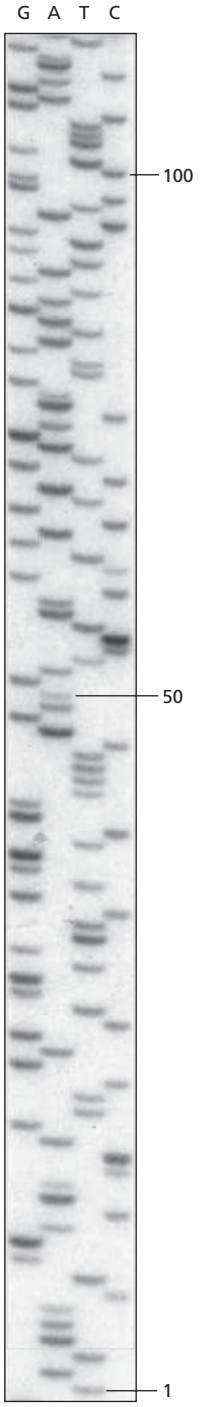


Figure 8–21 A dideoxy sequencing gel of a cloned segment of DNA (Problem 8–72). The lanes are labeled G, A, T, and C to indicate which ddNTP was included in the reaction.

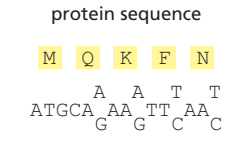


Figure 8–22 A degenerate oligonucleotide probe for the Factor VIII gene based on a stretch of amino acids from the protein (Problem 8–75). Because more than one DNA triplet can encode each amino acid, a number of different nucleotide sequences are possible for each amino acid sequence. During synthesis, a mixture of nucleotides is included at each ambiguous position (for example, A plus G at position 6). As a result, the mixture of synthesized oligonucleotides contains all possible sequences that might encode the specific amino acid segment. Although only one of these sequences in the genomic DNA will actually code for the protein, it is impossible to tell in advance which one it is. The mixture of the possible sequences—called a degenerate oligonucleotide probe—is used to search a genomic library for the gene.

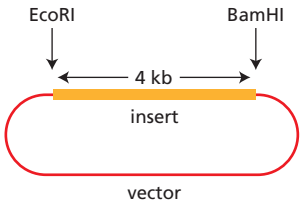


Figure 8–23 Recombinant plasmid containing a cloned DNA segment (Problem 8–76).

8–74 To prepare a genomic library, it is necessary to fragment the genome so that it can be cloned in a vector. A common method is to use a restriction nuclease.

A. How many different DNA fragments would you expect to obtain if you cleaved human genomic DNA with Sau3A (5′-GATC)? (Recall that there are 3.2×10^9 base pairs in the haploid human genome.) How many would you expect to get with EcoRI (5′-GAATTC)?

B. Human genomic libraries are often made from fragments obtained by cleaving human DNA with Sau3A in such a way that the DNA is only partially digested; that is, so that not all the Sau3A sites have been cleaved. What is a possible reason for doing this?

8–75 A degenerate set of oligonucleotide probes for the Factor VIII gene for blood clotting is shown in **Figure 8–22**. Each of these probes is only 15 nucleotides long. On average, how many exact matches to any single 15-nucleotide sequence would you expect to find in the human genome (3.2×10^9 base pairs)? How many matches to the collection of sequences in the degenerate oligonucleotide probe would you expect to find? How might you determine that a match corresponds to the Factor VIII gene?

DATA HANDLING

8–76 You have cloned a 4-kb segment of a gene into a plasmid vector (**Figure 8–23**) and now wish to prepare a restriction map of the gene in preparation for other DNA manipulations. Your advisor left instructions on how to do it, but she is now on vacation, so you are on your own. You follow her instructions, as outlined below.

1. Cut the plasmid with EcoRI.
2. Add a radioactive label to the EcoRI ends.
3. Cut the labeled DNA with BamHI.
4. Purify the insert away from the vector.
5. Digest the labeled insert briefly with a restriction nuclease so that on average each labeled molecule is cut about one time.
6. Repeat step 5 for several different restriction nucleases.
7. Run the partially digested samples side by side on an agarose gel.
8. Place the gel against x-ray film so that fragments with a radioactive end can expose the film to produce an autoradiograph.
9. Draw the restriction map.

Your biggest problem thus far has been step 5; however, by decreasing the amounts of nuclease and lowering the temperature, you were able to find conditions for partial digestion. You have now completed step 8, and your autoradiograph is shown in **Figure 8–24**.

Unfortunately, your advisor was not explicit about how to construct a map from the data in the autoradiograph. She is due back tomorrow. Will you figure it out in time?

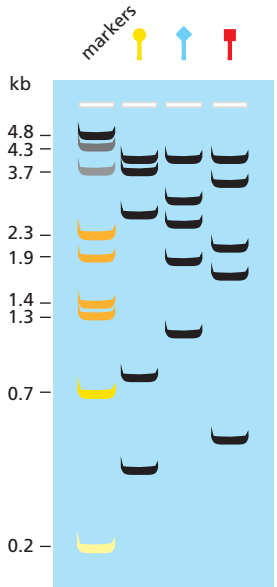


Figure 8–24 Autoradiograph showing the electrophoretic separation of the labeled fragments after partial digestion with the three restriction nucleases represented by the *symbols* (Problem 8–76). Numbers at the *left* indicate the sizes of a set of marker fragments in kilobases.

8–77 The DNA of certain animal viruses can integrate into a cell's DNA as shown schematically in **Figure 8–25**. You want to know the

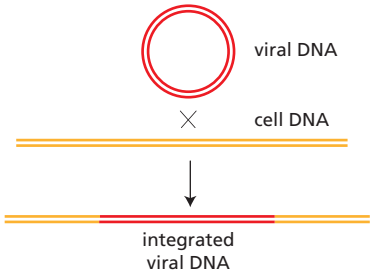


Figure 8–25 Integration of viral DNA into cell DNA (Problem 8–77).

structure of the viral genome as it exists in the integrated state. You digest samples of viral DNA and DNA from cells that contain the integrated virus with restriction nucleases that cut the viral DNA at known sites (**Figure 8–26A**). Subsequently, you separate the fragments by electrophoresis on agarose gels and visualize the bands that contain viral DNA by hybridization with a viral DNA probe. You obtain the patterns shown in **Figure 8–26B**.

From this information, decide in which of the five segments of the viral genome (labeled *a* to *e* in Figure 8–26A) the integration event occurred.

MEDICAL LINKS

8–78 Many mutations that cause human genetic diseases involve the substitution of one nucleotide for another, as is the case for sickle-cell anemia (**Figure 8–27A**). An assay based on ligation of oligonucleotides provides a rapid way to detect such specific single-nucleotide differences. This assay uses pairs of oligonucleotides: for each pair, one oligonucleotide is labeled with biotin and the other with a radioactive (or fluorescent) tag. In the assay shown in **Figure 8–27B** for the detection of the mutation responsible for sickle-cell anemia, two pairs of oligonucleotides are hybridized to DNA from an individual and incubated in the presence of DNA ligase. Biotinylated oligonucleotides are then bound to streptavidin on a solid support and any associated radioactivity is visualized by autoradiography, as shown in **Figure 8–27C**.

A. Do you expect the β^A and β^S oligonucleotides to hybridize to both β^A and β^S DNA?

B. How does this assay distinguish between β^A and β^S DNA?

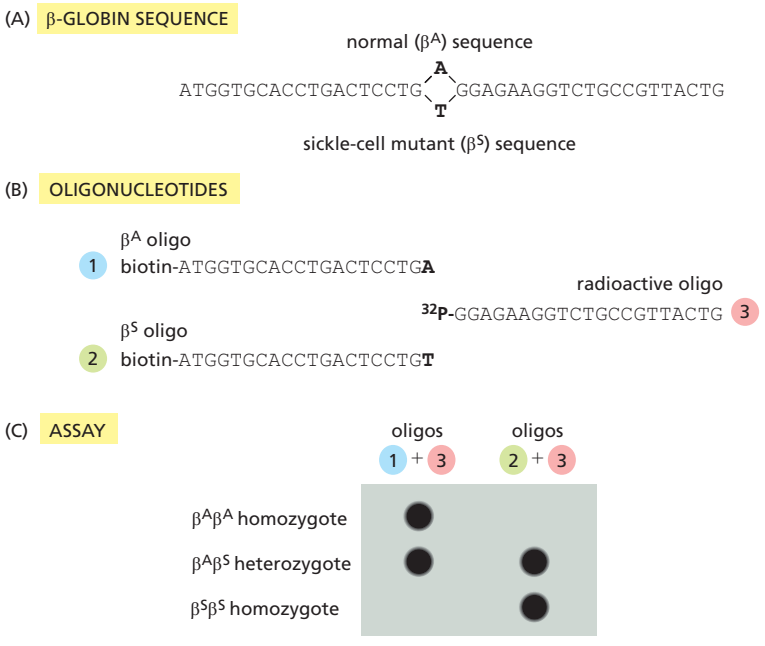


Figure 8–27 Oligonucleotide-ligation assay (Problem 8–78). (A) Sequence of the β-globin gene around the site of the sickle-cell (β^S) mutation. The normal β^A sequence carries an A at the central position; the sickle-cell mutant β^S sequence has a T instead. (B) Specific oligonucleotides for ligation assay. (C) Assays to detect the single-nucleotide difference between the β^A and β^S sequences. After hybridization to patient DNA, biotinylated oligonucleotides were collected in a spot on a sheet of filter paper and exposed to x-ray film to detect radioactivity, which turns the film black.

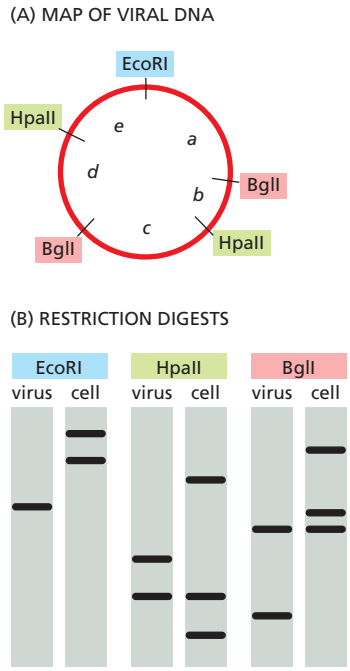


Figure 8–26 Viral and cell DNA digested with various restriction nucleases and hybridized to a viral DNA probe (Problem 8–77). (A) Restriction sites on the viral genome. The DNA segments defined by these sites are indicated by the letters *a* to *e*. (B) Restriction digests of viral DNA and cellular DNA. Agarose gels separate DNA fragments on the basis of size—the smaller the fragment, the farther it moves toward the *bottom* of the gel.

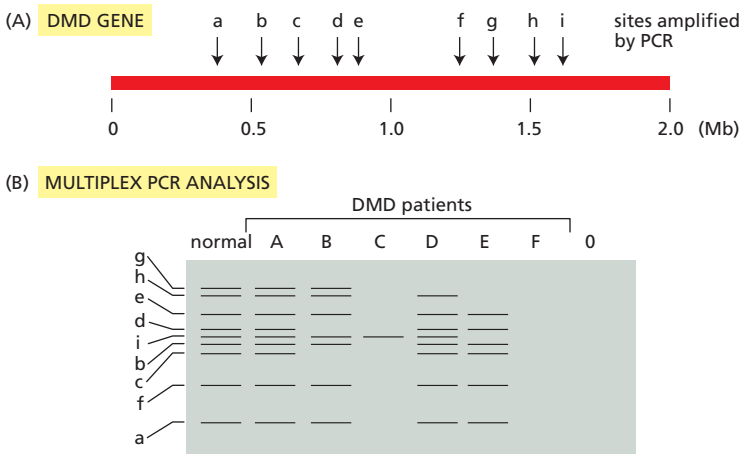


Figure 8–28 Multiplex PCR analysis of six DMD patients (Problem 8–79). (A) The DMD gene with the nine sites amplified by PCR indicated by *arrows*. The sizes of the PCR products are so small on this scale that their location is simply indicated. (B) Agarose gel display of amplified PCR products. "Normal" indicates a normal male. The lane marked "0" shows a negative control with no added DNA.

8–79 Duchenne's muscular dystrophy (DMD) is among the most common human genetic diseases, affecting approximately 1 in 3500 male births. One-third of all new cases arise via new mutations. The DMD gene, which is located on the X chromosome, is greater than 2 million base pairs in length and contains at least 70 exons. Large deletions account for about 60% of all cases of the disease, and they tend to be concentrated around two regions of the gene.

The very large size of the DMD gene complicates the analysis of mutations. One rapid approach, which can detect about 80% of all deletions, is termed multiplex PCR. It uses multiple pairs of PCR primers to amplify nine different segments of the gene in the two most common regions for deletions (**Figure 8–28A**). By arranging the PCR primers so that each pair gives a different size product, it is possible to amplify and analyze all nine segments in one PCR reaction. An example of multiplex PCR analysis of six unrelated DMD males is shown in **Figure 8–28B**.

A. Describe the extent of the deletions, if any, in each of the six DMD patients.

B. What additional control might you suggest to confirm your analysis of patient F?

STUDYING GENE EXPRESSION AND FUNCTION

TERMS TO LEARN

allele
chromatin immunoprecipitation
complementation test
conditional mutation
DNA microarray
epistasis analysis
genetic screen
genetics
genotype
green fluorescent protein (GFP)
haplotype block
phenotype
polymorphism
quantitative RT-PCR
reverse genetics
single-nucleotide polymorphism (SNP)
transgene
transgenic organism

DEFINITIONS

Match each definition below with its term from the list above.

8–80 A search through a large collection of mutants for a mutant with a particular phenotype.

8–81 One of a number of common sequence variants that coexist in the population.

8–82 One of a set of alternative forms of a gene.

8–83 The observable character of a cell or an organism.

8–84 Comparing the phenotypes of different combinations of mutations to determine the order in which the genes act.

8–85 Ancestral chromosome segment that has been inherited with little genetic rearrangement across generations.

8–86 Animal or plant that has been permanently engineered by gene deletion, gene insertion, or gene replacement.

8–87 The genetic constitution of an individual cell or organism.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

8–88 In an organism whose genome has been sequenced, identifying the mutant gene responsible for an interesting phenotype is as easy for mutations induced by chemical mutagenesis as it is for those generated by insertional mutagenesis.

8–89 Loss-of-function mutations are usually recessive.

8–90 If two mutations have a synthetic phenotype, it usually means that the mutations are in genes whose products operate in the same pathway.

THOUGHT PROBLEMS

8–91 Distinguish between the following genetic terms:

A. Locus and allele
B. Homozygous and heterozygous
C. Genotype and phenotype
D. Dominant and recessive

8–92 Explain the difference between a gain-of-function mutation and a dominant-negative mutation. Why are both these types of mutation usually dominant?

8–93 Discuss the following statement: "We would have no idea today of the importance of insulin as a regulatory hormone if its absence were not associated with the human disease diabetes. It is the dramatic consequences of its absence that focused early efforts on the identification of insulin and the study of its normal role in physiology."

8–94 What are single-nucleotide polymorphisms (SNPs), and how can they be used to locate a mutant gene?

8–95 Fatty acid synthase in mammalian cells is encoded by a single gene. This remarkable protein carries out seven distinct biochemical reactions. The mammalian fatty acid synthase gene is homologous to seven different *E. coli* genes, each of which encodes one of the functions of the mammalian protein. Do you think it is likely that the proteins in *E. coli* function together as a complex? Why or why not?

8–96 How does reverse genetics differ from standard genetics?

8–97 The cells in an individual animal contain nearly identical genomes. In an experiment, a tissue composed of multiple cell types is fixed and subjected to *in situ* hybridization with a DNA probe to a particular gene. To your surprise, the hybridization signal is much stronger in some cells than in others. Explain this result.

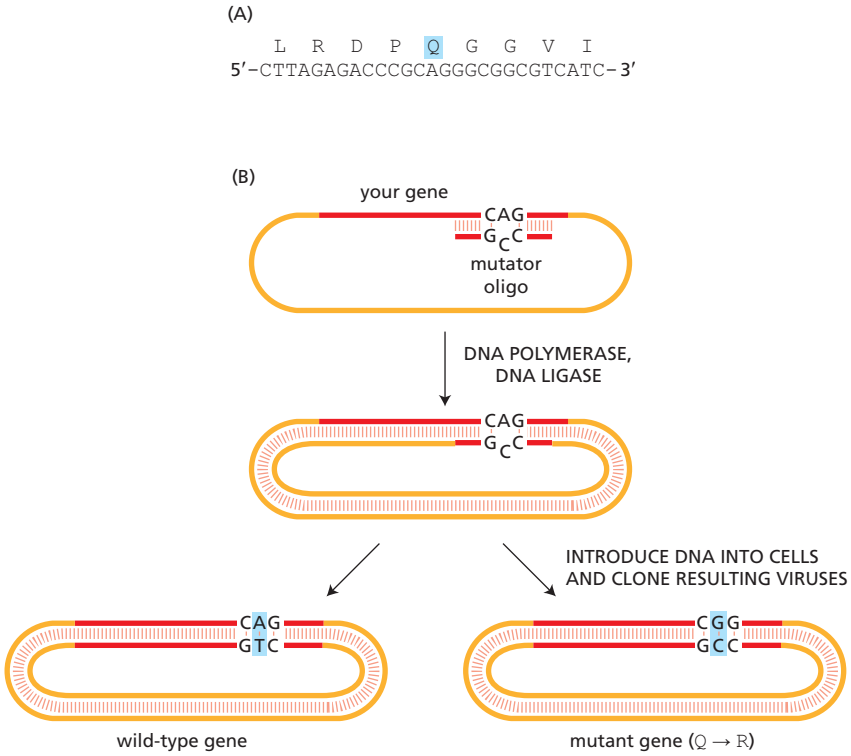


Figure 8–29 Site-directed mutagenesis (Problem 8–98). (A) Sequence of DNA and the encoded protein. (B) Conversion of normal gene into a mutant gene.

8–98 From previous work, you suspect that the glutamine (Q) in the protein segment in **Figure 8–29A** plays an important role at the active site. Your advisor wants you to alter the protein in three ways: change the glutamine to arginine (R) change the glutamine to glycine (G), and delete the glutamine from the protein. You plan to accomplish these mutational alterations on a version of your gene that is cloned into M13 viral DNA. You want to hybridize an appropriate oligonucleotide to the M13 viral DNA, so that when DNA polymerase extends the oligonucleotide around the single-stranded M13 circle, it will complete a strand that encodes the complement of the desired mutant protein. Design three 20-nucleotide-long oligonucleotides that could be hybridized to the cloned gene on single-stranded M13 viral DNA as the first step in effecting the mutational changes (**Figure 8–29B**).

8–99 You have just gotten back the results from an RNA-seq analysis of mRNA from liver. You had anticipated counting the number of reads of each mRNA to determine the relative abundance of different mRNAs. But you are puzzled because many of the mRNAs have given you results like those shown in **Figure 8–30**. How is it that different parts of an mRNA can be represented at different levels?

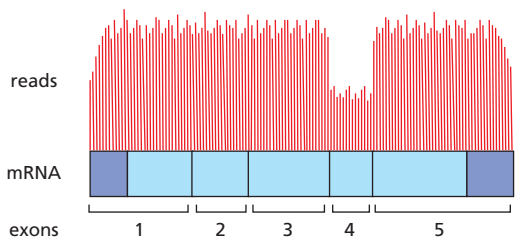


Figure 8–30 RNA-seq reads for a liver mRNA (Problem 8–99). The exon structure of the mRNA is indicated, with protein-coding segments indicated in *light blue* and untranslated regions in *dark blue*. The numbers of sequencing reads are indicated by the heights of the vertical lines above the mRNA.

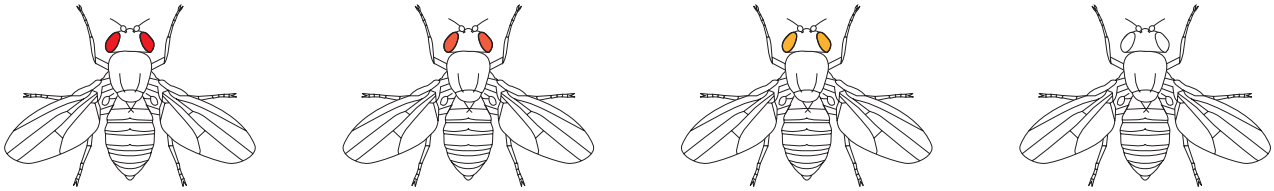


Figure 8–31 *Drosophila* with different color eyes (Problem 8–100). Wild-type flies with brick-red eyes are shown on the *left* and white-eyed flies are shown on the *right*. Flies with eye colors between red and white are shown in between.

DATA HANDLING

8–100 Early genetic studies in *Drosophila* laid the foundation for our current understanding of genes. *Drosophila* geneticists were able to generate mutant flies with a variety of easily observable phenotypic changes. Alterations from the fly's normal brick-red eye color have a venerable history because the very first mutant found by Thomas Hunt Morgan was a white-eyed fly (**Figure 8–31**). Since that time, a large number of mutant flies with intermediate eye colors have been isolated and given names that challenge your color sense: garnet, ruby, vermilion, cherry, coral, apricot, buff, and carnation. The mutations responsible for these eye-color phenotypes are recessive. To determine whether the mutations affected the same or different genes, flies homozygous for each mutation were bred to one another in pairs and the eye colors of their progeny were noted. In **Table 8–6**, brick-red wild-type eyes are shown as (+) and other colors are indicated as (–).

A. How is it that flies with two different eye colors—ruby and white, for example—give rise to progeny that all have brick-red eyes?
B. Which mutations affect different genes and which mutations are alleles of the same gene?
C. How can alleles of the same gene give different eye colors? That is to say, why don't all the mutations in the same gene give the same phenotype?

8–101 You have designed and constructed a DNA microarray that carries 20,000 allele-specific oligonucleotides (ASOs). These ASOs correspond to the wild-type and mutant alleles associated with 1000 human diseases. You have designed the microarray so the ASO that hybridizes to the mutant allele is located right below the ASO that hybridizes to the same site in the wild-type sequence. This arrangement is illustrated in **Figure 8–32** for ASOs that are specific for the sickle-cell allele (β^S) and the corresponding

TABLE 8–6 **Complementation analysis of *Drosophila* eye-color mutations** (Problem 8–100).

MUTATION	white	garnet	ruby	vermilion	cherry	coral	apricot	buff	carnation
White	–	+	+	+	–	–	–	–	+
Garnet		–	+	+	+	+	+	+	+
Ruby			–	+	+	+	+	+	+
Vermilion				–	+	+	+	+	+
Cherry					–	–	–	–	+
Coral						–	–	–	+
Apricot							–	–	+
Buff								–	+
Carnation									–

Brick-red eyes are indicated as (+). Other colors are indicated as (–).

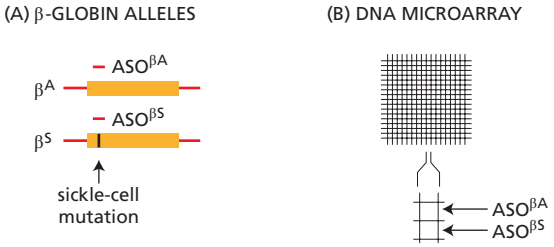


Figure 8–32 DNA microarray for detection of disease alleles (Problem 8–101). (A) The β-globin alleles. The wild-type β-globin gene (β^A) and the sickle-cell allele (β^S) are shown. The position of the sickle-cell mutation is shown by a *vertical line*. The ASOs are shown as *short red lines* arranged above the sites in the gene to which they hybridize. Because the ASOs are located at corresponding positions, each is specific for its allele: the wild-type ASO will not hybridize to the sickle-cell allele, nor will the sickle-cell ASO hybridize to the wild-type allele. (B) DNA microarray. A tiny section of the microarray is enlarged to illustrate the locations of the wild-type and sickle-cell ASOs.

site in the wild-type allele (β^A). ASO$^{\beta S}$ hybridizes to the sickle-cell mutation, and ASO$^{\beta A}$ hybridizes to the corresponding position in the wild-type allele. As a test of your microarray, you carry out hybridizations of DNA isolated from individuals who are homozygous for the wild-type allele, homozygous for the sickle-cell allele, or heterozygous for the wild-type and sickle-cell alleles. For each DNA sample, draw the expected patterns of hybridization to the globin ASOs on your microarray.

8–102 Now that news of your disease-specific DNA microarray has gotten around, you are being inundated with requests to analyze various samples. Just today you received requests from four physicians for help in the prenatal diagnosis of the same disease. Each of the pregnant mothers has a family history of this disease. You included on your array the five alleles known to cause this disease (**Figure 8–33**). Each of these alleles is recessive. You agree to help. You prepare samples of fetal DNA gotten by amniocentesis and hybridize them to your microarrays. Your data are shown in Figure 8–33C. Assuming that the five disease alleles shown in Figure 8–33A are the only ones in the human population, decide for each sample of DNA whether the individual will have the disease or not. Explain your reasoning.

8–103 You've heard about this cool technique for targeted recombination into embryonic stem (ES) cells that allows you to create a null allele or a conditional allele more or less at the same time. As your friend explained it to you, you first carry out standard gene targeting into ES cells by homologous recombination, as shown in **Figure 8–34**. The *Neo* gene, which codes for resistance to the antibiotic neomycin, allows selection for ES cells that have incorporated the vector. These cells can then be screened by Southern blotting for those that have undergone a targeted event. The really cool part is to flank the *Neo* gene and an adjacent exon or two

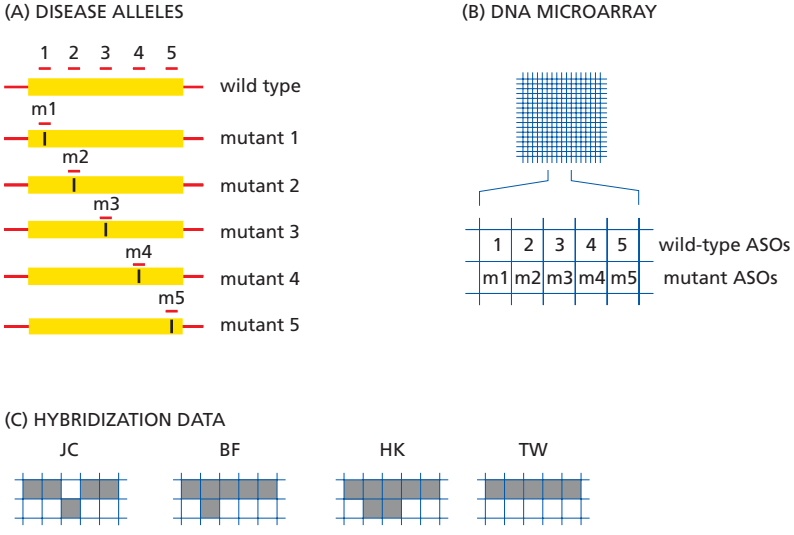


Figure 8–33 DNA microarray analysis of alleles present in prenatal samples (Problem 8–102). (A) Wild-type and disease alleles. *Vertical lines* indicate the sites of the mutations in the disease-causing alleles. The ASOs specific for the disease mutations are shown as *red lines* and labeled m1, m2, etc. The corresponding ASOs that hybridize to the wild-type gene at sites that correspond to the position of the mutations are labeled 1, 2, etc. (B) DNA microarray. The arrangement of wild-type and mutant ASOs is indicated. (C) Hybridization data. Samples of fetal DNA were hybridized to DNA arrays. *Dark spots* indicate sites where hybridization occurred. *Letters* identify the patients.

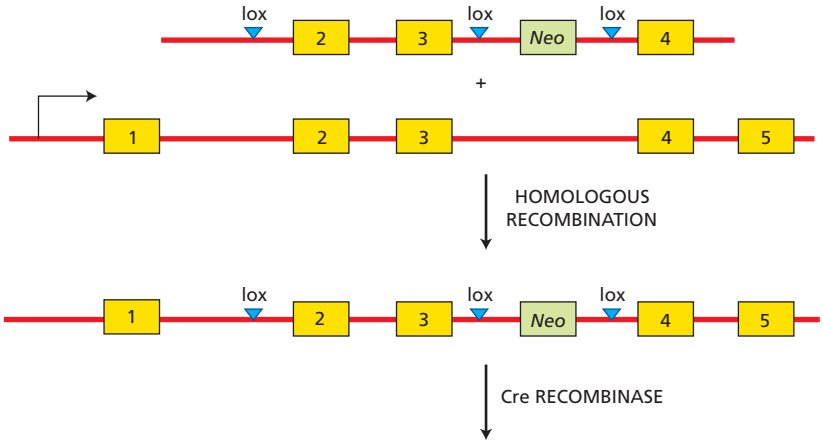


Figure 8–34 Targeted modification of a gene in mouse ES cells (Problem 8–103). Exons are shown as *boxes*; the promoter is identified by the *horizontal arrow*. The targeting vector is fully homologous to the gene, except for the presence of the three lox sites and the *Neo* gene, all of which are in introns. Cre recombinase, which can be introduced by transfection of a Cre-expression vector, catalyzes a site-specific recombination event between a pair of lox sites in about 20% of transfected cells.

with lox sites. This technique is commonly referred to as "floxing." Once the modified ES cells have been identified, they can be exposed to the Cre recombinase, which promotes site-specific recombination between pairs of lox sites. One advantage is that it allows you to get rid of the *Neo* gene and any bacterial DNA segments, which can sometimes influence the phenotype.

A. What possible products might you get from expression of Cre in modified ES cells that carry three lox sites, as indicated in Figure 8–34?
B. Which product(s) would be a null allele?
C. Which product(s) would have a pair of lox sites but be an otherwise normal allele?
D. If you had one mouse that expressed Cre under the control of a tissue-specific promoter, can you use the allele in part C (after you've put it into the germ line of a mouse) as a conditional allele; that is, one whose defect is expressed only in a particular tissue?

8–104 CRISPR/Cas9–guide RNA complexes hold enormous promise as aids for genome engineering in plants and animals. The CRISPR system is almost too good to be true. You want to test just how specific the Cas9–guide RNA complexes are; that is, whether they really recognize individual sites in the genome, which is the basis for their touted actions. You realize that you can test their specificity using the "DNA curtain" assay you have developed. You make the DNA curtain by tethering single molecules of bacteriophage lambda DNA (about 50,000 nucleotides) at one end, and then stretching them in the same direction by flowing buffer across the slide. You incubate the DNA curtain with a highly fluorescent version of Cas9—either loaded with the guide RNA or free—and visualize the distribution of the Cas9 by sensitive fluorescence microscopy, as shown in **Figure 8–35**.

A. Phage lambda DNA has a single site that perfectly matches the guide RNA. Does your experiment support the existence of a single site that is recognized by the Cas9–guide RNA complex? Explain your answer.

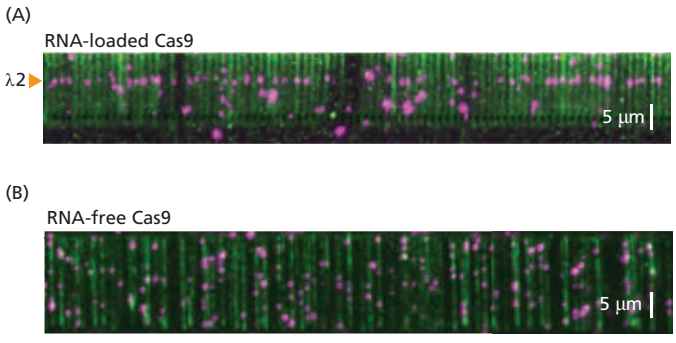


Figure 8–35 DNA curtain assay for target binding by Cas9–guide RNA (Problem 8–104). (A) DNA curtain incubated with Cas9–guide RNA that matches one site in the lambda genome. *Pink* spots indicate the sites where Cas9–guide RNA is located. (B) DNA curtains incubated with Cas9 in the absence of guide RNA.

B. One of the main concerns for using the CRISPR system is that the Cas9–guide RNA will bind to other sites that are related to the intended target (so-called off-target effects). Is there any evidence for off-target binding in your experiment? Do you think the results would be any different if you had a DNA curtain made from human chromosome 1 (about 250,000,000 nucleotides)?

MATHEMATICAL ANALYSIS OF CELL FUNCTIONS

TERMS TO LEARN

robustness
stochastic

DEFINITIONS

Match each definition below with its term from the list above.

8–105 The ability of biological regulatory systems to function normally in the face of frequent and sometimes extreme variations in external conditions or the concentrations or activities of key components.

8–106 Describes random variations in protein content of individual cells resulting in variations in cell phenotypes.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

8–107 To judge the biological importance of an interaction between protein *A* and protein *B*, we need to know quantitative details about their concentrations, affinities, and kinetic behaviors.

8–108 The association constant, K_a, is equal to 1 minus the dissociation constant, K_d; that is, $K_a = 1 - K_d$.

8–109 The rate of change in the concentration of any molecular species *X* is given by the balance between its rate of appearance and its rate of disappearance.

8–110 After a sudden increase in transcription, a protein with a slow rate of degradation will reach a new steady-state level more quickly than a protein with a rapid rate of degradation.

8–111 The *Lac* operon, which is turned on by a transcription activator and turned off by a transcription repressor, is a classic example of an AND NOT logic gate.

THOUGHT PROBLEMS

8–112 Consider the two network motifs illustrated in **Figure 8–36**. Both contain only negative regulation by repressors, and yet one is a negative feedback

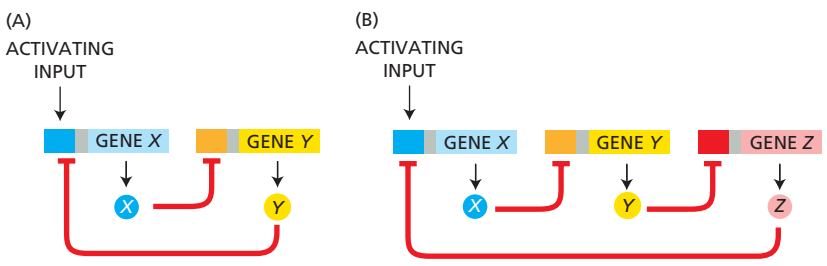


Figure 8–36 Two network motifs composed of transcriptional repressors (Problem 8–112). (A) A two-gene network. (B) A three-gene network.

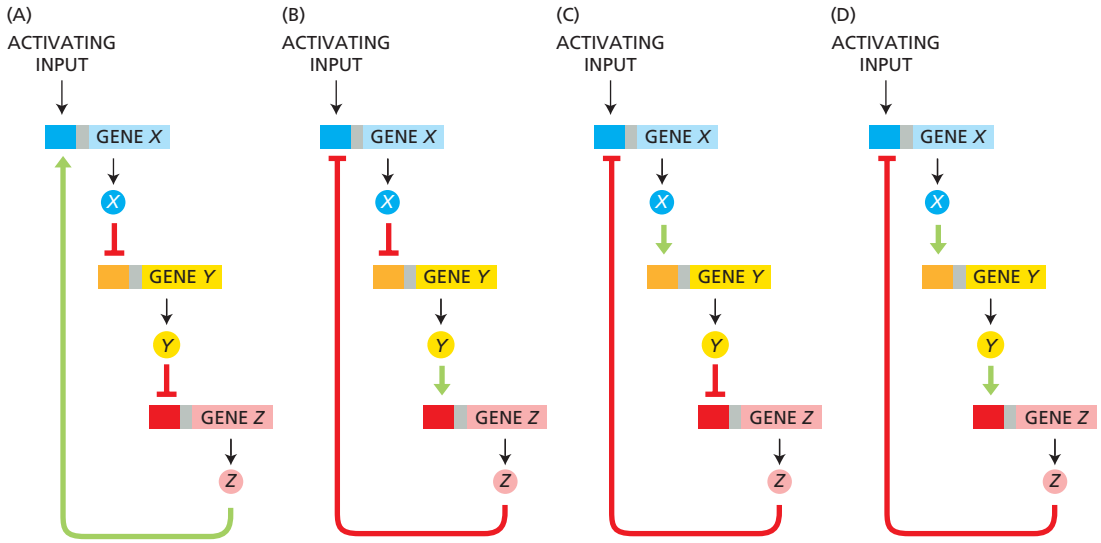


Figure 8–37 Network motifs composed of transcription activators and repressors (Problem 8–113).

loop and the other is a positive feedback loop. Which is the negative and which is the positive feedback loop? Explain how networks made up of negative regulatory elements can differ in their behavior.

8–113 Examine the network motifs in **Figure 8–37**. Decide which ones are negative feedback loops and which are positive. Explain your reasoning.

8–114 Imagine that a random perturbation positions a bistable system precisely at the boundary between two stable states (at the *orange dot* in **Figure 8–38**). How would the system respond?

8–115 For the network motif shown in **Figure 8–39A**, decide whether the pulse of protein *X* output (**Figure 8–39B**) requires that the equilibrium constant for the binding of transcription activator *A* (K_A) must be much greater or much less than the equilibrium constant for binding of the transcription repressor *R* (K_R). Explain your reasoning.

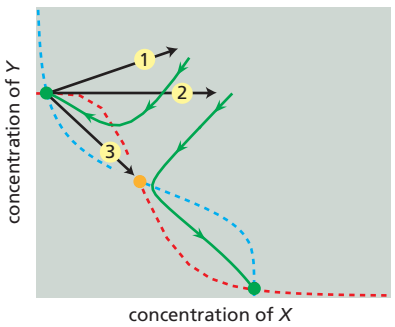


Figure 8–38 Perturbations of a bistable system (Problem 8–114). As shown by the *green lines*, after perturbation 1 the system returns to its original stable state (*green dot* at *left*), and after perturbation 2, the system moves to the other stable state (*green dot* at *right*). Perturbation 3 moves the system to the precise boundary between the two stable states *(orange dot)*.

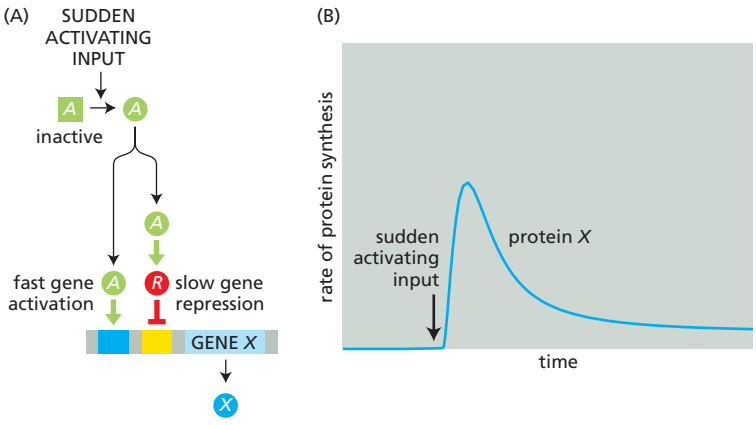


Figure 8–39 Network motif that generates a pulse of protein *X* (Problem 8–115). (A) Regulation of gene *X* by a transcription activator *(green circle)* and a transcription repressor *(red circle)*. (B) Pulse of protein *X* in response to an activating input.

CALCULATIONS

8–116 Consider the situation in which a transcription regulator (R) binds to the promoter (p_X) for gene X. If the concentration of a transcription regulator is 100 times $1/K$, where K is the equilibrium constant for the binding of the regulator to the promoter, what percentage of the promoters in a population of cells will be occupied? What percentage of promoters will be bound if $[R]$ is equal to $1/K$? If $[R]$ is 100-fold less than $1/K$? (The equation for bound fraction is $[R{:}p_X]/[p_X^T] = K[R]/(1 + K[R])$, where $[R{:}p_X]$ is the complex of the regulator with the promoter and $[p_X^T]$ is the total concentration of promoters.)

8–117 What is the molar concentration of a promoter sequence that is present at one copy per *E. coli*? Assume *E. coli* is a rod that is 0.8 μm in diameter and 3 μm in length. (The equation for the volume of a cylinder is $\pi r^2 h$.)

8–118 If the concentration of a transcription repressor is 100 molecules per *E. coli*, and it has a single binding site in the bacterial genome, what must the equilibrium constant be for the binding site to be 99% occupied?

8–119 The *Lac* repressor regulates the expression of a set of genes for lactose metabolism, which are adjacent to its binding site on the bacterial chromosome. In the absence of lactose in the medium, the binding of the repressor turns the genes off. When lactose is added, an inducer is generated that binds to the repressor and prevents it from binding to its DNA target, thereby turning on gene expression.

Inside *E. coli* there are about 10 molecules of *Lac* repressor (10^{-8} M) and 1 binding site (10^{-9} M) on the bacterial genome. The equilibrium constant, K, for binding of the repressor to its binding site is 10^{13} M^{-1}. In the presence of lactose, when an inducer of gene expression binds to the repressor, K for repressor binding to its DNA binding sites decreases to 10^{10} M^{-1}.

A. In a population of bacteria growing in the absence of lactose, what fraction of the binding sites would you expect to be bound by repressor?
B. In bacteria growing in the presence of lactose, what fraction of binding sites would you expect to be bound by the repressor?
C. Given the information in this problem, would you expect the inducer to turn on gene expression? Why or why not?
D. The *Lac* repressor binds nonspecifically to any sequence of DNA with a K of about 10^6 M^{-1}, which is a very low affinity. Can you suggest in a qualitative way how such low-affinity, nonspecific binding might alter the calculations in parts A and B and your conclusion in part C?

8–120 Equilibrium dialysis provides a simple method for determining the equilibrium constant for binding of a ligand (L) by a protein (Pr). The protein is confined inside a dialysis sac, formed by an artificial membrane with pores too tiny for the protein to enter, but which the much smaller ligand can freely permeate. The ligand, usually radiolabeled, is added to the solution surrounding the dialysis sac; after equilibrium has been established, the concentration of the ligand is measured in both compartments. The concentration in the external compartment is the concentration of free (unbound) ligand and the concentration in the dialysis sac is the sum of the bound (Pr–L) plus free ligand. By measuring these values after various initial ligand concentrations, the value of the equilibrium constant can be determined. By convention, the equilibrium is usually considered for the dissociation reaction (Pr–$L \longrightarrow Pr + L$), rather than the association reaction ($Pr + L \longrightarrow Pr$–$L$), and thus the equilibrium constant in this case is referred to as the dissociation constant, K_d.

It is useful to look at the transformation of the standard equilibrium relationship into the form commonly used to analyze the data. At equilibrium,

$$K_d = \frac{[Pr][L]}{[Pr\text{–}L]}$$

Given that the total protein concentration, $[Pr]_{TOT}$, is the sum of the concentration of bound protein $[Pr\text{–}L]$ and free protein $[Pr]$, we can substitute $[Pr]_{TOT} - [Pr\text{–}L]$ for $[Pr]$ and rearrange to give

$$[Pr\text{–}L] = \frac{[Pr]_{TOT}[L]}{K_d + [L]}$$

This is an equation for a rectangular hyperbola. It can be rearranged to give a linear form, as was first done by George Scatchard in 1947 (hence, graphs of such data are commonly known as Scatchard plots):

$$\frac{[Pr\text{–}L]}{[L]} = \frac{-[Pr\text{–}L]}{K_d} + \frac{[Pr]_{TOT}}{K_d}$$

At constant protein concentration and a variety of ligand concentrations, a plot of bound over free ligand ($[Pr\text{–}L]/[L]$) against bound ligand ($[Pr\text{–}L]$) gives a line with slope equal to $-1/K_d$ and an x-intercept equal to $[Pr]_{TOT}$, which is the total concentration of binding sites.

In the early 1960s, when the nature of the genetically identified repressors of bacterial gene expression had not been defined, Walter Gilbert and Benno Müller-Hill used equilibrium dialysis to measure the binding of an inducer of gene expression (IPTG) to the *Lac* repressor protein. They used radiolabeled IPTG and two partially purified preparations of *Lac* repressor protein: one from wild-type cells and the other from mutant cells in which induction of the lactose operon occurred at lower concentrations of IPTG. The mutant cells were assumed to carry a *Lac* repressor that bound IPTG more tightly. Their data are shown as a Scatchard plot in **Figure 8–40**.

A. What are the K_d values for the two lines shown in the figure?
B. Which line corresponds to the wild-type *Lac* repressor and which to the mutant (tighter IPTG-binding) repressor?

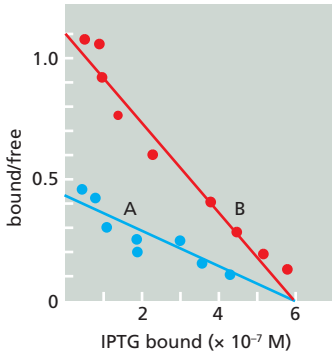


Figure 8–40 Scatchard plot of equilibrium dialysis data for the binding of IPTG to the *Lac* repressor (Problem 8–120).

DATA HANDLING

8–121 Detailed analysis of the regulatory region of the *Lac* operon has revealed surprising complexity. Instead of a single binding site for the *Lac* repressor, as might be expected, there are three sites termed operators: O_1, O_2, and O_3, arrayed along the DNA as shown in **Figure 8–41**. To probe the functions of these three sites, you make a series of constructs in which various combinations of operator sites are present. You examine their

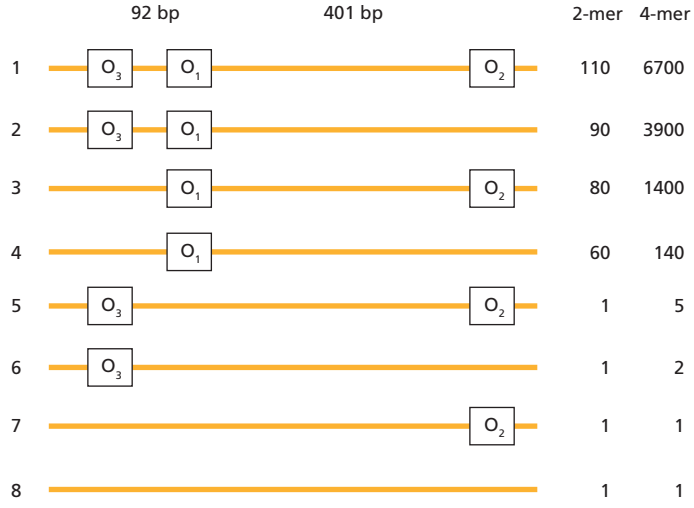


Figure 8–41 Repression of β-galactosidase by promoter regions that contain different combinations of *Lac* repressor binding sites (Problem 8–121). The base-pair (bp) separation of the three operator sites is shown. Numbers at *right* refer to the level of repression, with higher numbers indicating more effective repression by dimeric (2-mer) or tetrameric (4-mer) repressors.

ability to repress expression of β-galactosidase, using either tetrameric (wild type) or dimeric (mutant) forms of the *Lac* repressor. The dimeric form of the repressor can bind to a single operator (with the same affinity as the tetramer) with each monomer binding to half the site. The tetramer, the form normally expressed in cells, can bind to two sites simultaneously. When you measure repression of β-galactosidase expression, you find the results shown in Figure 8–41, with higher numbers indicating more effective repression.

A. Which single operator site is the most important for repression? How can you tell?
B. Do combinations of operator sites (Figure 8–41, constructs 1, 2, 3, and 5) substantially increase repression by the dimeric repressor? Do combinations of operator sites substantially increase repression by the tetrameric repressor? If the two repressors behave differently, offer an explanation for the difference.
C. The wild-type repressor binds O_3 very weakly when it is by itself on a segment of DNA. However, if O_1 is included on the same segment of DNA, the repressor binds O_3 quite well. How can that be?

MCAT STYLE

Passage 1 (Questions 8–122 to 8–126)

Cancer is caused by aberrant versions of our own genes. Chronic myelogenous leukemia, for example, is caused by a hybrid chromosome—the Philadelphia chromosome—that is formed by fusion of broken segments of chromosomes 9 and 22. By chance, this fusion links the *Bcr* gene from chromosome 22 to the *Abl* gene from chromosome 9. The *Bcr-Abl* fusion gene produces a chimeric protein that combines the protein kinase activity of Abl with an activating segment of Bcr to produce a hyperactive Bcr-Abl fusion protein that drives cells of the immune system to proliferate excessively.

Drugs that specifically inhibit such hyperactive kinases are revolutionizing cancer treatment. The first example of such a drug—imatinib—was discovered by searching for compounds that bind and inhibit the *Bcr-Abl* protein kinase. Imatinib caused rapid disappearance of cancer cells with minimal side effects. Typically, however, after a few months or years, the cancer returns, this time in a form that is resistant to the drug. The appearance of drug-resistant cancers is a major challenge for many inhibitor-based therapies.

8–122 Which of the following techniques would serve best as the basis for a rapid, highly sensitive blood test to detect circulating cancer cells that carry the *Bcr-Abl* gene?

A. DNA sequencing
B. Flow cytometry
C. PCR analysis
D. Western blotting

8–123 You hypothesize that drug resistance is caused by mutations in the fusion gene that block binding of imatinib to the Bcr-Abl protein. What would be your first step in testing this hypothesis?

A. Amplify the *Bcr-Abl* gene by PCR and sequence it to look for mutations in the gene.
B. Chemically modify imatinib to search for new drugs that will kill the resistant cells.
C. Determine whether the resistant cancer cells carry the Philadelphia chromosome.
D. Purify Bcr-Abl protein from resistant cancer cells and test whether it binds imatinib.

8–124 To test whether imatinib inhibits the activity of the Bcr-Abl kinases from imatinib-resistant cancer cells from patients, you will need a lot of the

Bcr-Abl protein. What would be the most rapid and direct way to obtain a large amount of the protein?

A. Clone the *Bcr-Abl* gene from a genomic DNA library into an expression vector and purify the protein after expression in bacteria.
B. Grow large amounts of drug-resistant cancer cells in culture and isolate the protein after SDS polyacrylamide-gel electrophoresis.
C. Grow large amounts of drug-resistant cancer cells in culture and purify the Bcr-Abl protein by column chromatography.
D. Use PCR to amplify the *Bcr-Abl* gene from cDNA, clone it into an expression vector, express the protein in bacteria, and then purify it.

8–125 As an alternative hypothesis, you consider that imatinib resistance arises from mutations outside the coding region of the *Bcr-Abl* gene that cause the gene to be expressed at abnormally high levels, thereby reducing the effectiveness of imatinib. Which one of the following techniques would you use to most rapidly test this hypothesis?

A. cDNA library analysis
B. *In situ* hybridization
C. Quantitative RT-PCR
D. Western blotting

8–126 If you find that there are no mutations in the *Bcr-Abl* coding region and the gene is not overexpressed, what would you do next to define the basis for imatinib resistance?

A. Look for mutations in regions neighboring the *Bcr-Abl* gene.
B. Sequence the genomic DNA from imatinib-resistant cells.
C. Use genetic analysis to identify the genes responsible for resistance.
D. Use genome-wide association studies to identify resistance genes.

Passage 2 (Questions 8–127 to 8–131)

Highly regulated proteolysis of cytoplasmic proteins plays an important role in many cellular events. Biochemical analysis uncovered the molecular machinery responsible for regulated proteolysis. Early studies established that damaged proteins added to cell lysates were rapidly destroyed in an ATP-dependent manner. Subsequently, it was shown that a small protein called ubiquitin was covalently attached to proteins before they were destroyed. Column chromatography was used to purify the proteins that attach ubiquitin to other proteins. These studies led to the discovery of a biochemical pathway in which ubiquitin is first activated via formation of a high-energy thioester bond with a protein called E1. E1 then passes the ubiquitin to another protein called E2. Finally, ubiquitin is covalently attached to the target protein. A multiprotein complex called E3 binds to both E2 and the target protein, which ensures that the ubiquitin is attached to the correct target.

Components of the ubiquitin machinery were independently identified in genetic screens for genes that regulate the cell cycle. These screens were based on the hypothesis that mutations in genes necessary for specific steps in the cell cycle should cause cells to arrest before completion of those steps. Thus, a mutation in a gene required for chromosome segregation should cause cells to arrest in mitosis before chromosomes have separated. Mutagenized yeast cells were screened for mutations that cause cells to arrest at specific points in the cell cycle, which led to a collection of cell-division control (*Cdc*) mutants. Three of these mutants—*Cdc16*, *Cdc23*, and *Cdc27*—arrested in mitosis before chromosome segregation. Subsequent work found that the protein products of these genes were required for proteolytic destruction of specific proteins, which triggers chromosome segregation.

8–127 As a first step toward identification of proteins that play a role in ATP-dependent proteolytic destruction, cell extracts were passed over an ion-exchange column. The ion-exchange column was then washed with buffer and eluted with high salt. This resulted in two fractions: proteins that bound to the column (bound fraction), and proteins that flowed

through the column (unbound fraction). No proteolytic activity could be detected in either of these fractions, even though the starting extract had robust activity. What should you do next?

A. Carry out additional purification steps and test for activity.
B. Combine bound and unbound fractions and test for activity.
C. Improve the sensitivity of the assay used to detect proteolysis.
D. Use a gel-filtration column instead of an ion-exchange column.
E. Give up and go home.

8–128 Analysis of the unbound fraction identified the small protein ubiquitin as a key factor. The investigators then sought to discover additional proteins in the cell lysates that work with ubiquitin in the proteolytic pathway. Which one of the following techniques do you suppose they applied?

A. A BLAST search
B. Affinity chromatography
C. Gel-filtration chromatography
D. SDS gel electrophoresis

8–129 To isolate yeast mutants that cannot traverse the cell-division cycle, what kind of mutation did the investigators need to obtain?

A. Conditional mutation
B. Gain-of-function mutation
C. Loss-of-function mutation
D. Null mutation

8–130 The *Cdc16*, *Cdc23*, and *Cdc27* mutants displayed identical phenotypes, which suggested that the encoded proteins work together to execute common functions. Investigators hypothesized that they exist in a multiprotein complex. What technique would you use to test this hypothesis most rapidly?

A. Co-immunoprecipitation
B. Ion-exchange chromatography
C. Nuclear magnetic resonance
D. X-ray diffraction

8–131 Cdc16, Cdc23, and Cdc27 were found to be components of the anaphase-promoting complex (APC). The APC is an E3 complex that targets specific proteins for destruction during mitosis. Investigators hypothesized that the APC triggers chromosome segregation by destroying a protein called securin, which inhibits chromosome segregation. To test this hypothesis, they arrested yeast cells in G_1 phase to synchronize the cell population. By releasing the cells from the G_1 arrest, they could study a population of cells going through the cell cycle in synchrony. Starting with the synchronized cells, which one of the following techniques would be most useful for testing whether securin is proteolytically destroyed during the cell cycle?

A. Hybridization
B. Immunoprecipitation
C. Mass spectrometry
D. Western blotting

CHAPTER 9

Visualizing Cells

LOOKING AT CELLS IN THE LIGHT MICROSCOPE

TERMS TO LEARN

bright-field microscope
cell doctrine
confocal microscope
dark-field microscope
differential-interference-contrast microscope
fluorescence microscope
fluorescence recovery after photobleaching (FRAP)
fluorescence resonance energy transfer (FRET)
green fluorescent protein (GFP)
image processing
ion-sensitive indicator
light microscope
limit of resolution
microelectrode
phase-contrast microscope
photoactivation
superresolution

DEFINITIONS

Match each definition below with its term from the list above.

9–1 Fluorescent protein (from a jellyfish) that is widely used as a marker for monitoring the movements of proteins in living cells.

9–2 The minimal separation between two objects at which they appear distinct.

9–3 The normal light microscope in which the image is obtained by simple transmission of light through the object being viewed.

9–4 Computer treatment of images gained from microscopy that reveals information not immediately visible to the eye.

9–5 Similar to a light microscope but the illuminating light is passed through one set of filters before the specimen, to select those wavelengths that excite the dye, and through another set of filters before it reaches the eye, to select only those wavelengths emitted when the dye fluoresces.

9–6 Type of light microscope that produces a clear image of a given plane within a solid object. It uses a laser beam as a pinpoint source of illumination and scans across the plane to produce a two-dimensional optical section.

9–7 Technique for monitoring the closeness of two fluorescently labeled molecules (and thus their interaction) in cells.

9–8 Piece of fine glass tubing, pulled to an even finer tip, that is used to inject electric current into cells.

IN THIS CHAPTER

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

9–9 Because the DNA double helix is only 2 nm wide—well below the limit of resolution of the light microscope—it is impossible to see chromosomes in living cells without special stains.

9–10 Monoclonal antibodies are superior to the mixture of antibodies in standard antisera raised against the same protein because they are absolutely specific for that protein.

9–11 Caged molecules can be introduced into a cell and then activated by a strong pulse of laser light at the precise time and cellular location chosen by the experimenter.

9–12 Superresolution techniques allow fluorescently tagged molecules to be imaged to accuracies an order of magnitude below the classic diffraction limit to resolution.

THOUGHT PROBLEMS

9–13 Examine the diagram of the light microscope in **Figure 9–1**. Identify and label the eyepiece, the condenser, the light source, the objective, and the specimen. At what two points in the light path is the image of the specimen magnified?

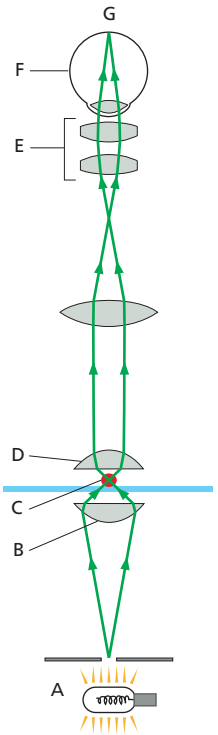


Figure 9–1 Schematic diagram of a light microscope (Problem 9–13).

9–14 Why is it important to keep dust, fingerprints, and other smudges off the lenses of a light microscope?

9–15 When light enters a medium with a different optical density, it bends in a direction that depends on the refractive indices of the two media. Air and glass, for example, have refractive indices of 1.00 and 1.51, respectively. When light enters glass—the medium with the higher refractive index—it bends *toward* a line drawn normal to the surface (**Figure 9–2A**). Conversely, when light exits glass into air, it bends *away* from the normal line (**Figure 9–2B**). Using these principles, draw the paths of two parallel light rays that pass through the hemispherical glass lens shown in **Figure 9–2C**. Will the two rays converge or diverge? Would the result be any different if the glass lens were flipped so that light entered the flat surface?

9–16 The diagrams in **Figure 9–3** show the paths of light rays passing through a specimen with a dry lens and with an oil-immersion lens. Offer an

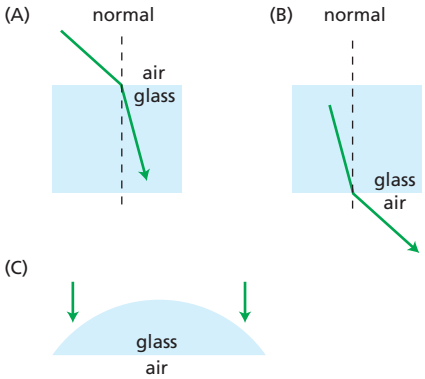


Figure 9–2 Refraction of light at air–glass interfaces (Problem 9–15). (A) A ray of light passing from air to glass. (B) A ray of light passing from glass to air. (C) Two parallel rays of light entering a glass lens.

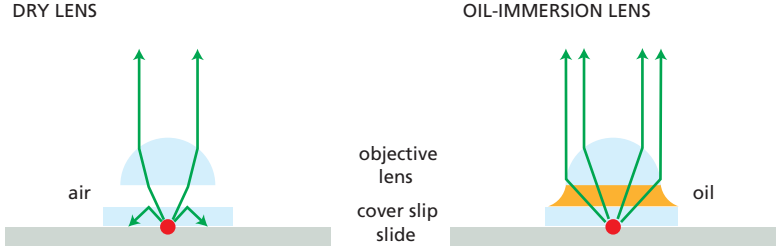


Figure 9–3 Paths of light rays through dry and oil-immersion lenses (Problem 9–16). The *red circle* at the origin of the light rays is the specimen.

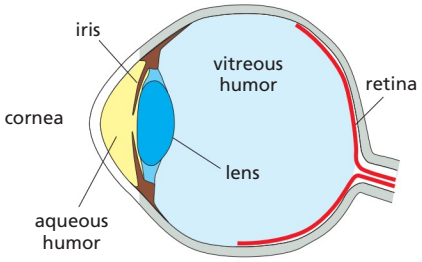


Figure 9–4 Diagram of the human eye (Problem 9–17).

explanation for why oil-immersion lenses should give better resolution. Air, glass, and oil have refractive indices of 1.00, 1.51, and 1.51, respectively.

9–17 Figure 9–4 shows a diagram of the human eye. The refractive indices of the components in the light path are: cornea 1.38, aqueous humor 1.33, crystalline lens 1.41, and vitreous humor 1.38. Where does the main refraction—the main focusing—occur? What role do you suppose the lens plays?

9–18 Why do humans see so poorly under water? And why do goggles help?

9–19 Reading through a beaker filled with clear glass balls is impossible (Figure 9–5). Do you suppose it would help to fill the beaker with water? With immersion oil? Explain your reasoning.

9–20 Examine the four photomicrographs of the same cell in Figure 9–6. Match each image to the technique listed below.

1. Bright-field microscopy
2. Dark-field microscopy
3. Nomarski differential-interference-contrast microscopy
4. Phase-contrast microscopy

9–21 Explain the difference between resolution and magnification.

9–22 Many fluorescent dyes that stain DNA require excitation by ultraviolet light. Hoechst 33342, for example, binds to DNA and absorbs light at 352 nm and emits at 461 nm. If you want to use this membrane-permeant dye in living cells, do you have to worry about ultraviolet light damage to the DNA, which absorbs light maximally at around 260 nm? Why or why not?

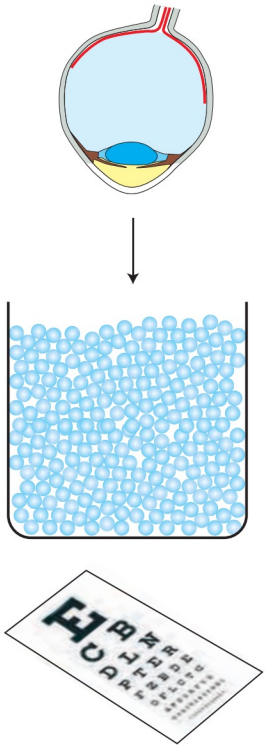


Figure 9–5 Viewing an eye chart through a beaker filled with clear glass balls (Problem 9–19).

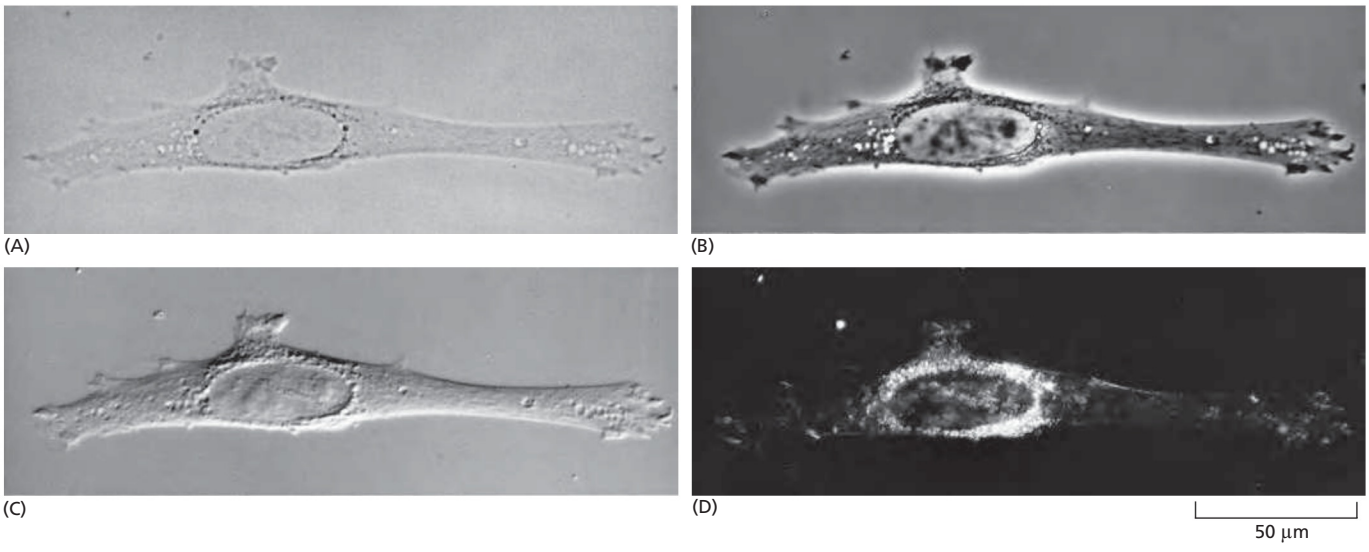


Figure 9–6 Four photomicrographs of the same cell (Problem 9–20).

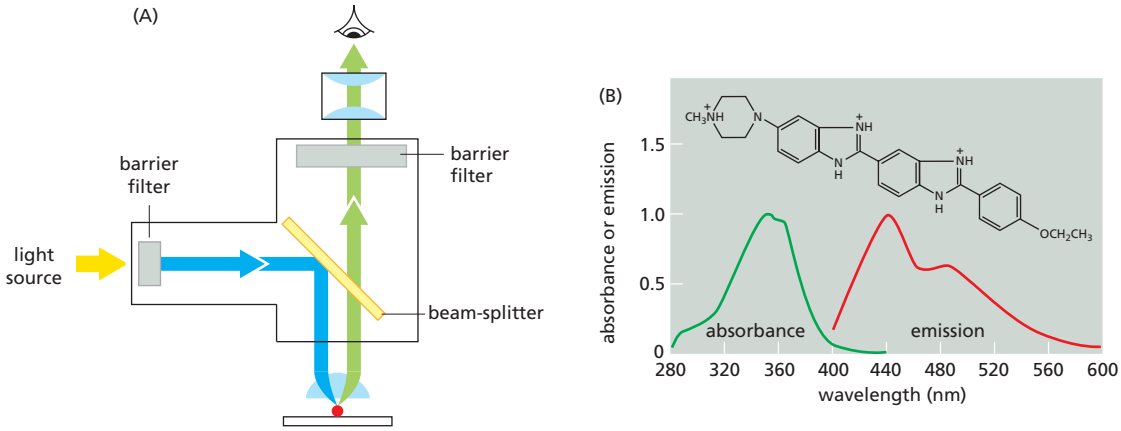


Figure 9–7 Fluorescence microscopy (Problem 9–24). (A) The light path through a fluorescence microscope. (B) Absorption and fluorescence emission spectra of Hoechst 33342 bound to DNA. The structure of Hoechst 33342 is shown above the spectra.

9–23 Why is it, do you suppose, that a fluorescent molecule, having absorbed a single photon of light at one wavelength, *always* emits a photon at a longer wavelength?

9–24 A fluorescence microscope, which is shown schematically in **Figure 9–7A**, uses two filters and a beam-splitting (dichroic) mirror to excite the sample and capture the emitted fluorescent light. Imagine that you wish to view the fluorescence of a sample that has been stained with Hoechst 33342, a common stain for DNA. The absorption and fluorescence emission spectra for Hoechst 33342 are shown in **Figure 9–7B**.

A. Which of the following commercially available barrier filters would you select to place between the light source and the sample? Which one would you place between the sample and the eyepiece?
   1. A filter that passes wavelengths between 300 nm and 380 nm.
   2. A filter that passes all wavelengths above 420 nm.
   3. A filter that passes wavelengths between 450 nm and 490 nm.
   4. A filter that passes all wavelengths above 515 nm.
   5. A filter that passes wavelengths between 510 nm and 560 nm.
   6. A filter that passes all wavelengths above 590 nm.

B. How would you design your beam-splitting mirror? Which wavelengths would be reflected and which would be transmitted?

9–25 Antibodies that bind to specific proteins are important tools for defining the locations of molecules in cells. The sensitivity of the primary antibody—the antibody that reacts with the target molecule—is often enhanced by using labeled secondary antibodies that bind to it. What are the advantages and disadvantages of using secondary antibodies that carry fluorescent tags versus those that carry bound enzymes?

9–26 The green fluorescent protein (GFP) was isolated as a cDNA from a species of jellyfish that glows green. When the cDNA for GFP was introduced into bacteria, the colonies they formed glowed pale green under ultraviolet light. In these early studies, the following pertinent observations provided important insights into how GFP becomes fluorescent.

1. When bacteria are grown anaerobically, they express normal amounts of GFP but it is not fluorescent.
2. The denatured GFP found in insoluble protein aggregates (inclusion bodies) in bacteria is not fluorescent.
3. The rate of appearance of fluorescence follows first-order kinetics, with a time constant that is independent of the concentration of GFP.

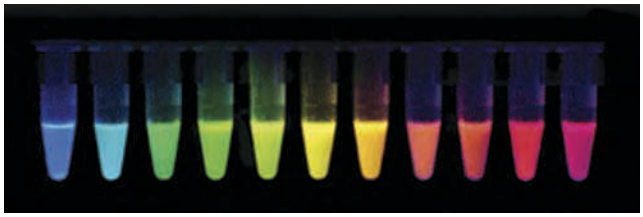


Figure 9–8 A rainbow of colors produced by modified GFPs (Problem 9–27).

4. Random mutations introduced into the cDNA coding for GFP produced some proteins with appreciably brighter fluorescence and some with different colors.

   Comment on what each of these observations says about GFP fluorescence.

9–27 **Figure 9–8** shows a series of modified GFPs that emit light in a range of colors. How do you suppose the exact same chromophore can fluoresce at so many different wavelengths?

CALCULATIONS

9–28 The resolving power of a light microscope depends on the width of the cone of light that illuminates the specimen, the wavelength of the light used, and the refractive index of the medium separating the specimen from the objective and condenser lenses, according to the formula

$$\text{resolution} = \frac{0.61\,\lambda}{n \sin\theta}$$

where λ equals the wavelength of light used, n is the refractive index, and θ is half the angular width of the cone of rays collected by the objective lens. Assuming an angular width of 120° ($\theta = 60°$), calculate the various resolutions you would expect if the sample were illuminated with violet light ($\lambda = 0.4$ μm) or red light ($\lambda = 0.7$ μm) in a refractive medium of air ($n = 1.00$) or oil ($n = 1.51$). Which of these conditions would give you the best resolution?

9–29 At a certain critical angle, which depends on the refractive indices of the two media, light from the optically denser medium will be bent sufficiently that it cannot escape and will be reflected back into the denser medium. The formula that describes refraction is

$$n_i \sin\theta_i = n_t \sin\theta_t$$

where n_i and n_t are the refractive indices of the incident and transmitting media, respectively, and θ_i and θ_t are the incident and transmitted angles, respectively, as shown in **Figure 9–9A**. For a glass–air interface, calculate the incident angle for a light ray that is bent so that it is parallel to the surface (**Figure 9–9B**). The refractive indices for air and glass are 1.00 and 1.51, respectively.

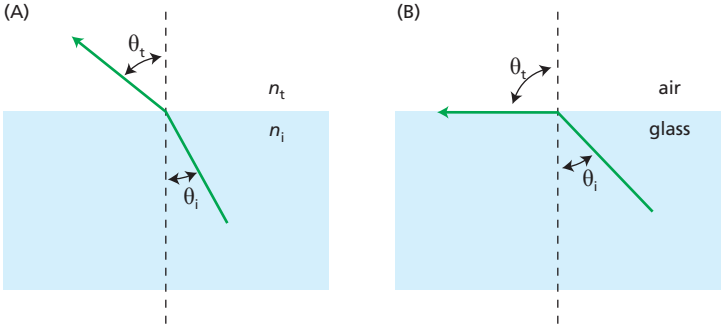


Figure 9–9 Refraction at the interface between media with different refractive indices (Problem 9–29). (A) The relationship between incident and transmitted angles for materials with different refractive indices such that $n_i > n_t$. (B) The incident angle for a glass–air interface such that the transmitted (bent) ray is parallel to the interface.

TABLE 9–1 Excitation properties of a few common biological fluorophores (Problem 9–30).

Fluorophore	Ope[a] absorption maximum (nm)	Tpe[b] absorption maximum (nm)	Emission maximum (nm)
DAPI	358	685	461
ER-Tracker	374	728	575
Mito-Tracker	579	1133	599
Alexa Fluor 488	491	985	515
FITC-IgG	490	947	525

[a]One-photon excitation; [b]Two-photon excitation.

DATA HANDLING

9–30 Fluorescent molecules can be excited by a single high-energy photon or by multiple lower-energy photons. A list of commonly used biological fluorophores is given in **Table 9–1**. Why do you suppose that the absorption maximum for two-photon excitation is about twice that for one-photon excitation?

9–31 You wish to attach fluorescent tags to two different proteins so that you can follow them independently. The excitation and emission spectra for cyan, green, and yellow fluorescent proteins (CFP, GFP, and YFP) are shown in **Figure 9–10**. Can you pick any pair of these proteins? Or are some pairs better than others? Explain your answer.

9–32 Consider a fluorescent detector designed to report the cellular location of active protein tyrosine kinases. A blue (cyan) fluorescent protein (CFP) and a yellow fluorescent protein (YFP) were fused to either end of a hybrid

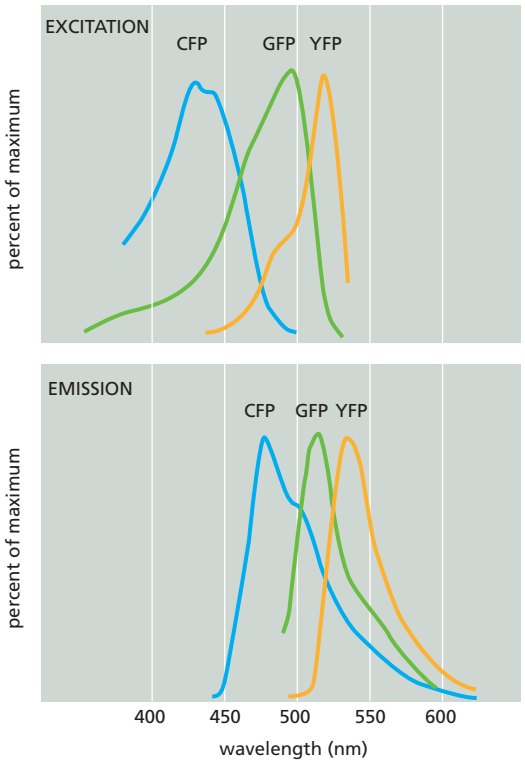


Figure 9–10 Excitation and emission spectra for CFP, GFP, and YFP (Problem 9–31).

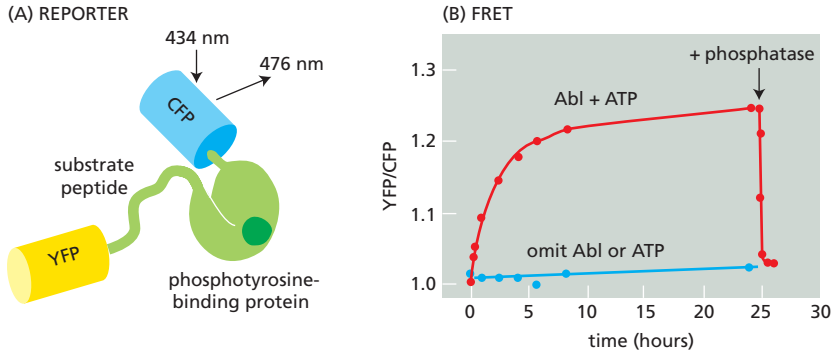


Figure 9–11 Fluorescent reporter protein designed to detect tyrosine phosphorylation (Problem 9–32). (A) Domain structure of reporter protein. Four domains are indicated: CFP, YFP, tyrosine kinase substrate peptide, and a phosphotyrosine-binding domain. (B) FRET assay. YFP/CFP is normalized to 1.0 at time zero. The reporter was incubated in the presence (or absence) of Abl and ATP for the indicated times. *Arrow* indicates time of addition of a tyrosine phosphatase.

protein domain. The hybrid protein segment consisted of a substrate peptide recognized by the Abl protein tyrosine kinase and a phosphotyrosine-binding domain (**Figure 9–11A**). Stimulation of the CFP domain does not cause emission by the YFP domain when the domains are separated. When the CFP and YFP domains are brought close together, fluorescence resonance energy transfer (FRET) allows excitation of CFP to stimulate emission by YFP. FRET shows up experimentally as an increase in the ratio of emission at 526 nm versus 476 nm (YFP/CFP) when CFP is excited by 434 nm light.

Incubation of the reporter protein with Abl protein tyrosine kinase in the presence of ATP gave an increase in YFP/CFP emission (**Figure 9–11B**). In the absence of ATP or the Abl protein, no FRET occurred. FRET was also eliminated by addition of a tyrosine phosphatase (Figure 9–11B). Describe as best you can how the reporter protein detects active Abl protein tyrosine kinase.

9–33 Cells activate Abl protein tyrosine kinase in response to platelet-derived growth factor (PDGF). PDGF binds to the PDGF receptor, which activates Src, which then activates Abl. It is unclear where in the cell Abl is active, but one of the consequences of PDGF stimulation is the appearance of membrane ruffles. To investigate this question, the reporter construct described in Problem 9–32 was transfected into cells, which were then stimulated by addition of PDGF. Using fluorescence microscopy, YFP emission in response to CFP excitation (FRET) was followed in different parts of the cell, with the results shown in **Figure 9–12**. What can you infer about the cellular distribution of active Abl protein tyrosine kinase in response to PDGF?

9–34 You are using a cameleon indicator to measure intracellular concentrations of Ca^{2+}. The indicator is composed of a central calmodulin domain, which converts from an extended form to a much more compact form upon calcium binding, and two flanking fluorescent proteins, with CFP

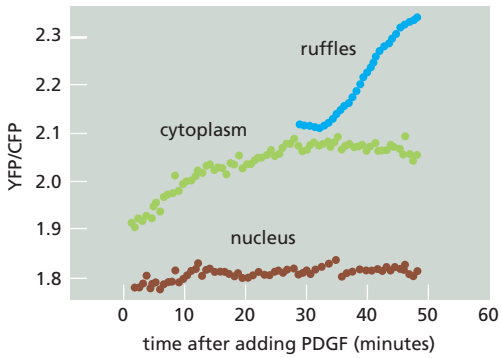


Figure 9–12 Time course of FRET in various parts of the cell after addition of PDGF (Problem 9–33). FRET was measured as the increase in the ratio of emission at 526 nm to that at 476 nm (YFP/CFP) when CFP was excited by 434 nm light.

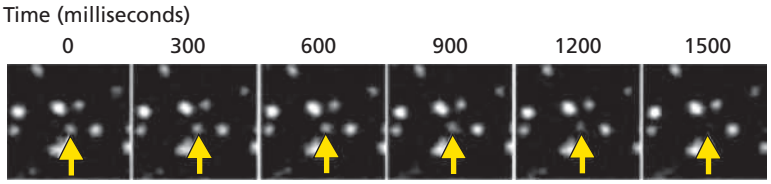


Figure 9–13 TIRF microscopy of cells expressing clathrin–GFP (Problem 9–35). Each frame in this time course represents 300 msec. The *yellow arrow* indicates a dot that disappears during the 1.5 sec observation period. Green dots appear *white* in this image.

attached at one end and YFP attached at the other. You have expressed this indicator in cells and now wish to measure intracellular changes in Ca^{2+} concentration in response to the biological process you are studying. The instructions say to excite the cameleon at 440 nm and to measure emission at 535 nm. How do you suppose the cameleon indicator works?

9–35 The formation of clathrin-coated vesicles is initiated by the assembly of a basketlike framework of clathrin on the underside of the cell membrane, which distorts the membrane into a shallow pit. These clathrin-coated pits ultimately pinch off to form clathrin-coated vesicles. You want to understand how pits are converted into vesicles, and have engineered a cell line that expresses clathrin–GFP. To focus on events at the membrane surface, you use total internal reflection fluorescence (TIRF) microscopy to examine the cell membrane and a thin slice of adjacent cytosol. You are excited to find that the samples show many green dots under the microscope and that some dots disappear over time (**Figure 9–13**).

A. Why do you suppose some dots remain unchanged, while others disappear?

B. You are concerned that the green dots may not be functional assemblies, but some kind of artifact. A colleague suggests that you test whether the green dots really represent clathrin-coated structures by incubating the cells with transferrin that is tagged with a red fluorophore. Transferrin, which carries iron ions, binds to its receptors on the cell surface and is taken into the cell in clathrin-coated vesicles. What might you expect to see if you carried out this experiment?

LOOKING AT CELLS AND MOLECULES IN THE ELECTRON MICROSCOPE

TERMS TO LEARN

cryoelectron microscopy
electron microscope (EM)
electron-microscope tomography
immunogold electron microscopy
negative staining
scanning electron microscope (SEM)
single-particle reconstruction

DEFINITIONS

Match each definition below with its term from the list above.

9–36 A contrast-enhancing technique for the electron microscope in which a heavy-metal salt is used to create a reverse, or negative, image of the object.

9–37 Type of microscope that uses a beam of electrons to create an image.

9–38 Electron microscopy technique in which the objects to be viewed, such as macromolecules and viruses, are rapidly frozen.

9–39 Type of electron microscope that produces an image of the surface of an object.

9–40 Electron microscopy technique in which cellular structures or molecules of interest are labeled with antibodies tagged with electron-dense gold particles, which show up as black spots on the image.

TRUE/FALSE

Decide whether this statement is true or false, and then explain why.

9–41 Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) can both be used to examine a structure in the interior of a thin section; TEM provides a projection view, while SEM captures electrons scattered from the structure and gives a more three-dimensional view.

THOUGHT PROBLEMS

9–42 A major challenge to electron microscopists from the beginning was to convince others that what they observed in micrographs truly reflected structures that were originally present in the living cell. Outline a current approach to this problem.

9–43 The technique of negative staining uses heavy metals such as uranium to provide contrast. If the heavy metals don't actually bind to defined biological structures (which they don't), how is it that they can help to make such structures visible?

9–44 Heavy metals can be used to highlight the surface features of a sample. In the technique called metal shadowing, a heavy metal such as platinum is evaporated at a shallow angle onto the specimen so as to deposit a thin film. The thickness of the film depends on the surface features of the sample and on their orientation relative to the source of platinum. By stabilizing the metal film and eliminating the original specimen, it is possible to image the metal replica of the sample by transmission electron microscopy, as shown in the micrographs in **Figure 9–14**.

It is sometimes difficult to tell bumps from pits in such micrographs just by looking at the pattern of shadows, as illustrated in Figure 9–14 for a set of shaded circles. In Figure 9–14A, the circles appear to be bumps; however, when the picture is simply turned upside down (Figure 9–14B), the circles seem to be pits. This is a classic illusion. The same illusion is present in metal shadowing, as shown in Figure 9–14C and D. In one micrograph, the membrane appears to be covered in bumps, while in the same micrograph, turned upside down, the membrane looks heavily pitted. Is it possible for an electron microscopist to be sure that one view is correct, or is it all arbitrary? Explain your reasoning.

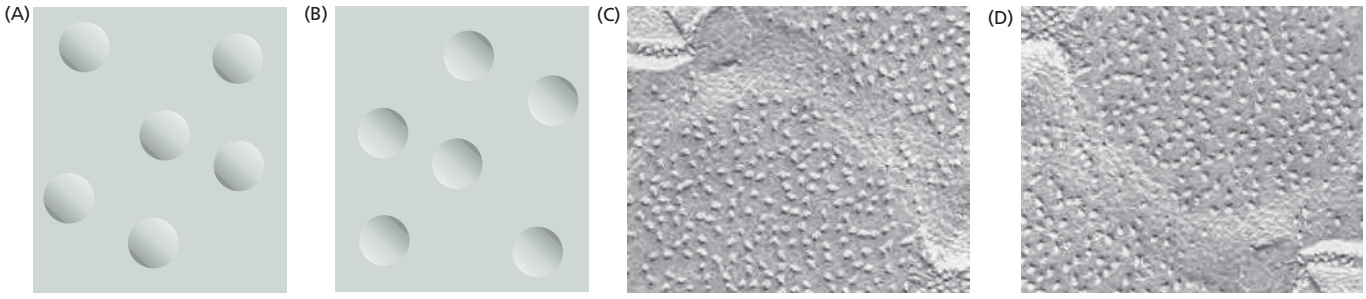


Figure 9–14 Bumps and pits (Problem 9–44). (A) Shaded circles that look like bumps. (B) Shaded circles that look like pits. (C) An electron micrograph oriented so that it appears to be covered with bumps. (D) The same electron micrograph oriented so that it appears to be covered with pits.

9–45 Nuclear pore complexes, which are large assemblages of more than 30 different proteins, mediate the exchange of macromolecules between the nucleus and cytoplasm. You have gently isolated nuclei from *Dictyostelium discoideum* and frozen them in vitreous ice for examination by cryoelectron tomography. You obtain a tomogram of a nucleus and combine the images from 267 nuclear pore complexes. You expect that individual nuclei will have been arrested in different states of transport because the isolated nuclei were shown to be competent for transport. Assuming that parts of the structure flex and move during the transport process, how do you suppose that averaging structures in different states will affect your final picture? Can you think of a way to improve the quality of the image you would get from this data set?

CALCULATIONS

9–46 The practical resolving power of modern electron microscopes is around 0.1 nm. The major reason for this constraint is the small numerical aperture ($n \sin \theta$), which is limited by θ (half the angular width of rays collected at the objective lens). Assuming that the wavelength (λ) of the electron is 0.004 nm and that the refractive index (n) is 1.0, calculate the value for θ. How does that value compare with a θ of 60°, which is typical for light microscopes?

$$\text{resolution} = \frac{0.61\,\lambda}{n \sin \theta}$$

DATA HANDLING

9–47 You are studying two proteins that you think may be components of gap junctions, which are structures in membranes that allow adjacent cells to exchange small molecules. When cells are very rapidly frozen and then cracked with a knife blade, the cells often fracture along membrane surfaces. When the freeze-fractured cells are viewed in the electron microscope, gap junctions show up clearly as specialized areas densely populated with membrane particles. To decide whether your proteins are components of gap junctions, you have prepared antibodies against each of them. To one antibody you've attached 15 nm gold particles; to the other, 10 nm gold particles. You prepare freeze-fractured cells for electron microscopy and then incubate them with your gold-tagged antibodies, with the results shown in **Figure 9–15**. Do these results indicate that both proteins are part of gap junctions? Why or why not?

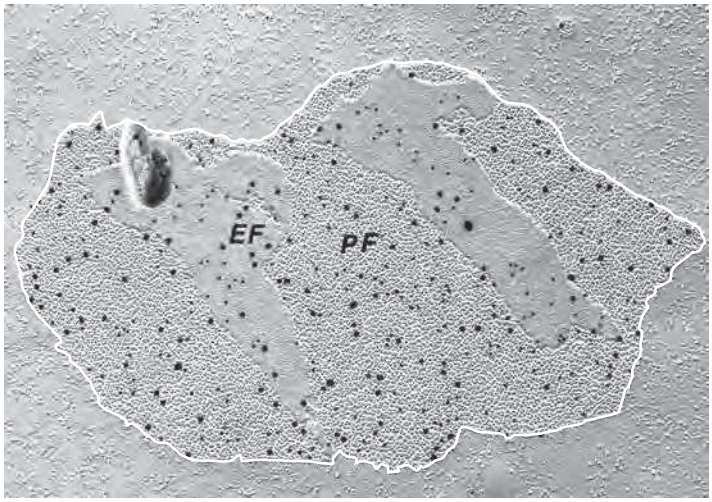


Figure 9–15 A freeze-fracture electron micrograph of a gap junction (Problem 9–47). The central densely packed area outlined in *white* is the gap junction. The two leaflets of the plasma membrane are indicated as EF (for external face) and PF (for protoplasmic face). Gold particles show up as *black dots*.

9–48 Aquaporin water channels, which are known to be located in the plasma membrane, play a major role in water metabolism and osmoregulation in many cells, including nerve cells of the brain and spinal cord. To determine their structural organization in the membrane, you have prepared a highly specific antibody against the protein AQP4, which forms the water channels in the astrocytes of the brain. You prepare a freeze-fractured sample from the brain, incubate it with gold-tagged antibodies against AQP4, and examine it by electron microscopy (**Figure 9–16**).

A. Are the gold particles (black dots) consistently associated with any particular structure?

B. Are there any examples of black dots that are not associated with these structures? Are there any examples of structures that do not have black dots? How do your answers to these questions affect your confidence that the structure you've identified is the aquaporin water channel?

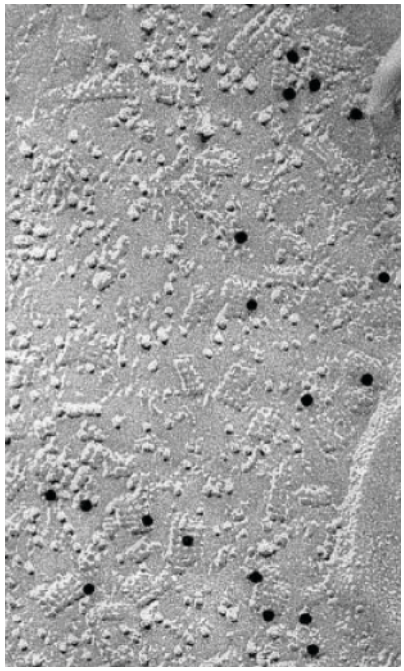


Figure 9–16 Freeze-fracture micrograph of an astrocyte membrane labeled with gold-tagged antibodies against AQP4 (Problem 9–48).

MCAT STYLE

Passage (Questions 9–49 to 9–51)

Many key advances in cell biology were dependent upon technical advances in microscopy. This was particularly true in the case of the cytoskeleton, the dynamic network of cytoplasmic fibers that is responsible for cell motility and chromosome movement during mitosis. The two key components of the cytoskeleton—actin filaments and microtubules—assemble by polymerization of individual subunits. Actin filaments and microtubules were first seen by electron microscopy, which showed that they have diameters of 7 nm and 25 nm, respectively, and can form very long polymers. Later development of light microscopy techniques for imaging individual microtubules and actin filaments under natural conditions revolutionized the field.

9–49 Imagine that you are developing techniques to visualize individual actin filaments by light microscopy for the first time. Which of the following approaches would you choose?

I. Assemble actin filaments from fluorescently tagged subunits and image them with a fluorescence microscope.

II. Visualize actin filaments by using differential-interference-contrast microscopy.

III. Combine standard light microscopic techniques with digital image processing to enhance resolution of actin filaments.

A. I
B. II
C. III
D. I, II, and III

9–50 What would be the apparent diameter of actin filaments imaged using the approach or approaches you selected in the previous question?

A. 7 nm
B. 50 nm
C. 100 nm
D. 200 nm

9–51 Microscopy has also played a key role in answering mechanistic questions about how enzymes work. Consider, for example, ATP synthase, the remarkable multisubunit enzyme responsible for ATP synthesis in the inner mitochondrial membrane. The structure of ATP synthase revealed a central stalk surrounded by other subunits that form a ring 10 nm in diameter. It was hypothesized that rotation of the stalk relative to the ring subunits—powered by proton flow across the inner membrane—generated sufficient mechanical energy to force the conformational changes needed to drive ATP synthesis. You would like to test this hypothesis by

directly visualizing rotation of the stalk relative to the ring. Which one of the following ideas do you think would be the best approach for detecting rotation of the stalk?

A. Attach a 2 μm-long actin filament assembled from fluorescent subunits to the stalk and view its rotation by fluorescence microscopy.
B. Attach green fluorescent protein (GFP) to the proteins in the ring and use fluorescence microscopy to observe rotation of the ring around the stalk.
C. Attach GFP to the stalk and RFP (red fluorescent protein) to the ring and observe their relative rotation by fluorescence microscopy.
D. Attach multiple fluorescently labeled antibodies to the stalk and then observe rotation of the stalk by fluorescence microscopy.

CHAPTER 10

Membrane Structure

IN THIS CHAPTER

THE LIPID BILAYER

TERMS TO LEARN

amphiphilic
cholesterol
ganglioside
glycolipid
hydrophilic
hydrophobic
lipid bilayer
lipid droplet
lipid raft
liposome
phosphoglyceride
phospholipid
plasma membrane

DEFINITIONS

Match the definition below with its term from the list above.

10–1 Artificial phospholipid bilayer vesicle formed from an aqueous suspension of phospholipid molecules.

10–2 Small region of the plasma membrane enriched in sphingolipids and cholesterol.

10–3 Any glycolipid having one or more sialic acid residues in its structure; especially abundant in the plasma membranes of nerve cells.

10–4 Having both hydrophobic and hydrophilic regions, as in a phospholipid or a detergent molecule.

10–5 The main type of phospholipid in animal cell membranes, with two fatty acids and a polar head group attached to a three-carbon glycerol backbone.

10–6 Lipid molecule with a characteristic four-ring steroid structure that is an important component of the plasma membranes of animal cells.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

10–7 Although lipid molecules are free to diffuse in the plane of the bilayer, they cannot flip-flop across the bilayer unless enzyme catalysts called phospholipid translocators are present in the membrane.

10–8 All of the common phospholipids—phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin—carry a positively charged moiety on their head group, but none carry a net positive charge.

10–9 Glycolipids are never found on the cytoplasmic face of membranes in living cells.

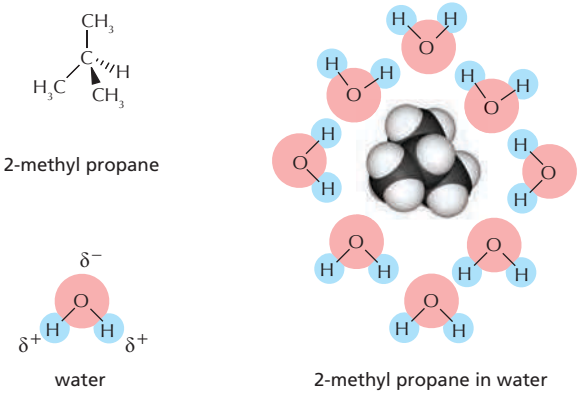


Figure 10–1 Icelike cage of water molecules around a hydrophobic solute (Problem 10–10).

THOUGHT PROBLEMS

10–10 Hydrophobic solutes are said to "force the adjacent water molecules to reorganize into icelike cages" (**Figure 10–1**). This statement seems paradoxical because water molecules do not interact with hydrophobic solutes. How could water molecules "know" about the presence of a hydrophobic solute and change their behavior to interact differently with one another? Discuss this seeming paradox and develop a clear concept of what is meant by an "icelike" cage. How does it compare to ice? Why would such a cagelike structure be energetically unfavorable relative to pure water?

10–11 When a lipid bilayer is torn, why does it not seal itself by forming a "hemi-micelle" cap at the edges, as shown in **Figure 10–2**?

10–12 Five students in your class always sit together in the front row. This could be because (1) they really like each other or (2) nobody else in your class wants to sit next to them. Which explanation holds for the assembly of a lipid bilayer? Explain your answer. If the lipid bilayer assembled for the opposite reason, how would its properties differ?

10–13 Predict the properties of a lipid bilayer in which all of the hydrocarbon chains were saturated. What would be the properties if all of the hydrocarbon chains were unsaturated?

10–14 What is meant by the term "two-dimensional fluid"?

10–15 Margarine is made from vegetable oil by a chemical process. Do you suppose this process converts saturated fatty acids to unsaturated ones, or vice versa? Explain your answer.

10–16 Which one of the phospholipids listed below is present in very small quantities in the plasma membranes of mammalian cells?

A. Phosphatidylcholine
B. Phosphatidylethanolamine
C. Phosphatidylinositol
D. Phosphatidylserine
E. Sphingomyelin

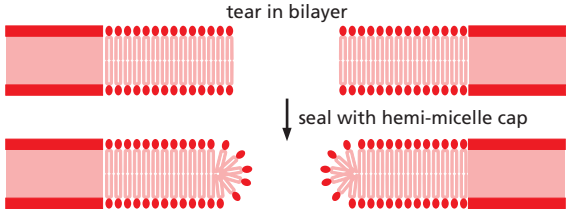


Figure 10–2 A torn lipid bilayer sealed with a hypothetical "hemi-micelle" cap (Problem 10–11).

10–17 Many snake venoms contain enzymes that cause red blood cells to lyse. Imagine that you have purified such an enzyme. When you add the purified enzyme to red blood cells, you find that in addition to cell lysis, choline with a phosphate group attached to it is released, as well as diacylglycerol (glycerol with two fatty acid chains attached). What molecule is cleaved by the enzyme to cause cell lysis?

10–18 Predict which of the following organisms will have the highest percentage of unsaturated fatty acid chains in their membranes. Explain your answer.

A. Antarctic fish
B. Desert iguana
C. Human being
D. Polar bear
E. Thermophilic bacterium

10–19 If lipid rafts form because membrane components such as sphingolipids and cholesterol molecules preferentially associate with one another, why do you think it is that they aggregate into multiple tiny rafts instead of into a single large one?

10–20 The lipid bilayers found in cells are fluid, yet asymmetrical in the composition of the monolayers. Is this a paradox? Explain your answer.

10–21 Phosphatidylserine, which is normally confined to the cytoplasmic monolayer of the plasma membrane lipid bilayer, is redistributed to the outer monolayer during apoptosis. How is this redistribution accomplished?

CALCULATIONS

10–22 If a lipid raft is typically 70 nm in diameter and each lipid molecule has a diameter of 0.5 nm, about how many lipid molecules would there be in a lipid raft composed entirely of lipid? At a ratio of 50 lipid molecules per protein molecule (50% protein by mass), how many proteins would be in a typical raft? (Neglect the loss of lipid from the raft that would be required to accommodate the protein.)

DATA HANDLING

10–23 A classic paper studied the behavior of lipids in the two monolayers of a membrane by labeling individual molecules with nitroxide groups, which are stable free radicals (**Figure 10–3**). These spin-labeled lipids can be detected by electron spin resonance (ESR) spectroscopy, a technique that does not harm living cells. Spin-labeled lipids are introduced into small lipid vesicles, which are then fused with cells, thereby transferring the labeled lipids into the plasma membrane.

The two spin-labeled phospholipids shown in Figure 10–3 were incorporated into intact human red blood cell membranes in this way. To determine whether they were introduced equally into the two monolayers of the bilayer, ascorbic acid (vitamin C), which is a water-soluble reducing agent that does not cross membranes, was added to the medium to destroy any nitroxide radicals exposed on the outside of the cell. The ESR signal was followed as a function of time in the presence and absence of ascorbic acid as indicated in **Figure 10–4A and B**.

A. Ignoring for the moment the difference in extent of loss of ESR signal, offer an explanation for why phospholipid 1 (Figure 10–4A) reacts faster with ascorbate than does phospholipid 2 (Figure 10–4B). Note that phospholipid 1 reaches a plateau in about 15 minutes, whereas phospholipid 2 takes almost an hour.

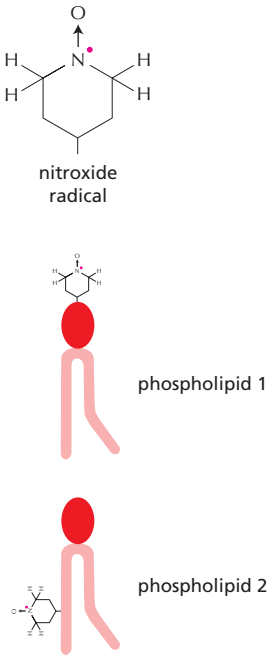


Figure 10–3 Structures of two nitroxide-labeled lipids (Problem 10–23). The nitroxide radical is shown at the *top*, and its position of attachment to the phospholipids is shown *below*.

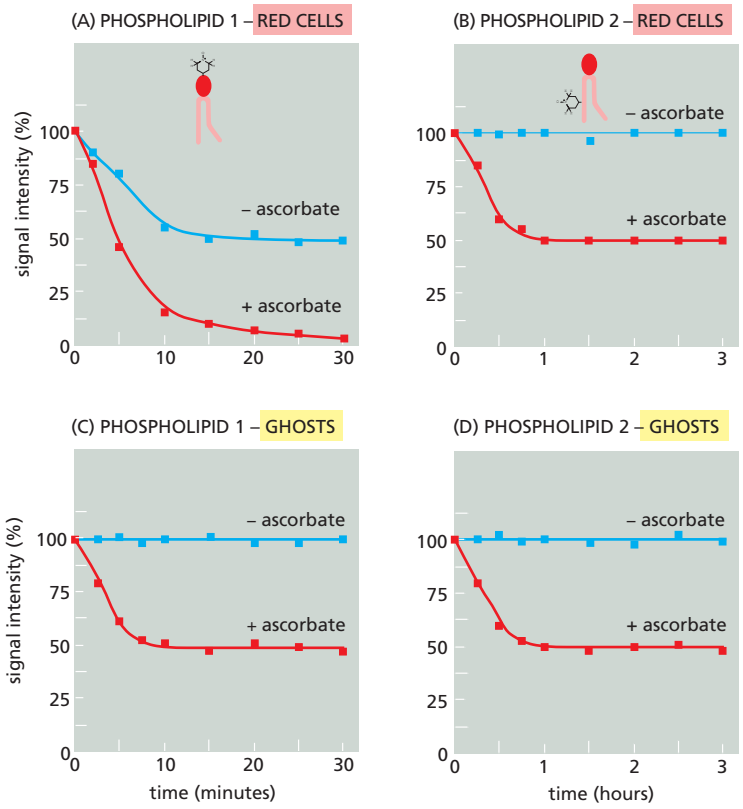


Figure 10–4 Decrease in ESR signal intensity as a function of time in red cells and red cell ghosts in the presence and absence of ascorbate (Problem 10–23). (A and B) Phospholipid 1 and phospholipid 2 in red cells. (C and D) Phospholipid 1 and phospholipid 2 in red cell ghosts.

B. To investigate the difference in extent of loss of ESR signal with the two phospholipids, the experiments were repeated using red cell ghosts that had been resealed to make them impermeable to ascorbate (**Figure 10–4C and D**). Resealed red cell ghosts are missing all of their cytoplasm, but have an intact plasma membrane. In these experiments, the loss of ESR signal for both phospholipids was negligible in the absence of ascorbate and reached a plateau at 50% in the presence of ascorbate. What do you suppose might account for the difference in extent of loss of ESR signal in experiments with red cell ghosts (Figure 10–4C and D) versus those with normal red cells (Figure 10–4A and B).

C. Were the spin-labeled phospholipids introduced equally into the two monolayers of the red cell membrane?

10–24 Fluorescence resonance energy transfer (FRET) has been used to investigate the existence of lipid rafts in living cells. To test for the presence of lipid rafts by FRET, you use two different cell lines: one that expresses a glycosylphosphatidylinositol (GPI)-anchored form of the folate receptor, and one that expresses the transmembrane-anchored form. Folate receptors can be made fluorescent by addition of a fluorescent folate analog. Cells tagged in this way show variation in fluorescence intensity over their surface because of chance variations in the density of labeled receptors. This allows different densities of receptors to be analyzed by examining different places in the same cell. The proximity of labeled receptors can be determined by FRET, which depends on the distance between receptors. The ratio of FRET to direct fluorescence gives different expectations for dispersed receptors versus receptors that are clustered together, as depicted in **Figure 10–5**.

A. Explain the basis for the difference between the graphs of these expectations.

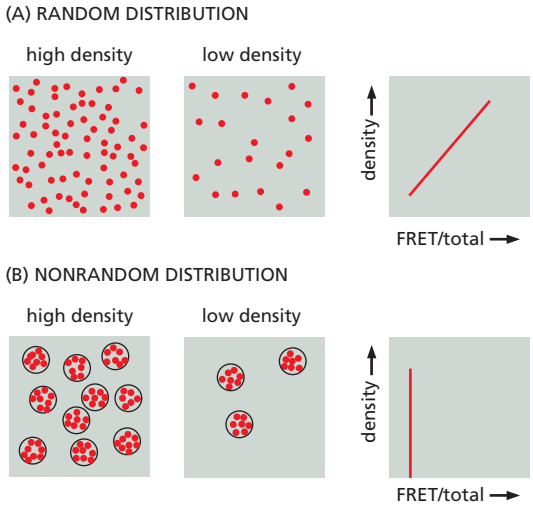


Figure 10–5 Expectations for FRET between tagged folate receptors at different densities of receptors (Problem 10–24). (A) Randomly distributed receptors. The *red dots* in the box represent fluorescent receptors. (B) Receptors clustered in microdomains. *Circles* represent microdomains such as lipid rafts. *Red dots* represent fluorescent receptors.

B. The actual experiments showed that transmembrane-anchored folate receptors followed the expectations shown in Figure 10–5A, whereas the GPI-anchored folate receptors followed those in Figure 10–5B. Do these experiments provide evidence for the existence of lipid rafts in the plasma membrane? Why or why not?

10–25 The asymmetric distribution of phospholipids in the two monolayers of the plasma membrane implies that very little spontaneous flip-flop occurs or, alternatively, that any spontaneous flip-flop is rapidly corrected by phospholipid translocators that return phospholipids to their appropriate monolayer. The rate of phospholipid flip-flop in the plasma membrane of intact red blood cells has been measured to decide between these alternatives.

One experimental measurement used the same two spin-labeled phospholipids described in Problem 10–23 (see Figure 10–3). To measure the rate of flip-flop from the cytoplasmic monolayer to the outer monolayer, red cells with spin-labeled phospholipids exclusively in the cytoplasmic monolayer were incubated for various times in the presence of ascorbate and the loss of ESR signal was followed. To measure the rate of flip-flop from the outer to the cytoplasmic monolayer, red cells with spin-labeled phospholipids exclusively in the outer monolayer were incubated for various times in the absence of ascorbate and the loss of ESR signal was followed. The results of these experiments are illustrated in **Figure 10–6**.

A. From the results in Figure 10–6, estimate the rate of flip-flop from the cytoplasmic to the outer monolayer, and from the outer to the cytoplasmic monolayer. A convenient way to express such rates is as the half-time of flip-flop—that is, the time it takes for half the phospholipids to flip-flop from one monolayer to the other.

B. From what you learned about the behavior of the two spin-labeled phospholipids in Problem 10–23, deduce which one was used to label the cytoplasmic monolayer of the intact red blood cells, and which one was used to label the outer monolayer.

C. Using the information in this problem, propose a method to generate intact red cells that contain spin-labeled phospholipids exclusively in the cytoplasmic monolayer, and a method to generate cells spin-labeled exclusively in the outer monolayer.

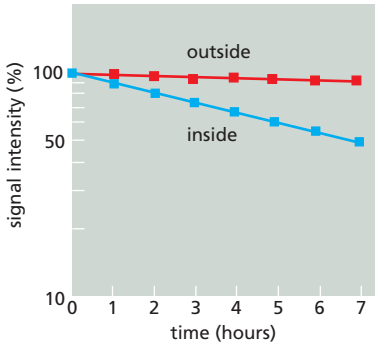


Figure 10–6 Decrease in ESR signal intensity of red blood cells containing spin-labeled phospholipids in the outer monolayer (outside) and cytoplasmic monolayer (inside) of the plasma membrane (Problem 10–25).

MEMBRANE PROTEINS

TERMS TO LEARN

bacteriorhodopsin
carbohydrate layer
cortex
detergent
glycosylphosphatidylinositol (GPI) anchor
lectin
lumen
membrane-associated protein
membrane-bending protein
membrane protein
multipass transmembrane protein
single-pass transmembrane protein
spectrin
transmembrane protein

DEFINITIONS

Match the definition below with its term from the list above.

10–26 Protein that binds tightly to a specific sugar.

10–27 The outer coat of a eukaryotic cell, composed of oligosaccharides linked to intrinsic plasma membrane glycoproteins and glycolipids, as well as proteins that have been secreted and reabsorbed onto the cell surface.

10–28 Abundant protein associated with the cytosolic side of the plasma membrane in red blood cells, forming a rigid network that supports the membrane.

10–29 Protein whose polypeptide chain crosses the lipid bilayer more than once.

10–30 Pigmented protein found in the plasma membrane of *Halobacterium halobium*, where it pumps protons out of the cell in response to light.

10–31 The complicated cytoskeletal network in the cytosol just beneath the plasma membrane.

10–32 Type of lipid linkage, formed as proteins pass through the endoplasmic reticulum, by which some proteins are attached to the noncytosolic surface of the membrane.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

10–33 Whereas all the carbohydrate in the plasma membrane faces outward on the external surface of the cell, all the carbohydrate on internal membranes faces toward the cytosol.

10–34 Human red blood cells contain no internal membranes other than the nuclear membrane.

10–35 Although membrane domains with different protein compositions are well known, there are at present no examples of membrane domains that differ in lipid composition.

THOUGHT PROBLEMS

10–36 Which of the arrangements of membrane-associated proteins indicated in **Figure 10–7** have been found in biological membranes?

10–37 Which one of the following statements correctly describes the mass ratio of lipids to proteins in membranes?

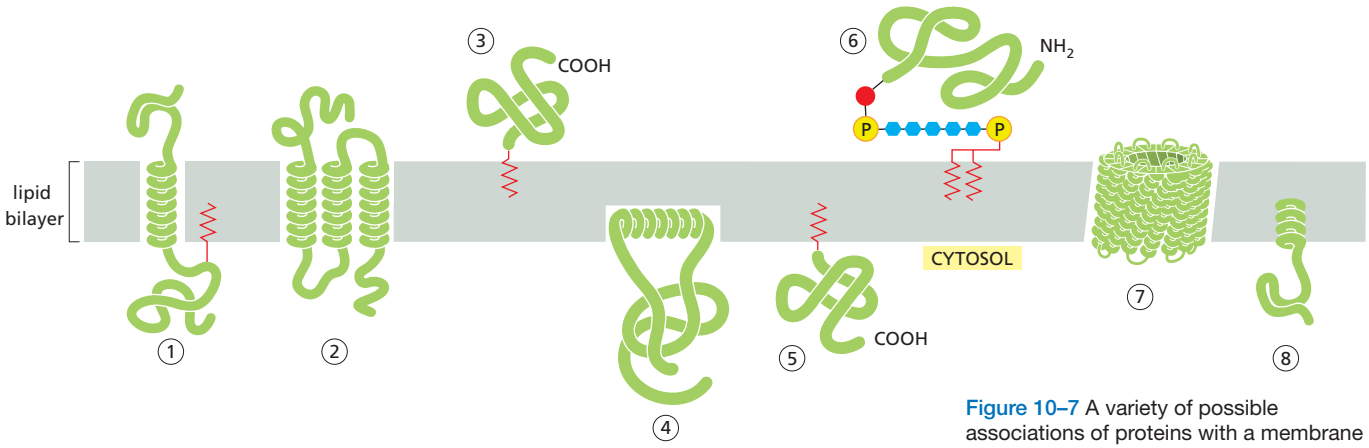


Figure 10–7 A variety of possible associations of proteins with a membrane (Problem 10–36).

A. The mass of lipids greatly exceeds the mass of proteins.
B. The mass of proteins greatly exceeds the mass of lipids.
C. The masses of lipids and proteins are about equal.
D. The mass ratio of lipids to proteins varies widely in different membranes.

10–38 Name the three general types of lipid anchors that are used to attach proteins to membranes.

10–39 Monomeric single-pass transmembrane proteins span a membrane with a single α helix that has characteristic chemical properties in the region of the bilayer. Which of the three 20-amino-acid sequences listed below is the most likely candidate for such a transmembrane segment? Explain the reasons for your choice. (See Table 8, page 966, for one-letter amino acid code; FAMILY VW is a convenient mnemonic for hydrophobic amino acids.)

A. I T L I Y F G V M A G V I G T I L L I S
B. I T P I Y F G P M A G V I G T P L L I S
C. I T E I Y F G R M A G V I G T D L L I S

10–40 Consider a transmembrane protein complex that forms a hydrophilic pore across the plasma membrane of a eukaryotic cell. The pore is made of five similar protein subunits, each of which contributes a membrane-spanning α helix to form the pore. Each α helix has hydrophilic amino acid side chains on one side of the helix and hydrophobic amino acid side chains on the opposite side. Propose a possible arrangement of these five α helices in the membrane.

10–41 Why is it that membrane-spanning protein segments are almost always α helices or β barrels, but never disordered chains?

10–42 You are studying the binding of proteins to the cytoplasmic face of cultured neuroblastoma cells and have found a method that gives a good yield of inside-out vesicles from the plasma membrane. Unfortunately, your preparations are contaminated with variable amounts of right-side-out vesicles. Nothing you have tried avoids this problem. A friend suggests that you pass your vesicles over an affinity column made of lectin coupled to solid beads. What is the point of your friend's suggestion?

10–43 Detergents are small amphiphilic molecules—one end hydrophobic and the other hydrophilic—that tend to form micelles in water. Examine the structures of sodium dodecyl sulfate (SDS) and Triton X-100 in **Figure 10–8** and explain why the black portions are hydrophilic and the blue sections are hydrophobic.

10–44 Why does a red blood cell membrane need proteins?

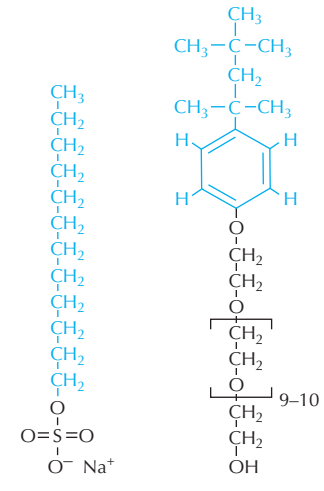


Figure 10–8 The structures of SDS and Triton X-100 (Problem 10–43).

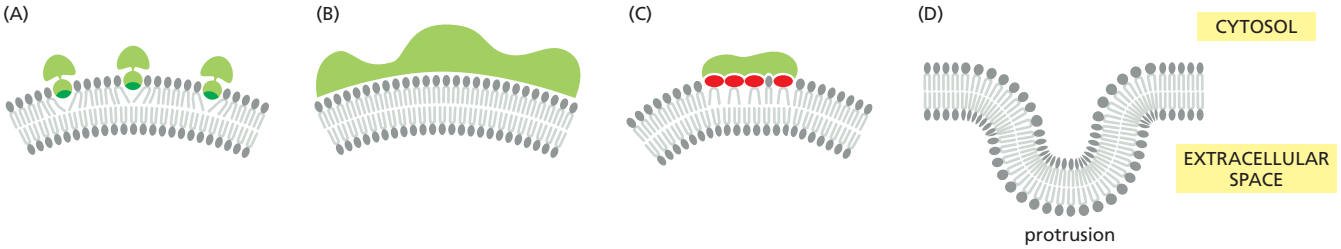


Figure 10–9 Bending of the plasma membrane by cytosolic proteins (Problem 10–47). (A) Insertion of a protein "finger" into the cytosolic leaflet of the membrane. (B) Binding of lipids to the curved surface of a membrane-binding protein. (C) Binding of membrane proteins to membrane lipids with large head groups. (D) A segment of the plasma membrane showing a protrusion.

10–45 Glycophorin, a protein in the plasma membrane of the red blood cell, normally exists as a homodimer that is held together entirely by interactions between its transmembrane domains. Since transmembrane domains are hydrophobic, how is it that they can associate with one another so specifically?

10–46 Describe the different methods that cells use to restrict proteins to specific regions of the plasma membrane. Is a membrane with many anchored proteins still fluid?

10–47 Three mechanisms by which membrane-binding proteins bend a membrane are illustrated in **Figure 10–9A–C**. As shown, each of these cytosolic membrane-bending proteins would induce an invagination of the plasma membrane. Could similar kinds of cytosolic proteins induce a protrusion of the plasma membrane (**Figure 10–9D**)? Which ones? Explain how they might work.

CALCULATIONS

10–48 In the membrane of a human red blood cell, the ratio of the mass of protein (average molecular weight 50,000) to phospholipid (average molecular weight 800) to cholesterol (molecular weight 386) is about 2:1:1. How many lipid molecules (phospholipid + cholesterol) are there for every protein molecule?

10–49 Estimates of the number of membrane-associated proteins per cell and the fraction of the plasma membrane occupied by such proteins provide a useful quantitative basis for understanding the structure of the plasma membrane. These calculations are straightforward for proteins in the plasma membrane of a red blood cell because red cells are readily isolated from blood and they contain no internal membranes to confuse the issue. Plasma membranes are prepared, the membrane-associated proteins are separated by SDS polyacrylamide-gel electrophoresis, and then they are stained with a dye (Coomassie Blue). Because the intensity of color is roughly proportional to the mass of protein present in a band, quantitative estimates can be made as shown in **Table 10–1**.

A. From the information in Table 10–1, calculate the number of molecules of spectrin, anion exchanger (AE1), and glycophorin in an individual red blood cell. Assume that 1 mL of red cell ghosts contains 10^{10} cells and 5 mg of total membrane protein.

TABLE 10–1 Proportion of stain associated with three membrane-associated proteins (Problem 10–49).

Protein	Molecular weight	Percent of stain
Spectrin	250,000	25
AE1	100,000	30
Glycophorin	30,000	2.3

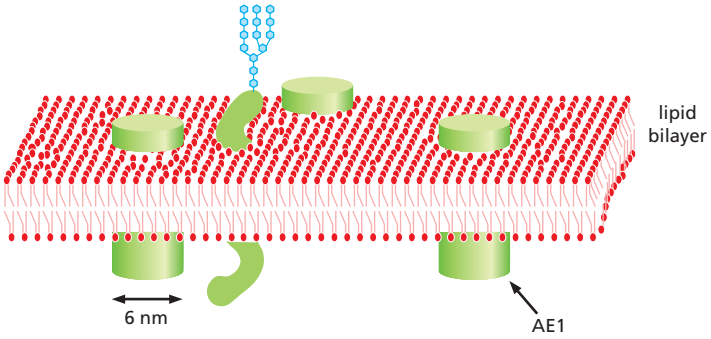


Figure 10–10 Schematic diagram of AE1, represented as a cylinder, in the plasma membrane (Problem 10–49). AE1 was originally identified as a prominent band ("band 3") after the membrane proteins from red cell ghosts were separated by SDS polyacrylamide-gel electrophoresis; it is often referred to as band 3 in the literature.

B. Calculate the fraction of the plasma membrane that is occupied by AE1. Assume that AE1 is a cylinder 3 nm in radius and 10 nm in height and is oriented in the membrane as shown in **Figure 10–10**. The total surface area of a red cell is 10^8 nm^2.

DATA HANDLING

10–50 Look carefully at the transmembrane anion exchanger (AE1) in **Figure 10–11A**. Imagine that you could mark all the AE1 proteins specifically with a fluorescent group and measure their mobility by fluorescence recovery after photobleaching (FRAP). You photobleach a small spot on the membrane and then measure the increase in fluorescence in that spot as fluorescent AE1 molecules diffuse into it from neighboring regions of the membrane. Sketch the recovery curve you would expect to see with time (**Figure 10–11B**).

MEDICAL LINKS

10–51 Pythagoras forbade his followers to eat fava beans. Beyond the political implications (Greeks voted with beans), there turns out to be a rational basis for this proscription. In the Middle Eastern population, defective forms of the gene encoding glucose 6-phosphate dehydrogenase (G6PD) are common. These mutant forms of the gene typically reduce G6PD activity to about 10% of normal. They have been selected for in the Middle East, and in other areas of the world where malaria is common, because they afford protection against the malarial parasite. G6PD controls the first step in the pathway for NADPH production. A lower-than-normal level of NADPH in red blood cells creates an environment unfavorable for growth of the protist *Plasmodium falciparum*, which causes malaria.

Although somewhat protected against malaria, G6PD-deficient individuals occasionally have other problems. NADPH is the principal agent required to keep the red cell cytosol in a properly reduced state, constantly converting transient disulfide bonds (–S–S–) back to sulfhydryls (–SH HS–). When a G6PD-deficient individual eats raw or undercooked fava beans, an oxidizing substance in the beans overwhelms the reducing capacity of the red cells, leading to a severe—sometimes life-threatening—hemolytic anemia. How do you suppose eating fava beans leads to anemia?

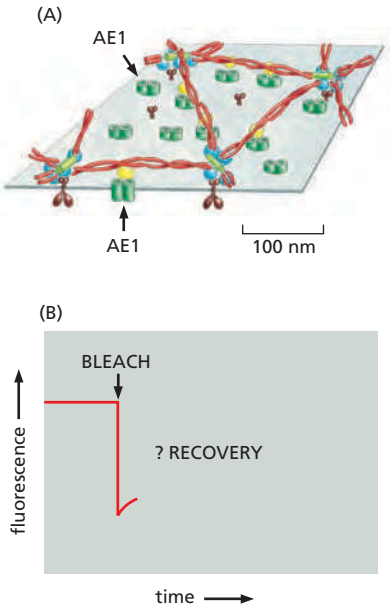


Figure 10–11 Mobility of membrane proteins (Problem 10–50). (A) Model of red blood cell membrane. (B) Recovery of fluorescence after photobleaching a red cell plasma membrane containing AE1 protein tagged with a fluorescent group.

MCAT STYLE

Passage 1 (Questions 10–52 to 10–56)

Cholesterol is transported in the bloodstream in lipoprotein particles. There are several classes of lipoprotein particles, but one of the most important classes is called low-density lipoprotein (LDL) particles. LDL particles are composed of a single molecule of a large protein called apolipoprotein, as well as thousands of

cholesterol molecules and phospholipids. Part of the apolipoprotein forms the surface of the particle and faces the aqueous phase, while other parts face the hydrophobic interior of the particle where they interact with cholesterol and the hydrophobic tails of the phospholipids. When cells need additional cholesterol, they produce a receptor protein on the surface of their plasma membrane that binds to apolipoprotein and initiates uptake of LDL particles into the cell via a process called endocytosis.

10–52 What is the most likely reason that LDL particles are used to transport cholesterol in the bloodstream?

A. Cholesterol in LDL particles easily inserts into lipid bilayers.
B. Cholesterol is largely insoluble in aqueous solutions.
C. Cholesterol is synthesized within LDL particles.
D. LDL particles can form lipid rafts in the lipid bilayer.

10–53 What best describes the class of protein that apolipoprotein belongs to?

A. Amphiphilic proteins
B. Cell-surface receptors
C. Membrane-associated proteins
D. Multipass transmembrane proteins

10–54 What is the most likely location of phospholipids in LDL particles?

A. Bound to hydrophilic domains of apolipoprotein
B. In the lipid bilayer that surrounds LDL particles
C. On the surface, interacting with the aqueous environment
D. Within the hydrophobic core of the particle

10–55 What kind of molecule on the surface of a cell would be most likely to serve as a receptor for LDL particles?

A. A cholesterol-binding protein
B. A glycolipid
C. A phospholipid
D. A transmembrane protein

10–56 What conditions would be likely to trigger increased uptake of LDL particles by cells?

A. Increased glycolipid synthesis
B. Increased plasma membrane growth
C. Increased protein secretion
D. Increased protein synthesis

Membrane Transport of Small Molecules and the Electrical Properties of Membranes

CHAPTER 11

IN THIS CHAPTER

PRINCIPLES OF MEMBRANE TRANSPORT

TERMS TO LEARN

active transport
channel
electrochemical gradient
membrane transport protein
passive transport
transporter

DEFINITIONS

Match each definition below with its term from the list above.

11–1 An aqueous pore in a lipid membrane, with walls made of protein, through which selected ions or molecules can pass.

11–2 The movement of a small molecule or ion across a membrane due to a difference in concentration or electrical charge.

11–3 General term for a membrane-embedded protein that serves as a carrier of ions or small molecules from one side of the membrane to the other.

11–4 Movement of a molecule across a membrane that is driven by ATP hydrolysis or other form of metabolic energy.

11–5 Driving force for ion movement that is due to differences in ion concentration and electrical charge on either side of the membrane.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

11–6 The plasma membrane is highly impermeable to all charged molecules.

11–7 Transport by transporters can be either active or passive, whereas transport by channels is always passive.

THOUGHT PROBLEMS

11–8 Order the molecules on the following list according to their ability to diffuse through a lipid bilayer, beginning with the one that crosses the bilayer most readily. Explain your order.

1. Ca^{2+}
2. CO_2
3. Ethanol
4. Glucose
5. RNA
6. H_2O

11–9 Why are the maximum rates of transport by transporters and channels thought to be so different?

11–10 A simple enzyme reaction can be represented by the equation

$$E + S \rightleftharpoons ES \rightarrow E + P$$

where E is the enzyme, S is the substrate, P is the product, and ES is the enzyme–substrate complex.

A. Write a corresponding equation describing a transporter (T) that mediates transport of a solute (S) down its concentration gradient.

B. The Michaelis–Menten equation for the simple enzyme reaction above is

$$\text{rate} = V_{max} \frac{[S]}{[S] + K_m}$$

where "rate" is the initial rate of the reaction, V_{max} is the maximum rate of the enzyme-catalyzed reaction, [S] is the concentration of substrate, and K_m is the Michaelis constant. Write the corresponding Michaelis–Menten equation for the process of solute transport by a transporter. What do rate, V_{max}, and K_m mean in the equation for transport?

C. Would these equations provide an appropriate description for channels? Why or why not?

11–11 Suppose a membrane contains a single passive transporter with a K_m of 0.1 mM for its solute. How effective would the transporter be at equalizing the concentrations of solute across the membrane if the starting concentrations were 0.01 mM inside and 0.05 mM outside? What if the concentrations were 100 mM inside and 500 mM outside?

11–12 How is it possible for some molecules to be at equilibrium across a biological membrane and yet not be at the same concentration on both sides?

CALCULATIONS

11–13 Brain cells, which depend on glucose for energy, use the glucose transporter GLUT3, which has a K_m of 1.5 mM. Liver cells, which store glucose (as glycogen) after a meal and release glucose between meals, use the glucose transporter GLUT2, which has a K_m of 15 mM.

A. Calculate the rate (as a percentage of V_{max}) of glucose uptake in brain cells and in liver cells at circulating glucose concentrations of 3 mM (starvation conditions), 5 mM (normal levels), and 7 mM (after a carbohydrate-rich meal). Rearranging the Michaelis–Menten equation gives

$$\frac{\text{rate}}{V_{max}} = \frac{[S]}{[S] + K_m}$$

B. Although the concentration of glucose in the general circulation normally doesn't rise much above 7 mM, the liver is exposed to much higher concentrations after a meal. The intestine delivers glucose into the portal circulation, which goes directly to the liver. In the portal circulation, the concentration of glucose can be as high as 15 mM. At what fraction of the maximum rate (V_{max}) do liver cells import glucose at this concentration?

C. Do these calculations fit with the physiological functions of brain and liver cells? Why or why not?

11–14 Cells use transporters to move nearly all metabolites across membranes. But how much faster is a transporter than simple diffusion? There is sufficient information available for glucose transporters to make a comparison. The normal circulating concentration of glucose in humans is 5 mM, whereas the intracellular concentration is usually very low. (For this problem assume the internal concentration of glucose is 0 mM.)

A. At what rate (molecules/sec) would glucose diffuse into a cell if there were no transporter? The permeability coefficient for glucose is

3×10^{-8} cm/sec. Assume a cell is a sphere with a diameter of 20 μm. The rate of diffusion equals the concentration difference multiplied by the permeability coefficient and the total surface area of the cell (surface area = $4\pi r^2$). (Remember to convert everything to compatible units so that the rate is molecules/sec.)

B. If in the same cell there are 10^5 GLUT3 molecules (K_m = 1.5 mM) in the plasma membrane, each of which can transport glucose at a maximum rate of 10^4 molecules per second, at what rate (molecules/sec) will glucose enter the cell? How much faster is transporter-mediated uptake of glucose than entry by simple diffusion?

DATA HANDLING

11–15 Cytochalasin B, which is often used as an inhibitor of actin-based motility systems, is also a very potent competitive inhibitor of D-glucose uptake into mammalian cells. When red blood cell ghosts (red cells, emptied of their cytoplasm) are incubated with ^{3}H-cytochalasin B and then irradiated with ultraviolet light, the cytochalasin becomes cross-linked to the glucose transporter, GLUT1. Cytochalasin is not cross-linked to the transporter if an excess of D-glucose is present during the labeling reaction; however, an excess of L-glucose (which is not transported) does not interfere with labeling. Why does an excess of D-glucose, but not L-glucose, prevent cross-linking of cytochalasin to GLUT1?

11–16 Insulin is a small protein hormone that binds to receptors in the plasma membranes of many cells. In fat cells, this binding dramatically increases the rate of uptake of glucose into the cells. The increase occurs within minutes and is not blocked by inhibitors of protein synthesis or of glycosylation. These results suggest that insulin increases the activity of the glucose transporter, GLUT4, in the plasma membrane, without increasing the total number of GLUT4 molecules in the cell.

The two experiments described below suggest a possible mechanism for this insulin effect. In the first experiment, the initial rate of glucose uptake in control and insulin-treated cells was measured, with the results shown in **Figure 11–1**. In the second experiment, the concentration of GLUT4 in fractionated membranes from control and insulin-treated cells was measured, using the binding of radioactive cytochalasin B as the assay (see Problem 11–15), as shown in **Table 11–1**.

A. Deduce the mechanism by which glucose transport through GLUT4 increases in insulin-treated cells.

B. Does insulin stimulation alter either the K_m or the V_{max} of GLUT4? How can you tell from these data?

TABLE 11–1 Amount of GLUT4 associated with the plasma membrane and internal membranes in the presence and absence of insulin (Problem 11–16).

Membrane fraction	Bound ^{3}H-cytochalasin B (cpm/mg protein)	
	Untreated cells (– insulin)	Treated cells (+ insulin)
Plasma membrane	890	4480
Internal membranes	4070	80

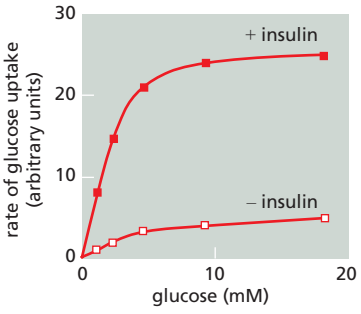


Figure 11–1 Rate of glucose uptake into cells in the presence and absence of insulin (Problem 11–16).

TRANSPORTERS AND ACTIVE MEMBRANE TRANSPORT

TERMS TO LEARN

ABC transporter
antiporter
Ca^{2+}-pump (Ca^{2+} ATPase)
multidrug resistance (MDR) protein
Na^+-K^+ pump (Na^+-K^+ ATPase)
P-type pump
symporter
transcellular transport
uniporter
V-type pump

DEFINITIONS

Match each definition below with its term from the list above.

11–17 Large superfamily of membrane transport proteins that use the energy of ATP hydrolysis to transfer peptides and a variety of small molecules across membranes.

11–18 Type of ABC transporter protein that can pump hydrophobic drugs (such as some anticancer drugs) out of the cytoplasm of eukaryotic cells.

11–19 Membrane carrier protein that transports two different ions or small molecules across a membrane in opposite directions, either simultaneously or in sequence.

11–20 Transport of solutes across an epithelium, by means of membrane transport proteins in the apical and basal surfaces of the epithelial cells.

11–21 Carrier protein that transports two types of solute across the membrane in the same direction.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

11–22 A symporter would function as an antiporter if its orientation in the membrane were reversed; that is, if the portion of the protein normally exposed to the cytosol faced the outside of the cell instead.

11–23 The co-transport of Na^+ and a solute into a cell, which harnesses the energy in the Na^+ gradient, is an example of primary active transport.

11–24 In response to depolarization of the muscle cell plasma membrane, the Ca^{2+}-pumps in the sarcoplasmic reticulum (SR) use the energy of ATP hydrolysis to move Ca^{2+} from the lumen of the SR to the cytosol to initiate muscle contraction.

THOUGHT PROBLEMS

11–25 Which of the ions listed in **Table 11–2** could be used to drive an electrically neutral coupled transport of a solute across the plasma membrane? Indicate the direction of movement of the listed ions (inward or outward) and indicate what sort of ion would be co-transported to preserve electrical neutrality.

Incidentally, there is a glaring intracellular deficiency of anions relative to cations in Table 11–2, yet cells are electrically neutral. What anions do you suppose are missing from this table?

11–26 A transmembrane protein has the following properties: it has two binding sites, one for solute A and one for solute B. The protein can undergo a conformational change to switch between two states: either both binding sites are exposed exclusively on one side of the membrane, or both are

TABLE 11–2 A comparison of ion concentrations inside and outside a typical mammalian cell (Problem 11–25).

Component	Intracellular concentration (mM)	Extracellular concentration (mM)
Cations		
Na^+	5–15	145
K^+	140	5
Mg^{2+}	0.5	1–2
Ca^{2+}	10^{-4}	1–2
H^+	7×10^{-5} ($10^{-7.2}$ M or pH 7.2)	4×10^{-5} ($10^{-7.4}$ M or pH 7.4)
Anions		
Cl^-	5–15	110

exposed exclusively on the other side of the membrane. The protein can switch between the two conformational states only if both binding sites are occupied or if both binding sites are empty, but cannot switch if only one binding site is occupied.

A. What kind of a transporter do these properties define?
B. Do you need to specify any additional properties to turn this protein into a transporter that couples the movement of solute A up its concentration gradient to the movement of solute B down its electrochemical gradient?
C. Write a set of rules like those in the body of this problem that defines the properties of an antiporter.

11–27 A model for a uniporter that could mediate passive transport of glucose down its concentration gradient is shown in **Figure 11–2**. How would you need to change the diagram to convert the transporter into a pump that transports glucose up its concentration gradient by hydrolyzing ATP? Explain the need for each of the steps in your new illustration.

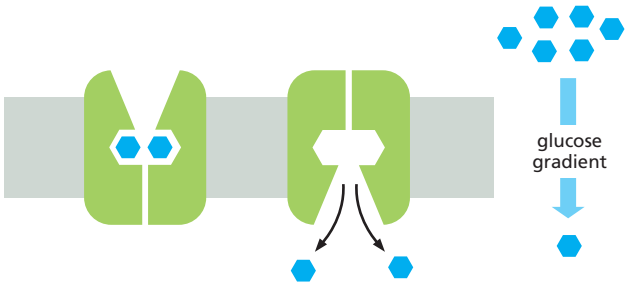


Figure 11–2 Hypothetical model showing how a conformational change in a transporter could mediate passive transport of glucose (Problem 11–27). The transition between the two conformational states is proposed to occur randomly and to be completely reversible, regardless of binding-site occupancy.

11–28 Ion transporters are "linked" together—not physically, but as a consequence of their actions. For example, cells can raise their intracellular pH, when it becomes too acidic, by exchanging external Na^+ for internal H^+, using a Na^+–H^+ antiporter. The change in internal Na^+ is then redressed using the Na^+-K^+ pump.

A. Can these two transporters, operating together, normalize both the H^+ and the Na^+ concentrations inside the cell?
B. Does the linked action of these two pumps cause imbalances in either the K^+ concentration or the membrane potential?

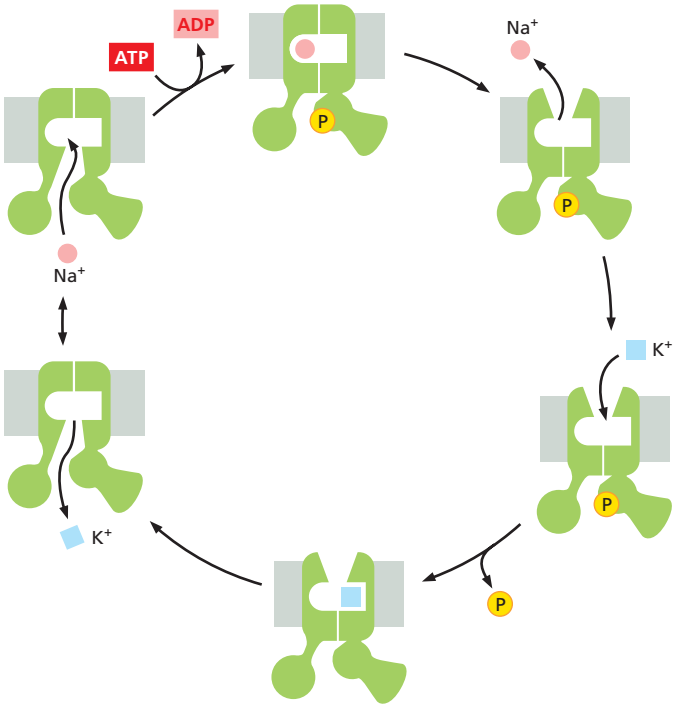


Figure 11–3 The Na^+-K^+ pump (Problem 11–29).

11–29 You have prepared lipid vesicles (spherical lipid bilayers) that contain Na^+-K^+ pumps as the sole membrane protein. Assume for the sake of simplicity that each pump transports one Na^+ one way and one K^+ the other way in each pumping cycle, as illustrated in **Figure 11–3**. All of the Na^+-K^+ pumps are oriented so that the portion of the molecule that normally faces the cytosol faces the outside of the vesicle. Predict what would happen under each of the following conditions.

A. The solution inside and outside the vesicles contains both Na^+ and K^+ ions, but no ATP.
B. The solution inside the vesicles contains both Na^+ and K^+ ions; the solution outside also contains both ions, as well as ATP.
C. The solution inside contains Na^+; the solution outside contains Na^+ and ATP.
D. The solution is as in B, but the Na^+-K^+ pump molecules are randomly oriented, some facing one direction, some the other.

CALCULATIONS

11–30 Microvilli increase the surface area of intestinal cells, providing more efficient absorption of nutrients. Microvilli are shown in profile and cross section in **Figure 11–4**. From the dimensions given in the figure, estimate the increase in surface area that microvilli provide (for the portion of the plasma membrane in contact with the lumen of the gut) relative to the corresponding surface of a cell with a "flat" plasma membrane.

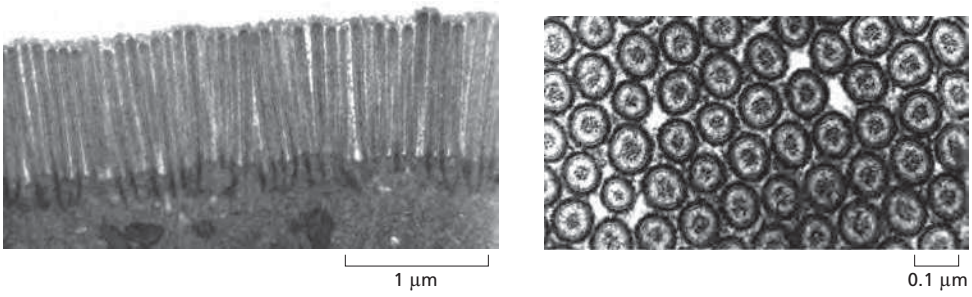


Figure 11–4 Microvilli of intestinal epithelial cells in profile and cross section (Problem 11–30).

11–31 How much energy does it take to pump substances across membranes? Or, to put it another way, since active transport is usually driven directly or indirectly by ATP, how steep a gradient can ATP hydrolysis maintain for a particular solute? For transport into the cell, the free-energy change (ΔG_{in}) per mole of solute moved across the plasma membrane is

$$\Delta G_{in} = -2.3RT \log \frac{C_o}{C_i} + zFV$$

where R = the gas constant, 8.3×10^{-3} kJ/K mole
T = the absolute temperature in K (37°C = 310 K)
C_o = solute concentration outside the cell
C_i = solute concentration inside the cell
z = the valence (charge) on the solute
F = Faraday's constant, 96 kJ/V mole
V = the membrane potential in volts (V)

Since $\Delta G_{in} = -\Delta G_{out}$, the free-energy change for transport out of the cell is

$$\Delta G_{out} = 2.3RT \log \frac{C_o}{C_i} - zFV$$

At equilibrium, where $\Delta G = 0$, the equations can be rearranged to the more familiar form known as the Nernst equation.

$$V = 2.3 \frac{RT}{zF} \log \frac{C_o}{C_i}$$

For the questions below, assume that hydrolysis of ATP to ADP and P_i proceeds with a ΔG of –50 kJ/mole; that is, ATP hydrolysis can drive active transport with a ΔG of +50 kJ/mole. Assume that V is –60 mV.

A. What is the maximum concentration gradient that can be achieved by the ATP-driven active transport into the cell of an uncharged molecule such as glucose, assuming that 1 ATP is hydrolyzed for each solute molecule that is transported?
B. What is the maximum concentration gradient that can be achieved by active transport of Ca^{2+} from the inside to the outside of the cell? How does this maximum compare with the actual concentration gradient observed in mammalian cells (see Table 11–2)?
C. Calculate how much energy it takes to drive the Na^+-K^+ pump. This remarkable molecular device transports five ions for every molecule of ATP that is hydrolyzed: 3 Na^+ out of the cell and 2 K^+ into the cell. The pump typically maintains internal Na^+ at 10 mM, external Na^+ at 145 mM, internal K^+ at 140 mM, and external K^+ at 5 mM. As shown in **Figure 11–5**, Na^+ is transported against the membrane potential, whereas K^+ is transported with it. (The ΔG for the overall reaction is equal to the sum of the ΔG values for transport of the individual ions.)
D. How efficient is the Na^+-K^+ pump? That is, what fraction of the energy available from ATP hydrolysis is used to drive transport?

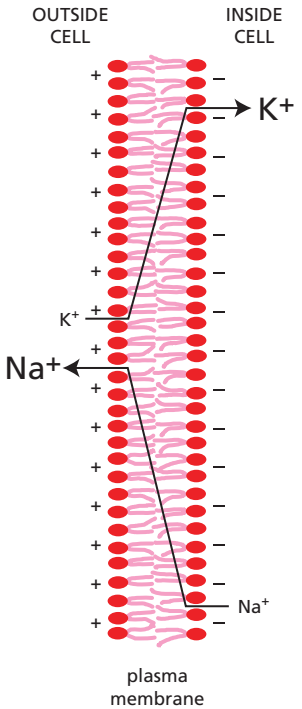


Figure 11–5 Na^+ and K^+ gradients and direction of pumping across the plasma membrane (Problem 11–31). *Large letters* symbolize high concentrations and *small letters* symbolize low concentrations. Both Na^+ and K^+ are pumped against chemical concentration gradients; however, Na^+ is pumped up the electrical gradient, whereas K^+ is pumped down the electrical gradient.

DATA HANDLING

11–32 If you have ever used the standard probe on a pH meter, you may well wonder how pH could possibly be measured in the tiny volumes inside cellular compartments. The recent development of pH-sensitive fluorophores has simplified this difficult task immensely. One such fluorescent indicator is a hydrophobic ester of SNARF-1, which can enter cells by passive diffusion and then is trapped inside after intracellular enzymes hydrolyze the ester bonds to liberate SNARF-1 (**Figure 11–6**). SNARF-1 absorbs light at 488 nm and emits fluorescent light with peaks at 580 nm and 640 nm. Emission spectra for SNARF-1 at pH 6.0 and pH 9.0 are

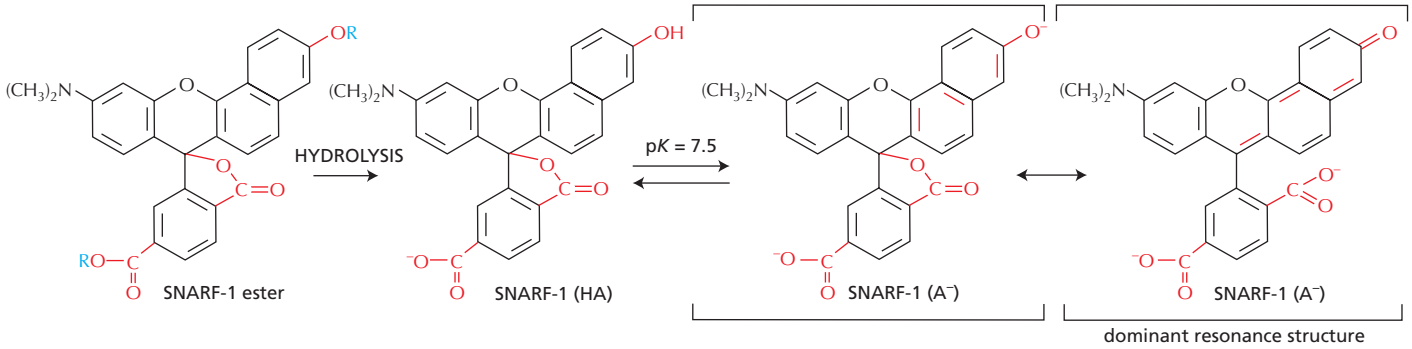


Figure 11–6 Structure of the ester and free forms of SNARF-1 (Problem 11–32). The blocking ester groups are shown as R. The acid (HA) and salt (A^-) forms of SNARF-1 are indicated. The two different resonance structures for the salt form of SNARF-1 are shown in *brackets*.

shown in **Figure 11–7**. The p*K* of SNARF-1 is 7.5.

A. Explain why the ester of SNARF-1 diffuses through membranes, whereas the cleaved form stays inside cells.
B. Why do you think there are two peaks of fluorescence (at 580 nm and at 640 nm) that change so dramatically in intensity with a change in pH (see Figure 11–7)? What features of SNARF-1 might be important in this?
C. What forms of SNARF-1 are present at pH 6.0 and what are their relative proportions? At pH 9.0? The Henderson–Hasselbalch equation describing the dissociation of a weak acid is pH = p*K* + log ([salt]/[acid]).
D. Sketch an approximate curve for the SNARF-1 emission spectrum inside a cell at pH 7.2. (All such curves pass through the point where the two curves in Figure 11–7 cross.)
E. Why do you suppose indicators such as SNARF-1 that have emission spectra with two peaks are preferred to those that have a single peak?

MEDICAL LINKS

11–33 CO_2 is removed from the body through the lungs in a process that is mediated by red blood cells, as summarized in **Figure 11–8**. Transport of CO_2 is coupled to the transport of O_2 through hemoglobin. Upon release of O_2 in the tissues, hemoglobin undergoes a conformational change that raises the p*K* of a histidine side chain, allowing it to bind an H^+, which is generated during hydration of CO_2 by the action of the enzyme carbonic anhydrase. This process occurs in reverse in the lungs when O_2 is bound to hemoglobin.

A. To what extent does the intracellular pH of the red blood cell vary during its movement from the tissues to the lungs and back, and why is this so?
B. In what form, and where, is the CO_2 during its movement from the tissues to the lungs?
C. How is it that the Cl^-–HCO_3^- exchanger operates in one direction in the tissues and in the opposite direction in the lungs?

11–34 A rise in the intracellular Ca^{2+} concentration causes muscle cells to contract. In addition to an ATP-driven Ca^{2+}-pump, heart muscle cells, which contract quickly and regularly, have an antiporter that exchanges Ca^{2+} for extracellular Na^+ across the plasma membrane. This antiporter rapidly pumps most of the entering Ca^{2+} ions back out of the cell, allowing the cell to relax. Ouabain and digitalis, drugs that are used in the treatment of patients with heart disease, make the heart contract more strongly. Both drugs function by partially inhibiting the Na^+-K^+ pump in the membrane of the heart muscle cell. Can you propose an explanation for the effects of these drugs in patients? What will happen if too much of either drug is taken?

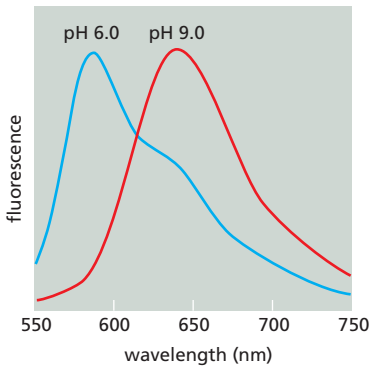


Figure 11–7 Emission spectra of SNARF-1 at pH 6.0 and pH 9.0 (Problem 11–32). SNARF-1 in solution at pH 6.0 or pH 9.0 was excited by light at 488 nm and the intensity of fluorescence was determined at wavelengths from 550 nm to 750 nm.

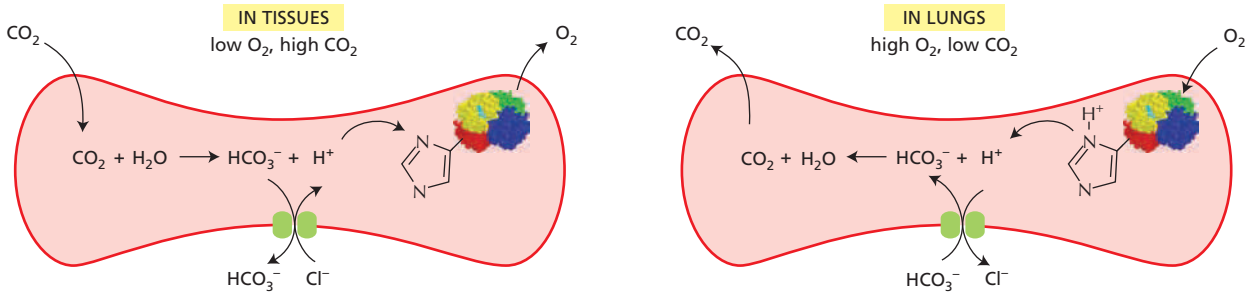


Figure 11–8 Red blood cell-mediated transport of CO_2 from the tissues to the lungs (Problem 11–33). Low and high O_2 and CO_2 refer to their concentrations, or partial pressures.

CHANNELS AND THE ELECTRICAL PROPERTIES OF MEMBRANES

TERMS TO LEARN

acetylcholine receptor
action potential
adaptation
AMPA receptor
aquaporin (water channel)
axon
Ca^{2+}-activated K^+ channel
channelrhodopsin
delayed K^+ channel
dendrite
depolarization
excitatory neurotransmitter
glial cell
inhibitory neurotransmitter
initial segment
ion channel
K^+ leak channel
long-term depression (LDP)
long-term potentiation (LTP)
mechanosensitive channel
membrane potential
metabotropic receptor
myelin sheath
Nernst equation
neuromuscular junction
neuron (nerve cell)
neurotransmitter
NMDA receptor
oligodendrocyte
optogenetics
patch-clamp recording
resting membrane potential
rapidly inactivating K^+ channel
Schwann cell
selectivity filter
synapse
synaptic plasticity
transmitter-gated ion channel
voltage-gated cation channel
voltage-gated K^+ channel
voltage-gated Na^+ channel

DEFINITIONS

Match each definition below with its term from the list above.

11–35 The adjustment in sensitivity of a cell or organism following repeated stimulation that allows a response even when there is a high background level of stimulation.

11–36 The long-lasting increase (days to weeks) in the sensitivity of certain synapses in the hippocampus that is induced by a short burst of repetitive firing in the presynaptic neurons.

11–37 Rapid, transient, self-propagating electrical signal in the plasma membrane of a cell such as a neuron or muscle cell: a nerve impulse.

11–38 Quantitative expression that relates the equilibrium ratio of concentrations of an ion on either side of a permeable membrane to the voltage difference across the membrane.

11–39 A photosensitive ion channel that opens in response to light.

11–40 Voltage difference across a membrane due to the slight excess of positive ions on one side and of negative ions on the other (typically –60 mV, inside negative, for an animal cell).

11–41 General term for a membrane protein that selectively allows cations such as Na^+ to cross a membrane in response to changes in membrane potential.

11–42 That part of an ion channel structure that determines which ions it can transport.

11–43 Insulating layer of specialized cell membrane wrapped around vertebrate axons.

11–44 A K^+-transporting ion channel in the plasma membrane of animal cells that remains open even in a "resting" cell.

11–45 Long, thin nerve cell process capable of rapidly conducting nerve impulses over long distances so as to deliver signals to other cells.

11–46 Transmembrane protein complex that forms a water-filled channel across the lipid bilayer through which specific inorganic ions can diffuse down their electrochemical gradients.

11–47 Specialized junction between a nerve cell and another cell, across which the nerve impulse is transferred, usually by a neurotransmitter, which is secreted by the nerve cell and diffuses to the target cell.

11–48 Small signaling molecule such as acetylcholine, glutamate, GABA, or glycine, secreted by a nerve cell at a chemical synapse to signal to the postsynaptic cell.

11–49 Technique in which the tip of a small glass electrode is sealed onto an area of cell membrane, thereby making it possible to record the flow of current through individual ion channels.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

11–50 Transporters saturate at high concentrations of the transported molecule when all their binding sites are occupied; channels, on the other hand, do not bind the ions they transport and thus the flux of ions through a channel does not saturate.

11–51 The membrane potential arises from movements of charge that leave ion concentrations practically unaffected, causing only a very slight discrepancy in the number of positive and negative ions on the two sides of the membrane.

11–52 The aggregate current crossing the membrane of an entire cell indicates the degree to which individual channels are open.

11–53 Transmitter-gated ion channels open in response to specific neurotransmitters in their environment but are insensitive to the membrane potential; therefore, they cannot by themselves (in the absence of ligand) generate an action potential.

11–54 When an action potential depolarizes the muscle cell membrane, the Ca^{2+}-pump is responsible for pumping Ca^{2+} from the sarcoplasmic reticulum into the cytosol to initiate muscle contraction.

THOUGHT PROBLEMS

11–55 According to Newton's laws of motion, an ion exposed to an electric field in a vacuum would experience a constant acceleration from the electric driving force, just as a falling body in a vacuum constantly accelerates due to gravity. In water, however, an ion moves at constant velocity in an electric field. Why do you suppose that is?

11–56 What two properties distinguish an ion channel from a simple aqueous pore?

11–57 You have prepared lipid vesicles that contain molecules of the K^+ leak channel, all oriented so that their cytosolic surface faces the outside of the vesicle. Predict how K^+ ions will move under the following conditions and what sort of membrane potential will develop.

A. Equal concentrations of K^+ ion are present inside and outside the vesicle.
B. K^+ ions are present only inside the vesicle.
C. K^+ ions are present only outside the vesicle.

11–58 If a frog egg and a red blood cell are placed in pure water, the red blood cell will swell and burst, but the frog egg will remain intact. Although a frog egg is about one million times larger than a red cell, they both have nearly identical internal concentrations of ions so that the same osmotic forces are at work in each. Why do you suppose red blood cells burst in water, while frog eggs do not?

11–59 Aquaporins allow water to move across a membrane, but prevent the passage of ions. How does the structure of the pore through which the water molecules move prevent passage of ions such as K^+, Na^+, Ca^+, and Cl^-? H^+ ions present a different problem because they move by relay along a chain of hydrogen-bonded water molecules (**Figure 11–9**). How does the pore prevent the relay of H^+ ions across the membrane?

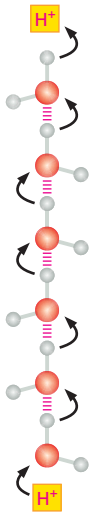


Figure 11–9 Rapid diffusion of H^+ ions by a molecular relay system involving the making and breaking of hydrogen bonds between adjacent water molecules (Problem 11–59).

11–60 Explain in 100 words or fewer how an action potential is passed along an axon.

11–61 The neurotransmitter acetylcholine is made in the cytosol and then transported into synaptic vesicles, where its concentration is more than 100-fold higher than in the cytosol. Synaptic vesicles isolated from neurons can take up additional acetylcholine if it is added to the solution in which they are suspended, but only in the presence of ATP. Na^+ ions are not required for acetylcholine uptake, but, curiously, raising the pH of the solution in which the synaptic vesicles are suspended increases acetylcholine uptake. Furthermore, transport is inhibited in the presence of drugs that make the membrane permeable to H^+ ions. Suggest a mechanism that is consistent with all these observations.

11–62 Excitatory neurotransmitters open Na^+ channels, while inhibitory neurotransmitters open either Cl^- or K^+ channels. Rationalize this observation in terms of the effects of these ions on the firing of an action potential.

11–63 Acetylcholine-gated cation channels do not discriminate among Na^+, K^+, and Ca^{2+} ions, allowing all to pass through freely. How is it, then, that when acetylcholine receptors in muscle cells open there is a large net influx principally of Na^+?

CALCULATIONS

11–64 In a subset of voltage-gated K^+ channels, the N-terminus of each subunit acts like a tethered ball that occludes the cytoplasmic end of the pore soon after it opens, thereby inactivating the channel. This "ball-and-chain" model for the rapid inactivation of voltage-gated K^+ channels has been elegantly supported for the *shaker* K^+ channel from *Drosophila melanogaster*. (The *shaker* K^+ channel in *Drosophila* is named after a mutant form that causes excitable behavior—even anesthetized flies keep twitching.) Deletion of the N-terminal amino acids from the normal *shaker* channel gives rise to a channel that opens in response to membrane depolarization but stays open (**Figure 11–10A**; 0 μM) instead of rapidly closing as the normal channel does. A peptide (MAAVAGLYGLGEDRQHRKKQ) that corresponds to the deleted N-terminus can partially inactivate the open channel at 50 μM and completely inactivate it at 100 μM (Figure 11–10A).

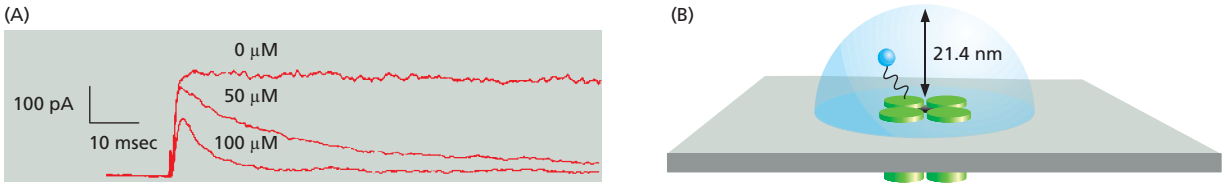


Figure 11–10 Inactivation of voltage-gated K^+ channels (Problem 11–64). (A) Patch-clamp recording of a defective *shaker* K^+ channel in the absence and presence of inactivating peptide. Current through the channel is indicated in picoamps (pA). (B) A "ball" tethered by a "chain" to a normal channel.

Is the concentration of free peptide (100 μM) required to inactivate the defective K^+ channel anywhere near the local concentration of the tethered ball on a normal channel? Assume that the tethered ball can explore a hemisphere [volume = $(2/3)\pi r^3$] with a radius of 21.4 nm, which is the length of the polypeptide "chain" (**Figure 11–10B**). Calculate the concentration for one ball in this hemisphere. How does that value compare with the concentration of free peptide needed to inactivate the channel?

11–65 If the resting membrane potential of a cell is –70 mV and the thickness of the lipid bilayer is 4.5 nm, what is the strength of the electric field across the membrane in V/cm? What do you suppose would happen if you applied this voltage to two metal electrodes separated by a 1 cm air gap?

11–66 The squid giant axon occupies a unique position in the history of our understanding of cell membrane potentials and nerve action. Its large size (0.2–1.0 mm in diameter and 5–10 cm in length) allowed electrodes, large by modern standards, to be inserted so that intracellular voltages could be measured. When an electrode is stuck into an intact giant axon, the membrane potential registers –70 mV. When the axon, suspended in a bath of seawater, is stimulated to conduct a nerve impulse, the membrane potential changes transiently from –70 mV to +40 mV.

The Nernst equation relates equilibrium ionic concentrations to the membrane potential.

$$V = 2.3\,\frac{RT}{zF}\,\log\,\frac{C_o}{C_i}$$

For univalent ions and at 20°C (293 K),

$$V = 58\text{ mV} \times \log\,\frac{C_o}{C_i}$$

A. Using this equation, calculate the potential across the resting membrane (1) assuming that it is due solely to K^+ and (2) assuming that it is due solely to Na^+. (The Na^+ and K^+ concentrations in the axon cytosol and in seawater are given in **Table 11–3**.) Which calculation is closer to the measured resting potential? Which calculation is closer to the measured action potential? Explain why these assumptions approximate the measured resting and action potentials.

B. If the solution bathing the squid giant axon is changed from seawater to artificial seawater in which NaCl is replaced with choline chloride, there is no effect on the resting potential, but the nerve no longer generates an action potential upon stimulation. What would you predict would happen to the magnitude of the action potential if the concentration of Na^+

TABLE 11–3 Ionic composition of seawater and of the cytosol in the squid giant axon (Problem 11–66).

Ion	Cytosol	Seawater
Na^+	65 mM	430 mM
K^+	344 mM	9 mM

in the external medium were reduced to a half or a quarter of its normal value, using choline chloride to maintain osmotic balance?

11–67 The number of Na^+ ions entering the squid giant axon during an action potential can be calculated from theory. Because the cell membrane separates positive and negative charges, it behaves like a capacitor. From the known capacitance of biological membranes, the number of ions that enter during an action potential can be calculated. Starting from a resting potential of –70 mV, it can be shown that 1.1×10^{-12} moles of Na^+ must enter the cell per cm^2 of membrane during an action potential.

To determine experimentally the number of entering Na^+ ions during an action potential, a squid giant axon (1 mm in diameter and 5 cm in length) was suspended in a solution containing radioactive Na^+ (specific activity = 2×10^{14} cpm/mole) and a single action potential was propagated down its length. When the cytoplasm was analyzed for radioactivity, a total of 340 cpm were found to have entered the axon.

A. How well does the experimental measurement match the theoretical calculation?
B. How many moles of K^+ must cross the membrane of the axon, and in which direction, to reestablish the resting potential after the action potential is over?
C. Given that the concentration of Na^+ inside the axon is 65 mM, calculate the fractional increase in internal Na^+ concentration that results from the passage of a single action potential down the axon.
D. At the other end of the spectrum of nerve sizes are small dendrites about 0.1 μm in diameter. Assuming the same length (5 cm), the same internal Na^+ concentration (65 mM), and the same resting and action potentials as for the squid giant axon, calculate the fractional increase in internal Na^+ concentration that would result from the passage of a single action potential down a dendrite.
E. Is the Na^+-K^+ pump more important for the continuing performance of a giant axon, or of a dendrite?

11–68 Acetylcholine-gated cation channels at the neuromuscular junction open in response to acetylcholine released by the nerve terminal and allow Na^+ ions to enter the muscle cell, which causes membrane depolarization and ultimately leads to muscle contraction.

A. Patch-clamp measurements show that young rat muscles have cation channels that respond to acetylcholine (**Figure 11–11**). How many kinds of channel are there? How can you tell?
B. For each kind of channel, calculate the number of ions that enter in one millisecond. (One ampere is a current of one coulomb per second; one pA equals 10^{-12} ampere. An ion with a single charge such as Na^+ carries a charge of 1.6×10^{-19} coulomb.)

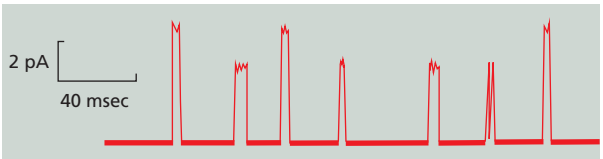


Figure 11–11 Patch-clamp measurements of acetylcholine-gated cation channels in young rat muscle (Problem 11–68).

DATA HANDLING

11–69 The *shaker* K^+ channel in *Drosophila* opens in response to membrane depolarization and then rapidly inactivates via a ball-and-chain mechanism (see Problem 11–64). The *shaker* K^+ channel assembles as a tetramer composed of four subunits, each with its own ball and chain. Do multiple balls in the tetrameric channel act together to inactivate the channel, or is one ball sufficient?

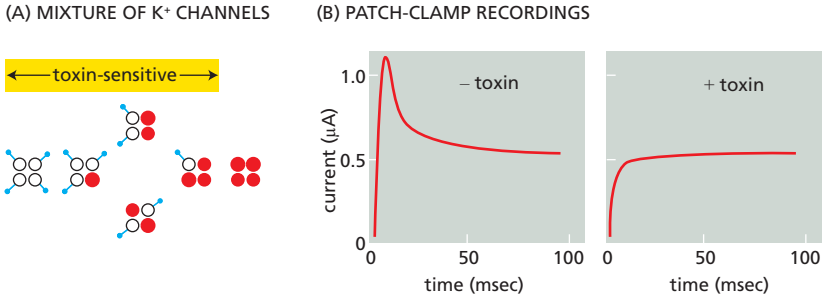


Figure 11–12 Analysis of the inactivation of the *shaker* K^+ channel (Problem 11–69). (A) Mixture of normal subunits with balls *(white)* and toxin-resistant subunits without balls *(red)*. If the two subunits were expressed in equal amounts, the different forms of the K^+ channel would be present in the ratio 1:4:6:4:1; however, higher levels of toxin-resistant subunit mRNA were used, significantly skewing the ratios in favor of channels with toxin-resistant subunits. (B) Patch-clamp recordings in the presence and absence of scorpion toxin.

This question has been answered by mixing subunits from two different forms of the K^+ channel: the normal subunits with balls, and scorpion-toxin-resistant subunits without balls (**Figure 11–12A**). Scorpion toxin prevents the opening of normal (toxin-sensitive) K^+ channels, but not channels composed entirely of toxin-resistant subunits. Moreover, hybrid channels containing even a single toxin-sensitive subunit fail to open in the presence of toxin.

Mixed K^+ channels were created by injecting a mixture of mRNAs for the two types of subunit into frog oocytes. After expression, patches of membrane containing several hundred channels were studied by patch-clamp recording after subjecting the membranes to depolarization in the absence or presence of scorpion toxin (**Figure 11–12B**).

A. Sketch the expected patch-clamp recordings, in the presence and absence of toxin, for a pure population of K^+ channels composed entirely of toxin-resistant subunits without balls.
B. Sketch the expected patch-clamp recordings, in the presence and absence of toxin, for a pure population of K^+ channels composed entirely of normal (toxin-sensitive) subunits with balls.
C. Sketch the expected patch-clamp recordings, in the presence and absence of toxin, for a mixed population of K^+ channels, 50% composed entirely of normal (toxin-sensitive) subunits with balls and 50% composed entirely of toxin-resistant subunits without balls.
D. The key observation in Figure 11–12B is that the final plateau values of the currents in the absence and presence of toxin are the same. Does this observation argue that a single ball can close a channel or that multiple balls must act in concert? (Think about what the curves would look like if one, two, three, or four balls were required to close a channel.)

11–70 A recording from a patch of membrane (**Figure 11–13A**) measured current through acetylcholine-gated ion channels, which open when acetylcholine is bound (**Figure 11–13B**). To obtain the recording, acetylcholine was added to the solution in the micropipette (Figure 11–13A). Describe what you can learn about the channels from this recording. How would the recording differ if acetylcholine were (a) omitted or (b) added only to the solution outside the micropipette?

11–71 One important parameter for understanding any particular membrane transport process is to know the number of copies of the specific transport protein present in the cell membrane. To measure the number of

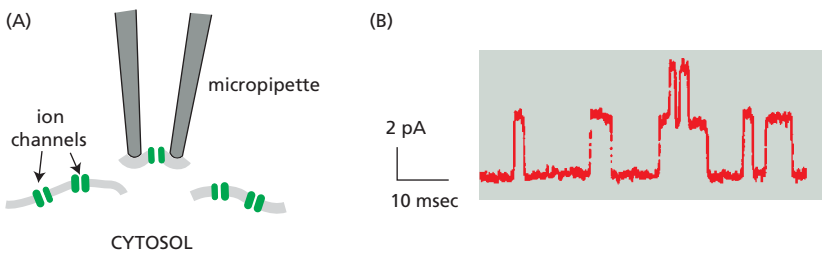


Figure 11–13 Analysis of acetylcholine-gated ion channels (Problem 11–70). (A) A micropipette with a patch of membrane. (B) Patch-clamp recording of current through acetylcholine-gated ion channels.

voltage-gated Na^+ channels in the rabbit vagus nerve, you use a potent toxin, saxitoxin, a shellfish poison that specifically inactivates the voltage-gated Na^+ channels in these nerve cells. Assuming that each channel binds one toxin molecule, the number of Na^+ channels in a segment of vagus nerve will be equal to the maximum number of bound toxin molecules.

You incubate identical segments of nerve for 8 hours with increasing amounts of ^{125}I-labeled toxin. You then wash the segments to remove unbound toxin and measure the radioactivity associated with the nerve segments to determine the toxin-binding curve (**Figure 11–14**, upper curve). You are puzzled because you expected to see binding reach a maximum (saturate) at high concentrations of toxin; however, no distinct end point was reached, even at higher concentrations of toxin than those shown in the figure. After careful thought, you design a control experiment in which the binding of labeled toxin is measured in the presence of a large molar excess of unlabeled toxin. The results of this experiment, which are shown in the lower curve in Figure 11–14, make everything clear and allow you to calculate the number of Na^+ channels in the membrane of the vagus nerve axon.

A. Why does binding of the labeled toxin not saturate? What is the point of the control experiment, and how does it work?

B. Given that 1 gram of vagus nerve has an axonal membrane area of 6000 cm^2 and assuming that the Na^+ channel is a cylinder with a diameter of 6 nm, calculate the number of Na^+ channels per square micrometer of axonal membrane and the fraction of the cell surface occupied by the channel. (Use 100 pmol as the amount of toxin specifically bound to the receptor.)

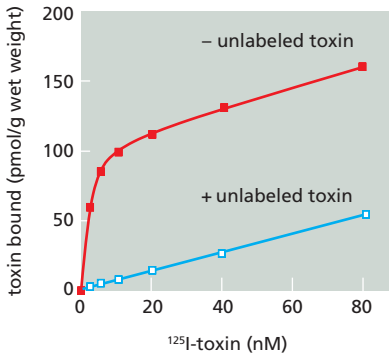


Figure 11–14 Toxin-binding curves in the presence and absence of unlabeled saxitoxin (Problem 11–71).

MEDICAL LINKS

11–72 To make antibodies against the acetylcholine receptor from the electric organ of electric eels, you inject the purified receptor into mice. You note an interesting correlation: mice with high levels of antibodies against the receptor appear weak and sluggish; those with low levels are lively. You suspect that the antibodies against the eel acetylcholine receptors are reacting with the mouse acetylcholine receptors, causing many of the receptors to be destroyed. Since a reduction in the number of acetylcholine receptors is also the basis for the human autoimmune disease myasthenia gravis, you wonder whether an injection of the drug neostigmine into the mice might give them a temporary restoration of strength, as it does for myasthenic patients. Neostigmine inhibits acetylcholinesterase, the enzyme responsible for hydrolysis of acetylcholine in the synaptic cleft. Sure enough, when you inject your mice with neostigmine, they immediately become very active. Propose an explanation for how neostigmine restores temporary function to a neuromuscular synapse with a reduced number of acetylcholine receptors.

11–73 The ion channels for neurotransmitters such as acetylcholine, serotonin, GABA, and glycine have similar overall structures. Each class comprises an extremely diverse set of channel subtypes, with different ligand affinities, different channel conductances, and different rates of opening and closing. Why is such extreme diversity a good thing from the standpoint of the pharmaceutical industry?

MCAT STYLE

Passage 1 (Questions 11–74 to 11–76)

Numerous interesting drugs and toxins act on channels and transporters. One such drug is scopolamine, which is used to treat vertigo, motion sickness, and

smooth muscle spasms. Scopolamine was first isolated from Jimson Weed, which produces a beautiful white flower—often painted by Georgia O'Keeffe. Jimson Weed is also called Sacred Datura, and was used by Native Americans for religious purposes because it can produce hallucinations and time distortions. Imagine that you are the first to purify scopolamine and are trying to determine how it works. You find that if you incubate isolated muscle cells with scopolamine, subsequent addition of acetylcholine no longer causes membrane depolarization and cell contraction, as it does in the absence of scopolamine.

11–74 Which one of the following statements best explains how scopolamine might exert its effects?

A. It binds to the acetylcholine-gated Na^+ channel and inhibits its opening.
B. It inhibits opening of the Ca^{2+} channel in the sarcoplasmic reticulum.
C. It inhibits transporters that import Na^+ during an action potential.
D. It inhibits voltage-gated K^+ channels during an action potential.

11–75 Scorpion α-toxin, a component of scorpion venom, dramatically prolongs the change in membrane potential during the firing of a nerve impulse (**Figure 11–15**). Which one of the following hypotheses best explains how α-toxin exerts its effects?

A. It accelerates the opening of voltage-gated K^+ channels.
B. It opens the voltage-gated Na^+ channel at a lower voltage.
C. It promotes the opening of a ligand-gated Na^+ channel.
D. It slows the inactivation of the voltage-gated Na^+ channel.

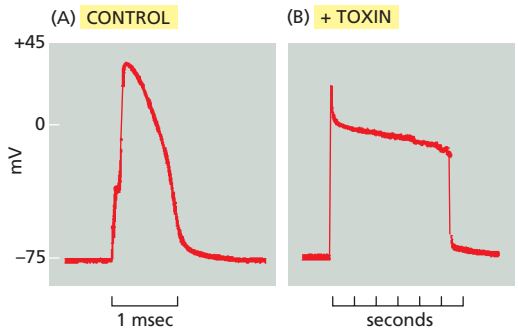


Figure 11–15 Effects of scorpion α-toxin on the duration of an action potential (Problem 11–75).

11–76 Extracts from the African plant *Stropthantus gratus* were once used to make poison arrowheads for hunting. The active compound in the extract is extremely toxic; when concentrated and placed on an arrowhead, it can kill a hippopotamus. Imagine that you treat cells with the compound and observe that they begin to swell and eventually burst. Which one of the following actions would best explain the effects of the toxic compound?

A. Closing of K^+ leak channels.
B. Closing of Na^+ channels.
C. Inhibition of glucose transporters.
D. Inhibition of the Na^+-K^+ pump.

Passage 2 (Questions 11–77 to 11–78)

Severe diarrhea associated with diseases such as cholera and dysentery was the leading cause of infant mortality world-wide prior to 1980. Severe diarrhea produces such a rapid fluid loss that the individual becomes so dehydrated that organs fail. When it invades the gut, *Vibrio cholerae*, the bacterium responsible for cholera, produces a toxin that hyperactivates the cystic fibrosis transmembrane regulator (CFTR). Hyperactivation of CFTR increases the movement of Cl^- ions into the lumen of the intestine, which creates an ionic imbalance. As a result, water rushes into the intestine from surrounding tissues, leading to diarrhea and

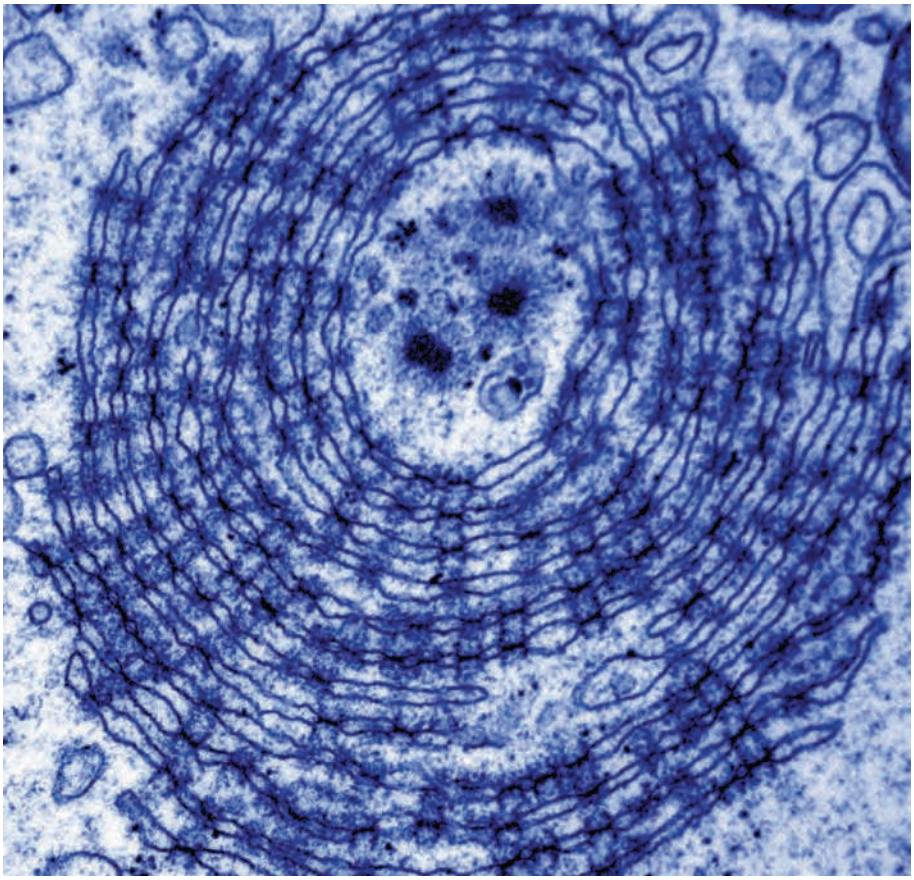
rapid water loss. Mutations that inactivate CFTR cause cystic fibrosis. In this case, decreased flow of Cl^- to the lining of the lungs leads to an ionic imbalance that dehydrates the mucus, which clogs the air passages.

11–77 Analysis of CFTR led to some surprises. Although CFTR is homologous to ABC transporters, which use ATP hydrolysis to pump solutes in or out of the cell, it shows unusual behavior. Which one of the following statements would suggest that CFTR is not a typical member of the ABC transporter family?

A. CFTR binds to Cl^- to move the ions across membranes.
B. CFTR can move Cl^- against a concentration gradient.
C. CFTR produces a robust Cl^- current across membranes.
D. CFTR requires ATP to move C^- across membranes.

11–78 The loss of fluids in severe diarrhea can be effectively treated by oral rehydration therapy, which has been called one of the greatest medical advances of the twentieth century because it dramatically reduces deaths caused by dehydration. Oral rehydration therapy is simple and cheap: affected individuals drink a solution of 90 mM NaCl and 110 mM glucose. Which of the following statements best describes how oral rehydration therapy works?

A. Co-transport of Na^+ and glucose via a symporter increases cell osmolarity and water uptake.
B. Glucose powers the Na^+-K^+ pump, which pumps Na^+ into the cell to increase cell osmolarity.
C. In the gut, the low osmolarity rehydration solution drives water into cells lining the intestine.
D. The salt and glucose in the rehydration solution kills *Vibrio cholerae*, normalizing cell osmolarity.



Annulate Lamellae in a Human Oocyte. These cytoplasmic whorls of double membranes punctured by nuclear pore complexes are found in many oocytes and other cells, but their significance and possible function(s) are still very mysterious. Do they represent stockpiles of nuclear membranes? Are the dark blobs at the center stored maternal mRNA? They are difficult to purify and study biochemically, and compared to other intracellular membranous structures have received scant attention from cell biologists.

Intracellular Compartments and Protein Sorting

CHAPTER 12

IN THIS CHAPTER

THE COMPARTMENTALIZATION OF CELLS

TERMS TO LEARN

cytoplasm	organelle	signal sequence
cytosol	protein translocation	sorting signal
gated transport	signal patch	vesicular transport
lumen	signal peptidase	

DEFINITIONS

Match the definition below with its term from the list above.

12–1 Contents of a cell that are contained within its plasma membrane but, in the case of eukaryotic cells, outside the nucleus.

12–2 Protein sorting signal that consists of a specific three-dimensional arrangement of atoms on the folded protein's surface.

12–3 Contents of the main compartment of the cell, excluding the nucleus and membrane-bounded compartments such as endoplasmic reticulum and mitochondria.

12–4 Movement of proteins through nuclear pore complexes between the cytosol and the nucleus.

12–5 Membrane-enclosed compartment in a eukaryotic cell that has a distinct structure, macromolecular composition, and function.

12–6 Protein sorting signal that consists of a short continuous sequence of amino acids.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

12–7 The biological membranes that partition the cell into functionally distinct compartments are impermeable.

12–8 Like the lumen of the endoplasmic reticulum (ER), the interior of the nucleus is topologically equivalent to the outside of the cell.

12–9 ER-bound and free ribosomes, which are structurally and functionally identical, differ only in the proteins they happen to be making at a particular time.

12–10 Each signal sequence specifies a particular destination in the cell.

THOUGHT PROBLEMS

12–11 Discuss the following statement: "The plasma membrane is only a minor component of most eukaryotic cells."

12–12 Is it really true that *all* human cells contain the same basic set of membrane-enclosed organelles? Do you know of any examples of human cells that do not have a complete set of organelles?

12–13 When cells are treated with drugs that depolymerize microtubules, the Golgi apparatus is fragmented into small vesicles and dispersed throughout each cell. When such drugs are removed, cells typically recover and grow normally. If cells that have recovered from such treatment are examined by electron microscopy, they are found to contain a perfectly normal-looking Golgi apparatus. Does this mean that the Golgi apparatus has been synthesized anew from scratch? If not, how do you suppose it might have happened?

12–14 Why do eukaryotic cells require a nucleus as a separate compartment when prokaryotic cells manage perfectly well without?

12–15 What is the fate of a protein with no sorting signal?

12–16 Protein synthesis in a liver cell occurs nearly exclusively on free ribosomes in the cytosol and on ribosomes that are bound to the ER membrane. (A small fraction of total protein synthesis is directed by the mitochondrial genome and occurs on ribosomes in the mitochondrial matrix.) Which type of protein synthesis—in the cytosol or on the ER—do you think is responsible for the majority of protein synthesis in a liver cell? Assume that the average density and lifetimes of proteins are about the same in all compartments. Explain the basis for your answer. Would your answer change if you took into account that some proteins are secreted from liver cells?

12–17 List the organelles in an animal cell that obtain their proteins via gated transport, via transmembrane transport, or via vesicular transport.

12–18 Imagine that you have engineered a set of genes, each encoding a protein with a pair of conflicting signal sequences that specify different compartments. If the genes were expressed in a cell, predict which signal would win out for the following combinations. Explain your reasoning.

A. Signals for import into nucleus and import into ER.
B. Signals for import into peroxisomes and import into ER.
C. Signals for import into mitochondria and retention in ER.
D. Signals for import into nucleus and export from nucleus.

12–19 If you think of the protein as a traveler, what kind of vehicle would best describe the sorting receptor: a private car, a taxi, or a bus? Explain your choice.

CALCULATIONS

12–20 A typical animal cell is said to contain some 10 billion protein molecules that need to be sorted into their proper compartments. That's a lot of proteins. Can 10 billion protein molecules even fit into a cell? An average protein encoded by the human genome is 450 amino acids in length, and the average mass of an amino acid in a protein is 110 daltons. Given that the average density of a protein is 1.4 g/cm^3, what fraction of the volume of a cell would 10 billion average protein molecules occupy? Consider a liver cell, which has a volume of about 5000 μm^3, and a pancreatic exocrine cell, which has a volume of about 1000 μm^3.

12–21 The lipid bilayer, which is 5 nm thick, occupies about 60% of the volume of a typical cell membrane. (Lipids and proteins contribute equally on a mass basis, but lipids are less dense and therefore account for more of the volume.) For liver cells and pancreatic exocrine cells, the total area of all cell membranes is estimated at about 110,000 μm^2 and 13,000 μm^2,

respectively. What fraction of the total volumes of these cells is accounted for by lipid bilayers? The volumes of liver cells and pancreatic exocrine cells are about 5000 μm^3 and 1000 μm^3, respectively.

12–22 The rough ER is the site of synthesis of many classes of membrane proteins. Some of these proteins remain in the ER, whereas others are sorted to compartments such as the Golgi apparatus, lysosomes, and the plasma membrane. One measure of the difficulty of the sorting problem is the degree of "purification" that must be achieved during transport from the ER to the other compartments. For example, if membrane proteins bound for the plasma membrane represented 90% of all proteins in the ER, then only a small degree of purification would be needed (and the sorting problem would appear relatively easy). On the other hand, if plasma membrane proteins represented only 0.01% of the proteins in the ER, a very large degree of purification would be required (and the sorting problem would appear correspondingly more difficult).

What is the magnitude of the sorting problem? What fraction of the membrane proteins in the ER are destined for other compartments? A few simple considerations allow one to answer these questions. Assume that all proteins on their way to other compartments remain in the ER for 30 minutes on average before exiting, and that the ratio of proteins to lipids in the membranes of all compartments is the same.

A. In a typical growing cell that is dividing once every 24 hours, the equivalent of one new plasma membrane must transit the ER every day. If the ER membrane is 20 times the area of a plasma membrane, what is the ratio of plasma membrane proteins to other membrane proteins in the ER?
B. If in the same cell the Golgi membrane is three times the area of the plasma membrane, what is the ratio of Golgi membrane proteins to other membrane proteins in the ER?
C. If the membranes of all other compartments (lysosomes, endosomes, inner nuclear membrane, and secretory vesicles) that receive membrane proteins from the ER are equal in total area to the area of the plasma membrane, what fraction of the membrane proteins in the ER of this cell are permanent residents of the ER membrane?

DATA HANDLING

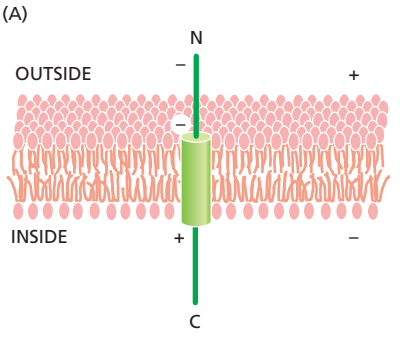
12–23 Although the vast majority of transmembrane proteins insert into membranes with the help of dedicated protein-translocation machines, a few proteins can insert into membranes on their own. Such proteins may provide a window into how membrane insertion occurred in the days before complex translocators had evolved.

You are studying a protein that inserts itself into the bacterial membrane independent of the normal translocation machinery. This protein has an N-terminal, 18-amino-acid hydrophilic segment that is located on the outside of the membrane, a 19-amino-acid hydrophobic transmembrane segment flanked by negatively and positively charged amino acids, and a C-terminal domain that resides inside the cell (**Figure 12–1A**). If the protein is properly inserted in the membrane, the N-terminal segment is exposed externally where it can be clipped off by a protease, allowing you to quantify insertion.

To examine the roles of the hydrophobic segment and its flanking charges, you construct a set of modified genes that express mutant proteins with altered charges in the N-terminal segment, altered lengths of the hydrophobic segment, and combinations of the two (**Figure 12–1B**). For each gene you measure the fraction of the total protein that is cleaved by the protease, which is the fraction that was inserted correctly (Figure 12–1B). To assess the contribution of the normal membrane potential (positive outside, negative inside), you repeat the measurements in the

presence of CCCP, an ionophore that eliminates the charge on the membrane (Figure 12–1B).

A. Which of the two N-terminal negative charges is the more important for insertion of the protein in the presence of the normal membrane potential (minus CCCP)? Explain your reasoning.
B. In the presence of the membrane potential (minus CCCP), is the hydrophobic segment or the N-terminal charge more important for insertion of the protein into the membrane? Explain your reasoning.
C. In the absence of the membrane potential (plus CCCP), is the hydrophobic segment or the N-terminal charge more important for insertion of the protein into the membrane? Explain your reasoning.



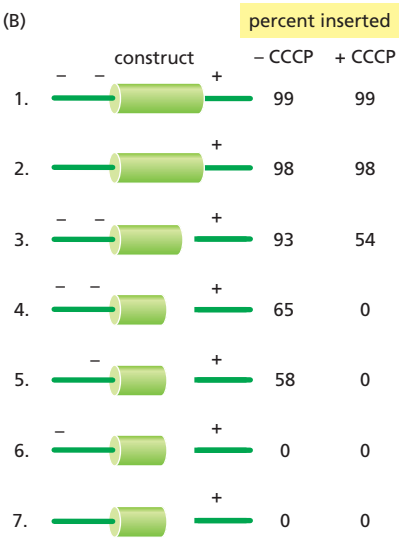


Figure 12–1 Insertion of a small protein into the bacterial membrane (Problem 12–23). (A) Normal orientation of the protein in the membrane. (B) Mutant proteins used to investigate the contributions of the N-terminal negative charges and length of the hydrophobic segment to membrane insertion. The presence of negative charges is indicated by –; *green cylinders* indicate the length of α-helical, transmembrane hydrophobic segments; deletions of the hydrophobic segments are shown as *gaps*. Percent inserted refers to the proportion of the total protein whose N-termini are sensitive to protease digestion.

THE TRANSPORT OF MOLECULES BETWEEN THE NUCLEUS AND THE CYTOSOL

TERMS TO LEARN

inner nuclear membrane
nuclear envelope
nuclear export receptor
nuclear export signal
nuclear import receptor
nuclear lamin
nuclear lamina
nuclear localization signal
nuclear pore complex (NPC)
nuclear transport receptor
nucleoporin
outer nuclear membrane
Ran

DEFINITIONS

Match the definition below with its term from the list above.

12–24 Sorting signal contained in the structure of macromolecules and complexes that are transported from the nucleus to the cytosol through nuclear pore complexes.

12–25 Large multiprotein structure forming a channel through the nuclear envelope that allows selected molecules to move between nucleus and cytoplasm.

12–26 Monomeric GTPase present in both cytosol and nucleus that is required for the active transport of macromolecules into and out of the nucleus through nuclear pore complexes.

12–27 Fibrous meshwork of proteins on the inner surface of the inner nuclear membrane.

12–28 Protein that binds nuclear localization signals and facilitates the transport of proteins with these signals from the cytosol into the nucleus through nuclear pore complexes.

12–29 Sorting signal found in proteins destined for the nucleus and which enable their selective transport into the nucleus from the cytosol through the nuclear pore complexes.

12–30 The portion of the nuclear envelope that is continuous with the endoplasmic reticulum and is studded with ribosomes on its cytosolic surface.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

12–31 The nuclear membrane is freely permeable to ions and other small molecules under 5000 daltons.

12–32 To avoid the inevitable collisions that would occur if two-way traffic through a single pore were allowed, nuclear pore complexes are specialized so that some mediate import while others mediate export.

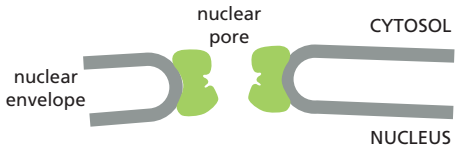


Figure 12–2 Cross section through a nuclear pore complex, showing continuity of inner and outer nuclear membranes (Problem 12–35).

12–33 Some proteins are kept out of the nucleus, until needed, by inactivating their nuclear localization signals by phosphorylation.

12–34 All cytosolic proteins have nuclear export signals that allow them to be removed from the nucleus when it reassembles after mitosis.

THOUGHT PROBLEMS

12–35 As shown in **Figure 12–2**, the inner and outer nuclear membranes form a continuous sheet, connecting through the nuclear pores. Continuity implies that membrane proteins can move freely between the two nuclear membranes by diffusing through the bilayer at the nuclear pores. Yet the inner and outer nuclear membranes have different protein compositions, as befits their different functions. How do you suppose this apparent paradox is reconciled?

12–36 How is it that a single nuclear pore complex can efficiently transport proteins that possess different kinds of nuclear localization signal?

12–37 How do you suppose that proteins with a nuclear export signal get into the nucleus?

12–38 Your advisor is explaining his latest results in your weekly lab meeting. By fusing his protein of interest to green fluorescent protein (GFP), he has shown that it is located entirely in the nucleus. But he wonders if it is a true nuclear protein or a shuttling protein that just spends most of its time in the nucleus. He is unsure how to resolve this issue. Having just read an article about how a similar problem was answered, you suggest that he make a heterokaryon by fusing cells that are expressing his tagged protein with an excess of cells that are not expressing it. You tell him that in the presence of a protein synthesis inhibitor to block new synthesis of the tagged protein, he can resolve the issue by examining fused cells with two nuclei. He gives you a puzzled look and asks, "How does that help?" You tell him what he has so often told you: "Think about it."

A. How would examining the two nuclei in a heterokaryon answer the question? What results would you expect if the protein were a true nuclear protein? What would you expect if it were a shuttling protein?

B. Why did you suggest that a protein synthesis inhibitor would be needed in this experiment?

12–39 Nuclear localization signals are not cleaved off after transport into the nucleus, whereas the signal sequences for import into other organelles are often removed after import. Why do you suppose it is critical that nuclear localization signals remain attached to their proteins?

12–40 To test the hypothesis that the directionality of transport across the nuclear membrane is determined primarily by the gradient of the Ran-GDP outside the nucleus and Ran-GTP inside the nucleus, you decide to reverse the gradient to see if you can force the import of a protein that is normally exported from the nucleus. You add a well-defined nuclear export substrate, fluorescent BSA coupled with a nuclear export signal (NES-BSA), to the standard permeabilized cell assay. Sure enough, it is excluded from the nuclei (**Figure 12–3A**). Now you add Crm1, the nuclear export receptor that recognizes the export signal, and RanQ69L-GTP, a

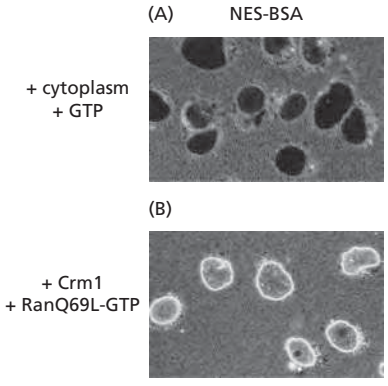


Figure 12–3 Directionality of nuclear transport (Problem 12–40). (A) Exclusion of fluorescent NES-BSA from the nucleus in a standard import assay. (B) Import of fluorescent NES-BSA in the presence of Crm1 and RanQ69L-GTP.

mutant form of Ran that cannot hydrolyze GTP. With these additions, the tagged BSA now enters the nuclei (**Figure 12–3B**). Unlike conventional nuclear import, which concentrates imported proteins in the nucleus, the concentration of NES-BSA in the nucleus in the import assay is no higher than in the surrounding cytoplasm.

A. Why doesn't NES-BSA accumulate to a higher concentration in the nucleus than in the cytoplasm in these experiments?

B. In a standard nuclear import assay with added cytoplasm and GTP, proteins with a nuclear localization signal accumulate to essentially 100% in the nucleus. How is it that the standard assay allows 100% accumulation in the nucleus?

CALCULATIONS

12–41 By following the increase in nuclear fluorescence over time in the forced nuclear import experiments in Figure 12–3B, the authors were able to show that nuclear fluorescence reached half its maximal value in 60 seconds. Since the added concentration of NES-BSA was 0.3 μM, its concentration in the nucleus after 60 seconds was 0.15 μM. Given that the volume of the nucleus is 500 fL and that each nucleus contains 3000 nuclear pores, calculate the rate of import of NES-BSA per pore in this experiment. (For this calculation, neglect export of NES-BSA.) Is your answer physiologically reasonable? Why or why not?

12–42 A nuclear pore can dilate to accommodate a gold particle 26 nm in diameter. If it could accommodate a spherical protein of the same dimensions, what would the protein's molecular mass (g/mole) be? [Assume that the density of the protein is 1.4 g/cm^3. The volume of a sphere is $(4/3)\pi r^3$.]

12–43 Assuming that 32 million histone octamers are required to package the human genome, how many histone molecules must be transported per second per nuclear pore complex in cells whose nuclei contain 3000 nuclear pores and are dividing once per day?

12–44 The nuclear pore complex (NPC) creates a barrier to the free exchange of molecules between the nucleus and cytoplasm, but in a way that remains mysterious. In yeast, for example, the central pore has a diameter of 35 nm and is 30 nm long, which is somewhat smaller than its vertebrate counterpart. Even so, it is large enough to accommodate virtually all components of the cytosol. Yet the pore allows passive diffusion of molecules only up to about 40 kd; entry of anything larger requires help from a nuclear import receptor. Selective permeability is controlled by protein components of the NPC that have unstructured, polar tails extending into the central pore. These tails are characterized by periodic repeats of the hydrophobic amino acids phenylalanine (F) and glycine (G).

At high enough concentration (50 mM), the FG-repeat domains of these proteins can form a gel, with a meshwork of interactions between the hydrophobic FG repeats (**Figure 12–4A**). These gels allow passive diffusion of small molecules, but they prevent entry of larger proteins such as the fluorescent protein mCherry fused to maltose binding protein

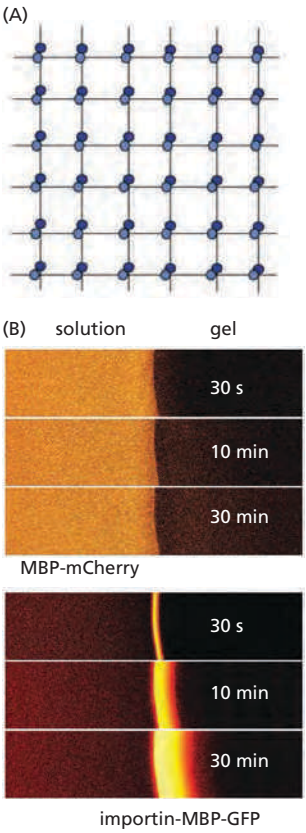


Figure 12–4 FG-repeat gel and influx of proteins into the nucleus (Problem 12–44). (A) Cartoon of the meshwork (gel) formed by pairwise interactions between hydrophobic FG repeats. For FG-repeats separated by 17 amino acids, as is typical, the mesh formed by extended amino acid side chains would correspond to about 4 nm on a side, which would be large enough to account for the characteristic passive diffusion of proteins through nuclear pores. (B) Diffusion of MBP-mCherry and importin-MBP-GFP into a gel of FG-repeats. In each group, the solution is shown at *left* and the gel at *right*. The bright areas indicate regions that contain the fluorescent proteins.

(MBP) (**Figure 12–4B**). (The fusion to MBP makes mCherry too large to enter the nucleus by passive diffusion.) However, if the nuclear import receptor, importin, is fused to a similar protein, MBP-GFP, the importin-MBP-GFP fusion readily enters the gel (Figure 12–4B).

A. FG-repeats only form gels *in vitro* at relatively high concentration (50 mM). Is this concentration reasonable for FG repeats in the NPC core? In yeast, there are about 5000 FG-repeats in each NPC. Given the dimensions of the yeast nuclear pore (35 nm diameter and 30 nm length), calculate the concentration of FG-repeats in the cylindrical volume of the pore. Is this concentration comparable to the one used *in vitro*?

B. Is diffusion of importin-MBP-GFP through the FG-repeat gel fast enough to account for the efficient flow of materials between the nucleus and cytosol? From experiments of the type shown in Figure 12–4B, the diffusion coefficient (D) of importin-MBP-GFP through the FG-repeat gel was determined to be about 0.1 μm^2/sec. The equation for diffusion is $t = x^2/2D$, where t is time and x is distance. Calculate the time it would take importin-MBP-GFP to diffuse through a yeast nuclear pore (30 nm) if the pore consisted of a gel of FG-repeats. Does this time seem fast enough for the needs of a eukaryotic cell?

DATA HANDLING

12–45 Before nuclear pore complexes were well understood, it was unclear whether nuclear proteins diffused passively into the nucleus and accumulated there by binding to residents of the nucleus such as chromosomes, or whether they were actively imported and accumulated regardless of their affinity for nuclear components.

A classic experiment that addressed this problem used several forms of radioactive nucleoplasmin, which is a large pentameric protein involved in chromatin assembly. In this experiment, either the intact protein or the nucleoplasmin heads, tails, or heads with a single tail were injected into the cytoplasm of a frog oocyte or into the nucleus (**Figure 12–5**). All forms of nucleoplasmin, except heads, accumulated in the nucleus when injected into the cytoplasm, and all forms were retained in the nucleus when injected there.

A. What portion of the nucleoplasmin molecule is responsible for localization in the nucleus?

B. How do these experiments distinguish between active transport, in which a nuclear localization signal triggers transport by the nuclear pore complex, and passive diffusion, in which a binding site for a nuclear component allows accumulation in the nucleus?

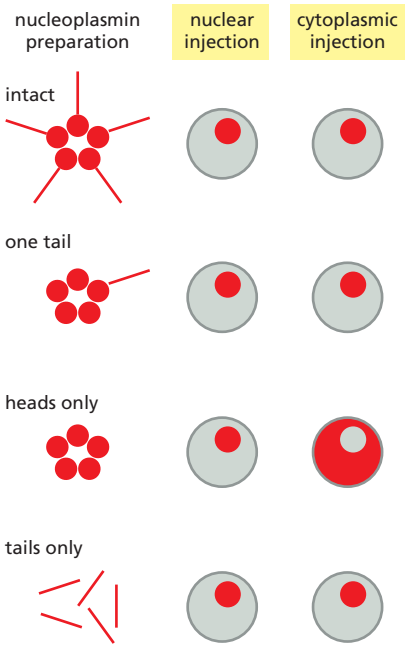


Figure 12–5 Cellular location of injected nucleoplasmin and nucleoplasmin components (Problem 12–45). Schematic diagrams of autoradiographs show the cytoplasm and nucleus with the location of nucleoplasmin indicated by the *red* areas.

12–46 You have just joined a laboratory that is analyzing the nuclear transport machinery in yeast. Your advisor, who is known for her extraordinarily clever ideas, has given you a project with enormous potential. In principle, it would allow a genetic selection for conditional-lethal mutants in the nuclear transport apparatus.

She gave you two plasmids. Each plasmid consists of a hybrid gene under the control of a regulatable promoter (**Figure 12–6**). The hybrid gene is a fusion between a gene whose product is normally imported into the nucleus and the gene for the restriction nuclease EcoRI. The plasmid pNL^+ contains a functional nuclear localization signal (NLS); the plasmid pNL^- does not have an NLS. The promoter, which is from the yeast *Gal1* gene, allows transcription of the hybrid gene only when the sugar galactose is present in the growth medium.

Following her instructions, you introduce the plasmids into yeast (in the absence of galactose) and then assay the transformed yeast in medium containing glucose and in medium containing galactose. Your results are shown in **Table 12–1**. You don't remember what your advisor

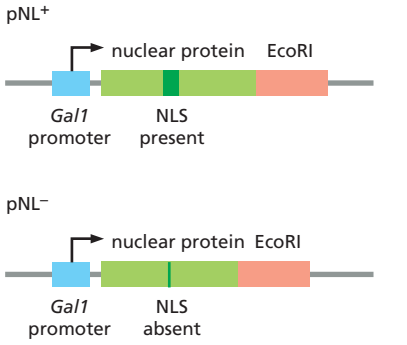


Figure 12–6 Two plasmids for investigating nuclear localization in yeast (Problem 12–46). Plasmids are shown as linear molecules for clarity. *Arrows* indicate direction of transcription from the *Gal1* promoter.

TABLE 12–1 Results of proliferation experiments with yeast carrying plasmids pNL⁺ or pNL⁻ (Problem 12–46).

Plasmid	Glucose medium	Galactose medium
pNL⁺	proliferation	death
pNL⁻	proliferation	proliferation

told you to expect, but you know you will be expected to explain these results at the weekly lab meeting.

Why do yeasts with the pNL⁺ plasmid proliferate in the presence of glucose but die in the presence of galactose, whereas yeasts with the pNL⁻ plasmid proliferate in both media?

How might you use this system for a selection assay to isolate cells defective in nuclear transport?

12–47 Purified importin in the presence of Ran and GTP promotes uptake of a labeled substrate into nuclei (**Figure 12–7A**). No uptake occurs in the absence of GTP, and Ran alone is unable to promote nuclear uptake. Importin by itself causes a GTP-independent accumulation of substrate at the nuclear periphery, but does not promote nuclear uptake (Figure 12–7A).

To define the steps in the uptake pathway, you first incubate nuclei with substrate in the presence of importin. You then wash away free importin and substrate and incubate a second time with Ran and GTP (**Figure 12–7B**).

A. Why do you think the substrate accumulates at the nuclear periphery, as is seen in the absence of GTP or with importin alone in the presence of GTP?

B. To the extent these data allow, define the order of events that leads to uptake of substrate into the nucleus.

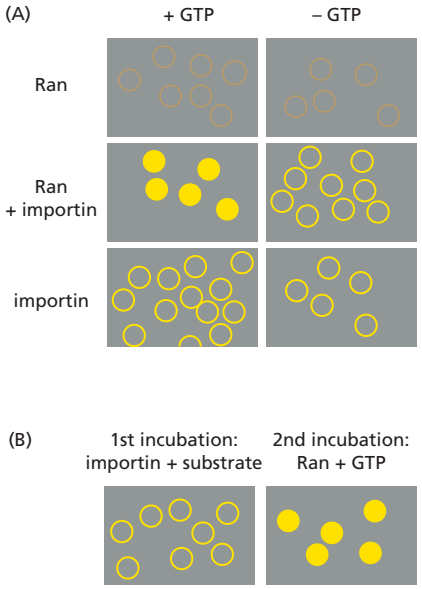


Figure 12–7 Effects of Ran, importin, and GTP on nuclear uptake of fluorescein-labeled substrate (Problem 12–47). (A) Comparison of various combinations of Ran and importin in the presence and absence of GTP. (B) Two-stage incubation of cell remnants with importin and substrate and then with Ran and GTP. *Circles* are the nuclei; *very light circles* are nuclei without bound substrate.

12–48 The structures of Ran-GDP and Ran-GTP (actually Ran-GppNp, a stable GTP analog) are strikingly different, as shown in **Figure 12–8**. Not surprisingly, Ran-GDP binds to a different set of proteins than does Ran-GTP.

To look at the uptake of Ran itself into the nuclei of permeabilized cells, you attach a red fluorescent tag to a cysteine side chain in Ran to make it visible. This modified Ran supports normal nuclear uptake. Fluorescent Ran-GDP is taken up by nuclei only if cytoplasm is added, whereas a mutant form, RanQ69L-GTP, which is unable to hydrolyze GTP, is not taken up in the presence or absence of cytoplasm. To identify the cytoplasmic protein that is crucial for Ran-GDP uptake, you construct affinity columns with bound Ran-GDP or bound RanQ69L-GTP and pass

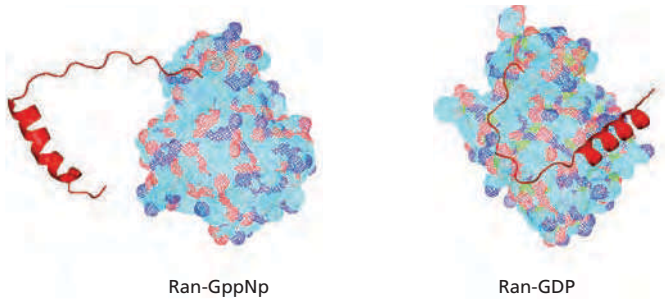


Figure 12–8 Structures of Ran-GDP and Ran-GppNp (Problem 12–48). The segment of Ran shown in *red* displays two dramatically different conformations depending on whether GDP or the GTP analog is bound.

cytoplasm through them. Cytoplasm passed over a Ran-GDP column no longer supports nuclear uptake of Ran-GDP, whereas cytoplasm passed over a column of RanQ69L-GTP retains this activity. You elute the bound proteins from each column and analyze them on an SDS polyacrylamide gel, looking for differences that might identify the factor that is required for nuclear uptake of Ran (**Figure 12–9**).

A. Why did you use RanQ69L-GTP instead of Ran-GTP in these experiments? Could you have used Ran-GppNp instead of RanQ69L-GTP to achieve the same purpose?
B. Which of the many proteins eluted from the two different affinity columns is a likely candidate for the factor that promotes nuclear import of Ran-GDP?
C. What other protein or proteins would you predict the Ran-GDP import factor would bind in order to carry out its function?
D. How might you confirm that the factor you have identified is necessary for promoting the nuclear uptake of Ran?

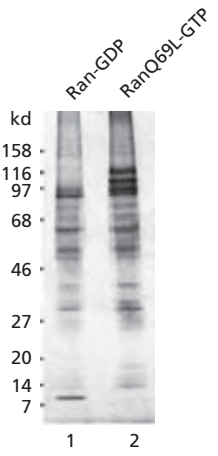


Figure 12–9 Proteins eluted from Ran-GDP (lane 1) and RanQ69L-GTP (lane 2) affinity columns (Problem 12–48). The molecular masses of marker proteins are shown on the *left*.

12–49 The broad-spectrum antibiotic leptomycin B inhibits nuclear export, but how does it work? In the yeast *S. pombe*, resistance to leptomycin B can arise by mutations in the *Crm1* gene, which encodes a nuclear export receptor for proteins with leucine-rich nuclear export signals. To look at nuclear export directly, you modify the green fluorescent protein (GFP) by adding a nuclear export signal (NES). In both wild-type and mutant cells that are resistant to leptomycin B, NES-GFP is found exclusively in the cytoplasm in the absence of leptomycin B (**Figure 12–10**). In the presence of leptomycin B, however, NES-GFP is present in the nuclei of wild-type cells, but in the cytoplasm of mutant cells (Figure 12–10). Is this result the one you would expect if leptomycin B blocked nuclear export? Why or why not?

12–50 Frog oocytes are a useful experimental system for studying nuclear export because they are large cells with large nuclei. It is easy (with practice) to inject oocytes with labeled RNA and to separate the nucleus and cytoplasm to follow the fate of the injected label. You inject a mixture of various ^{32}P-labeled RNA molecules into the nucleus in the presence and absence of leptomycin B to study its effect on nuclear export of RNA. Immediately after injection and three hours later, you analyze total (T), cytoplasmic (C), and nuclear (N) contents by polyacrylamide-gel electrophoresis and autoradiography (**Figure 12–11**).

A. How good was your injection technique? Did you actually inject into the nucleus? Did you rip apart the nuclear envelope when you injected the RNAs? How do you know?
B. Which, if any, of the RNAs are normally exported from the nucleus?
C. Is the export of any of the RNAs inhibited by leptomycin B? What does your answer imply about export of this collection of RNAs?

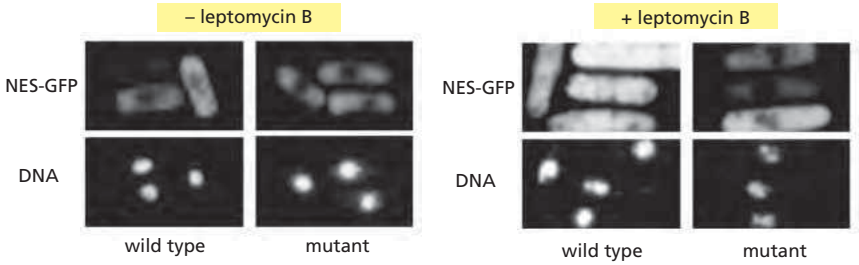


Figure 12–10 Distribution of NES-GFP in *S. pombe* in the presence and absence of leptomycin B (Problem 12–49). *Light areas* in the NES-GFP panels show the position of GFP. *Light areas* in the DNA panels result from a stain that binds to DNA and marks the position of the nuclei in the cells in the NES-GFP panels.

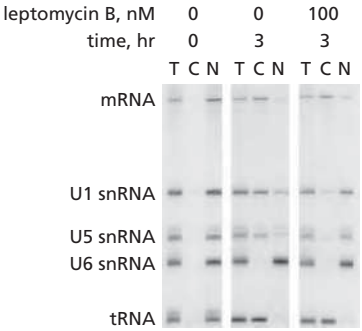


Figure 12–11 Effects of leptomycin B on export of various RNAs from frog oocyte nuclei (Problem 12–50). Total (T), cytoplasmic (C), and nuclear (N) fractions are indicated.

THE TRANSPORT OF PROTEINS INTO MITOCHONDRIA AND CHLOROPLASTS

TERMS TO LEARN

chloroplast
inner membrane
intermembrane space
matrix space
mitochondria
mitochondrial hsp70
mitochondrial precursor protein
outer membrane
OXA complex
porin
protein translocator
SAM complex
stroma
thylakoid
TIM complex
TOM complex

DEFINITIONS

Match the definition below with its term from the list above.

12–51 Membrane-enclosed organelles, about the size of bacteria, that carry out oxidative phosphorylation and produce most of the ATP in eukaryotic cells.

12–52 Part of a multisubunit protein assembly that is bound to the matrix side of the TIM23 complex and acts as a motor to pull the precursor protein into the matrix space.

12–53 Multisubunit protein assembly that transports proteins across the mitochondrial outer membrane.

12–54 The matrix space of a chloroplast.

12–55 The membrane of a mitochondrion that encloses the matrix and is folded into cristae.

12–56 Central subcompartment of a mitochondrion, enclosed by the inner mitochondrial membrane.

12–57 Flattened sac of membrane in a chloroplast that contains the protein subunits of the photosynthetic system and of the ATP synthase.

12–58 Protein encoded by a nuclear gene, synthesized in the cytosol, and subsequently transported into mitochondria.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

12–59 The TOM complex is required for the import of all nucleus-encoded mitochondrial proteins.

12–60 The two signal sequences required for transport of nucleus-encoded proteins into the mitochondrial inner membrane via the TIM23 complex are cleaved off the protein in different mitochondrial compartments.

12–61 Import of proteins into mitochondria and chloroplasts is very similar; even the individual components of their transport machinery are homologous, as befits their common evolutionary origin.

THOUGHT PROBLEMS

12–62 To aid your studies of protein import into mitochondria, you treat yeast cells with cycloheximide, which blocks ribosome movement along mRNA. When you examine these cells in the electron microscope, you are surprised to find cytosolic ribosomes attached to the outside of the mitochondria. You have never seen attached ribosomes in the absence

of cycloheximide. To investigate this phenomenon further, you prepare mitochondria from cells that have been treated with cycloheximide and then extract the mRNA that is bound to the ribosomes associated with the mitochondria. You translate this mRNA *in vitro* and compare the protein products with similarly translated mRNA from the cytosol. The results are clear-cut: the mitochondria-associated ribosomes are translating mRNAs that encode mitochondrial proteins.

You are astounded! Here, clearly visible in the electron micrographs, seems to be proof that protein import into mitochondria occurs during translation. How might you reconcile this result with the prevailing view that mitochondrial proteins are imported only after they have been synthesized and released from ribosomes?

12–63 You have made a peptide that contains a functional mitochondrial import signal. Would you expect the addition of an excess of this peptide to affect the import of mitochondrial proteins? Why or why not?

12–64 Components of the TIM complexes, the multisubunit protein translocators in the mitochondrial inner membrane, are much less abundant than those of the TOM complex. They were initially identified using a genetic trick. The yeast *Ura3* gene, whose product is an enzyme that is normally located in the cytosol where it is essential for synthesis of uracil, was modified so that the protein carried an import signal for the mitochondrial matrix. A population of cells carrying the modified *Ura3* gene in place of the normal gene was then grown in the absence of uracil. Most cells died, but the rare cells that grew were shown to be defective for import into the mitochondrial matrix. Explain how this selection identifies cells with defects in components required for import into the mitochondrial matrix. Why don't normal cells with the modified *Ura3* gene grow in the absence of uracil? Why do cells that are defective for mitochondrial import grow in the absence of uracil?

12–65 Mitochondria normally provide cells with most of the ATP they require to meet their energy needs. Mitochondria that cannot import proteins are defective for ATP synthesis. How is it that cells with import-defective mitochondria can survive at all? How do they get the ATP they need to function?

12–66 Describe in a general way how you might use radiolabeled proteins and proteases to study import processes in isolated, intact mitochondria. What sorts of experimental controls might you include to ensure that the results you obtain mean what you think they do?

12–67 If the enzyme dihydrofolate reductase (DHFR), which is normally located in the cytosol, is engineered to carry a mitochondrial targeting sequence at its N-terminus, it is efficiently imported into mitochondria. If the modified DHFR is first incubated with methotrexate, which binds tightly to the active site, the enzyme remains in the cytosol. How do you suppose that the binding of methotrexate interferes with mitochondrial import?

12–68 Why do mitochondria need a special translocator to import proteins across the outer membrane, when the membrane has already has large pores formed by porins?

CALCULATIONS

12–69 The vast majority of mitochondrial proteins are imported through the outer membrane by the multisubunit protein translocators known as TOM complexes. Since the number of TOM complexes in yeast mitochondria is known (10 pmole/mg of mitochondrial protein), it is possible to calculate whether a co-translational mechanism could account

for the bulk of mitochondrial protein import. If mitochondrial proteins were all imported co-translationally, then 10 pmol of TOM complexes would need to import 1 mg of protein each generation. Given that mitochondria double every 3 hours and that the rate of protein synthesis is 3 amino acids per second (which is therefore the maximum rate of import through a single TOM complex), how many milligrams of mitochondrial protein could 10 pmol of TOM complexes import in one generation? (On average, an amino acid has a mass of 110 daltons.)

DATA HANDLING

12–70 Barnase is a 110-amino-acid bacterial ribonuclease that is often used as a model for studies of protein folding and unfolding. It forms a compact folded structure that has a high energy of activation for unfolding (about 85 kJ/mole). Can such a protein be imported into mitochondria? To the N-terminus of barnase, you add 35, 65, or 95 amino acids from the N-terminus of pre-cytochrome b_2, all of which include the cytochrome's mitochondrial import signal. N35-barnase is not imported, N65-barnase is imported at a low rate, and N95-barnase is imported very efficiently into isolated mitochondria. None of these N-terminal extensions have any measurable effect on the stability of the barnase domain. If these proteins are denatured before testing for import, they are all imported at the same high rate. How do you suppose that longer N-terminal extensions facilitate the import of barnase?

12–71 Are proteins imported into mitochondria as completely unfolded polypeptide chains, or can the translocation apparatus accommodate fully or partially folded structures? That is, is the protein sucked up like a noodle, or is it swallowed whole, as a python devours its prey? It is possible to engineer cysteine amino acids into barnase and then cross-link them to make disulfide bonds either between C5 and C78 or between C43 and C80 (**Figure 12–12**). Import of N95-barnase (see Problem 12–70) was tested in the presence and absence of disulfide cross-links at these two positions. Its import was unaffected by either cross-link. By contrast, import of N65-barnase was blocked by the C5–C78 cross-link but unaffected by the C43–C80 cross-link. Do these results allow you to distinguish between import of extended polypeptide chains or of folded structures? Why or why not?

12–72 Tim23 is a key component of the mitochondrial TIM23 protein translocator complex. The N-terminal half of Tim23 is hydrophilic, while the C-terminal half is hydrophobic and probably spans the membrane four times, as suggested by the hydropathy plot in **Figure 12–13A**. To determine the arrangement of Tim23 in mitochondrial membranes, a protease was added to intact mitochondria or to mitoplasts, which are mitochondria

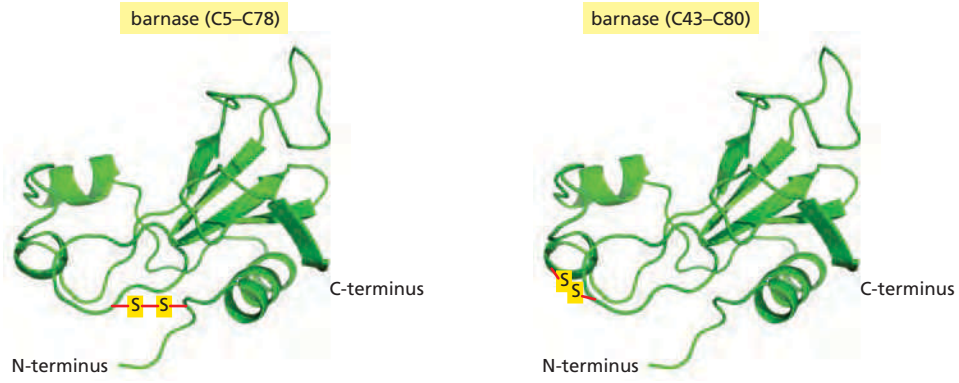


Figure 12–12 Structures of barnase molecules with disulfide bonds between cysteines at positions 5 and 78 (C5–C78) or between positions 43 and 80 (C43–C80) (Problem 12–71).

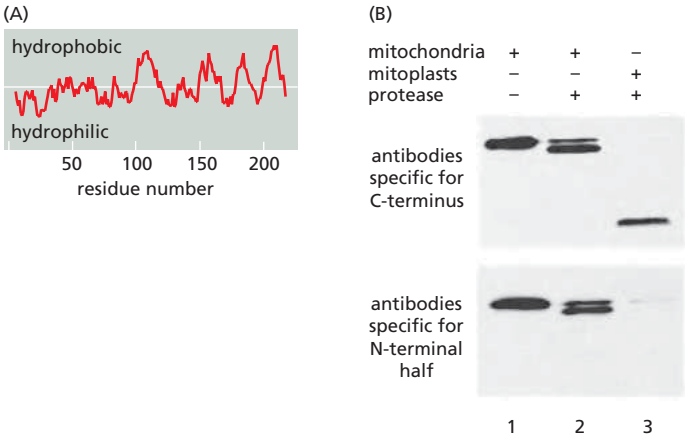


Figure 12–13 Arrangement of Tim23 in mitochondrial membranes (Problem 12–72). (A) Hydropathy plot for Tim23. (B) Sensitivity of Tim23 in mitochondria and mitoplasts to digestion with a protease.

from which the outer membranes have been removed. The mobility of Tim23 was detected on SDS polyacrylamide gels by immunoblotting with antibodies specific for the N-terminal half or for the extreme C-terminus of Tim23 (**Figure 12–13B**). Normal-sized Tim23 was present in both mitochondria and mitoplasts (as shown for mitochondria in Figure 12–13B), but Tim23 was partially digested when mitochondria and mitoplasts were treated with a protease (Figure 12–13B).

A. Is Tim23 an integral component of the inner or outer mitochondrial membrane? Explain your reasoning.
B. To the extent the information in this problem allows, diagram the arrangement of Tim23 in mitochondrial membranes.

PEROXISOMES

TERMS TO LEARN

peroxin peroxisome

DEFINITIONS

Match the definition below with its term from the list above.

12–73 Small membrane-bounded organelle that uses molecular oxygen to oxidize organic molecules.

12–74 One of several proteins that are involved in protein import into peroxisomes.

TRUE/FALSE

Decide whether this statement is true or false, and then explain why.

12–75 Peroxisomes are found in only a few specialized types of eukaryotic cell.

THOUGHT PROBLEMS

12–76 Catalase, an enzyme normally found in peroxisomes, is present in normal amounts in cells that do not have visible peroxisomes. It is possible to determine the location of catalase in such cells using immunofluorescence microscopy with antibodies specific for catalase. Fluorescence micrographs of normal cells and peroxisome-deficient cells are shown in **Figure 12–14**. Where is catalase located in cells without peroxisomes (Figure 12–14B)? Why does catalase show up as small dots of fluorescence in normal cells (Figure 12–14A)?

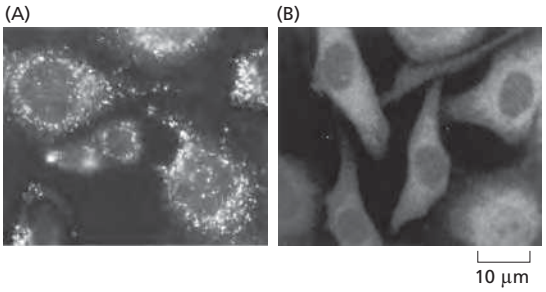


Figure 12–14 Location of catalase in cells as determined by immunofluorescence microscopy (Problem 12–76). (A) Normal cells. (B) Peroxisome-deficient cells. Cells were reacted with antibodies specific for catalase, washed, and then stained with a fluorescein-labeled second antibody that is specific for the catalase-specific antibody. The two panels are at the same magnification; a 10 μm scale bar is shown in (B).

12–77 Cells with functional peroxisomes incorporate 9-(1′-pyrene)nonanol (P9OH) into membrane lipids. Exposure of such cells to ultraviolet (UV) light causes cell death because excitation of the pyrene moiety generates reactive oxygen species, which are toxic to cells. Cells that do not make peroxisomes lack a critical enzyme responsible for incorporating P9OH into membrane lipids. How might you make use of P9OH to select for cells that are missing peroxisomes?

DATA HANDLING

12–78 You have isolated two mutant cell lines that lack typical peroxisomes. When you test these cells for peroxisomal enzymes, you find that catalase activity is virtually the same as in normal cells. By contrast, acyl CoA oxidase activity is absent in both mutant cell lines. To investigate the acyl CoA oxidase deficiency, you perform a pulse-chase experiment: you grow cells for 1 hour in medium containing ^{35}S-methionine, then transfer them to unlabeled medium and immunoprecipitate acyl CoA oxidase at various times after transfer (**Figure 12–15**). You observe two forms of oxidase in normal cells, but only one in the mutant cell lines. To clarify the relationship between the 75 kd and 53 kd forms of the oxidase, you isolate mRNA from wild-type and the mutant cell lines, translate it *in vitro,* and immunoprecipitate acyl CoA oxidase. All three sources of mRNA give similar levels of the 75 kd form, but none of the 53 kd form.

A. How do you think the two forms of acyl CoA oxidase in normal cells are related? Which one, if either, do you suppose is the active enzyme?

B. Why do the mutant cells have only the 75 kd form of acyl CoA oxidase, and why do you think it disappears during the pulse-chase experiment? If you had done a similar experiment with catalase, do you suppose it would have behaved the same way?

12–79 Proteins that are imported into the peroxisome matrix using a C-terminal tripeptide signal are recognized by the cytosolic receptor Pex5, which docks at a complex of proteins in the peroxisomal membrane. Delivery by a cytosolic receptor distinguishes peroxisomal import from import into mitochondria, chloroplasts, and the endoplasmic reticulum. Moreover, unlike import into those organelles, peroxisomes can import fully folded proteins and protein oligomers. You wish to distinguish three potential modes of action for Pex5: (1) it delivers its cargo to the peroxisomal membrane but remains cytosolic; (2) it enters the peroxisome along with its cargo; or (3) it cycles between the cytosol and the peroxisomal matrix.

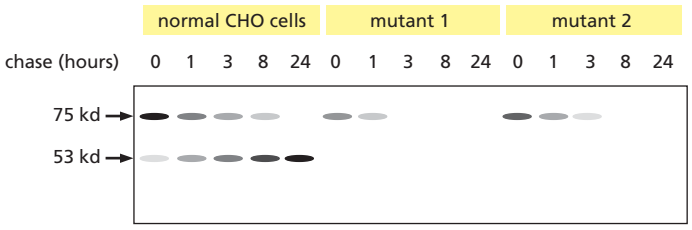


Figure 12–15 Pulse-chase experiments with normal Chinese hamster ovary (CHO) cells and mutant cells (Problem 12–78).

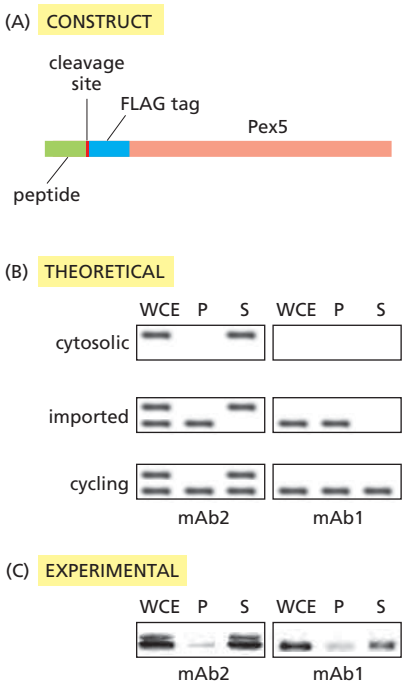


Figure 12–16 Mechanism of Pex5-mediated peroxisomal import (Problem 12–79). (A) Modified Pex5. (B) Expectations for different mechanisms of Pex5-mediated peroxisomal import. (C) Analysis of transfection of the modified *Pex5* gene into cells. "WCE" stands for whole-cell extract, "P" for pellet, and "S" for supernatant. Proteins were detected by immunoblotting using mAb1 or mAb2, followed by reaction with a second antibody that binds mAb1 and mAb2 and also carries bound horseradish peroxidase, which then converts an added precursor into a light-emitting molecule that can be detected on photographic film. *Black bands* correspond to sites where the film has been exposed by the light-emitting molecule. In (B), uncleaved Pex5 *(upper band)* and cleaved Pex5 *(lower band)* are separated by a larger distance than they are in the experiment (C) for clarity.

To define the mechanism for Pex5-mediated import, you modify the *Pex5* gene to encode an N-terminal peptide segment that includes a cleavage site for a protease localized exclusively to the matrix of the peroxisome (**Figure 12–16A**). Immediately adjacent to the cleavage site is a sequence of amino acids (the so-called FLAG tag) that can be recognized by commercial antibodies. One antibody, mAb2, binds the FLAG tag in any context, whereas another, mAb1, binds the FLAG tag only when it is at the N-terminus and thus will detect only cleaved Pex5. By preparing a whole-cell extract (WCE) and fractionating it into a pellet (P), which contains the peroxisomes, and supernatant (S), which contains the cytosol, you can distinguish the three possible mechanisms of Pex5-mediated import—cytosolic, imported, cycling—by using the mAb1 and mAb2 antibodies, as shown in **Figure 12–16B**.

When you express the modified *Pex5* gene in cells, prepare cell fractions, separate the proteins by electrophoresis, and react them with mAb1 and mAb2 antibodies, you obtain the results shown in **Figure 12–16C**.

A. Explain the theoretical results (Figure 12–16B) expected for each of the three possible mechanisms of Pex5-mediated import.
B. Based on the results in Figure 12–16C, how does Pex5 mediate the import of proteins into the matrix of the peroxisome? Explain your reasoning.
C. Pex5-mediated import into peroxisomes resembles most closely import into what other cellular organelle?

MEDICAL LINKS

12–80 Primary hyperoxaluria type 1 (PH1) is a lethal autosomal recessive disease caused by a deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT). About one-third of PH1 patients possess significant levels of AGT protein and enzyme activity. Analysis of these patients shows that their AGT contains two critical single amino acid changes: one that interferes with peroxisomal targeting and a second that allows the N-terminus to form an amphiphilic α helix with a positively charged side. Where do you suppose this mutant AGT is found in cells from these patients? Explain your reasoning.

12–81 Trypanosomes are single-celled parasites that cause sleeping sickness when they infect humans. Trypanosomes from humans carry the enzymes for a portion of the glycolytic pathway in a peroxisomelike organelle, termed the glycosome. By contrast, trypanosomes from the tsetse fly—the intermediate host—carry out glycolysis entirely in the cytosol. This intriguing difference has alerted the interest of the pharmaceutical company that employs you. Your company wishes to exploit this difference to control the disease.

You decide to study the enzyme phosphoglycerate kinase (PGK) because it is in the affected portion of the glycolytic pathway. Trypanosomes from the tsetse fly express PGK entirely in the cytosol, whereas trypanosomes from humans express 90% of the total PGK activity in glycosomes and only 10% in the cytosol. When you clone *PGK* genes from

trypanosomes, you find three forms that differ slightly from one another. Exploiting these small differences, you design three oligonucleotides that hybridize specifically to the mRNAs from each gene. Using these oligonucleotides as probes, you determine which genes are expressed by trypanosomes from humans and from tsetse flies. The results are shown in **Figure 12–17**.

A. Which *PGK* genes are expressed in trypanosomes from humans? Which are expressed in trypanosomes from tsetse flies?
B. Which *PGK* gene probably encodes the glycosomal form of PGK?
C. Do you think that the minor cytosolic PGK activity in trypanosomes from humans is due to inaccurate sorting into glycosomes? Explain your answer.

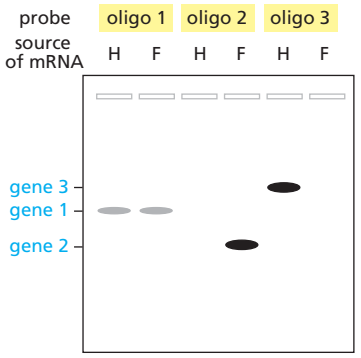


Figure 12–17 Hybridization of specific oligonucleotide probes (oligos) to mRNA isolated from trypanosomes from humans (H) and tsetse flies (F) (Problem 12–81). The intensity of the bands on the autoradiograph reflects the concentrations of the mRNAs.

THE ENDOPLASMIC RETICULUM

TERMS TO LEARN

BiP
calnexin
calreticulin
co-translational
dolichol
endoplasmic reticulum (ER)
ER lumen
ER resident protein
ER retention signal
ER signal sequence
ER tail-anchored protein
free ribosome
glycoprotein
GPI anchor
membrane-bound ribosome
microsome
multipass transmembrane protein
polyribosome
post-translational
protein glycosylation
rough ER
Sec61 complex
signal-recognition particle (SRP)
single-pass transmembrane protein
smooth ER
SRP receptor
start-transfer signal
stop-transfer signal
translocon
unfolded protein response

DEFINITIONS

Match the definition below with its term from the list above.

12–82 Region of the ER not associated with ribosomes.

12–83 Type of lipid linkage by which some proteins are bound to the membrane.

12–84 Labyrinthine membrane-enclosed compartment in the cytoplasm of eukaryotic cells, where lipids are synthesized and membrane-bound proteins and secretory proteins are made.

12–85 Ribonucleoprotein complex that binds an ER signal sequence on a partially synthesized polypeptide chain and directs the polypeptide and its attached ribosome to the ER.

12–86 Hydrophobic amino acid sequence that halts translocation of a polypeptide chain through the ER membrane, thus anchoring the protein chain in the membrane.

12–87 Describes import of a protein into the ER before the polypeptide chain is completely synthesized.

12–88 Short amino acid sequence on a protein that keeps it in the ER.

12–89 Cellular action triggered by an accumulation of misfolded proteins in the ER.

12–90 The protein translocator that forms a water-filled pore in the ER

membrane, allowing passage of a polypeptide chain as it is being synthesized by membrane-bound ribosomes.

12–91 Any protein with one or more oligosaccharide chains covalently linked to amino acid side chains.

12–92 Ribosome in the cytosol that is unattached to any membrane.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

12–93 The signal peptide binds to a hydrophobic site on the ribosome causing a pause in protein synthesis, which resumes when SRP binds to the signal peptide.

12–94 Nascent polypeptide chains are transferred across the ER membrane through a pore in the Sec61 protein translocator complex.

12–95 In multipass transmembrane proteins, the odd-numbered transmembrane segments (counting from the N-terminus) act as start-transfer signals and the even-numbered segments act as stop-transfer signals.

12–96 The ER lumen contains a mixture of thiol-containing reducing agents that prevent the formation of S–S linkages (disulfide bonds) by maintaining the cysteine side chains of luminal proteins in reduced (–SH) form.

THOUGHT PROBLEMS

12–97 Explain how an mRNA molecule can remain attached to the ER membrane while the individual ribosomes translating it are released and rejoin the cytosolic pool of ribosomes after each round of translation.

12–98 Why are cytosolic hsp70 chaperone proteins required for import of proteins into mitochondria and chloroplasts, but not for co-translational import into the ER?

12–99 Where would you expect to see microsomes in an electron micrograph of a liver cell?

12–100 Compare and contrast protein import into the ER and into the nucleus. List at least two major differences in the mechanisms and speculate on why the nuclear mechanism might not work for ER import and vice versa.

12–101 Four membrane proteins are represented schematically in **Figure 12–18**. The boxes represent membrane-spanning segments and the arrows represent sites for cleavage of the signal sequence. Predict how each of the mature proteins will be arranged across the membrane of the ER. Indicate

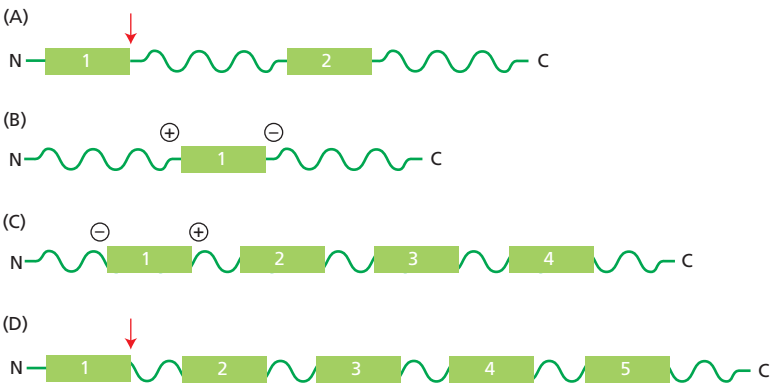


Figure 12–18 Distribution of the membrane-spanning segments in proteins to be inserted into the ER membrane (Problem 12–101). *Boxes* represent membrane-spanning segments and *arrows* indicate sites at which signal sequences are cleaved. The *pluses* and *minuses* indicate the charged amino acids at the ends of some transmembrane segments.

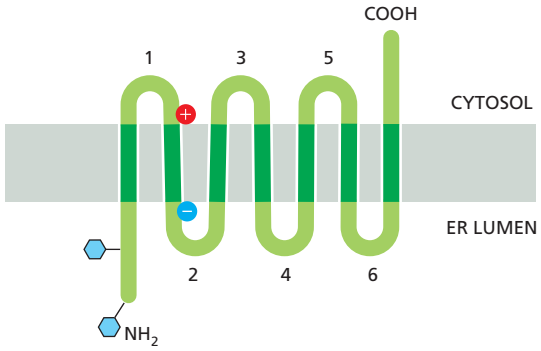


Figure 12–19 Arrangement of a multipass transmembrane protein in the ER membrane (Problem 12–102). *Blue hexagons* represent covalently attached oligosaccharides. The positions of positively and negatively charged amino acids flanking the second transmembrane segment are shown.

clearly the N- and C-termini relative to the cytosol and the lumen of the ER, and label each box as a start-transfer or stop-transfer signal.

12–102 Examine the multipass transmembrane protein shown in **Figure 12–19**. What would you predict would be the effect of converting the first hydrophobic transmembrane segment to a hydrophilic segment? Sketch the arrangement of the modified protein in the ER membrane.

12–103 Why might it be advantageous to add a preassembled block of 14 sugars to a protein in the ER, rather than building the sugar chains step-by-step on the surface of the protein by the sequential addition of sugars by individual enzymes?

12–104 Outline the steps by which misfolded proteins in the ER trigger synthesis of additional ER chaperone proteins. How does this response benefit the cell?

12–105 All new phospholipids are added to the cytosolic leaflet of the ER membrane, yet the ER membrane has a symmetrical distribution of different phospholipids in its two leaflets. By contrast, the plasma membrane, which receives all its membrane components ultimately from the ER, has a very asymmetrical distribution of phospholipids in the two leaflets of its lipid bilayer. How is the symmetry generated in the ER membrane, and how is the asymmetry generated and maintained in the plasma membrane?

DATA HANDLING

12–106 Translocation of proteins across rough microsomal membranes can be judged by several experimental criteria: (1) the newly synthesized proteins are protected from added proteases, unless detergents are present to solubilize the lipid bilayer; (2) the newly synthesized proteins are glycosylated by oligosaccharide transferases, which are localized exclusively to the lumen of the ER; (3) the signal peptides are cleaved by signal peptidase, which is active only on the luminal side of the ER membrane.

Use these criteria to decide whether a protein is translocated across rough microsomal membranes. The mRNA is translated into protein in a cell-free system in the absence or presence of microsomes. Samples of newly synthesized proteins are treated in four different ways: (1) no treatment, (2) addition of a protease, (3) addition of a protease and detergent, and (4) disruption of microsomes and addition of endoglycosidase H (endo H), which removes *N*-linked sugars that are added in the ER. An electrophoretic analysis of these samples is shown in **Figure 12–20**.

A. Explain the experimental results that are seen in the absence of microsomes (Figure 12–20, lanes 1 to 4).

B. Using the three criteria outlined in the problem, decide whether the experimental results in the presence of microsomes (Figure 12–20, lanes 5 to 8) indicate that the protein is translocated across microsomal

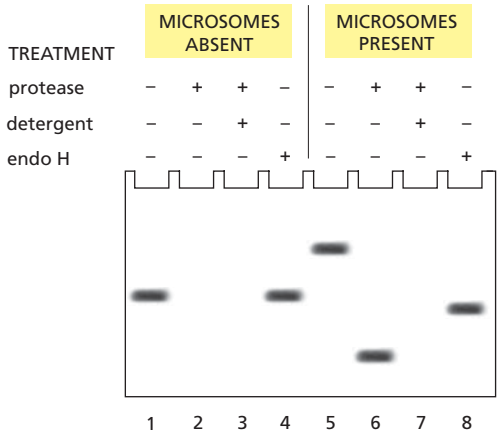


Figure 12–20 Results of translation of a pure mRNA in the presence and absence of microsomal membranes (Problem 12–106). Treatments of the products of translation before electrophoresis are indicated at the *top* of each lane. Electrophoresis was on an SDS polyacrylamide gel, which separates proteins on the basis of size, with lower molecular weight proteins migrating farther down the gel.

membranes. How would you account for the migration of the proteins in Figure 12–20, lanes 5, 6, and 8?

C. Is the protein anchored in the membrane, or is it translocated all the way through the membrane?

12–107 Things are not going well. You've just had a brief but tense meeting with your research advisor that you will never forget, and it is clear that your future in his lab is in doubt. He is not fond of your habit of working late and sleeping late; he thinks it is at the root of your lack of productivity (as he perceives it). A few days after the meeting, you roll in at the bright and early hour of noon, to be informed by your student colleagues that your advisor is "looking all over for you." You are certain the axe is going to fall. But it turns out he is excited, not upset. He's just heard a seminar that reminded him of the note you'd left on his desk the month before, describing a selection scheme you had crafted (late one night, of course) for isolating mutants in the ER translocation machinery. You are completely flabbergasted.

You quickly settle on the details for the selection, which involves fusing an ER import signal to the N-terminus of the *His4* gene product. His4 is a cytosolic enzyme that converts histidinol to histidine. Yeast strains that are defective for an early step in the histidine biosynthetic pathway can grow on added histidinol, if His4 is present. You decide to look for temperature-sensitive (*ts*) mutants, which are normal at 24°C but defective at 37°C. Using a strain that expresses His4 with an ER import signal, you select for cells that grow on histidinol at 30°C and screen them for ones that die at 37°C. The first mutant you isolate is in the *Sec61* gene (later shown to encode a principal component of the translocator through which ribosomes insert nascent proteins across the ER membrane.) You are back in your advisor's good graces!

A. Why is it that normal cells with the modified His4 cannot grow on histidinol, whereas cells with a defective ER-import apparatus can?

B. Why did the selection scheme set out to find *ts* mutants? Why was selection applied at the intermediate temperature of 30°C, rather than at 24°C or 37°C?

12–108 A classic paper describes a genetic method for determining the organization of a bacterial protein in the membrane of *E. coli*. The hydropathy plot of the protein in **Figure 12–21** indicated three potential membrane-spanning segments. Hybrid fusion proteins of different lengths, some with internal deletions, were made with the membrane protein at the N-terminus and alkaline phosphatase at the C-terminus (**Figure 12–22**). Alkaline phosphatase is easy to assay in whole cells and has no significant

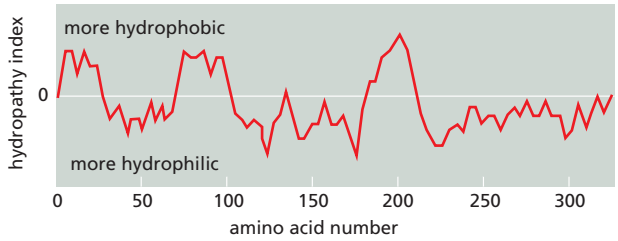


Figure 12–21 Hydropathy plot of a membrane protein (Problem 12–108). The three hydrophobic peaks indicate the positions of three potential membrane-spanning segments.

hydrophobic stretches. Moreover, when it is on the cytoplasmic side of the membrane its activity is low, and when it is on the external side of the membrane (in the periplasmic space) its activity is high. The assayed levels of alkaline phosphatase activity are indicated (HIGH or LOW) in Figure 12–22.

A. How is the protein organized in the membrane? Explain how the results with the fusion proteins indicate this arrangement.

B. How is the organization of the membrane protein altered by the deletion? Are your measurements of alkaline phosphatase activity in the internally deleted plasmids consistent with the altered arrangement?

12–109 Mitochondria and peroxisomes, as opposed to most other cellular membranes, acquire new phospholipids via phospholipid exchange proteins. One such protein, PC exchange protein, specifically transfers phosphatidylcholine (PC) between membranes. Its activity can be measured by mixing red blood cell ghosts (intact plasma membranes with cytoplasm removed) with synthetic phospholipid vesicles containing radioactively labeled PC in both monolayers of the vesicle bilayer. After incubation at 37°C, the mixture is centrifuged briefly so that ghosts form a pellet, whereas the vesicles stay in the supernatant. The amount of exchange is determined by measuring the radioactivity in the pellet.

Figure 12–23 shows the result of an experiment along these lines, using labeled (donor) vesicles with an outer radius of 10.5 nm and a bilayer 4.0 nm in thickness. No transfer occurred in the absence of the exchange protein, but in its presence up to 70% of the labeled PC in the vesicles could be transferred to the red cell membranes.

Several control experiments were performed to explore the reason why only 70% of the label in donor vesicles was transferred.

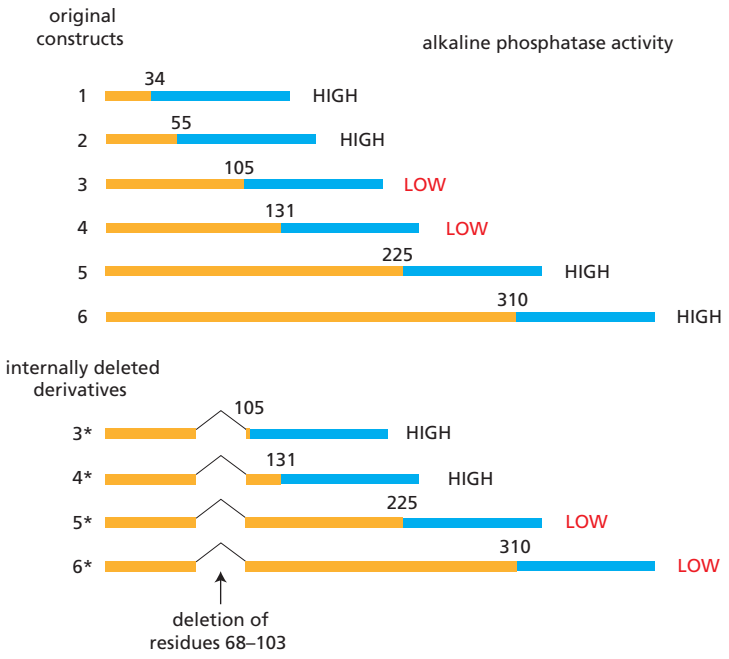


Figure 12–22 Structures of hybrid proteins used to determine the organization of a membrane protein (Problem 12–108). The membrane protein *(orange segment)* is at the N-terminus and alkaline phosphatase *(blue segment)* is at the C-terminus of the protein. The *inverted V* indicates the site from which amino acids were deleted from modified hybrid proteins. The most C-terminal amino acid of the membrane protein is *numbered* in each hybrid protein. The activity of alkaline phosphatase in each hybrid protein is shown on the *right*.

1. Five times as many membranes from red cell ghosts were included in the incubation: the transfer still stopped at the same point.
2. Fresh exchange protein was added after 1 hour: it caused no further transfer.
3. The labeled lipids remaining in donor vesicles at the end of the reaction were extracted and made into fresh vesicles: 70% of the label in these vesicles was exchangeable.

When the red cell ghosts that were labeled in this experiment were used as donor membranes in the reverse experiment (that is, transfer of PC from red cell membranes to synthetic vesicles), 96% of the label could be transferred to the acceptor vesicles.

A. What possible explanations for the 70% limit do each of the three control experiments eliminate?
B. What do you think is the explanation for the 70% limit? (Hint: the area of the outer surface of these small donor vesicles is about 2.6 times larger than the area of the inner surface.)
C. Why do you think that almost 100% of the label in the red cell membrane can be transferred back to the vesicle?

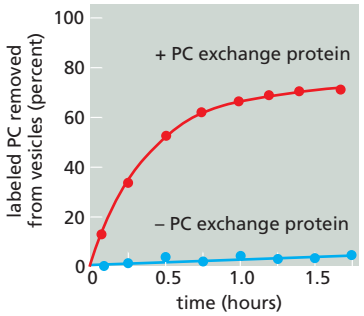


Figure 12–23 Transfer of labeled PC from donor vesicles to red cell membranes by PC exchange protein (Problem 12–109).

MCAT STYLE

Passage 1 (Questions 12–110 to 12–112)

You are studying a transcription factor that controls entry into the cell cycle. It is present in the nucleus in nondividing cells, but rapidly exits the nucleus when cells are stimulated to begin cell division. You hypothesize that the transcription factor represses expression of genes that drive entry into the cell cycle, and that transport of the repressor out of the nucleus is a critical step that helps initiate the cell cycle. While searching for the signals that control this step, you find that the protein is phosphorylated by a protein kinase at a specific serine. Since phosphorylation of proteins is an important mode of regulation, you investigate further. To do so, you create a mutant version of the transcription factor in which the serine that is the target for phosphorylation is changed to an alanine, which cannot be phosphorylated. When you express this mutant version of the transcription factor in the cell, you find that very little of it can be detected in the nucleus.

12–110 Transport of the transcription factor into the nucleus most likely requires:

I. Binding to a nuclear import receptor
II. Direct participation of Ran-GDP
III. Involvement of the Sec61 complex

A. I
B. I and II
C. I and III
D. I, II, and III

12–111 Which one of the following hypotheses best explains how phosphorylation regulates nuclear localization of the transcription factor?

A. Phosphorylation causes a conformational change that exposes a nuclear export signal.
B. Phosphorylation near a nuclear export signal blocks binding of a nuclear export factor.
C. Phosphorylation near a nuclear localization signal blocks binding of a nuclear import factor.
D. Phosphorylation promotes binding of the transcription factor to Ran-GTP.

12–112 How might you test the best hypothesis from the previous question?

A. Addition of a nuclear import signal to the mutant transcription factor should allow it to accumulate in the nucleus.
B. Deletion of the nuclear export signal from the mutant transcription factor should allow it to accumulate in the nucleus.

C. Deletion of the Ran-GDP binding site from the mutant transcription factor should allow it to accumulate in the nucleus.
D. Inactivation of the Ran-GTPase should allow the normal—but not the mutant—transcription factor to enter the nucleus.

Passage 2 (Questions 12–113 to 12–115)

When cells are broken open, the endoplasmic reticulum (ER) fragments into spherical vesicles coated with ribosomes, which are called rough microsomes. These rough microsomes can be purified and used to form a lipid bilayer that separates two liquid-filled chambers. One side of the lipid bilayer is coated with ribosomes and corresponds to the cytosolic side of the ER, while the other corresponds to the luminal side of the ER. The chambers on each side of the membrane can be monitored to detect conductance of salt ions across the membrane. No conductance can be detected across the rough microsomal membrane as it is isolated from cells. However, treatment of the ribosome-coated side of the membrane with puromycin, a compound that causes release of growing peptide chains from ribosomes, causes a large increase in conductance. Further treatment with high-salt buffers causes the membrane to be impermeable to ions again.

12–113 Association of ribosomes with the endoplasmic reticulum requires:
I. Signal-recognition particle
II. Chaperones
III. Protein translation

A. I
B. I and III
C. II and III
D. I, II, and III

12–114 Which one of the following statements best explains the increase in conductance caused by puromycin?
A. Premature termination of peptide synthesis by puromycin leads to improperly folded proteins that activate the unfolded protein response.
B. Release of the growing peptide exposes the water-filled pore that is used for translocation of proteins across the ER membrane.
C. Release of the peptide allows the signal-recognition particle to open a channel for translocation of peptides across the ER membrane.
D. Treatment with puromycin opens channels in the membrane that are normally used to release Ca^{2+} from the ER.

12–115 Why does washing the membrane with a buffer containing high concentrations of salt block the conductance of the microsomal membrane?
A. High salt causes the Ca^{2+} channel to close.
B. High salt increases the resistance of the membrane.
C. High-salt removal of ribosomes closes the pore.
D. High salt shuts off the unfolded protein response.

CHAPTER 13

Intracellular Membrane Traffic

THE MOLECULAR MECHANISMS OF MEMBRANE TRANSPORT AND THE MAINTENANCE OF COMPARTMENTAL DIVERSITY

TERMS TO LEARN

adaptor protein
ARF protein
cargo
clathrin
clathrin-coated vesicle
coated vesicle
coat-recruitment GTPase
COPI-coated vesicle
COPII-coated vesicle
dynamin
lumen
NSF
phosphatidylinositide (PIP)
Rab cascade
Rab effector
Rab protein
Sar1 protein
SNARE protein (SNARE)
transport vesicle
t-SNARE
v-SNARE

DEFINITIONS

Match each definition below with its term from the list above.

13–1 General term for a membrane-enclosed container that moves material between membrane-enclosed compartments within the cell.

13–2 Any of a large family of monomeric GTPases present in the plasma membrane and organelle membranes that confer specificity on vesicle docking.

13–3 A protein that mediates binding between the clathrin coat and transmembrane proteins, including transmembrane cargo receptors.

13–4 Cytosolic GTPase that binds to the neck of a clathrin-coated vesicle and helps it to pinch off from the membrane.

13–5 The protein that catalyzes the disassembly of the helical domains of paired SNARE proteins.

13–6 Coated vesicle that transports material from the plasma membrane and between endosomal and Golgi compartments.

13–7 The coat-recruitment GTPase responsible for both COPI coat assembly and clathrin coat assembly at Golgi membranes.

13–8 Protein that facilitates vesicle transport, docking, and membrane fusion once it is bound by an activated Rab protein.

13–9 General term for a member of the large family of proteins that catalyze the membrane fusion reactions in membrane transport.

13–10 The interior space of a membrane-enclosed compartment.

13–11 General term for a transport vesicle that carries a distinctive cage of proteins covering its cytosolic surface.

IN THIS CHAPTER

13–12 The coat-recruitment GTPase responsible for COPII coat assembly at the ER membrane.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

13–13 In all events involving fusion of a vesicle to a target membrane, the cytosolic leaflets of the vesicle and target bilayers always fuse together, as do the leaflets that are not in contact with the cytosol.

13–14 Complementary Rab proteins on transport vesicles and target membranes bind to one another to allow transport vesicles to dock selectively at their appropriate target membranes.

THOUGHT PROBLEMS

13–15 In a nondividing cell such as a liver cell, why must the flow of membrane between compartments be balanced, so that the retrieval pathways match the outward flow? Would you expect the same balanced flow in a gut epithelial cell, which is actively dividing?

13–16 The diagram in **Figure 13–1** shows the various intracellular compartments involved in the biosynthetic-secretory, endocytic, and retrieval pathways.

A. Label the various compartments in the diagram.
B. Indicate on the arrows whether the indicated flow is part of the biosynthetic-secretory pathway, the endocytic pathway, or a retrieval pathway.

13–17 Discuss the following analogy: "Cargo receptors competing to be transported by the coated-pit system can be compared to skiers joining a cable-car network. Entry is permitted to ticket holders only, but there is no guarantee of who is found with whom in a particular cable car, although all travelers, hopefully, will reach the next station."

13–18 The clathrin coat on a vesicle is made up of numerous triskelions that form a cage 60–200 nm in diameter, composed of both pentagonal and hexagonal faces, just like C60 fullerene (**Figure 13–2**). Sketch the location of an individual triskelion in the clathrin-coated vesicle in Figure 13–2B.

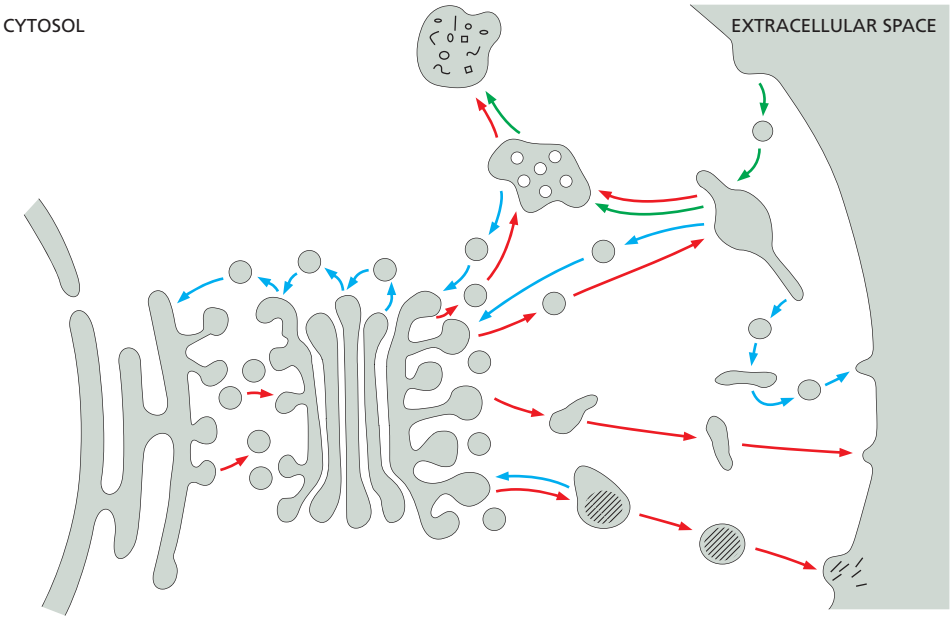


Figure 13–1 The intracellular compartments in the biosynthetic-secretory, endocytic, and retrieval pathways, with flow between compartments indicated (Problem 13–16).

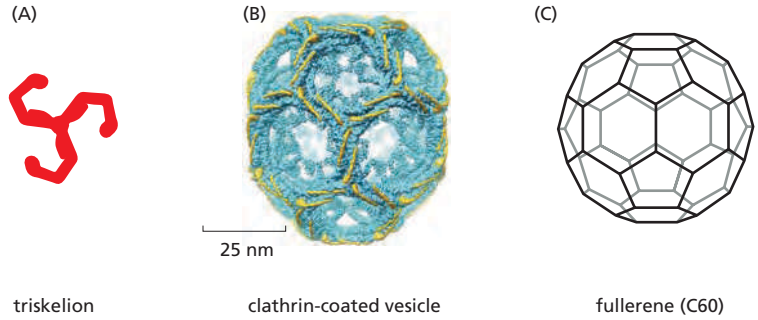


Figure 13–2 Structure of a clathrin coat (Problem 13–18). (A) A triskelion subunit. (B) A clathrin-coated vesicle. (C) A C60 fullerene.

At what point in its structure does a triskelion have to be most flexible to accommodate changes in size of the vesicle? At what point does it have to be most flexible to fit into both the pentagonal and hexagonal faces?

13–19 Yeast, and many other organisms, make a single type of clathrin heavy chain and a single type of clathrin light chain; thus, they make a single kind of clathrin coat. How is it, then, that a single clathrin coat can be used for three different transport pathways—Golgi to late endosomes, plasma membrane to early endosomes, and immature secretory vesicles to Golgi—that each involves different specialized cargo proteins?

13–20 Imagine that ARF1 protein was mutated so that it could not hydrolyze GTP, regardless of its binding partners. Would you expect COPI-coated vesicles to form normally? How would you expect transport mediated by COPI-coated vesicles to be affected? If this were the only form of ARF1 in a cell, would you expect it to be lethal? Explain your answers.

13–21 How can it possibly be true that complementary pairs of specific SNAREs uniquely mark vesicles and their target membranes? After vesicle fusion, the target membrane will contain a mixture of t-SNAREs and v-SNAREs. Initially, these SNAREs will be tightly bound to one another, but NSF can pry them apart, reactivating them. What do you suppose prevents target membranes from accumulating a population of v-SNAREs equal to or greater than their population of t-SNAREs?

13–22 Viruses are the ultimate scavengers—a necessary consequence of their small genomes. Wherever possible, they make use of the cell's machinery to accomplish the steps involved in their own reproduction. Many different viruses have membrane coverings. These so-called enveloped viruses gain access to the cytosol by fusing with a cell membrane. Why do you suppose that each of these viruses encodes its own special fusion protein, rather than making use of a cell's SNAREs?

CALCULATIONS

13–23 For fusion of a vesicle with its target membrane to occur, the membranes have to be brought to within 1.5 nm so that the two bilayers can join (**Figure 13–3**). Assuming that the relevant portions of the two membranes at the fusion site are circular regions 1.5 nm in diameter, calculate the number of water molecules that would remain between the membranes. (Water is 55.5 M and the volume of a cylinder is $\pi r^2 h$.) Given that an average phospholipid occupies a membrane surface area of 0.2 nm^2, how many phospholipids would be present in each of the opposing monolayers at the fusion site? Are there sufficient water molecules to bind to the hydrophilic head groups of this number of phospholipids? (It is estimated that 10–12 water molecules are normally associated with each phospholipid head group at the exposed surface of a membrane.)

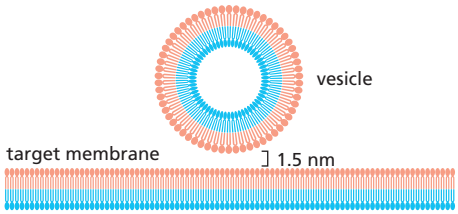


Figure13–3 Close approach of a vesicle and its target membrane in preparation for fusion (Problem 13–23).

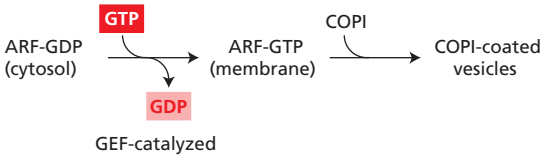


Figure 13–4 Normal pathway for formation of COPI-coated vesicles (Problem 13–24). The small GTPase ARF carries a bound GDP in its cytosolic form. In response to a guanine nucleotide exchange factor (GEF), ARF releases GDP and picks up GTP. Binding of GTP causes a conformational change that exposes a fatty acid tail on ARF, which promotes binding of ARF-GTP to the membrane. COPI subunits bind to ARF-GTP to form COPI-coated vesicles.

DATA HANDLING

13–24 When the fungal metabolite brefeldin A is added to cells, the Golgi apparatus largely disappears and the Golgi proteins intermix with those in the ER. Brefeldin-A treatment also causes the rapid dissociation of some Golgi-associated peripheral membrane proteins, including subunits of the COPI coat. These observations imply that brefeldin A prevents transport involving COPI-coated vesicles by blocking the assembly of coats, and thus the budding of transport vesicles. In principle, brefeldin A could block formation of COPI-coated vesicles at any point in the normal scheme for assembly, which is shown in **Figure 13–4**. The following observations identify the point of action of brefeldin A.

1. ARF with bound GTPγS (a nonhydrolyzable analog of GTP) causes COPI-coated vesicles to form when added to Golgi membranes. Formation of vesicles in this way is not affected by brefeldin A.
2. ARF with bound GDP exchanges the GDP for GTP when added to Golgi membranes. This exchange reaction does not occur in the presence of brefeldin A. The essential component in the Golgi membrane is sensitive to trypsin digestion, suggesting that it is a protein.

Given these experimental observations, how do you think brefeldin A blocks formation of COPI-coated vesicles?

13–25 Small GTPases are generally active in the GTP-bound state and inactive when the GTP is hydrolyzed to GDP. In the absence of a GTPase-activating protein (GAP), small GTPases typically hydrolyze GTP very slowly. The mechanism by which a GAP stimulates GTP hydrolysis is known for the small GTPase Ras. When Ras-GAP binds to Ras, it alters the conformation of Ras and provides a critical, catalytic arginine "finger" that stabilizes the transition state for GTP hydrolysis, thereby stimulating hydrolysis by several orders of magnitude.

During assembly of COPI-coated vesicles, ARF1—a small GTPase—binds to ARF1-GAP, which locks ARF1 into its active catalytic conformation but does not supply the catalytic arginine. Since COPI subunits also bind to ARF1, you wonder if they might affect GTP hydrolysis. To test this possibility, you mix ARF1, ARF1-GAP, and COPI subunits in various combinations and measure GTP hydrolysis (**Table 13–1**).

How would you interpret these results? How might you further test your conclusions?

13–26 SNAREs exist as complementary partners that carry out membrane fusions between appropriate vesicles and their target membranes. In this way, a vesicle with a particular variety of v-SNARE will fuse only with a

TABLE 13–1 Rates of GTP hydrolysis by various combinations of ARF1, ARF1-GAP, and COPI subunits (Problem 13–25).

Components added	Rate of GTP hydrolysis
ARF1	0
ARF1 + ARF1-GAP	1
ARF1 + COPI subunits	0
ARF1 + ARF1-GAP + COPI subunits	1000

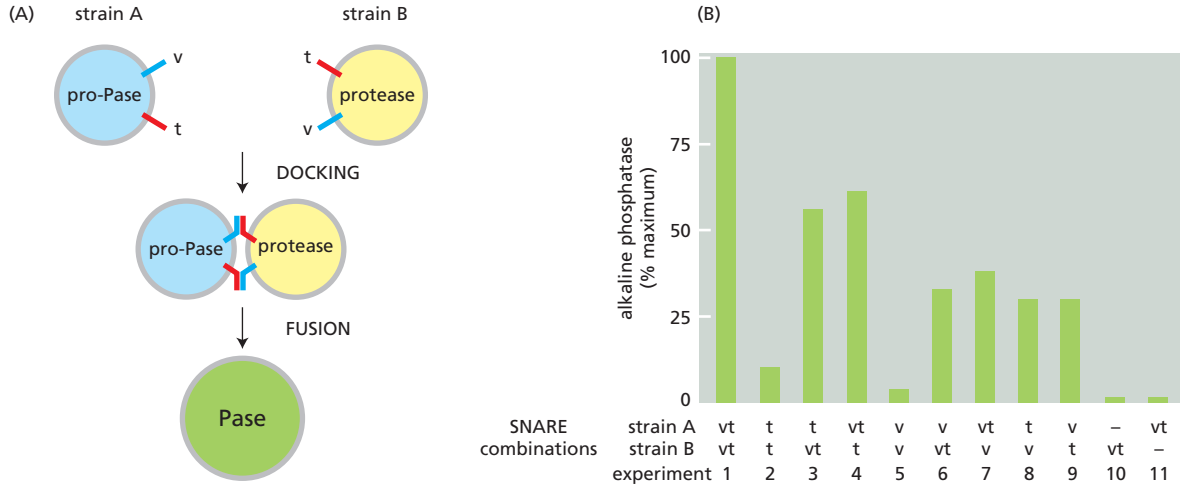


Figure 13–5 SNARE requirements for vesicle fusion (Problem 13–26). (A) Scheme for measuring the fusion of vacuolar vesicles. (B) Results of fusions of vesicles with different combinations of v-SNAREs and t-SNAREs. The SNAREs present on the vesicles of the two strains are indicated as v (v-SNARE) and t (t-SNARE).

membrane that carries the complementary t-SNARE. In some instances, however, fusions of identical membranes (homotypic fusions) are known to occur. For example, when a yeast cell forms a bud, vesicles derived from the mother cell's vacuole move into the bud where they fuse with one another to form a new vacuole. These vesicles carry both v-SNAREs and t-SNAREs. Are both types of SNAREs essential for this homotypic fusion event?

To test this point, you have developed an ingenious assay for fusion of vacuolar vesicles. You prepare vesicles from two different mutant strains of yeast: strain B has a defective gene for vacuolar alkaline phosphatase (Pase); strain A is defective for the protease that converts the precursor of alkaline phosphatase (pro-Pase) into its active form (Pase) (**Figure 13–5A**). Neither strain has active alkaline phosphatase, but when extracts of the strains are mixed, vesicle fusion generates active alkaline phosphatase, which can be easily measured (Figure 13–5).

Now you delete the genes for the vacuolar v-SNARE, t-SNARE, or both in each of the two yeast strains. You prepare vacuolar vesicles from each and test them for their ability to fuse, as measured by the alkaline phosphatase assay (**Figure 13–5B**).

What do these data say about the requirements for v-SNAREs and t-SNAREs in the fusion of vacuolar vesicles? Does it matter which kind of SNARE is on which vesicle?

13–27 You wish to identify the target proteins that are bound by NSF and its two accessory proteins. You incubate purified NSF and its accessory proteins with a crude detergent extract of synaptic membranes, and then add NSF-specific antibodies that are attached to beads. By centrifuging the mixture, you can readily separate the beads, and any attached proteins, from the rest of the crude extract. The proteins attached to the beads can be analyzed by SDS polyacrylamide-gel electrophoresis. When the incubation is carried out in the presence or absence of ATP, you find that NSF alone is present on the beads. If you incubate in the presence of ATPγS, a nonhydrolyzable analog of ATP, the beads bring down NSF, its accessory proteins, syntaxin, SNAP25, and synaptobrevin. What is the substrate for NSF and its accessory proteins? Why does the experiment work when ATPγS is present, but not in the presence or absence of ATP?

13–28 The *Sec4* gene of budding yeast encodes a small GTPase that plays an essential role in the secretion pathway that forms the daughter bud. Normally, about 80% of the Sec4 protein is found on the cytosolic surface of transport vesicles and 20% is free in the cytosol. When

temperature-sensitive *Sec4* mutants of yeast (*Sec4*^{ts}) are incubated at high temperature, growth ceases and small vesicles accumulate in the daughter bud.

To define the role of Sec4 in secretion, you engineer two specific *Sec4* mutants based on the way other small GTPases work. One mutant, *Sec4-ccΔ*, lacks two cysteines at its C-terminus, which you expect will prevent attachment of the fatty acid required for membrane binding. The second mutant, *Sec4N133I*, encodes an isoleucine in place of the normal asparagine at position 133; you expect that this protein will be locked into its active state, even though it should not be able to bind GTP or GDP.

You find that Sec4-ccΔ binds GTP but remains entirely cytosolic with none bound to vesicles. When expressed at high levels in yeast that also have a normal *Sec4* gene, it does not inhibit their growth. In contrast, Sec4N133I is located almost entirely on vesicles, and when it is expressed at high levels in normal yeast, it completely inhibits growth, and the yeast are found to be packed with small vesicles.

A. Do you think Sec4 is required for formation of vesicles, for vesicle fusion with target membranes, or for both? Based on its function, would you guess it was analogous to mammalian ARF, Sar1, or Rab proteins?
B. Using your knowledge of the way the analogous mammalian protein works, outline how you think normal Sec4 functions in vesicle formation and fusion. Why is some Sec4 free in the cytosol of wild-type cells? How does removal of the C-terminal cysteines prevent Sec4-ccΔ from carrying out its function?
C. Why do you think expression of Sec4N133I inhibits growth of the yeast that also express normal Sec4?

TRANSPORT FROM THE ER THROUGH THE GOLGI APPARATUS

TERMS TO LEARN

cis face
cis Golgi network (CGN)
cisternal maturation model
complex oligosaccharide
Golgi apparatus (Golgi complex)
high-mannose oligosaccharide
O-linked glycosylation
proteoglycan
trans face
trans Golgi network (TGN)
vesicle transport model

DEFINITIONS

Match each definition below with its term from the list above.

13–29 The hypothesis that new cisternae form continuously at the cis face of the Golgi and then migrate through the stack as they mature.

13–30 Molecule consisting of one or more glycosaminoglycan chains attached to a core protein.

13–31 The side of the Golgi stack at which material enters the organelle.

13–32 Chain of sugars attached to a glycoprotein that is generated by initially trimming the original oligosaccharide attached in the ER and by then adding other sugars.

13–33 Membrane-enclosed organelle in eukaryotic cells in which proteins and lipids transferred from the ER are modified and sorted.

13–34 Chain of sugars attached to a glycoprotein that contains many mannose residues.

13–35 Meshwork of interconnected cisternae and tubules on the side of the Golgi stack at which material is transferred out of the Golgi.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

13–36 There is one strict requirement for the exit of a protein from the ER: it must be correctly folded.

13–37 All of the glycoproteins and glycolipids in intracellular membranes have their oligosaccharide chains facing the lumenal side, and all those in the plasma membrane have their oligosaccharide chains facing the outside of the cell.

13–38 The Golgi apparatus confers the heaviest glycosylation of all on proteoglycan core proteins, which are converted into proteoglycans by the addition of one or more *O*-linked glycosaminoglycan chains.

THOUGHT PROBLEMS

13–39 How is it that soluble proteins in the ER can be selectively recruited into vesicles destined for the Golgi?

13–40 Isn't quality control always a good thing? How can quality control in the ER be detrimental to cystic fibrosis patients?

13–41 The C-terminal 40 amino acids of three ER resident proteins—calnexin, calreticulin, and HMG CoA reductase—are shown in **Figure 13–6**. Decide for each protein whether it is likely to be a transmembrane protein or a soluble one. Explain your answers.

13–42 If you were to remove the ER retrieval signal from protein disulfide isomerase (PDI), which is normally a soluble resident of the ER lumen, where would you expect the modified PDI to be located?

13–43 The KDEL receptor must shuttle back and forth between the ER and the Golgi apparatus in order to accomplish its task of ensuring that soluble ER proteins are retained in the ER lumen. In which compartment does the KDEL receptor bind its ligands more tightly? In which compartment does it bind its ligands more weakly? What is thought to be the basis for its different binding affinities in the two compartments? If you were designing the system, in which compartment would you have the highest concentration of KDEL receptor? Would you predict that the KDEL receptor, which is a transmembrane protein, would itself possess an ER retrieval signal?

13–44 When the KDEL retrieval signal is added to rat growth hormone or human chorionic gonadotropin, two proteins that are normally secreted, the proteins are still secreted, but about six times more slowly. If the C-terminal L in the signal is changed to V, the proteins are once again secreted at their normal rate. By contrast, bona fide ER resident proteins rarely, if ever, are secreted from the cell; they are usually captured and returned very efficiently. How is it, do you suppose, that normal resident proteins with a KDEL signal are efficiently retained in the ER, whereas secreted proteins to which a KDEL signal has been added are not efficiently retained? Is this what you would expect if the KDEL signal and the KDEL receptor accounted entirely for retention of soluble proteins in the ER?

Calnexin C-terminus

```
...KDKGDEEEEGEEKLEEKQKSDAEEDGGTVSQEEEDRKPKAEEDEILNRSPRNRKPRRE
```

Calreticulin

```
...KQDEEQRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL
```

HMG CoA reductase

```
...PGENARQLARIVCGTVMAGELSLMAALAAGHLVKSHMIHNRSKINLQDLQGACTKKTA
```

Figure 13–6 C-terminal amino acids of proteins that are residents of the ER (Problem 13–41).

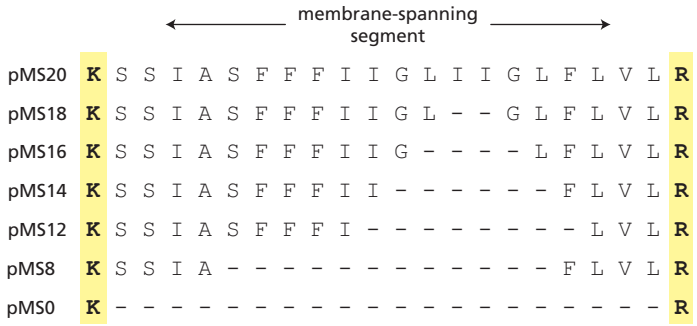


Figure 13–7 The membrane-spanning domains of normal and mutant VSV G proteins (Problem 13–46). Plasmid numbers indicate the number of amino acids in the membrane-spanning segment; for example, pMS20 contains the wild type, 20-amino-acid segment. *Dashed lines* indicate amino acids that are missing in the other plasmids. *Highlighted letters* indicate the basic amino acids that flank the membrane-spanning segment.

13–45 Cells have evolved a set of complicated pathways for addition of carbohydrates to proteins, implying that carbohydrates serve important functions. List at least three functions that carbohydrates on proteins are known to carry out.

DATA HANDLING

13–46 The vesicular stomatitis virus (VSV) G protein is a typical membrane glycoprotein. In addition to its signal peptide, which is removed after import into the ER, the G protein contains a single membrane-spanning segment that anchors the protein in the plasma membrane. The membrane-spanning segment consists of 20 uncharged and mostly hydrophobic amino acids that are flanked by basic amino acids (**Figure 13–7**). Twenty amino acids arranged in an α helix are just sufficient to span the 3-nm thickness of the lipid bilayer of the membrane.

To test the length requirements for membrane-spanning segments, you modify a cloned version of the VSV G protein to generate a series of mutants in which the membrane-spanning segment is shorter, as indicated in Figure 13–7. When you introduce the modified plasmids into cultured cells, roughly the same amount of G protein is synthesized from each mutant as from wild-type cells. You analyze the cellular distribution of the altered G proteins in several ways.

1. You examine the cellular location of the modified VSV G proteins by immunofluorescence microscopy, using G-protein-specific antibodies tagged with fluorescein.
2. You characterize the attached oligosaccharide chains by digestion with endoglycosidase H (Endo H), which cleaves off *N*-linked oligosaccharides until the first mannose is removed in the medial portion of the Golgi apparatus (see Figure 13–8, Problem 13–47).
3. You determine whether the altered VSV G proteins retain the small C-terminal cytoplasmic domain (which characterizes the normal G protein) by treating isolated microsomes with a protease. In the normal VSV G protein, this domain is sensitive to protease treatment and is removed.

The results of these experiments are summarized in **Table 13–2**.

A. To the extent these data allow, deduce the intracellular location of each altered VSV G protein that fails to reach the plasma membrane.
B. For the VSV G protein, what is the minimum length of the membrane-spanning segment that is sufficient to anchor the protein in the membrane?
C. What is the minimum length of the membrane-spanning segment that is consistent with proper sorting of the G protein? How is it that transmembrane segments shorter than this can make it to the Golgi apparatus, but then not be able to exit?

13–47 You have isolated several mutant cell lines that are defective in their ability to add carbohydrate to exported proteins. Using an easily purified

TABLE 13–2 Results of experiments characterizing the cellular distribution of G proteins from normal and mutant cells (Problem 13–46).

Plasmid	Cellular location	Endo H treatment	Protease treatment
pMS20	plasma membrane	resistant	sensitive
pMS18	plasma membrane	resistant	sensitive
pMS16	plasma membrane	resistant	sensitive
pMS14	plasma membrane	resistant	sensitive
pMS12	intracellular	+/– resistant	sensitive
pMS8	intracellular	sensitive	sensitive
pMS0	intracellular	sensitive	resistant

protein that carries only *N*-linked complex oligosaccharides, you have analyzed the sugar monomers that are added in the different mutant cells. Each mutant is unique in the kinds and numbers of different sugars contained in its *N*-linked oligosaccharides (**Table 13–3**).

A. Arrange the mutants in the order that corresponds to the steps in the pathway for processing *N*-linked oligosaccharides (**Figure 13–8**). (Assume that each mutant cell line is defective for a single enzyme required to construct the *N*-linked oligosaccharide.)
B. Which of these mutants are defective in processing events that occur in the ER? Which mutants are defective in processing events that occur in the Golgi?
C. Which of the mutants are likely to be defective in a processing enzyme that is directly responsible for modifying *N*-linked oligosaccharides? Which mutants might not be defective in a processing enzyme, but rather in another enzyme that affects oligosaccharide processing indirectly?

TABLE 13–3 Analysis of the sugars present in the *N*-linked oligosaccharides from wild-type and mutant cell lines defective in oligosaccharide processing (Problem 13–47).

Cell line	Man	GlcNAc	Gal	NANA	Glc
Wild type	3	5	3	3	0
Mutant A	3	5	0	0	0
Mutant B	5	3	0	0	0
Mutant C	9	2	0	0	3
Mutant D	9	2	0	0	0
Mutant E	5	2	0	0	0
Mutant F	3	3	0	0	0
Mutant G	8	2	0	0	0
Mutant H	9	2	0	0	2
Mutant I	3	5	3	0	0

Abbreviations: Man = mannose; GlcNAc = *N*-acetylglucosamine; Gal = galactose; NANA = *N*-acetylneuraminic acid, or sialic acid; Glc = glucose. Numbers indicate the number of sugar monomers in the oligosaccharide.

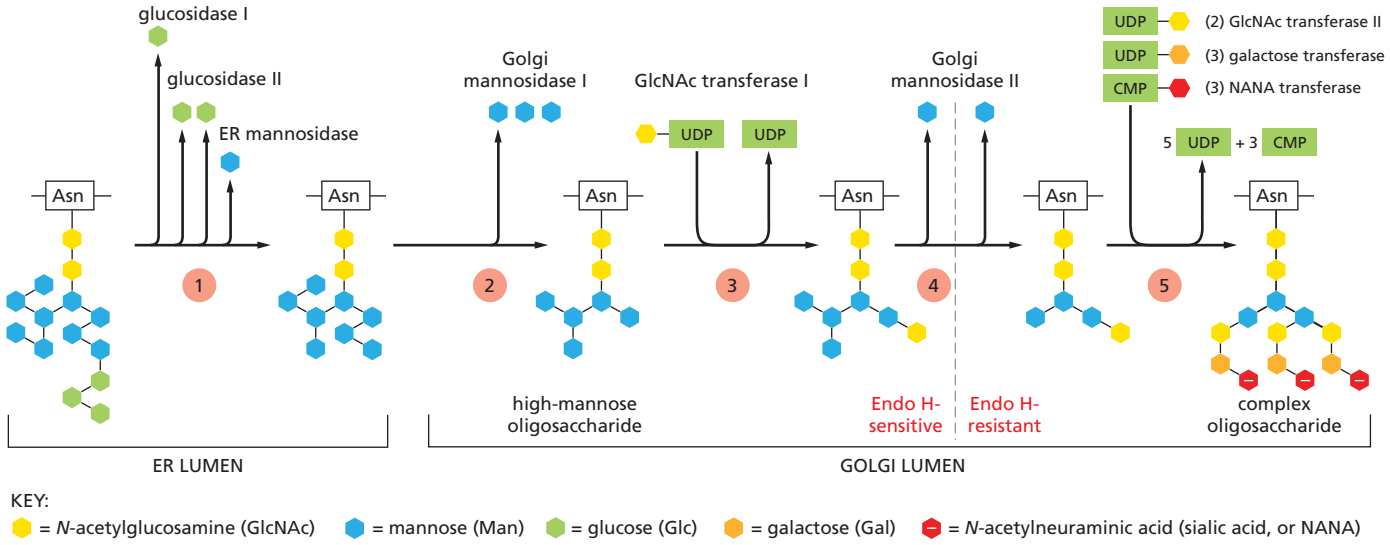


Figure 13–8 Oligosaccharide processing in the ER and the Golgi apparatus (Problem 13–47).

13–48 Two extreme models—vesicle transport and cisternal maturation—have been proposed to account for the movement of molecules across the polarized structure of the Golgi apparatus. In the vesicle transport model, the individual Golgi cisternae remain in place as proteins move through them (**Figure 13–9A**). By contrast, in the cisternal maturation model, the individual Golgi cisternae move across the stack, carrying the proteins with them (**Figure 13–9B**). Transport vesicles serve critical functions in both models, but their roles are distinctly different. Describe the roles of the transport vesicles in each of the two models. Comment specifically on the roles of vesicles in the forward movement of proteins across the Golgi stack, in the retention of Golgi resident proteins in individual cisternae, and on the return of escaped ER proteins to the ER.

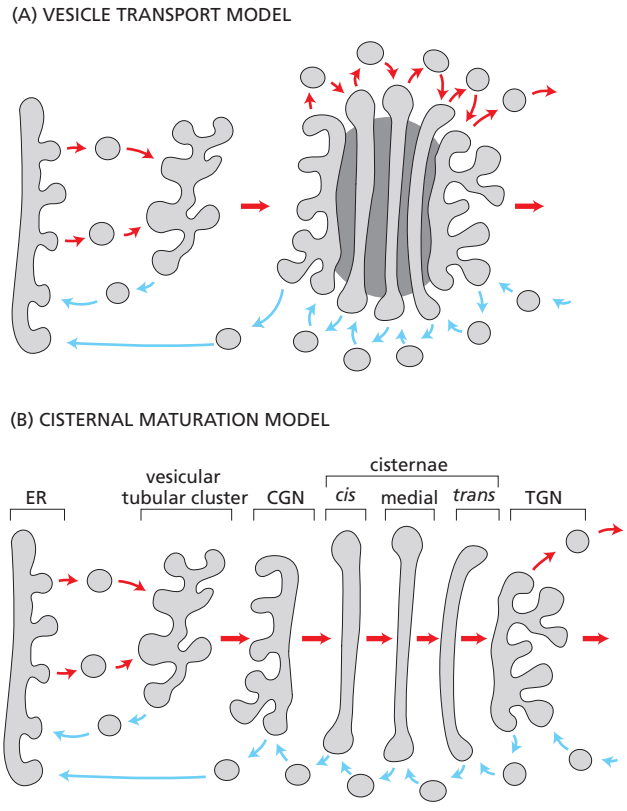


Figure 13–9 Two models for the movement of molecules through the Golgi apparatus (Problem 13–48). (A) The vesicle transport model. (B) The cisternal maturation model. In (B), the individual cisternae have been separated for illustration purposes. ER, endoplasmic reticulum; CGN, *cis* Golgi network; TGN, *trans* Golgi network.

TABLE 13–4 Addition of galactose to VSV G protein after fusion of VSV-infected cells with uninfected cells (Problem 13–49).

Infected cells	Uninfected cells	Precipitate	Supernatant
Mutant cells	Wild-type cells	45%	55%
Mutant cells	Mutant cells	5%	95%
Wild-type cells	Wild-type cells	85%	15%

The percentage of radioactivity in the precipitate indicates the fraction of GlcNac-labeled G protein that has acquired galactose.

13–49 One early test of the vesicle transport and cisternal maturation models (see Figure 13–9) looked for the movement of a protein between Golgi cisternae. This study made use of mutant cells that cannot add galactose to proteins, which normally occurs in the *trans* compartment of the Golgi (see Figure 13–8). The mutant cells were infected with vesicular stomatitis virus (VSV) to provide a convenient marker protein, the viral G protein. At an appropriate point in the infection, an inhibitor of protein synthesis was added to stop further synthesis of G protein. The infected cells were then incubated briefly with a radioactive precursor of GlcNAc, which is added only in the medial cisterna of the Golgi (see Figure 13–8). Next, the infected mutant cells were fused with uninfected wild-type cells to form a common cytoplasm containing both wild-type and mutant Golgi stacks. After a few minutes, the cells were dissolved with detergent and all the VSV G protein was captured using G-protein-specific antibodies. After separation from the antibodies, the G proteins carrying galactose were precipitated, using a lectin that binds galactose. The radioactivity in the precipitate and in the supernatant was measured. The results of this experiment along with control experiments (which used mutant cells only or wild-type cells only) are shown in **Table 13–4**.

A. Between which two compartments of the Golgi apparatus is the movement of proteins being tested in this experiment? Explain your answer.
B. If proteins moved through the Golgi apparatus by cisternal maturation, what would you predict for the results of this experiment? If proteins moved through the Golgi via vesicular transport, what would you predict?
C. Which model is supported by the results in Table 13–4?

MEDICAL LINKS

13–50 Processing of *N*-linked oligosaccharides is not uniform among species. Most mammals, with the exception of humans and Old World primates, occasionally add galactose—in place of an *N*-acetylneuraminic acid—to a galactose, forming a terminal Gal(α1–3)Gal disaccharide on some branches of an *N*-linked oligosaccharide. How does this explain the preferred use of Old World primates for production of recombinant proteins for therapeutic use in humans?

TRANSPORT FROM THE *TRANS* GOLGI NETWORK TO LYSOSOMES

TERMS TO LEARN

acid hydrolase
autophagosome
autophagy
lysosomal storage disease
lysosome
M6P receptor protein
vacuole

DEFINITIONS

Match each definition below with its term from the list above.

13–51 Digestion of obsolete parts of the cell by the cell's own lysosomes.

13–52 Very large, fluid-filled vesicle found in most plant and fungal cells, typically occupying more than 30% of the cell volume.

13–53 Membrane-enclosed organelle in eukaryotic cells that contains digestive enzymes, which are typically most active at the acidic pH found in the lumen.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

13–54 Lysosomal membranes contain a proton pump that utilizes the energy of ATP hydrolysis to pump protons out of the lysosome, thereby maintaining the lumen at a low pH.

13–55 Late endosomes are converted to mature lysosomes by the loss of distinct endosomal membrane proteins and a further decrease in their internal pH.

13–56 If cells were treated with a weak base such as ammonia or chloroquine, which raises the pH of organelles toward neutrality, M6P receptors would be expected to accumulate in the Golgi because they could not bind to the lysosomal enzymes.

THOUGHT PROBLEMS

13–57 How does the low pH of lysosomes protect the rest of the cell from lysosomal enzymes in case the lysosome breaks?

13–58 Imagine that an autophagosome is formed by engulfment of a mitochondrion by the ER membrane. How many layers of membrane would separate the matrix of the mitochondrion from the cytosol outside the autophagosome? Identify the source of each membrane and the spaces between the membranes.

13–59 The principal pathway for transport of lysosomal hydrolases from the *trans* Golgi network (pH 6.6) to the late endosomes (pH 6) and for the recycling of M6P receptors back to the Golgi depends on the pH difference between those two compartments. From what you know about M6P receptor binding and recycling and the pathways for delivery of material to lysosomes, describe the consequences of changing the pH in those two compartments.

A. What do you suppose would happen if the pH in late endosomes were raised to pH 6.6?

B. What do you suppose would happen if the pH in the *trans* Golgi network were lowered to pH 6?

13–60 Melanosomes are specialized lysosomes that store pigments for eventual release by exocytosis. Various cells such as skin and hair cells then take up the pigment, which accounts for their characteristic pigmentation. Mouse mutants that have defective melanosomes often have pale or unusual coat colors. One such light-colored mouse, the *Mocha* mouse (**Figure 13–10**), has a defect in the gene for one of the subunits of the adaptor protein complex AP3, which is associated with coated vesicles budding from the *trans* Golgi network. How might the loss of AP3 cause a defect in melanosomes?

Figure 13–10 A normal mouse and the *Mocha* mouse (Problem 13–60). In addition to its light coat color, the *Mocha* mouse has a poor sense of balance.

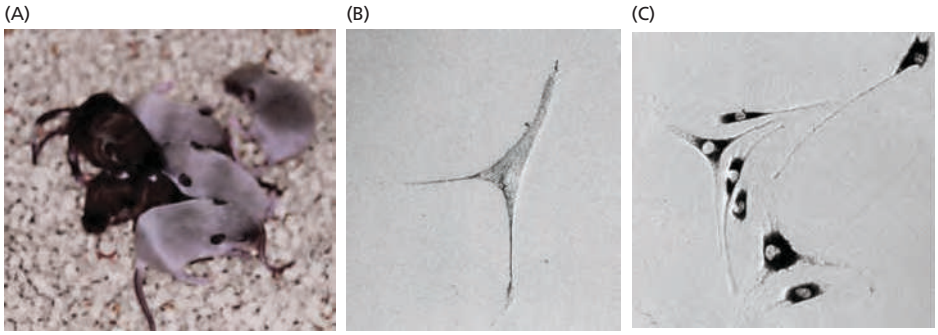


Figure 13–11 Pigmentation defects in *Ashen* mice (Problem 13–61). (A) Normally pigmented mice and pale *Ashen* mice. (B) A melanocyte from a normal mouse. (C) Melanocytes from an *Ashen* mouse.

DATA HANDLING

13–61 More than 50 different genes are known to affect coat color in mice. Three of them—*Dilute*, *Leaden*, and *Ashen*—are grouped together because of their highly similar phenotypes. Although these mice have normal melanosomes in their melanocytes, the pigment in the melanosomes is not delivered correctly to hair cells, giving rise to pale coats, as shown for *Ashen* mice in **Figure 13–11A**. *Dilute* mice lack an unconventional myosin heavy chain, MyoVa, which interacts with a microtubule-based transport motor. *Ashen* mice carry a mutation in the gene for the Rab protein, Rab27a, which associates with melanosomes. *Leaden* mice are missing melanophilin (Mlph), which is a modular protein with individual domains that bind to MyoVa, to Rab27a, and to actin filaments in the cell cortex.

Melanocytes from normal mice have a characteristic branch morphology (**Figure 13–11B**) and normally discharge their melanosomes near the tips of the branches. As shown in **Figure 13–11C**, melanocytes from *Ashen* mice have a normal morphology but their melanosomes surround the nucleus. Melanocytes from *Dilute* mice and *Leaden* mice have the same appearance as those from *Ashen* mice. Try to put these observations together to formulate a hypothesis to account for the normal delivery of melanosomes to the tips of the melanocyte branches, and for the defects in melanosome function in these mice.

MEDICAL LINKS

13–62 Patients with I-cell disease are missing the enzyme GlcNAc phosphotransferase, which catalyzes the first of the two steps required for addition of phosphate to mannose to create the M6P marker (see Figure 13–12). In the absence of the M6P marker, the M6P receptor cannot bind to the protein and deliver it to a lysosome. How do you suppose that the lysosomes in some cells from these patients—liver cells, for example—acquire a normal complement of lysosomal enzymes?

13–63 Patients with Hunter's syndrome or with Hurler's syndrome rarely live beyond their teens. These patients accumulate glycosaminoglycans in lysosomes due to the lack of specific lysosomal enzymes necessary for their degradation. When cells from patients with the two syndromes are fused, glycosaminoglycans are degraded properly, indicating that the cells are missing different degradative enzymes. Even if the cells are just cultured together, they still correct each other's defects. Most surprising of all, the medium from a culture of Hurler's cells corrects the defect in Hunter's cells (and vice versa). The corrective factors in the media are inactivated by treatment with proteases, by treatment with periodate, which destroys carbohydrate, and by treatment with alkaline phosphatase, which removes phosphates.

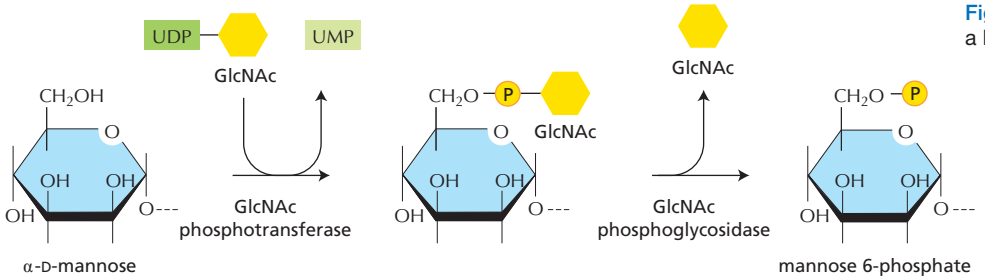


Figure 13–12 Synthesis of M6P marker on a lysosomal hydrolase (Problem 13–64).

A. What do you suppose the corrective factors are? Beginning with the donor patient's cells, describe the route by which the factors reach the medium and subsequently enter the recipient cells to correct the lysosomal defects.
B. Why do you suppose the treatments with protease, periodate, and alkaline phosphatase inactivate the corrective factors?
C. Would you expect a similar sort of correction scheme to work for mutant cytosolic enzymes?

13–64 Children with I-cell disease synthesize perfectly good lysosomal enzymes, but they are secreted outside the cell instead of being sorted to lysosomes. The mistake occurs because the cells lack GlcNAc phosphotransferase, which is required to create the M6P marker that is essential for proper sorting (**Figure 13–12**). In principle, I-cell disease could also be caused by deficiencies in GlcNAc phosphoglycosidase, which removes GlcNAc to expose M6P (Figure 13–12), or in the M6P receptor itself. Thus, there are three potential kinds of I-cell disease, which could be distinguished by the ability of various culture supernatants to correct defects in mutant cells. Imagine that you have three cell lines (A, B, and C), each of which derives from a patient with one of the three hypothetical I-cell diseases. Experiments with supernatants from these cell lines give the results below.

1. The supernatant from normal cells corrects the defects in B and C but not the defect in A.
2. The supernatant from A corrects the defect in Hurler's cells, which are missing a specific lysosomal enzyme, but the supernatants from B and C do not.
3. If the supernatants from the mutant cells are first treated with phosphoglycosidase to remove GlcNAc, then the supernatants from A and C correct the defect in Hurler's cells, but the supernatant from B does not.

From these results, deduce the nature of the defect in each of the mutant cell lines.

TRANSPORT INTO THE CELL FROM THE PLASMA MEMBRANE: ENDOCYTOSIS

TERMS TO LEARN

caveola
caveolin
clathrin-coated pit
early endosome
endocytic vesicle
endocytosis
endosome maturation
ESCRT protein complexes
late endosome
low-density lipoprotein (LDL)
macrophage
macropinocytosis
multivesicular body
neutrophil
phagocytosis
phagosome
pinocytosis
receptor-mediated endocytosis
recycling endosome
transcytosis
transferrin receptor

DEFINITIONS

Match each definition below with its term from the list above.

13–65 General term for the process by which cells take up macromolecules, particulate substances, and even other cells into membrane-enclosed vesicles.

13–66 Complex vesicle with invaginating buds and internal vesicles involved in the maturation of early endosomes into late endosomes.

13–67 Phagocytic cell—derived from a hematopoietic stem cell—that ingests invading microorganisms and plays an important role in scavenging senescent cells and apoptotic cells.

13–68 Type of endocytosis in which soluble materials are taken up from the environment and incorporated into vesicles for digestion.

13–69 Invagination that forms from lipid rafts at the cell surface and buds off internally to form a pinocytic vesicle.

13–70 Region of plasma membrane of animal cells that is covered with the protein clathrin on its cytosolic face; it will bud off from the membrane to form an intracellular vesicle.

13–71 Process by which macromolecules bind to complementary transmembrane receptor proteins, accumulate in coated pits, and then enter the cells as receptor–macromolecule complexes in clathrin-coated vesicles.

13–72 One of a family of structural proteins in caveolae that are unusual because they extend multiple hydrophobic loops into the membrane from the cytosolic side, but do not cross the membrane.

13–73 Membrane-enclosed compartment just beneath the plasma membrane, to which external molecules are first delivered by endocytosis.

13–74 Specialized form of endocytosis in which a cell uses large endocytic vesicles to ingest large particles such as microorganisms and dead cells.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

13–75 Any particle that is bound to the surface of a phagocyte will be ingested by phagocytosis.

13–76 Like the LDL receptor, most of the more than 25 different receptors known to participate in receptor-mediated endocytosis enter coated pits only after they have bound their specific ligands.

13–77 All the molecules that enter early endosomes ultimately reach late endosomes, where they become mixed with newly synthesized acid hydrolases and end up in lysosomes.

13–78 During transcytosis, vesicles that form from coated pits on the apical surface fuse with the plasma membrane on the basolateral surface, and in that way transport molecules across the epithelium.

THOUGHT PROBLEMS

13–79 A macrophage ingests the equivalent of 100% of its plasma membrane each half hour by endocytosis. What is the rate at which membrane is returned by exocytosis?

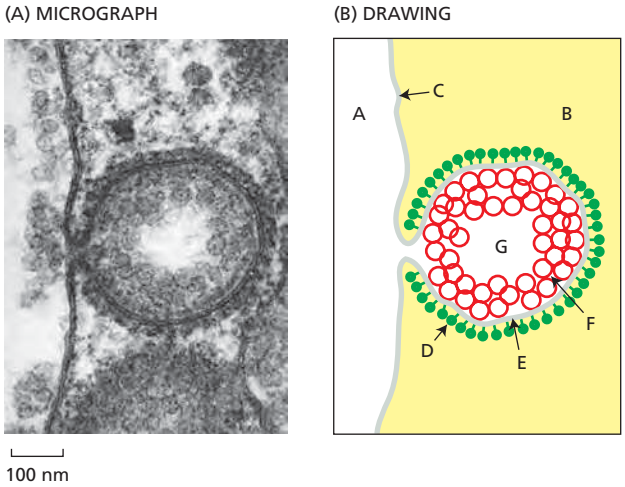


Figure 13–13 A coated pit about to bud from the membrane (Problem 13–80). (A) An electron micrograph. (B) A schematic drawing.

13–80 The electron micrograph in **Figure 13–13A** is illustrated schematically by the drawing in **Figure 13–13B**. Name the structures that are labeled in the drawing.

13–81 Caveolae are thought to form from lipid rafts, which are patches of the plasma membrane that are especially rich in cholesterol and glycosphingolipids. Caveolae may collect cargo proteins by virtue of the lipid composition of their membrane, rather than by assembly of a cytosolic protein coat. What might you predict would be a characteristic of the structure of transmembrane proteins that collect in caveolae?

13–82 Iron (Fe) is an essential trace metal that is needed by all cells. It is required, for example, for the synthesis of the heme groups that are part of cytochromes and hemoglobin. Iron is taken into cells via a two-component system. The soluble protein transferrin circulates in the bloodstream, and the transferrin receptor is a membrane protein that is continually endocytosed and recycled to the plasma membrane. Fe ions bind to transferrin at neutral pH but not at acidic pH. Transferrin binds to the transferrin receptor at neutral pH only when it has bound an Fe ion, but it binds to the receptor at acidic pH even in the absence of bound iron. From these properties, describe how iron is taken up, and discuss the advantages of this elaborate scheme.

CALCULATIONS

13–83 Cells take up extracellular molecules by receptor-mediated endocytosis and by fluid-phase endocytosis. A classic paper compared the efficiencies of these two pathways by incubating human cells for various periods of time in a range of concentrations of either ^{125}I-labeled epidermal growth factor (EGF), to measure receptor-mediated endocytosis, or horseradish peroxidase (HRP), to measure fluid-phase endocytosis. Both EGF and HRP were found to be present in small vesicles with an internal radius of 20 nm. The uptake of HRP was linear (**Figure 13–14A**), while that of EGF was initially linear but reached a plateau at higher concentrations (**Figure 13–14B**).

A. Explain why the shapes of the curves in Figure 13–14 are different for HRP and EGF.
B. From the curves in Figure 13–14, estimate the difference in the uptake rates for HRP and EGF when both are present at 40 nM. What would the difference be if both were present at 40 μM?
C. Calculate the average number of HRP molecules that get taken up by each endocytic vesicle (radius 20 nm) when the medium contains 40 μM HRP. [The volume of a sphere is $(4/3)\pi r^3$.]

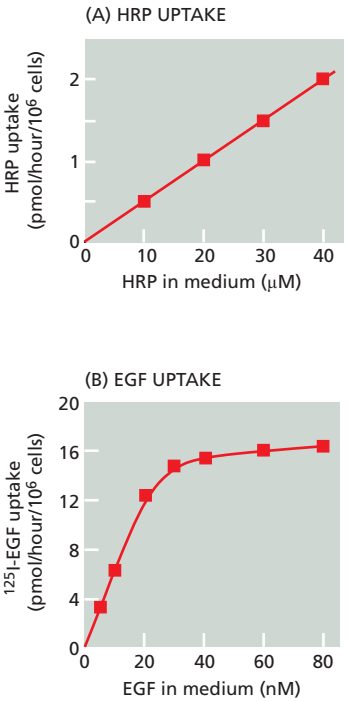


Figure 13–14 Uptake of HRP and EGF as a function of their concentration in the medium (Problem 13–83).

D. The scientists who did these experiments said at the time, "These calculations clearly illustrate how cells can internalize EGF by endocytosis while excluding all but insignificant quantities of extracellular fluid." What do you think they meant?

13–84 A ligand for receptor-mediated endocytosis circulates at a concentration of 1 nM (10^{-9} M). It is taken up in coated vesicles with a volume of 1.66×10^{-18} L (about 150 nm in diameter). On average, there are 10 of its receptors in each coated vesicle. If all the receptors were bound to the ligand, how much more concentrated would the ligand be in the vesicle than it was in the extracellular fluid? What would the dissociation constant (K_d) for the receptor–ligand binding need to be in order to concentrate the ligand 1000-fold in the vesicle? (You may wish to review the discussion of K_d in Problem 3–86.)

13–85 The recycling of transferrin receptors has been studied by labeling the receptors on the cell surface and following their fate at 0°C and 37°C. A sample of intact cells at 0°C was reacted with radioactive iodine under conditions that label cell-surface proteins. If these cells were kept on ice and incubated in the presence of trypsin, which destroys the receptors without damaging the integrity of the cell, the radioactive transferrin receptors were completely degraded. If the cells were first warmed to 37°C for 1 hour and then treated with trypsin on ice, about 70% of the initial radioactivity was resistant to trypsin. At both temperatures, most of the receptors were not labeled and most remained intact, as apparent from a protein stain.

A second sample of cells that had been surface-labeled at 0°C and incubated at 37°C for 1 hour was analyzed with transferrin-specific antibodies, which identify transferrin receptors via their linkage to Fe–transferrin complexes. If intact cells were reacted with antibody, 0.54% of the labeled proteins were bound by antibody. If the cells were first dissolved in detergent, 1.76% of the labeled proteins were bound by antibody.

A. When the cells were kept on ice, why did trypsin treatment destroy the labeled transferrin receptors, but not the majority of receptors? Why did most of the labeled receptors become resistant to trypsin when the cells were incubated at 37°C?

B. What fraction of all the transferrin receptors is on the cell surface after a 1-hour incubation at 37°C? Do the two experimental approaches agree?

MEDICAL LINKS

13–86 Cholesterol is an essential component of the plasma membrane, but people who have very high levels of cholesterol in their blood (hypercholesterolemia) tend to have heart attacks. Blood cholesterol is carried in the form of cholesterol esters in low-density lipoprotein (LDL) particles. LDL binds to a high-affinity receptor on the cell surface, enters the cell via a coated pit, and ends up in lysosomes. There its protein coat is degraded, and cholesterol esters are released and hydrolyzed to cholesterol. The released cholesterol enters the cytosol and inhibits the enzyme HMG CoA reductase, which controls the first unique step in cholesterol biosynthesis. Patients with severe hypercholesterolemia cannot remove LDL from the blood. As a result, their cells do not turn off normal cholesterol synthesis, which makes the problem worse.

LDL metabolism can be conveniently divided into three stages experimentally: binding of LDL to the cell surface, internalization of LDL, and regulation of cholesterol synthesis by LDL. Skin cells from a normal person and two patients suffering from severe familial hypercholesterolemia were grown in culture and tested for LDL binding, LDL internalization, and LDL regulation of cholesterol synthesis. The results are shown in **Figure 13–15**.

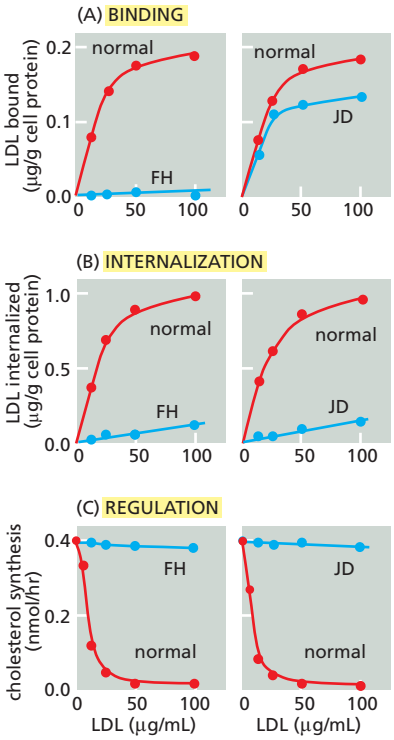


Figure 13–15 LDL metabolism in normal cells and in cells from patients with severe familial hypercholesterolemia (Problem 13–86). (A) Surface binding of LDL. Assays at 4°C allow binding but not internalization. (B) Internalization of LDL. After binding at 4°C, the cells are warmed to 37°C. Binding and uptake of LDL can be followed by labeling LDL either with ferritin particles, which can be seen by electron microscopy, or with radioactive iodine, which can be measured in a gamma counter. (C) Regulation of cholesterol synthesis by LDL.

A. In Figure 13–15A, the surface binding of LDL by normal cells is compared with LDL binding by cells from patients FH and JD. Why does binding by normal cells and by JD's cells reach a plateau? What explanation can you suggest for the lack of LDL binding by FH's cells?
B. In Figure 13–15B, internalization of LDL by normal cells increases as the external LDL concentration is increased, reaching a plateau 5-fold higher than the amount of externally bound LDL. Why does LDL enter cells from patients FH or JD at such a slow rate?
C. In Figure 13–15C, the regulation of cholesterol synthesis by LDL in normal cells is compared with that in cells from FH and JD. Why does increasing the external LDL concentration inhibit cholesterol synthesis in normal cells, but affect it only slightly in cells from FH or JD?
D. How would you expect the rate of cholesterol synthesis to be affected if normal cells and cells from FH or JD were incubated with cholesterol itself? (Free cholesterol crosses the plasma membrane by diffusion.)

13–87 What is wrong with JD's metabolism of LDL? As discussed in Problem 13–86, JD's cells bind LDL with the same affinity as normal cells and in almost the same amounts, but the binding does not lead to internalization of LDL. Two classes of explanation could account for JD's problem:

1. JD's LDL receptors are defective in a way that prevents internalization, even though the LDL-binding domains on the cell surface are unaffected.
2. JD's LDL receptors are entirely normal, but there is a mutation in the cellular internalization machinery such that loaded LDL receptors cannot be brought in.

To distinguish between these explanations, JD's parents were studied. It is known that an autosomal gene encodes the receptor that binds LDL. Thus, each parent must have donated one defective gene to JD. JD's mother suffered from mildly elevated blood cholesterol. Her cells bound only half as much LDL as normal cells, but the bound LDL was internalized at the same rate as in normal cells. JD's father also had mild hypercholesterolemia, but his cells bound even more LDL than normal cells. Of the bound LDL, less than half the label could be internalized; the rest remained on the cell surface.

The association of this family's LDL receptors with coated pits was studied by electron microscopy (EM), using LDL that was labeled with ferritin. The results are shown in **Table 13–5**.

A. Why does JD's mother have mild hypercholesterolemia? Based on the LDL binding and internalization studies, and on the EM observations, decide what kind of defective LDL receptor gene she passed to JD.

TABLE 13–5 Distribution of LDL receptors on the surface of cells from JD and his parents as compared with normal individuals (Problem 13–87).

	Number of LDL receptors	
Individual	In pits	Outside pits
Normal male	186	195
Normal female	186	165
JD	10	342
JD's father	112	444
JD's mother	91	87

B. Why does JD's father have mild hypercholesterolemia? Based on the LDL binding and internalization studies, and on the EM observations, decide what kind of defective LDL receptor gene he passed to JD.
C. Can you account for JD's hypercholesterolemia from the behavior of the LDL receptors in his parents? In particular, how is it that JD binds nearly a normal amount of LDL, but has severe hypercholesterolemia?
D. At the beginning of this problem, two possible explanations—defective receptor or defective internalization machinery—were proposed to account for the lack of internalization by JD's LDL receptors in the face of nearly normal LDL binding. Do these studies allow you to decide between these alternative explanations?

TRANSPORT FROM THE *TRANS* GOLGI NETWORK TO THE CELL EXTERIOR: EXOCYTOSIS

TERMS TO LEARN

constitutive secretory pathway
default pathway
exocytosis
regulated secretory pathway
secretory vesicle
synaptic vesicle

DEFINITIONS

Match each definition below with its term from the list above.

13–88 Specialized class of tiny secretory vesicles that store neurotransmitter molecules.

13–89 Pathway for exocytosis that operates continuously in all cells.

13–90 Membrane-enclosed organelle in which molecules destined to be exported are stored prior to release.

13–91 Process involving fusion of vesicles with the plasma membrane.

13–92 Pathway for exocytosis that operates mainly in cells specialized for secreting products rapidly on demand.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

13–93 When a foreign gene encoding a secretory protein is introduced into a secretory cell that normally does not make the protein, the alien secretory protein is not packaged into secretory vesicles.

13–94 Once a secretory vesicle is properly positioned beneath the plasma membrane, it will immediately fuse with the membrane and release its contents to the cell exterior.

THOUGHT PROBLEMS

13–95 In a cell capable of regulated secretion, what are the three main classes of protein that must be separated before they leave the *trans* Golgi network?

13–96 You are interested in exocytosis and endocytosis in a line of cultured liver cells that secrete albumin and take up transferrin. To distinguish between these events, you tag transferrin with colloidal gold and prepare ferritin-labeled antibodies that are specific for albumin. You add the tagged transferrin to the medium, and then after a few minutes you fix the cells, prepare thin sections, and react them with ferritin-labeled antibodies against albumin. Colloidal gold and ferritin are both electron-dense and

therefore readily visible when viewed by electron microscopy; moreover, they can be easily distinguished from one another on the basis of size.

A. Will this experiment allow you to identify vesicles in the exocytic and endocytic pathways? How?
B. Not all the gold-labeled vesicles are clathrin-coated. Why?

13–97 What would you expect to happen in cells that secrete large amounts of protein through the regulated secretory pathway, if the ionic conditions in the ER lumen could be changed to resemble those of the *trans* Golgi network?

13–98 Dynamin was first identified as a microtubule-binding protein, and its sequence indicated that it was a GTPase. The key to its function came from neurobiological studies in *Drosophila*. *Shibire* mutant flies, which carry a mutation in the dynamin gene, are rapidly paralyzed when the temperature is elevated. They recover quickly once the temperature is lowered. The complete paralysis at the elevated temperature suggested that synaptic transmission between nerve and muscle cells was blocked. Electron micrographs of synapses of the paralyzed flies showed a loss of synaptic vesicles and a tremendously increased number of coated pits relative to normal synapses (**Figure 13–16**).

Suggest an explanation for the paralysis shown by the *shibire* mutant flies, and indicate why signal transmission at a synapse might require dynamin.

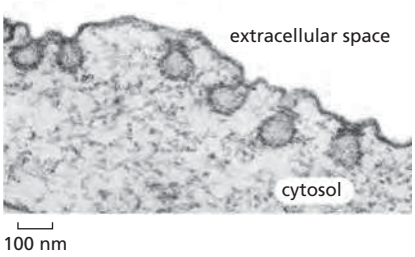


Figure 13–16 Electron micrograph of a nerve terminal from a *shibire* mutant fly at elevated temperature (Problem 13–98).

DATA HANDLING

13–99 Proteins without special signals are transported between cisternae in Golgi stacks and onward to the plasma membrane via the nonselective constitutive secretory pathway, or the default pathway, as it is commonly known. This transport is also sometimes referred to as bulk transport because the Golgi contents do not become concentrated in the vesicles. Given that transport in clathrin-coated vesicles is so highly concentrating, you are skeptical that no concentration occurs in the default pathway for secretion.

To determine whether vesicles in the default pathway concentrate their contents, you infect cells with vesicular stomatitis virus (VSV) and follow the viral G protein, which is transported by the default pathway. Your idea, an ambitious one, is to compare the concentration of G protein in the lumen of the Golgi stacks with that in the associated transport vesicles. You intend to measure G-protein concentration by preparing thin sections of VSV-infected cells and incubating them with G-protein-specific antibodies tagged with gold particles. Since the gold particles are visible in electron micrographs as small black dots, it is relatively straightforward to count dots in the lumens of transport vesicles (fully formed and just budding) and of the Golgi apparatus. You make two estimates of G-protein concentration: (1) the number of gold particles per cross-sectional area (surface density) and (2) the number of gold particles per linear length of membrane (linear density). Your results are shown in **Table 13–6**.

Do the vesicles involved in the default pathway concentrate their contents or not? Explain your reasoning.

13–100 Insulin is synthesized as a pre-pro-protein in the β cells of the pancreas. Its pre-peptide is cleaved off after it enters the ER lumen. To define the cellular location at which its pro-peptide is removed, you have prepared two antibodies: one that is specific for pro-insulin, and one that is specific for insulin. You have tagged the anti-pro-insulin antibody with a red fluorophore and the anti-insulin antibody with a green fluorophore, so you can follow them independently in the same cell. When you incubate

TABLE 13–6 Relative densities of G protein in Golgi and vesicle lumens and membranes (Problem 13–99).

Source of Golgi	Site measured	Parameter measured	Mean density
Uninfected cells	Whole Golgi	Surface density	5/μm^2
Infected cells	Whole Golgi	Surface density	271/μm^2
Infected cells	Golgi buds and vesicles	Surface density	233/μm^2
Infected cells	Golgi cisternal membranes	Linear density	6/μm
Infected cells	Golgi buds and vesicles	Linear density	4/μm

a pancreatic β cell with a mixture of your two antibodies, you obtain the results shown in **Table 13–7**. In what cellular compartment is the propeptide removed from pro-insulin?

13–101 Polarized epithelial cells must make an extra sorting decision since their plasma membranes are divided into apical and basolateral domains, which are populated by distinctive sets of proteins. Proteins destined for the apical or basolateral domains seem to travel there directly from the *trans* Golgi network. One way to sort proteins to these domains would be to use a specific sorting signal for one group of proteins, which would then be actively recognized and directed to one domain, and to allow the rest to travel via a default pathway to the other domain.

Consider the following experiment to identify the default pathway. The cloned genes for several foreign proteins were engineered by recombinant DNA techniques so that they could be expressed in the polarized epithelial cell line MDCK. These proteins are secreted in other types of cells, but are not normally expressed in MDCK cells. The cloned genes were introduced into the polarized MDCK cells, and their sites of secretion were assayed. Although the cells remained polarized, the foreign proteins were delivered in roughly equal amounts to the apical and basolateral domains.

TABLE 13–7 Fluorescence associated with various compartments of β cells after reaction with fluorescent antibodies directed against pro-insulin and insulin (Problem 13–100).

Compartment	Fluorescence
cis Golgi network	Red
Endoplasmic reticulum	Red
Golgi cisternae	Red
Immature secretory vesicles	Yellow
Lysosomes	None
Mature secretory vesicles	Green
Mitochondria	None
Nucleus	None
trans Golgi network	Red

A. What is the expected result of this experiment, based on the hypothesis that targeting to one domain of the plasma membrane is actively signaled and targeting to the other domain is via a default pathway?
B. Do these results support the concept of a default pathway as outlined above?

13–102 Neurons are difficult to study because of their excessively branched structure and long, thin dendrites, as shown in **Figure 13–17**. Fluorescently tagged antibodies are powerful tools for investigating certain aspects of neuron structure. Synaptic vesicles, for example, were shown to be concentrated in the presynaptic cells at nerve synapses in this way. A culture of neurons was first exposed for 1 hour to a fluorescently tagged antibody specific for the lumenal domain of synaptotagmin, a transmembrane protein that resides exclusively in the membranes of synaptic vesicles. The culture was then washed thoroughly to remove all synaptotagmin antibodies. When the culture was examined by fluorescence microscopy, dots of color from the synaptotagmin-specific antibody were found to mark the positions of the synaptic vesicles in the nerve terminals.

If antibodies do not cross intact membranes, how do you suppose the synaptic vesicles get labeled? When the procedure was repeated using an antibody specific for the cytoplasmic domain of synaptotagmin, the nerve terminals did not become labeled. Explain the results with the two different antibodies for synaptotagmin.

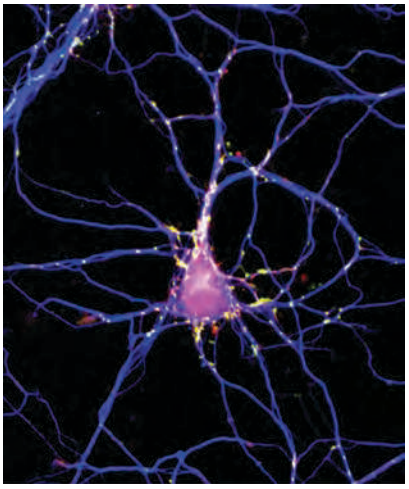


Figure 13–17 A hippocampal neuron (Problem 13–102).

13–103 The original version of the SNARE hypothesis suggested that the ATP-dependent disassembly of SNAREs by NSF provided the energy necessary for membrane fusion, and thus that NSF should act at the last step in secretion. More recent evidence suggests that NSF acts at an earlier step to prime the vesicle for secretion, and that SNAREs alone are sufficient to catalyze membrane fusion at the last step in secretion. It is important to know what really happens, and you have the means at hand to answer the question.

Using a whole-cell patch-clamp protocol (**Figure 13–18A**), you can diffuse cytosolic components into the cell through the pipet and assay exocytosis by changes in capacitance, which is a measure of the increase in the area of the plasma membrane. To control precisely the timing of vesicle fusion, you enclose Ca^{2+} in a photosensitive chemical "cage," from which it can be released with a flash of light. In response to Ca^{2+} release, there is a rapid burst of vesicle fusion (indicated by a rapid rise in capacitance), followed by a longer, slower fusion process (**Figure 13–18B**, –NEM). The initial burst represents the fusion of vesicles that were just waiting for the Ca^{2+} trigger. Because Ca^{2+} is rapidly removed from the cell, the procedure can be repeated with a second flash of light 2 minutes later; it yields the same rapid and slow components (**Figure 13–18C**, –NEM).

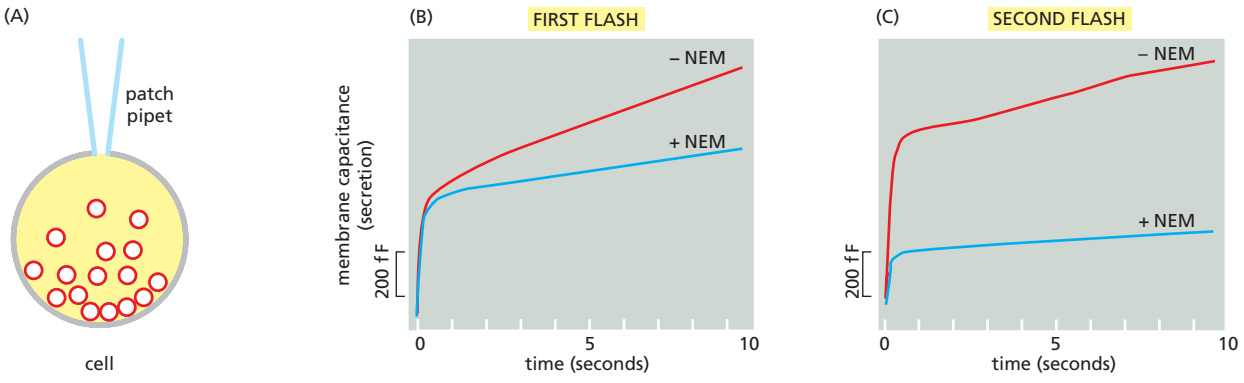


Figure 13–18 Analysis of the role of NSF in vesicle fusion (Problem 13–103). (A) Whole-cell patch-clamp. (B) Responses to the first flash-mediated release of Ca^{2+}. (C) Reponses to the second flash-mediated release of Ca^{2+}. Secretion was measured as an increase in membrane capacitance (in femtoFarads, fF) in the absence (–NEM) or presence (+NEM) of an inhibitor of NSF.

To test the role of NSF in vesicle fusion, you first diffuse *N*-ethylmaleimide (NEM) into cells to inhibit NSF. (The name "NSF" stands for NEM-sensitive factor.) You then repeat the flash protocols as before. In response to the first flash, the rapid component is unaffected, but the slow component is decreased (Figure 13–18B, +NEM). In response to the second flash, both components are inhibited (Figure 13–18C, +NEM).

A. What do you suppose the slow component of the fusion process represents?
B. Why does inhibition of NSF affect the slow component after both flashes, but inhibit the rapid component only after the second flash?
C. Which of the alternatives for the role of NSF in vesicle fusion—acting at the last step or an early step—do these experiments support? Explain your reasoning.
D. Propose a model for the molecular role of NSF in fusion of secretory vesicles.

MEDICAL LINKS

13–104 Antitrypsin, which inhibits certain proteases, is normally secreted into the bloodstream by liver cells. Antitrypsin is absent from the bloodstream of patients who carry a mutation that results in a single amino acid change in the protein. Antitrypsin deficiency causes a variety of severe problems, particularly in lung tissue, because of uncontrolled protease activity. Surprisingly, when the mutant antitrypsin is synthesized in the laboratory, it is as active as the normal antitrypsin at inhibiting proteases. Why then does the mutation cause the disease? Think of more than one possibility and suggest ways in which you could distinguish among them.

MCAT STYLE

Passage 1 (Questions 13–105 to 13–107)

Ricin is one of the most toxic substances known: less than 2 mg injected into the bloodstream will kill an adult human. Ricin is produced by the castor bean plant as a 65 kd protein heterodimer composed of an A chain and a B chain. The B chain is a lectin that binds to carbohydrates on the cell surface. The A chain is an enzyme that modifies a highly conserved site in rRNA, leading to inhibition of translation. After entering the cell, ricin eventually ends up in the lumen of the endoplasmic reticulum (ER), and from there it moves into the cytosol, where it inactivates ribosomes.

13–105 What is the most likely mechanism by which ricin enters the cell?
A. Binding to clathrin proteins
B. Entry through pore complexes
C. Interaction with SNARE proteins
D. Internalization via endocytosis

13–106 Which one of the following is required in order for ricin to be delivered to the ER?
A. *N*-ethylmaleimide-sensitive factor (NSF)
B. Golgi-derived COPI-coated vesicles
C. Mannose 6-phosphate (M6P) receptors
D. The Sar1 monomeric GTPase

13–107 Which one of the following describes the most likely scenario for how ricin gets into the cytosol?
A. Ricin has a signal sequence that allows it to be transported across the ER membrane into the cytosol.
B. Ricin is packaged into vesicles that form vesicular tubular clusters, which release proteins into the cytosol.

C. Ricin is transported from the ER to the Golgi apparatus to the lysosome, where it is released into the cytosol.
D. Ricin, by mimicking an unfolded protein, is tagged for transport across the ER membrane into the cytosol.

Passage 2 (Questions 13–108 to 13–111)

The molecular machinery responsible for vesicle fusion was discovered via biochemical analysis of vesicle transport. These experiments used a virus that hijacks the host-cell machinery to insert a viral coat protein into the ER membrane. From there, the viral protein is transported through the Golgi apparatus to the cell surface, where it is packaged into new virus particles that bud off the cell surface. In the mature virus, the viral protein is exposed to the outside of the virus and forms part of the viral coat. As the viral protein moves through the Golgi apparatus, it is modified with *N*-acetylglucosamine (GlcNAc).

The investigators used the virus to infect a mutant cell line that is incapable of modifying the viral protein with GlcNAc. The Golgi apparatus was then isolated from the infected cells. This "donor" Golgi apparatus was mixed with "acceptor" Golgi apparatus isolated from uninfected wild-type cells. The investigators hypothesized that transport of vesicles from the donor to acceptor Golgi apparatuses would move the viral protein into the wild-type acceptor Golgi apparatus, leading to addition of GlcNAc. Thus, vesicle traffic between Golgi stacks could be detected simply by assaying for the GlcNAc modification on the viral protein.

In addition to purifying donor and acceptor Golgi apparatuses, the investigators made a preparation of soluble cytosolic proteins by breaking open cells and removing membrane-bound organelles and other large particles by centrifugation. When the donor and acceptor Golgi apparatuses were mixed in the absence of cytosol, no transport of the viral protein could be detected. However, when ATP and soluble cytosolic proteins were included, the investigators detected transport of viral protein between the Golgi stacks. To identify proteins required for vesicle transport, the investigators treated the soluble cytoplasmic extract with *N*-ethylmaleimide (NEM), which covalently modifies lysine residues, inactivating some proteins. This process inactivated the activity in the extract, which allowed the investigators to purify a protein from untreated extracts that could be added back to NEM-treated extract to rescue vesicle transport. This protein was called NEM-sensitive factor (NSF). NSF was found to be a soluble protein that can hydrolyze ATP.

NSF was found to bind to the membranes of the Golgi apparatus in the presence of soluble proteins called "soluble NSF attachment proteins" (SNAPs). Complexes of NSF and SNAP formed only in the absence of ATP; addition of ATP caused rapid release of NSF. If Golgi membranes with bound NSF were solubilized by addition of nonionic detergent, NSF was found to be associated with a large protein complex. To identify proteins that bind to NSF, the investigators attached an antibody that recognizes NSF to beads. The beads were then used to bind detergent-solubilized NSF—with its associated complex of proteins—from bovine brain extracts. After washing with buffer to remove unbound proteins, ATP was added to release the proteins that bind to NSF and SNAP. These proteins were called SNAP receptors (SNAREs). Two of the proteins the investigators identified—syntaxin and synaptobrevin—had already been found in other studies of neuronal cell exocytosis. SNAREs were hypothesized to be the minimal machinery necessary for membrane fusion events in the secretory pathway. To test this, a v-SNARE and a t-SNARE were expressed in bacteria, purified, and reconstituted into lipid vesicles. When vesicles bearing the v-SNARE were mixed with those containing the t-SNARE, the vesicles fused. NSF and ATP were not required for fusion.

13–108 The investigators hypothesized that the viral protein was transported between the Golgi stacks inside vesicles. An alternative hypothesis, however, was that the viral protein was released from one Golgi apparatus and taken up by the other, without being packaged into vesicles. Which

of the following experiments would best distinguish between these two hypotheses?

A. Add a protease to the system and determine whether the viral protein is degraded.
B. Determine whether transport still occurs when clathrin is removed from the extract.
C. Test for association of GlcNAc-modified viral protein with membranes by centrifugation.
D. Test whether transport between Golgi stacks is blocked by addition of a detergent.

13–109 To learn more about the function of NSF, the investigators used a cytoplasmic extract that lacked NSF activity. To the extract, they added donor Golgi apparatus, purified NSF, and ATP. They then added NEM to inactivate NSF, followed by acceptor membranes. No vesicle transport was detected in this situation. What does this experiment tell you about the function of NSF?

A. NSF is required for packaging viral protein into donor vesicles.
B. NSF is required for formation of donor vesicles from donor Golgi.
C. NSF is required for fusion of donor vesicles to the acceptor Golgi.
D. NSF is required for release of viral protein into the acceptor Golgi.

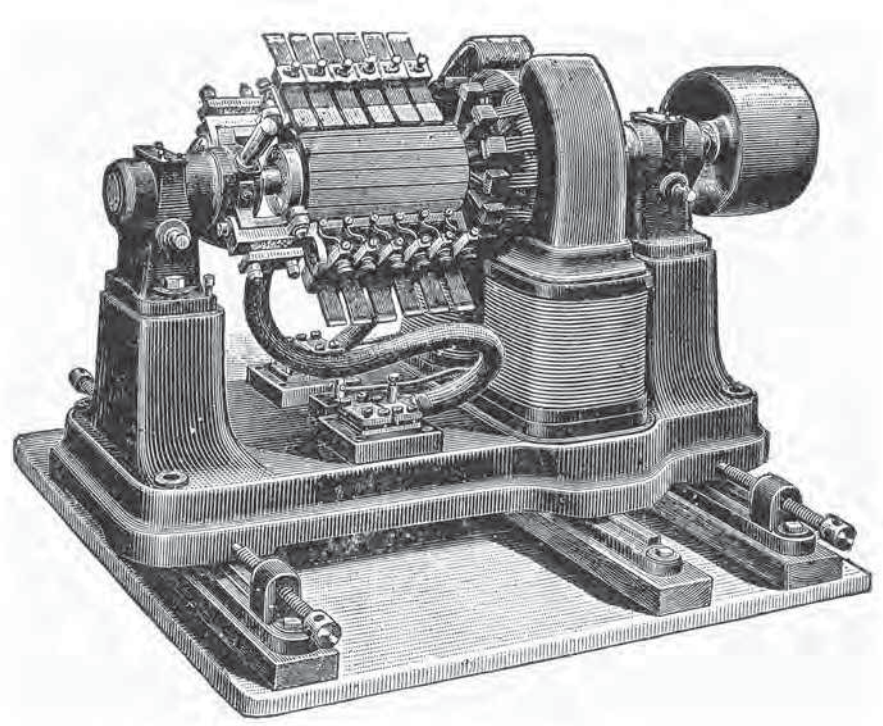
13–110 Which of the following statements about synaptobrevin and syntaxin are consistent with what we now know about the key functions of SNAREs?

I. Botulinum toxin proteolytically cleaves synaptobrevin, leading to muscle paralysis.
II. Synaptobrevin induces NSF to hydrolyze ATP to provide the energy for membrane fusion.
III. Synaptobrevin is on the vesicle membrane; syntaxin is on the plasma membrane.

A. I
B. I and II
C. I and III
D. II and III

13–111 Which one of the following statements best explains why NSF and ATP were required for vesicle fusion in the original reconstituted system for transport of a viral protein between Golgi stacks, but were not required for fusion of lipid vesicles containing a v-SNARE and a t-SNARE?

A. Fusion of lipid vesicles requires no extra energy in the presence of high concentrations of purified SNAREs; however, energy from ATP hydrolysis is required to fuse vesicles with natural membranes.
B. In the fusion of natural membranes, NSF and ATP are required to remove a cytosolic inhibitor that binds to SNARE proteins; the fusion of lipid vesicles occurs without ATP because the inhibitor is absent.
C. NSF uses ATP hydrolysis to move proteins out of the way, so that natural vesicles can dock on their target membrane, but ATP hydrolysis is not needed to dock lipid vesicles containing purified SNAREs.
D. NSF uses ATP to pull apart tight complexes between v-SNAREs and t-SNAREs to prime them for fusion, a process that is not required when the SNAREs are already segregated into different lipid vesicles.



High-Current Copper-Brush Commutated Dynamo.
According to Wikipedia, this large, belt-driven, high-current dynamo produced 310 amperes at 7 volts. Mitochondria are often referred to as the “power plants” of the cell. The pictured machine converted mechanical energy into electrical energy; mitochondria convert electrical energy into chemical energy.

CHAPTER 14

Energy Conversion: Mitochondria and Chloroplasts

IN THIS CHAPTER

THE MITOCHONDRION

TERMS TO LEARN

chemiosmotic coupling
cristae
electrochemical gradient
inner mitochondrial membrane
intermembrane space
mitochondrial matrix
mitochondria
outer mitochondrial membrane
oxidative phosphorylation
proton-motive force
respiratory chain

DEFINITIONS

Match each definition below with its term from the list above.

14–1 The subcompartment formed between the inner and outer mitochondrial membranes.

14–2 The mitochondrial electron-transport chain, which generates the proton gradient across the inner mitochondrial membrane that powers ATP synthase, producing most of the cell's ATP.

14–3 Mechanism by which a pH gradient across a membrane is used to drive an energy-requiring process, such as ATP production or the rotation of bacterial flagella.

14–4 Process in bacteria and mitochondria in which ATP formation is driven by the transfer of electrons from food molecules to molecular oxygen, with the intermediate generation of a proton gradient across a membrane.

14–5 The result of a combined pH gradient and membrane potential.

14–6 A sievelike membrane surrounding mitochondria that is permeable to all molecules of 5000 daltons or less.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

14–7 The intermembrane space is chemically equivalent to the cytosol with respect to small molecules due to the many specialized transport proteins in the mitochondrial outer membrane.

14–8 The most important contribution of the citric acid cycle to energy metabolism is the extraction of high-energy electrons during the oxidation of acetyl CoA to CO_2.

14–9 Each respiratory enzyme complex in the electron-transport chain has a greater affinity for electrons than its predecessors, so that electrons pass

sequentially from one complex to another until they are finally transferred to oxygen, which has the greatest electron affinity of all.

THOUGHT PROBLEMS

14–10 Mitochondria in liver cells appear to move freely in the cytosol, whereas those in cardiac muscle are immobilized at positions between adjacent myofibrils. Do you suppose these differences are a trivial consequence of cell architecture or do they reflect some underlying functional advantage? Explain your answer.

14–11 Electron micrographs show that mitochondria in heart muscle have a much higher density of cristae than mitochondria in skin cells. Why do you suppose this should be?

14–12 In the 1860s, Louis Pasteur noticed that when he added O_2 to a culture of yeast growing anaerobically on glucose, the rate of glucose consumption declined dramatically. Explain the basis for this result, which is known as the Pasteur effect.

14–13 The citric acid cycle generates NADH and $FADH_2$, which are then used in the process of oxidative phosphorylation to make ATP. If the citric acid cycle, which does not use oxygen, and oxidative phosphorylation are separate processes, as they are, then why is it that the citric acid cycle stops almost immediately upon removal of O_2?

14–14 When dinitrophenol (DNP) is added to mitochondria, the inner membrane becomes permeable to protons. When the drug valinomycin is added to mitochondria, the inner membrane becomes permeable to K^+.

A. How will the electrochemical gradient change in response to DNP?
B. How will it change in response to valinomycin?

14–15 Several coupled transport processes that occur across the inner mitochondrial membrane are illustrated in **Figure 14–1**. For each, decide whether transport is with the electrochemical gradient, against it, or unaffected by it. For those transport processes that are affected by the gradient, identify which component of the gradient (the difference in pH or the membrane potential) affects transport.

CALCULATIONS

14–16 Heart muscle gets most of the ATP needed to power its continual contractions through oxidative phosphorylation. When oxidizing glucose to CO_2, heart muscle consumes O_2 at a rate of 10 μmol/min per g of tissue, in order to replace the ATP used in contraction and give a steady-state ATP concentration of 5 μmol/g of tissue. At this rate, how many seconds would it take the heart to consume an amount of ATP equal to its steady-state levels? (Complete oxidation of one molecule of glucose to CO_2 yields 30 ATP, 26 of which are derived by oxidative phosphorylation using the 12 pairs of electrons captured in the electron carriers NADH and $FADH_2$.)

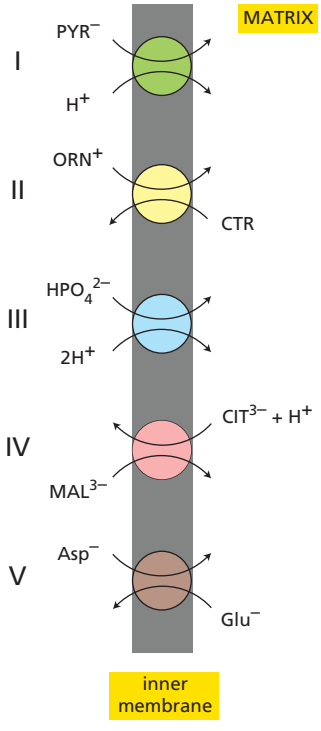


Figure 14–1 Five coupled transport processes that occur across the inner mitochondrial membrane (Problem 14–15). PYR is pyruvate; ORN is ornithine; CTR is citrulline; CIT is citrate; MAL is malate; Asp is aspartic acid; and Glu is glutamic acid.

THE PROTON PUMPS OF THE ELECTRON-TRANSPORT CHAIN

TERMS TO LEARN

cytochrome
cytochrome *c* oxidase complex
cytochrome *c* reductase
iron–sulfur center
NADH dehydrogenase complex
quinone (Q)
redox pairs
redox potential
redox reaction

DEFINITIONS

Match each definition below with its term from the list above.

14–17 An electron-driven proton pump in the respiratory chain that accepts electrons from cytochrome *c* and generates water using molecular oxygen as an electron acceptor.

14–18 Colored, heme-containing protein that transfers electrons during cellular respiration.

14–19 Electron-transporting group consisting of either two or four iron atoms bound to an equal number of sulfur atoms.

14–20 A reaction in which one component becomes oxidized and the other reduced.

14–21 The affinity of a redox pair for electrons, generally measured as the voltage difference between an equimolar mixture of the pair and a standard reference.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

14–22 Most cytochromes have a higher redox potential (higher affinity for electrons) than iron–sulfur centers, which is why the cytochromes tend to serve as electron carriers near the O_2 end of the respiratory chain.

14–23 The three respiratory enzyme complexes in the mitochondrial inner membrane tend to associate with each other in ways that facilitate the correct transfer of electrons between appropriate complexes.

THOUGHT PROBLEMS

14–24 Both H^+ and Ca^{2+} are ions that move through the cytosol. Why is the movement of H^+ ions so much faster than that of Ca^{2+} ions? How do you suppose the speed of these two ions would be affected by freezing the solution? Would you expect them to move faster or slower? Explain your answer.

14–25 Distinguish between a hydrogen atom, a proton, a hydride ion, and a hydrogen molecule.

14–26 The half reactions for some of the carriers in the respiratory chain are given in **Table 14–1**. From their E_0' values, what would you guess is their order in the chain? What would you need to know before you were more certain of their order?

14–27 The cytochrome *c* oxidase complex is strongly inhibited by cyanide, which binds to the Fe^{3+} form of cytochrome a_3. Cyanide kills at very low concentration because of its effects on the cytochrome *c* oxidase complex. Carbon monoxide (CO) also inhibits the cytochrome *c* oxidase

TABLE 14–1 Standard redox potentials for electron carriers in the respiratory chain (Problem 14–26).

Half reaction	E_0' (V)
ubiquinone + 2 H^+ + 2 e^- ⟶ ubiquinol	0.045
cytochrome *b* (Fe^{3+}) + e^- ⟶ cytochrome *b* (Fe^{2+})	0.077
cytochrome c_1 (Fe^{3+}) + e^- ⟶ cytochrome c_1 (Fe^{2+})	0.22
cytochrome *c* (Fe^{3+}) + e^- ⟶ cytochrome *c* (Fe^{2+})	0.25
cytochrome *a* (Fe^{3+}) + e^- ⟶ cytochrome *a* (Fe^{2+})	0.29
cytochrome a_3 (Fe^{3+}) + e^- ⟶ cytochrome a_3 (Fe^{2+})	0.55

complex by binding to cytochrome a_3, but it binds to the Fe^{2+} form. CO kills only at much higher doses than cyanide, not because of its binding to the cytochrome *c* oxidase complex, but because it binds to the heme group of hemoglobin, which also carries an Fe^{2+}. In a sense, by mopping up CO, hemoglobin protects cytochrome *c* oxidase from being inhibited by CO at low concentrations. One treatment for cyanide poisoning—if administered quickly enough—is to give sodium nitrite, which oxidizes Fe^{2+} to Fe^{3+}. How do you suppose sodium nitrite protects against the effects of cyanide?

14–28 The two different diffusible electron carriers, ubiquinone and cytochrome *c*, shuttle electrons between the three protein complexes of the electron-transport chain. In principle, could the same diffusible carrier be used for both steps? If not, why not? If it could, what characteristics would it need to possess and what would be the disadvantages of such a situation?

14–29 If you were to impose an artificially large electrochemical gradient across the mitochondrial inner membrane, would you expect electrons to flow up the respiratory chain, in the reverse of their normal direction? Why or why not?

14–30 Some bacteria have become specialized to live in an alkaline environment at pH 10. They maintain their internal environment at pH 7. Why is it that they cannot exploit the pH difference across their membrane to get ATP for free using a standard ATP synthase? Can you suggest an engineering modification to ATP synthase that would allow it to generate ATP from proton flow in such an environment?

CALCULATIONS

14–31 One of the problems in understanding redox reactions is coming to grips with the language. Consider the reduction of pyruvate by NADH:

$$\text{pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{lactate} + NAD^+$$

In redox reactions, oxidation and reduction necessarily occur together; however, it is convenient to list the two halves of a redox reaction separately. By convention, each half reaction is written as a reduction: oxidant + e^- ⟶ reductant. For the reduction of pyruvate by NADH the half reactions are

$$\text{pyruvate} + 2\,H^+ + 2e^- \longrightarrow \text{lactate} \qquad E_0' = -0.19\ \text{V}$$

$$NAD^+ + H^+ + 2e^- \longrightarrow \text{NADH} \qquad E_0' = -0.32\ \text{V}$$

where E_0' is the standard redox potential and refers to a reaction occurring under standard conditions (25°C or 298 K, all concentrations at 1 M, and pH 7). To obtain the overall equation for reduction of pyruvate by NADH, it is necessary to reverse the NAD^+/NADH half reaction and change the sign of E_0':

$$\text{pyruvate} + 2\,H^+ + 2e^- \longrightarrow \text{lactate} \qquad E_0' = -0.19\ \text{V}$$

$$\text{NADH} \longrightarrow NAD^+ + H^+ + 2e^- \qquad E_0' = +0.32\ \text{V}$$

Summing these two half reactions and their E_0' values gives the overall equation and its $\Delta E_0'$ value:

$$\text{pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{lactate} + NAD^+ \qquad \Delta E_0' = +0.13\ \text{V}$$

When a redox reaction takes place under nonstandard conditions, the tendency to donate electrons (ΔE) is equal to $\Delta E_0'$ modified by a concentration term:

$$\Delta E = \Delta E_0' - \frac{2.3\,RT}{nF} \log \frac{[\text{lactate}][NAD^+]}{[\text{pyruvate}][\text{NADH}]}$$

where $R = 8.3 \times 10^{-3}$ kJ/K mole, T = temperature in kelvins, n = the number of electrons transferred, and $F = 96$ kJ/V mole.

ΔG is related to ΔE by the equation

$$\Delta G = -nF\,\Delta E$$

Since the signs of ΔG and ΔE are opposite, a favorable redox reaction has a positive ΔE and a negative ΔG.

A. Calculate ΔG for reduction of pyruvate to lactate at 37°C with all reactants and products at a concentration of 1 M.
B. Calculate ΔG for the reaction at 37°C under conditions where the concentrations of pyruvate and lactate are equal and the concentrations of NAD^+ and NADH are equal.
C. What would the concentration term need to be for this reaction to have a ΔG of zero at 37°C?
D. Under normal conditions in vascular smooth muscle (at 37°C), the concentration ratio of NAD^+ to NADH is 1000, the concentration of lactate is 0.77 μmol/g, and the concentration of pyruvate is 0.15 μmol/g. What is ΔG for reduction of pyruvate to lactate under these conditions?

14–32 *Thiobacillus ferrooxidans*, a bacterium that lives on slag heaps at pH 2, is used by the mining industry to recover copper and uranium from low-grade ore by an acid leaching process. The bacteria oxidize Fe^{2+} to produce Fe^{3+}, which in turn oxidizes (and solubilizes) these minor components of the ore. It is remarkable that the bacterium can live in such an environment. It does so by exploiting the pH difference between the environment and its cytoplasm (pH 6.5) to drive synthesis of ATP and NADPH, which it can then use to fix CO_2 and nitrogen. In order to keep its cytoplasmic pH constant, *T. ferrooxidans* uses electrons from Fe^{2+} to reduce O_2 to water, thereby removing the protons:

$$4\,Fe^{2+} + O_2 + 4\,H^+ \longrightarrow 4\,Fe^{3+} + 2\,H_2O$$

What are the energetics of these processes? Is the flow of electrons from Fe^{2+} to O_2 energetically favorable? How difficult is it to reduce $NADP^+$ using electrons from Fe^{2+}? These are key questions for understanding how *T. ferrooxidans* manages to thrive in such an unlikely niche.

A. What are ΔE and ΔG for the reduction of O_2 by Fe^{2+}, assuming that the reaction occurs under standard conditions? The half reactions are

$$Fe^{3+} + e^- \longrightarrow Fe^{2+} \qquad E_0' = 0.77\ \text{V}$$

$$O_2 + 4\,H^+ + 4e^- \longrightarrow 2\,H_2O \qquad E_0' = 0.82\ \text{V}$$

B. Write a balanced equation for the reduction of $NADP^+ + H^+$ by Fe^{2+}. What is ΔG for this reaction under standard conditions? The half reaction for $NADP^+$ is

$$NADP^+ + H^+ + 2e^- \longrightarrow NADPH \qquad E_0' = -0.32\ V$$

What is ΔG for the reduction of $NADP^+ + H^+$ by Fe^{2+} if the concentrations of Fe^{3+} and Fe^{2+} are equal, the concentration of NADPH is 10-fold greater than that of $NADP^+$, and the temperature is 310 K? (Note: adjusting the number of atoms and electrons in order to balance the chemical equation does not affect E_0' or $\Delta E_0'$ values. It does, however, affect ΔE by its influence on the concentration term; each concentration term must be raised to an exponent equal to the number of atoms or molecules used in the balanced equation.)

14–33 What is the standard free-energy change ($\Delta G°$) associated with transfer of electrons from NADH to O_2, according to the balanced equation below?

$$O_2 + 2\ NADH + 2\ H^+ \rightleftharpoons 2\ H_2O + 2\ NAD^+$$

The half reactions are

$$O_2 + 4\ H^+ + 4e^- \longrightarrow 2\ H_2O \qquad E_0' = 0.82V$$

$$NAD^+ + H+ + 2e^- \longrightarrow NADH \qquad E_0' = -0.32\ V$$

A common way of writing this equation is

$$\tfrac{1}{2}\ O_2 + NADH + H^+ \rightleftharpoons H_2O + NAD^+$$

What is $\Delta G°$ for this equation? Do the two calculations give the same answer? Explain why they do or don't.

DATA HANDLING

14–34 In 1925, David Keilin used a simple spectroscope to observe the characteristic absorption bands of the cytochromes that make up the electron-transport chain in mitochondria. A spectroscope passes a very bright light through the sample of interest and then through a prism to display the spectrum from red to blue. If molecules in the sample absorb light of particular wavelengths, dark bands will interrupt the colors of the rainbow. Keilin found that tissues from a wide variety of animals all showed the pattern in **Figure 14–2**. (This pattern had actually been observed several decades before by an Irish physician named MacMunn, but he thought all the bands were due to a single pigment. His work was all but forgotten by the 1920s.)

The different heat stabilities of the individual absorption bands and their different intensities in different tissues led Keilin to conclude that the absorption pattern was due to three components, which he labeled cytochromes *a*, *b*, and *c* (Figure 14–2). His key discovery was that the absorption bands disappeared when oxygen was introduced (**Figure 14–3A**) and then reappeared when the samples became anaerobic

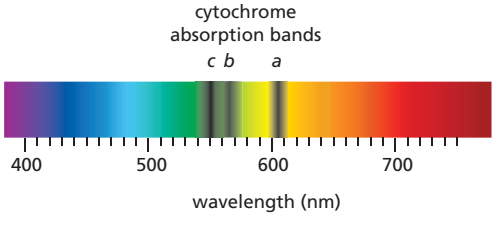


Figure 14–2 Cytochrome absorption bands (Problem 14–34).

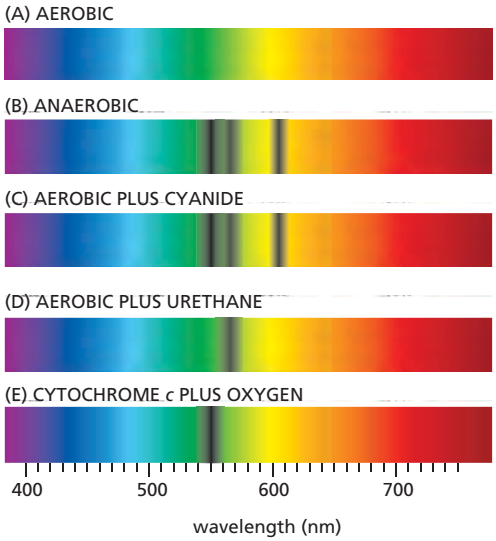


Figure 14–3 Cytochrome absorption bands under a variety of experimental conditions (Problem 14–34).

(**Figure 14–3B**). He later confessed, "This visual perception of an intracellular respiratory process was one of the most impressive spectacles I have witnessed in the course of my work."

Keilin subsequently discovered that cyanide prevented the bands from disappearing when oxygen was introduced (**Figure 14–3C**). When urethane (an inhibitor of electron transport that is no longer used) was added, bands *a* and *c* disappeared in the presence of oxygen, but band *b* remained (**Figure 14–3D**). Finally, using cytochrome *c* extracted from dried yeast, he showed that the band due to cytochrome *c* remained when oxygen was present (**Figure 14–3E**).

A. Is it the reduced (electron-rich) or the oxidized (electron-poor) forms of the cytochromes that give rise to the bands that Keilin observed?
B. From Keilin's observations, deduce the order in which the three cytochromes carry electrons from intracellular substrates to oxygen.
C. One of Keilin's early observations was that the presence of excess glucose prevented the disappearance of the absorption bands when oxygen was added. How do you think that rapid glucose oxidation to CO_2 might explain this observation?

14–35 If isolated mitochondria are incubated with a source of electrons such as succinate, but without oxygen, electrons enter the respiratory chain, reducing each of the electron carriers almost completely. When oxygen is then introduced, the carriers become oxidized at different rates (**Figure 14–4**). How does this result allow you to order the electron carriers in the respiratory chain? What is their order?

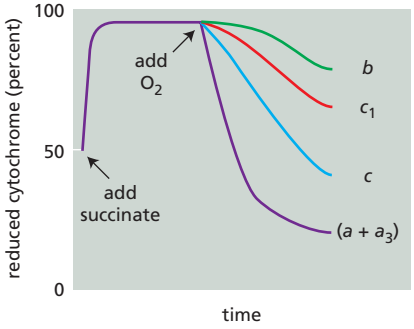


Figure 14–4 Rapid spectrophotometric analysis of the rates of oxidation of electron carriers in the respiratory chain (Problem 14–35). Cytochromes *a* and a_3 cannot be distinguished and thus are listed as cytochrome ($a + a_3$).

14–36 Inhibitors have provided extremely useful tools for analyzing mitochondrial function. **Figure 14–5** shows three distinct patterns of oxygen electrode traces obtained using a variety of inhibitors. In all experiments, mitochondria were added to a phosphate-buffered solution containing succinate as the sole source of electrons for the respiratory chain. After a short interval, ADP was added followed by an inhibitor, as indicated in Figure 14–5. The rates of oxygen consumption at various times during the experiment are shown by downward-sloping lines, with faster rates of consumption shown by steeper lines.

A. Based on the descriptions of the inhibitors in **Table 14–2**, assign each inhibitor to one of the oxygen traces in Figure 14–5. All these inhibitors stop ATP synthesis.
B. Using the same experimental protocol indicated in Figure 14–5, sketch

TABLE 14–2 Effects of a variety of inhibitors of mitochondrial function (Problem 14–36).

Inhibitor	Function
1. FCCP	Makes membranes permeable to protons
2. Malonate	Prevents oxidation of succinate
3. Cyanide	Inhibits the cytochrome *c* oxidase complex
4. Atractyloside	Inhibits the ADP/ATP transporter
5. Oligomycin	Inhibits ATP synthase
6. Butylmalonate	Blocks mitochondrial uptake of succinate

the oxygen traces that you would expect for the sequential addition of the pairs of inhibitors in the list below.

1. FCCP followed by cyanide
2. FCCP followed by oligomycin
3. Oligomycin followed by FCCP

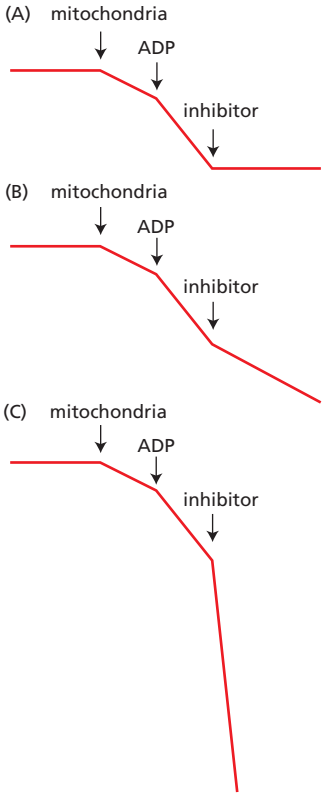


Figure 14–5 Oxygen traces showing three patterns of inhibitor effects on oxygen consumption by mitochondria (Problem 14–36).

MEDICAL LINKS

14–37 Normally, the flow of electrons to O_2 is tightly linked to the production of ATP via the electrochemical gradient. If ATP synthase is inhibited, for example, electrons do not flow down the electron-transport chain and respiration ceases. Since the 1940s, several substances—such as 2,4-dinitrophenol—have been known to uncouple electron flow from ATP synthesis. Dinitrophenol was once prescribed as a diet drug to aid in weight loss. How would an uncoupler of oxidative phosphorylation promote weight loss? Why do you suppose dinitrophenol is no longer prescribed?

ATP PRODUCTION IN MITOCHONDRIA

TERM TO LEARN

ATP synthase

DEFINITIONS

Match the definition below with its term from the list above.

14–38 Enzyme in the inner membrane of a mitochondrion that catalyzes the formation of ATP from ADP and inorganic phosphate.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

14–39 An average person contains 50 kg of ATP, which is required to meet their daily energy needs.

14–40 The number of *c* subunits in the rotor ring of ATP synthase defines how many protons need to pass through the turbine to make each molecule of ATP.

THOUGHT PROBLEMS

14–41 The respiratory chain is relatively inaccessible to experimental manipulation in intact mitochondria. After disrupting mitochondria with ultrasound, however, it is possible to isolate functional submitochondrial particles, which consist of broken cristae that have resealed inside-out into small closed vesicles. In these vesicles, the components that originally

faced the matrix are now exposed to the surrounding medium. How do you suppose such an arrangement might aid in the study of electron transport and ATP synthesis?

14–42 As electrons move down the respiratory chain, protons are pumped across the inner membrane. Are those protons confined to the intermembrane space? Why or why not?

14–43 You have reconstituted into the membranes of the same vesicles purified bacteriorhodopsin, which is a light-driven H^+ pump from a photosynthetic bacterium, and purified ATP synthase from ox heart mitochondria. Assume that all molecules of bacteriorhodopsin and ATP synthase are oriented as shown in **Figure 14–6**, so that protons are pumped into the vesicle and ATP synthesis occurs on the outer surface.

A. If you add ADP and phosphate to the external medium and shine light into the suspension of vesicles, would you expect ATP to be generated? Why or why not?
B. If you prepared the vesicles without being careful to remove all the detergent, which makes the bilayer leaky to protons, would you expect ATP to be synthesized?
C. If the ATP synthase molecules were randomly oriented so that about half faced the outside of the vesicle and half faced the inside, would you expect ATP to be synthesized? If the bacteriorhodopsin molecules were randomly oriented, would you expect ATP to be synthesized? Explain your answers.
D. You tell a friend over dinner about your new experiments. He questions the validity of an approach that utilizes components from so widely divergent, unrelated organisms. As he so succinctly puts it, "Why would anybody want to mix vanilla pudding with brake fluid?" Defend your approach against his criticism.

14–44 An elongated arm—the stator—links the catalytic head group (the $\alpha_3\beta_3$ complex) of the ATP synthase to the membrane-embedded rotor component. Attached to the rotor is a stalk (the axle-like γ subunit) that turns inside the head group to force the conformational changes that lead to ATP synthesis. If the stator were missing, would ATP be synthesized in response to the proton flow? Why or why not?

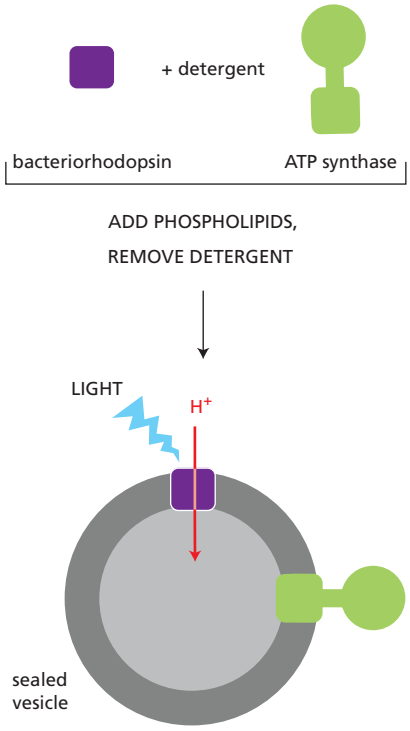


Figure 14–6 Reconstitution of bacteriorhodopsin and ATP synthase into lipid vesicles (Problem 14–43).

CALCULATIONS

14–45 In actively respiring liver mitochondria, the pH in the matrix is about half a pH unit higher than it is in the cytosol. Assuming that the cytosol is at pH 7 and the matrix is a sphere with a diameter of 1 μm [$V = (4/3)\,\pi r^3$], calculate the total number of protons in the matrix of a respiring liver mitochondrion. If the matrix began at pH 7 (equal to that in the cytosol), how many protons would have to be pumped out to establish a matrix pH of 7.5 (a difference of 0.5 pH unit)?

14–46 The relationship of free-energy change (ΔG) to the concentrations of reactants and products is important because it predicts the direction of spontaneous chemical reactions. Familiarity with this relationship is essential for understanding energy conversions in cells. Consider, for example, the hydrolysis of ATP to ADP and inorganic phosphate (P_i):

$$ATP + H_2O \longrightarrow ADP + P_i$$

The free-energy change due to ATP hydrolysis is

$$\Delta G = \Delta G^\circ + RT \ln \frac{[ADP][P_i]}{[ATP]}$$

$$= \Delta G^\circ + 2.3\,RT \log \frac{[ADP][P_i]}{[ATP]}$$

where the concentrations are expressed as molarities (by convention, the concentration of water is not included in the expression). R is the gas constant (8.3×10^{-3} kJ/K mole), T is temperature (assume 37°C, which is 310 K), and $\Delta G°$ is the standard free-energy change (–30.5 kJ/mole for ATP hydrolysis to ADP and $P_{i)}$.

A. Calculate ΔG for ATP hydrolysis when the concentrations of ATP, ADP, and P_i are all equal to 1 M. What is ΔG when the concentrations of ATP, ADP, and P_i are all equal to 1 mM?

B. In a resting muscle, the concentrations of ATP, ADP, and P_i are approximately 5 mM, 1 mM, and 10 mM, respectively. What is ΔG for ATP hydrolysis in resting muscle?

C. What will ΔG equal when the hydrolysis reaction reaches equilibrium? At $[P_i] = 10$ mM, what will be the ratio of [ATP] to [ADP] at equilibrium?

D. Show that, at constant $[P_i]$, ΔG decreases by 5.9 kJ/mole for every 10-fold increase in the ratio of [ATP] to [ADP], regardless of the value of $\Delta G°$. (For example, ΔG decreases by 11.8 kJ/mole for a 100-fold increase, by 17.7 kJ/mole for a 1000-fold increase, and so on.)

DATA HANDLING

14–47 ATP synthase is the world's smallest rotary motor. Passage of H^+ ions through the membrane-embedded portion of ATP synthase (the F_o component) causes rotation of the single, central, axle-like γ subunit inside the head group. The tripartite head is composed of the three αβ dimers, the β subunit of which is responsible for synthesis of ATP. The rotation of the γ subunit induces conformational changes in the αβ dimers that allow ADP and P_i to be converted into ATP. A variety of indirect evidence had suggested rotary catalysis by ATP synthase, but seeing is believing.

To demonstrate rotary motion, a modified form of the $\alpha_3\beta_3\gamma$ complex was used. The β subunits were modified so they could be firmly anchored to a solid support and the γ subunit was modified (on the end that normally inserts into the F_o component in the inner membrane) so that a fluorescently tagged, readily visible filament of actin could be attached (**Figure 14–7A**). This arrangement allows rotations of the γ subunit to be visualized as revolutions of the long actin filament. In these experiments, ATP synthase was studied in the reverse of its normal mechanism by allowing it to hydrolyze ATP. At low ATP concentrations, the actin filament was observed to revolve in steps of 120° and then pause for variable lengths of time, as shown in **Figure 14–7B**.

A. Why does the actin filament revolve in steps with pauses in between? What does this rotation correspond to in terms of the structure of the $\alpha_3\beta_3\gamma$ complex?

B. In its normal mode of operation inside the cell, how many ATP molecules do you suppose would be synthesized for each complete 360° rotation of the γ subunit? Explain your answer.

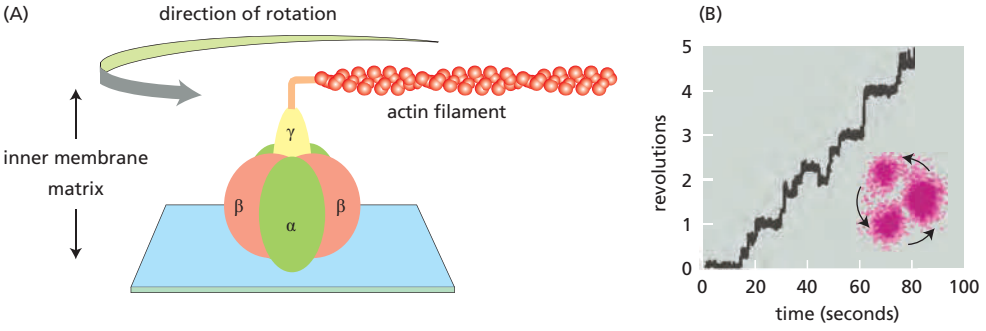


Figure 14–7 Experimental set-up for observing rotation of the γ subunit of ATP synthase (Problem 14–47). (A) The immobilized $\alpha_3\beta_3\gamma$ complex. The β subunits are anchored to a solid support and a fluorescent actin filament is attached to the γ subunit. (B) Stepwise revolution of the actin filament. The indicated trace is a typical example from one experiment. The inset shows the positions in the revolution at which the actin filament pauses.

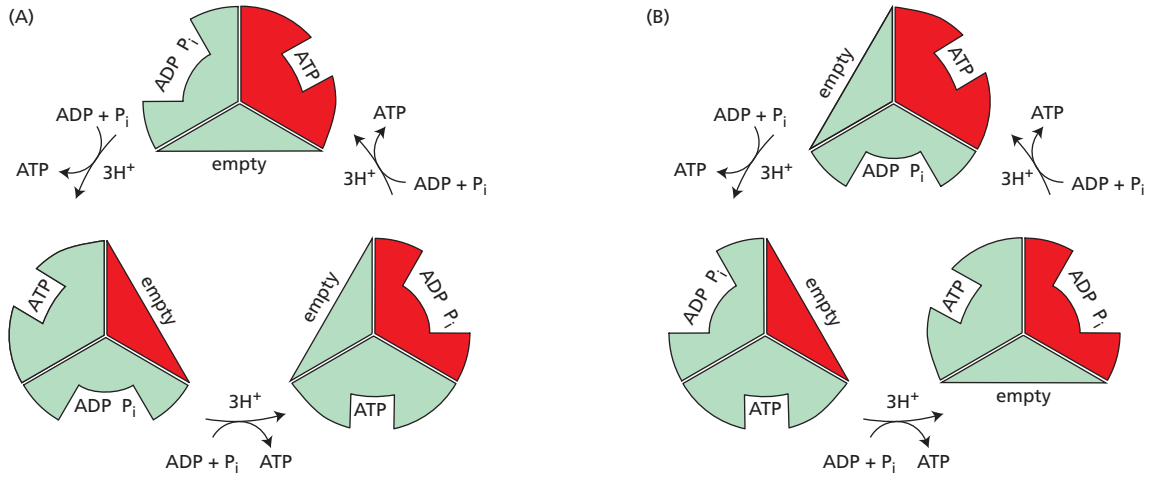


Figure 14–8 The two possible arrangements of conformations of the three αβ dimers in ATP synthase, along with the linked conformational changes driven by proton flow (Problem 14–48). One αβ dimer is colored *red* to emphasize that its position remains fixed as it changes conformation in response to proton flow. The perspective illustrated is from the *inner* membrane, looking at the *underside* of the tripartite $\alpha_3\beta_3$ complex.

14–48 The three αβ dimers in each ATP synthase normally exist in three different conformations: one empty, one with ADP and P_i bound, and one with ATP bound. The conformational changes are driven sequentially by rotation of the γ subunit, which in turn is driven by the flow of protons through the ATP synthase. As viewed from the inner membrane, looking at the underside of the $\alpha_3\beta_3$ complex, these sites could be arranged in either of two ways around the central γ subunit (**Figure 14–8**). The sequential, linked conformational changes driven by proton flow are also shown for the two arrangements in the figure. In Problem 14–47, the revolutions of the attached actin filaments during ATP hydrolysis were shown to be counterclockwise when viewed from the same perspective (see Figure 14–7). Which of the two arrangements of conformations of αβ dimers shown in Figure 14–8 is correct? Explain your answer.

14–49 A manuscript has been submitted to a prestigious scientific journal. In it the authors describe an experiment using an immobilized $\alpha_3\beta_3\gamma$ complex with an attached actin filament like that shown in Figure 14–7A. The authors show that they can mechanically rotate the γ subunit by applying force to the actin filament. Moreover, in the presence of ADP and phosphate, each 120° clockwise rotation of the γ subunit is accompanied by the synthesis of one molecule of ATP. Is this result at all reasonable? What would such an observation imply about the mechanism of ATP synthase? Should this manuscript be considered for publication in one of the best journals?

14–50 The ADP/ATP transporter in the mitochondrial inner membrane can exchange ATP for ATP, ADP for ADP, and ATP for ADP. Even though mitochondria can transport both ADP and ATP, there is a strong bias in favor of exchange of external ADP for internal ATP in actively respiring mitochondria. You suspect that this bias is due to the conversion of ADP into ATP inside the mitochondrion. ATP synthesis would continually reduce the internal concentration of ADP and thereby create a favorable concentration gradient for import of ADP. The same process would increase the internal concentration of ATP, thereby creating favorable conditions for export of ATP.

To test your hypothesis, you conduct experiments on isolated mitochondria. In the absence of substrate (when the mitochondria are not respiring and the membrane is uncharged), you find that ADP and ATP are taken up at the same rate. When you add substrate, the mitochondria begin to respire, and ADP enters mitochondria at a much faster rate than ATP. As you expected, when you add dinitrophenol, which collapses the pH gradient, along with the substrate, ADP and ATP again enter at

TABLE 14–3 Entry of ADP and ATP into isolated mitochondria (Problem 14–50).

Experiment	Substrate	Inhibitor	Relative rates of entry
1	Absent	None	ADP = ATP
2	Present	None	ADP > ATP
3	Present	Dinitrophenol	ADP = ATP
4	Present	Oligomycin	ADP > ATP
In all cases, the initial rates of entry of ATP and ADP were measured.			

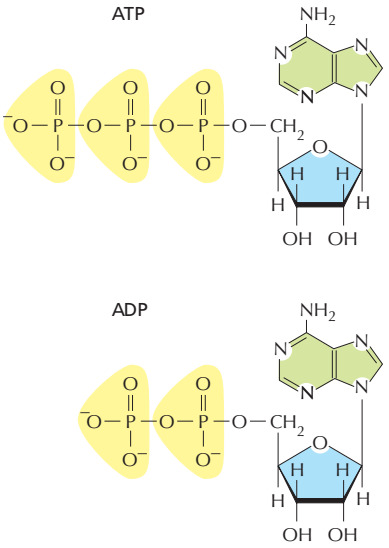


Figure 14–9 Structures of ATP and ADP (Problem 14–50).

the same rate. When you add an inhibitor of ATP synthase (oligomycin) along with the substrate, ADP is taken up much faster than ATP. Your results are summarized in Table 14–3. You are puzzled by the results with oligomycin, since your hypothesis predicted that the rates of uptake would be equal.

When you show the results to your advisor, she compliments you on your fine experiments and agrees that they disprove the hypothesis. She suggests that you examine the structures of ATP and ADP (Figure 14–9) if you wish to understand the behavior of the ADP/ATP transporter. What is the correct explanation for the biased exchange by the ADP/ATP transporter under some of the experimental conditions and an unbiased exchange under others?

CHLOROPLASTS AND PHOTOSYNTHESIS

TERMS TO LEARN

antenna complex
carbon fixation
carbon-fixation reactions
charge separation
chlorophyll
chloroplast
photochemical reaction center
photosynthetic electron transfer
photosystem
stroma
thylakoid membrane

DEFINITIONS

Match each definition below with its term from the list above.

14–51 Light-driven reactions in photosynthesis in which electrons move along the electron-transport chain in the thylakoid membrane.

14–52 Part of a photosystem that captures light energy and channels it into the photochemical reaction center.

14–53 Process by which green plants incorporate carbon atoms from atmospheric carbon dioxide into sugars.

14–54 The part of a photosystem that converts light energy into chemical energy.

14–55 Organelle in green algae and plants that contains chlorophyll and carries out photosynthesis.

14–56 Light-absorbing green pigment that plays a central role in photosynthesis.

14–57 The large space that surrounds the inner chloroplast membrane.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

14–58 In a general way, one might view the chloroplast as a greatly enlarged mitochondrion in which the cristae have been pinched off to form a series of interconnected submitochondrial particles in the matrix space.

14–59 When a molecule of chlorophyll in an antenna complex absorbs a photon, the excited electron is rapidly transferred from one chlorophyll molecule to another until it reaches the photochemical reaction center.

THOUGHT PROBLEMS

14–60 Both mitochondria and chloroplasts use electron transport to pump protons, creating an electrochemical proton gradient, which drives ATP synthesis. Are protons pumped across the same (analogous) membranes in the two organelles? Is ATP synthesized in the analogous compartments? Explain your answers.

14–61 A suspension of the green alga *Chlamydomonas* is actively carrying out photosynthesis in the presence of light and CO_2. If you turned off the light, how would you expect the amounts of ribulose 1,5-bisphosphate and 3-phosphoglycerate to change over the next minute? How about if you left the light on but removed the CO_2?

14–62 Why are plants green?

14–63 Treatment of chloroplasts with the herbicide DCMU stops O_2 evolution and photophosphorylation. If an artificial electron acceptor is added that accepts electrons from plastoquinone (Q), oxygen evolution is restored but not photophosphorylation. Propose a site at which DCMU acts in the flow of electrons through photosystems II and I (**Figure 14–10**). Explain your reasoning. Why is DCMU an herbicide?

14–64 In chloroplasts, protons are pumped out of the stroma across the thylakoid membrane, whereas in mitochondria, they are pumped out of the matrix across the crista membrane. Explain how this arrangement allows chloroplasts to generate a larger proton gradient across the thylakoid membrane than mitochondria can generate across the inner membrane.

14–65 Unlike mitochondria, chloroplasts do not have a transporter that allows them to export ATP to the cytosol. How, then, does the rest of the cell get the ATP it needs to survive?

CALCULATIONS

14–66 How much energy is available in visible light? How much energy does sunlight deliver to Earth? How efficient are plants at converting light energy into chemical energy? The answers to these questions provide an important backdrop to the subject of photosynthesis.

Each quantum or photon of light has energy $h\nu$, where h is Planck's constant (6.6×10^{-37} kJ sec/photon) and ν is the frequency in sec^{-1}. The frequency of light is equal to c/λ, where c is the speed of light (3.0×10^{17} nm/sec) and λ is the wavelength in nm. Thus, the energy (E) of a photon is

$$E = h\nu = hc/\lambda$$

A. Calculate the energy of a mole of photons (6×10^{23} photons/mole) at 400 nm (violet light), at 680 nm (red light), and at 800 nm (near-infrared light).

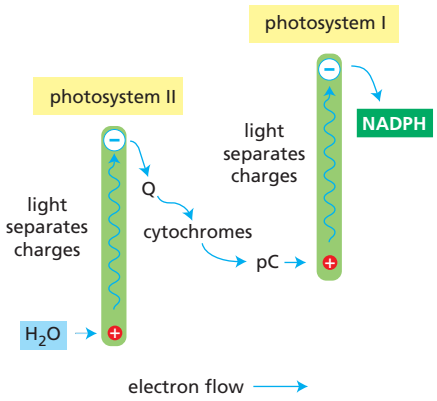


Figure 14–10 Flow of electrons through photosystems II and I during photosynthesis in chloroplasts (Problem 14–63). Electrons from photosystem II flow to plastoquinone (Q), then to the cytochrome b_6-f complex (cytochromes), and then to plastocyanin (pC), after which they enter photosystem I. The protons pumped by the cytochrome b_6-f complex generate an electrochemical gradient, which is used to drive ATP synthesis.

B. Bright sunlight strikes Earth at the rate of about 1.3 kJ/sec per square meter. Assuming for the sake of calculation that sunlight consists of monochromatic light of wavelength 680 nm, how many seconds would it take for a mole of photons to strike a square meter?
C. Assuming that it takes eight photons to fix one molecule of CO_2 as carbohydrate under optimal conditions (8–10 photons is the currently accepted value), calculate how long it would take a tomato plant with a leaf area of 1 square meter to make a mole of glucose from CO_2. Assume that photons strike the leaf at the rate calculated above and, furthermore, that all the photons are absorbed and used to fix CO_2.
D. If it takes 468 kJ/mole to fix a mole of CO_2 into carbohydrate, what is the efficiency of conversion of light energy into chemical energy after photon capture? Assume again that eight photons of red light (680 nm) are required to fix one molecule of CO_2.

14–67 What fraction of the free energy of light at 700 nm is captured when a chlorophyll molecule (P700) at the photochemical reaction center in photosystem I absorbs a photon? The equation for calculating the free energy available in one photon of light is given in Problem 14–66. If one assumes standard conditions, the captured free energy ($\Delta G = -nF\Delta E_0'$) can be calculated from the standard redox potential for $P700^*$ (excited) $\rightarrow$ P700 (ground state), which can be gotten from the half reactions:

$$P700^+ + e^- \longrightarrow P700 \qquad E_0' = 0.4\ V$$

$$P700^+ + e^- \longrightarrow P700^* \qquad E_0' = -1.2\ V$$

14–68 The balanced equation for production of NADPH by the Z scheme of photophosphorylation is

$$2\ H_2O + 2\ NADP^+ \longrightarrow 2\ NADPH + 2\ H^+ + O_2$$

How many photons must be absorbed to generate two NADPH and a molecule of O_2? (Assume one photon excites one electron.)

14–69 *T. ferrooxidans*, the slag-heap bacterium that lives at pH 2, fixes CO_2 like photosynthetic organisms but uses the abundant Fe^{2+} in its environment as a source of electrons instead of H_2O. *T. ferrooxidans* oxidizes Fe^{2+} to Fe^{3+} to reduce $NADP^+$ to NADPH, a very unfavorable reaction with a ΔE of about –1.1 V. It does so by coupling production of NADPH to the energy of the natural proton gradient across its membrane, which has a free-energy change (ΔG) of –26.8 kJ/mole H^+. What is the smallest number of protons to the nearest integer that would be required to drive the reduction of $NADP^+$ by Fe^{2+}? How do you suppose proton flow is mechanistically coupled to the reduction of $NADP^+$?

DATA HANDLING

14–70 Careful experiments comparing absorption and action spectra of plants ultimately led to the notion that two photosystems cooperate in chloroplasts. The absorption spectrum is the amount of light captured by photosynthetic pigments at different wavelengths. The action spectrum is the rate of photosynthesis (for example, O_2 evolution or CO_2 fixation) resulting from the capture of photons.

T.W. Engelmann, who used simple equipment and an ingenious experimental design, probably made the first measurement of an action spectrum in 1882. He placed a filamentous green alga into a test tube along with a suspension of oxygen-seeking bacteria. He allowed the bacteria to use up the available oxygen and then illuminated the alga with light that had been passed through a prism to form a spectrum. After a short time he observed the results shown in **Figure 14–11**. Sketch the

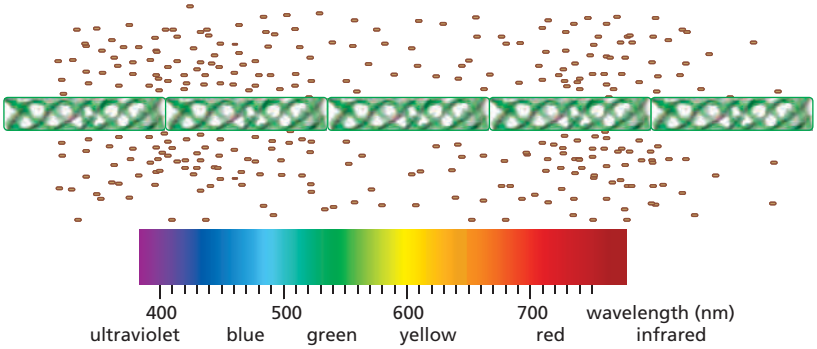


Figure 14–11 Experiment to measure the action spectrum of a filamentous green alga (Problem 14–70). Bacteria, which are indicated by the *brown rectangles*, were distributed evenly throughout the test tube at the beginning of the experiment.

action spectrum (O_2 evolved at different wavelengths of light) for this alga and explain how this experiment works.

14–71 The most compelling early evidence for the Z scheme of photosynthesis came from measuring the oxidation states of the cytochromes in algae under different regimes of illumination (**Figure 14–12**). Illumination with light at 680 nm caused oxidation of cytochromes (indicated by the upward trace in Figure 14–12A). Additional illumination with light at 562 nm caused reduction of the cytochromes (indicated by the downward trace in Figure 14–12A). When the lights were then turned off, both effects were reversed (Figure 14–12A). In the presence of the herbicide DCMU (see Problem 14–63), no reduction with 562-nm light occurred (Figure 14–12B).

A. In these algae, which wavelength stimulates photosystem I and which stimulates photosystem II?

B. How do these results support the Z scheme for photosynthesis; that is, how do they support the idea that there are two photosystems that are linked by cytochromes?

C. On which side of the cytochromes does DCMU block electron transport—on the side nearer photosystem I or the side nearer photosystem II?

14–72 Photosystem II accepts electrons from water, generating O_2, and donates them via the electron-transport chain to photosystem I. Each photon absorbed by photosystem II transfers only a single electron, and yet four

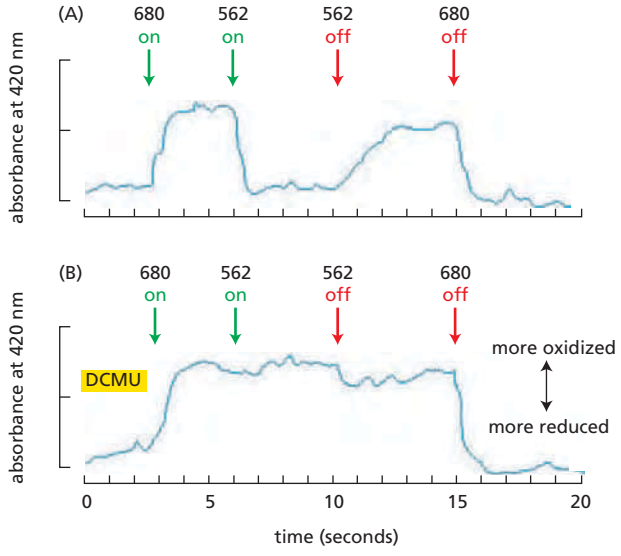


Figure 14–12 Oxidation state of cytochromes after illumination of algae with light of different wavelengths (Problem 14–71). (A) In the absence of DCMU. (B) In the presence of DCMU. An *upward* trace indicates oxidation of the cytochromes; a *downward* trace indicates reduction of the cytochromes.

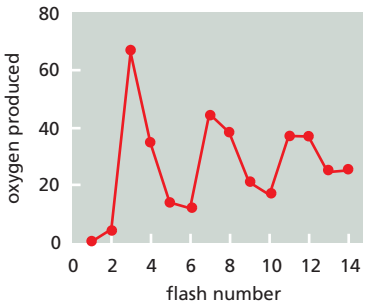


Figure 14–13 Oxygen evolution by spinach chloroplasts in response to saturating flashes of light (Problem 14–72). The chloroplasts were placed in the dark for 40 minutes prior to the experiment to allow them to come to the same "ground" state. Oxygen production is expressed in arbitrary units.

electrons must be removed from water to generate a molecule of O_2. Thus, four photons are required to produce a molecule of O_2:

$$2\,H_2O + 4\,h\nu \longrightarrow 4e^- + 4\,H^+ + O_2$$

How do four photons cooperate in the production of O_2? Is it necessary that four photons arrive at a single reaction center simultaneously? Can four activated reaction centers cooperate to produce a molecule of O_2? Or is there some sort of "gear wheel" that collects the four electrons from H_2O and transfers them one at a time to a reaction center?

To investigate this problem, you expose dark-adapted spinach chloroplasts to a series of brief saturating flashes of light (2 μsec) separated by short periods of darkness (0.3 sec) and measure the production of O_2 that results from each flash. Under this lighting regime, most photosystems capture a photon during each flash. As shown in **Figure 14–13**, O_2 is produced with a distinct periodicity: the first burst of O_2 occurs on the third flash, and subsequent peaks occur every fourth flash thereafter. If you first inhibit 97% of the photosystem II reaction centers with DCMU and then repeat the experiment, you observe the same periodicity of O_2 production, but the peaks are only 3% of the uninhibited values.

A. How do these results distinguish among the three possibilities posed at the outset (simultaneous action, cooperation among reaction centers, and a gear wheel)?
B. Why do you think it is that the first burst of O_2 occurs after the third flash, whereas additional peaks occur at four-flash intervals? (Consider what this observation implies about the dark-adapted state of the chloroplasts.)
C. Can you suggest a reason why the periodicity in O_2 production becomes less pronounced with increasing flash number?

14–73 In an insightful experiment performed in the 1960s, chloroplasts were first soaked in an acidic solution at pH 4, so that the stroma and thylakoid space became acidified (**Figure 14–14**). They were then transferred to a basic solution (pH 8). This rapidly increased the pH of the stroma to 8, while the thylakoid space temporarily remained at pH 4. A burst of ATP synthesis was observed, and the pH difference between the thylakoid space and the stroma quickly disappeared.

A. Explain why these conditions lead to ATP synthesis.
B. Is light needed for the experiment to work? Why or why not?
C. What would happen if the solutions were switched so that the first incubation was in the pH 8 solution and the second one was in the pH 4 solution? Explain your answer.
D. Does this experiment support the chemiosmotic model, or raise questions about it?

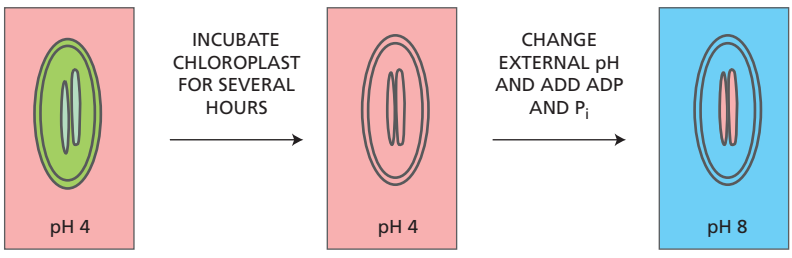


Figure 14–14 Soaking chloroplasts in acidic and basic solutions (Problem 14–73). *Pink* areas are at pH 4.

THE GENETIC SYSTEMS OF MITOCHONDRIA AND CHLOROPLASTS

TERM TO LEARN

maternal inheritance

DEFINITIONS

Match the definition below with its term from the list above.

14–74 Pattern of mitochondrial inheritance in higher animals that arises because the egg cells always contribute much more cytoplasm to the zygote than does the sperm.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

14–75 The mitochondrial genetic code differs slightly from the nuclear code, but it is identical in mitochondria from all species that have been examined.

14–76 The presence of introns in organellar genes is not surprising since similar introns have been found in related genes from bacteria whose ancestors are thought to have given rise to mitochondria and chloroplasts.

14–77 Mutations that are inherited according to Mendelian rules affect nuclear genes; mutations whose inheritance violates Mendelian rules are likely to affect organelle genes.

THOUGHT PROBLEMS

14–78 You have discovered a remarkable, one-celled protozoan that lives in an anaerobic environment. It has caught your attention because it has absolutely no mitochondria, an exceedingly rare condition among eukaryotes. If you could show that this organism derives from an ancient lineage that split off from the rest of eukaryotes before mitochondria were acquired, it would truly be a momentous discovery. You sequence the organism's genome so you can make detailed comparisons. It is clear from sequence comparisons that your organism does indeed derive from an ancient lineage. But here and there, scattered around the genome, are bits of DNA that in aggregate resemble the bacterial genome from which mitochondria evolved. Propose a plausible evolutionary history for your organism.

14–79 At the cellular level, evolutionary theories are particularly difficult to test since fossil evidence is lacking. The possible evolutionary origins of mitochondria and chloroplasts must be sought in living organisms. Fortunately, living forms resembling the ancestral types thought to have established an endosymbiotic relationship that led to the origin of mitochondria and chloroplasts can be found today. For example, the plasma membrane of the free-living aerobic bacterium *Paracoccus denitrificans* contains a respiratory chain that is nearly identical to the respiratory chain of mammalian mitochondria—both in the types of respiratory components present and in its sensitivity to respiratory inhibitors. Indeed, no significant feature of the mammalian respiratory chain is absent from *Paracoccus. Paracoccus* effectively assembles in a single organism all those features of the mitochondrial inner membrane that are otherwise distributed at random among other aerobic bacteria.

Figure 14–15 A variegated leaf of *Aucuba japonica* with green and yellow patches (Problem 14–80).

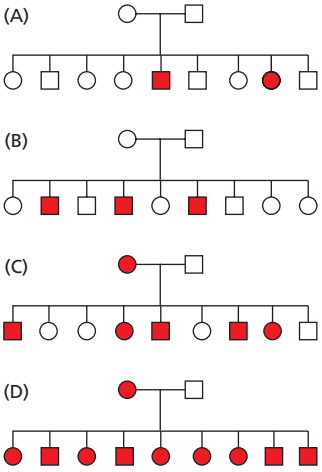


Figure 14–16 Hypothetical pedigrees representing four patterns of inheritance (Problem 14–81). Males are shown as *squares*; females as *circles*. Affected individuals are shown as *red symbols*; unaffected individuals are shown as *white symbols*.

Imagine that you are a protoeukaryotic cell looking out for your evolutionary future. You have been observing proto-*Paracoccus* and are amazed at its incredibly efficient use of oxygen in generating ATP. With such a source of energy, your horizons would be unlimited. You plot to hijack a proto-*Paracoccus* and make it work for you and your descendants. You plan to take it into your cytoplasm, feed it any nutrients it needs, and harvest the ATP. Accordingly, one dark night, you trap a wandering proto-*Paracoccus*, surround it with your plasma membrane, and imprison it in a new cytoplasmic compartment. To your relief, the proto-*Paracoccus* seems to enjoy its new environment. After a day of waiting, however, you feel as sluggish as ever. What has gone wrong with your scheme?

14–80 Examine the variegated leaf shown in **Figure 14–15**. Yellow patches surrounded by green are common, but there are no green patches surrounded by yellow. Propose an explanation for this phenomenon.

14–81 The pedigrees in **Figure 14–16** show one example each of the following types of mutation: mitochondrial mutation, autosomal recessive mutation, autosomal dominant mutation, and X-linked recessive mutation. In each family, the parents have had nine children. Assign each pedigree to one type of mutation. Explain the basis for your assignments.

DATA HANDLING

14–82 It is well accepted that transfer of DNA from organellar genomes to nuclear genomes is common during evolution. Do transfers between organellar genomes also occur? One experiment to search for genetic transfers between organellar genomes used a defined restriction fragment from spinach chloroplasts, which carried information for the gene for the large subunit of ribulose bisphosphate carboxylase. This gene has no known mitochondrial counterpart. Thus, if a portion of the chloroplast DNA in the restriction fragment were transferred to the mitochondrial genome, it would show up as a hybridizing band at a novel position. Mitochondrial and chloroplast DNAs were prepared from zucchini, corn, spinach, and pea. All these DNAs were digested with the same restriction nuclease, and the resulting fragments were separated by electrophoresis. The fragments were then transferred to a filter and hybridized to a radioactive preparation of the spinach fragment. A schematic representation of the autoradiograph is shown in **Figure 14–17**.

A. It is very difficult to prepare mitochondrial DNA that is not contaminated to some extent with chloroplast DNA. How do these experiments control for contamination of the mitochondrial DNA preparation by chloroplast DNA?

B. Which of these plant mitochondrial DNAs appear to have acquired chloroplast DNA?

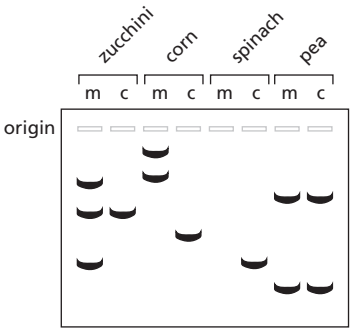


Figure 14–17 Patterns of hybridization of a probe from spinach chloroplast DNA to mitochondrial and chloroplast DNAs from zucchini, corn, spinach, and pea (Problem 14–82). Lanes labeled *m* contain mitochondrial DNA; lanes labeled *c* contain chloroplast DNA. Restriction fragments to which the probe hybridized are shown as *dark bands*.

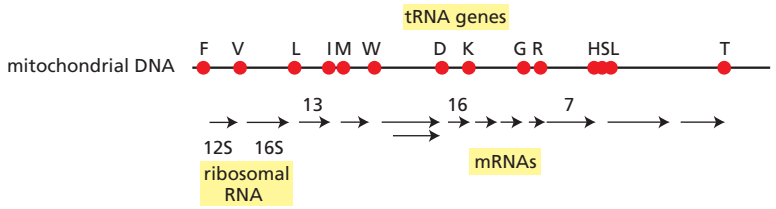


Figure 14–18 Transcription map of human mitochondrial DNA (Problem 14–83). Individual tRNA genes are indicated by *red circles*; the amino acids they carry are shown in the one-letter code. The three mRNAs whose detailed sequences are shown in Figure 14–19 are indicated by *number*.

14–83 The majority of mRNAs, tRNAs, and rRNAs in human mitochondria are transcribed from one strand of the genome. These RNAs are all present initially on one very long transcript, which is 93% of the length of the DNA strand. During mitochondrial protein synthesis, these RNAs function as separate, independent species of RNA. The relationship of the individual RNAs to the primary transcript and many of the special features of the mitochondrial genetic system have been revealed by comparing the sequences of the RNAs with the nucleotide sequence of the genome. An overview of the transcription map is shown in **Figure 14–18**.

Three segments of the nucleotide sequence of the human mitochondrial genome are shown in **Figure 14–19** along with the three mRNAs that are generated from those regions. The nucleotides that encode tRNA species are highlighted; the amino acids encoded by the mRNAs are indicated below the center base of the codon.

A. In terms of codon usage and mRNA structure, in what two ways does initiation of protein synthesis in mitochondria differ from initiation in the cytoplasm?

B. In what two ways are the termination codons for protein synthesis in mitochondria unusual? (The termination codons are shown in Figure 14–19 as asterisks.)

C. Does the arrangement of tRNA and mRNA sequences in the genome suggest a possible mechanism for processing the primary transcript into individual RNA species?

14–84 A friend of yours has been studying a pair of mutants in the fungus *Neurospora*, which she has whimsically named *poky* and *puny*. Both mutants grow at about the same rate, but much more slowly than wild type. Your friend has been unable to find any supplement that improves their growth rates. Her biochemical analysis shows that each mutant displays a different abnormal pattern of cytochrome absorption. To characterize the mutants genetically, she crossed them to wild type and to each other and tested the growth rates of the progeny. She has come to you because she is puzzled by the results.

She explains that haploid nuclei from the two parents fuse during a *Neurospora* mating and then divide meiotically to produce four haploid spores, which can be readily tested for their growth rates. The parents

Figure 14–19 Arrangements of tRNA and mRNA sequences at three places on the human mitochondrial genome (Problem 14–83). *Highlighted* sequences indicate tRNA genes. The sequences of the mRNAs are shown in *blue* below the corresponding genes. The middle portions of the mRNAs and their genes are indicated by *dots*. The 5′ ends of the sequences are shown at the *left*. The 5′ ends of the mRNAs are unmodified and the 3′ ends have poly-A tails. The encoded protein sequences are indicated in *green* below the mRNAs, with the letter for the amino acid immediately under the center nucleotide of the codon. An asterisk (*) indicates a termination codon.

TABLE 14–4 Genetic analysis of *Neurospora* mutants (Problem 14–84).

Cross	Protoperithecial parent	Fertilizing parent	Spore counts	
			Fast growth	Slow growth
1	*poky*	wild	0	1749
2	wild	*poky*	1334	0
3	*puny*	wild	850	799
4	wild	*puny*	793	801
5	*poky*	*puny*	0	1831
6	*puny*	*poky*	754	710
7	wild	wild	1515	0
8	*poky*	*poky*	0	1389
9	*puny*	*puny*	0	1588

contribute unequally to the diploid: one parent (the protoperithecial parent) donates a nucleus and the cytoplasm; the other (the fertilizing parent) contributes little more than a nucleus—much like egg and sperm in higher organisms. As shown in **Table 14–4**, the "order" of the crosses sometimes makes a difference: this is a result she has not seen before.

Can you help your friend understand these results?

MCAT STYLE

Passage 1 (Questions 14–85 to 14–87)

Scientists discovered the mechanism for ATP production via glycolysis decades before they understood the mechanism that generates ATP via oxidative phosphorylation. In glycolysis, ATP production is directly linked to the chemical reactions that break glucose down into two molecules of pyruvate. Early studies of oxidative phosphorylation suggested that transport of high-energy electrons down a cascade of electron acceptors generated the energy for ATP production. By analogy with glycolysis, it was thought that production of ATP would be directly linked to high-energy chemical intermediates produced during electron transport. Despite much effort, such compounds were never found. However, a number of experiments suggested an alternative hypothesis, in which the energy captured during electron transport was used to pump protons (H^+) across the membrane, generating a gradient of protons that was subsequently used to drive ATP synthesis. The proposed indirect linkage between electron transport and ATP production was known as the chemiosmotic hypothesis for oxidative phosphorylation. This hypothesis proved to be correct.

14–85 Electron transport is carried out by a series of large multiprotein complexes. Early work found that these complexes were in some way associated with the inner mitochondrial membrane, although their function and the nature of their association with the membrane were poorly understood. Which one of the following observations regarding the electron-transport complexes would have been most consistent with the chemiosmotic hypothesis?

A. Efficient electron transport can be detected in purified preparations of mitochondrial membranes.
B. Proteins in each electron-transport complex are exposed on both sides of the inner mitochondrial membrane.

C. The electron-transport complexes must be embedded in the membrane to accept electrons.
D. The proteins in each electron-transport complex are exposed only to the matrix side of the membrane.

14–86 In one experiment, mitochondrial membranes were mechanically broken into pieces by subjecting them to high-frequency sound waves. Which one of the following observations would have been most consistent with the chemiosmotic hypothesis?

A. Adding a source of electrons to the fragmented membranes yielded ATP.
B. Electron-transport complexes were associated with the fragmented membranes.
C. Fragmented membranes could transport electrons, but could not generate ATP.
D. Fragmented membranes produced protons in response to electron transport.

14–87 Which of the following experiments would have provided the clearest proof of the chemiosmotic hypothesis?

A. A decrease in pH inside the mitochondrial matrix could generate ATP in the complete absence of electron transport.
B. ATP production in intact mitochondria requires both the entire electron-transport chain and the ATP synthase complex.
C. Bacteriorhodopsin, which transports protons across membranes in response to light, could replace the electron-transport chain.
D. Reconstitution of the electron-transport chain in membranes was sufficient to transport protons across the membrane.

Our Colleague, the Late Julian Lewis, Semaphores the Letter H.
You may like to consider the similarities and differences between this human mode of communication and the signaling networks used by cells.

CHAPTER
15

Cell Signaling

IN THIS CHAPTER

PRINCIPLES OF CELL SIGNALING

TERMS TO LEARN

adaptation (desensitization)
adaptor
contact-dependent signaling
endocrine cell
extracellular signal molecule
GTPase-activating protein (GAP)
GTP-binding protein
guanine nucleotide exchange factor (GEF)
hormone
interaction domain
ion-channel-coupled receptor
kinase cascade
local mediator
monomeric GTPase
neurotransmitter
paracrine signaling
phosphorylation
primary cilium
protein kinase
protein phosphatase
receptor
scaffold protein
second messenger
serine/threonine kinase
synaptic signaling
tyrosine kinase

DEFINITIONS

Match each definition below with its term from the list above.

15–1 Protein that binds to a GTP-binding protein and activates it by stimulating release of tightly bound GDP, thereby allowing it to bind GTP.

15–2 General term for a protein that binds a specific extracellular molecule (ligand) and initiates a response in the cell.

15–3 Alteration of sensitivity following repeated stimulation, reducing a cell's response to that level of stimulus.

15–4 Compact protein module that binds to a particular structural motif in another protein (or lipid) molecule with which the signaling protein interacts.

15–5 Short-range cell–cell communication via secreted local mediators that act on adjacent cells.

15–6 A signal relay chain involving multiple protein kinases, each of which is activated by phosphorylation and then phosphorylates the next protein kinase in the sequence.

15–7 Small molecule that is formed in the cytosol, or released into it, in response to an extracellular signal and that helps to relay the signal to the interior of the cell.

15–8 Specialized animal cell that secretes a hormone into the blood.

15–9 Molecule from outside the cell that communicates the behavior or actions of other cells in the environment and elicits an appropriate response.

15–10 Enzyme that transfers the terminal phosphate group of ATP to a specific amino acid of a target protein.

15–11 Small signal molecule secreted by the presynaptic nerve cell at a chemical synapse to relay the signal to the postsynaptic cell.

15–12 Protein that binds to a GTP-binding protein and inactivates it by stimulating its GTPase activity so that its bound GTP is hydrolyzed to GDP.

15–13 Protein that organizes groups of interacting intracellular signaling proteins into signaling complexes.

15–14 Cell–cell communication in which the signal molecule remains bound to the signaling cell and only influences cells that physically touch it.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

15–15 There is no fundamental distinction between signaling molecules that bind to cell-surface receptors and those that bind to intracellular receptors.

15–16 All second messengers are water-soluble and diffuse freely through the cytosol.

THOUGHT PROBLEMS

15–17 Compare and contrast signaling by neurons to signaling by endocrine cells. What are the relative advantages of these two mechanisms for cellular communication?

15–18 Cells communicate in ways that resemble human communication. Decide which of the following forms of human communication are analogous to autocrine, paracrine, endocrine, and synaptic signaling by cells.

A. A telephone conversation
B. Talking to people at a cocktail party
C. A radio announcement
D. Talking to yourself

15–19 How is it that different cells can respond in different ways to exactly the same signaling molecule even when they have identical receptors?

15–20 Working out the order in which the individual components in a signaling pathway act is an essential step in defining the pathway. Imagine that two protein kinases, PK1 and PK2, act sequentially in a kinase cascade. When either kinase is completely inactivated, cells do not respond to the normal extracellular signal. By contrast, cells containing a mutant form of PK1 that is permanently active respond even in the absence of an extracellular signal. Doubly mutant cells that contain inactivated PK2 and permanently active PK1 respond in the absence of a signal.

In the normal kinase cascade, does PK1 activate PK2 or does PK2 activate PK1? What outcome would you have predicted for a doubly mutant cell line with an activating mutation in PK2 and an inactivating mutation in PK1? Explain your reasoning.

15–21 Why do you suppose that phosphorylation/dephosphorylation, as opposed to allosteric binding of small molecules, for example, has evolved to play such a prominent role in switching proteins on and off in signaling pathways?

15–22 The two main classes of molecular switches involve changes in phosphorylation state or changes in guanine nucleotide binding. Comment on the following statement. "In the regulation of molecular switches, protein kinases and guanine nucleotide exchange factors (GEFs) always turn proteins on, whereas protein phosphatases and GTPase-activating proteins (GAPs) always turn proteins off."

15–23 Consider a signaling pathway that proceeds through three protein kinases that are sequentially activated by phosphorylation. In one case, the kinases are held in a signaling complex by a scaffold protein; in the other, the kinases are freely diffusing (**Figure 15–1**). Discuss the properties of these two types of organization in terms of signal amplification, speed, and potential for cross-talk between signaling pathways.

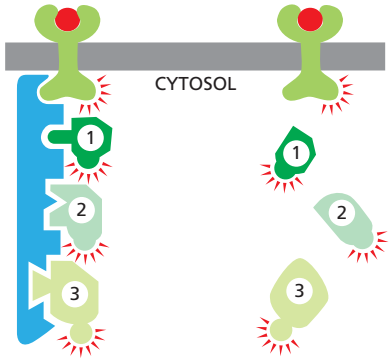


Figure 15–1 A protein kinase cascade organized by a scaffolding protein or composed of freely diffusing components (Problem 15–23).

15–24 Proteins in signaling pathways use a variety of binding domains to assemble into signaling complexes. Match the following domains with their binding targets. (A binding target can be used more than once.)

A. PH domain	1. phosphorylated tyrosines
B. PTB domain	2. proline-rich sequences
C. SH2 domain	3. phosphorylated inositol phospholipids
D. SH3 domain	

15–25 Describe three ways in which a gradual increase in an extracellular signal can be sharpened by the target cell to produce an abrupt or nearly all-or-none response.

CALCULATIONS

15–26 Suppose that the circulating concentration of hormone is 10^{-10} M and the K_d for binding to its receptor is 10^{-8} M. What fraction of the receptors will have hormone bound? If a meaningful physiological response occurs when 50% of the receptors have bound a hormone molecule, how much will the concentration of hormone have to rise to elicit a response? Recall that the fraction of receptors (R) bound to hormone (H) to form a receptor–hormone complex (R–H) is $[R\text{–}H]/([R] + [R\text{–}H]) = [R\text{–}H]/[R]_{TOT} = [H]/([H] + K_d)$.

15–27 Radioimmunoassay (RIA) is a powerful tool for quantifying virtually any substance of biological interest because it is sensitive, accurate, and fast. RIA technology arose from studies on adult-onset diabetes. Some patients had antibodies with high affinity for insulin, and RIA was developed as a method to distinguish free insulin from antibody-bound insulin.

How can high-affinity antibodies be exploited to measure low concentrations of insulin? When a small amount of insulin-specific antiserum is mixed with an equally small amount of very highly radioactive insulin, some binds and some remains free according to the equilibrium.

$$\text{Insulin (I)} + \text{Antibody (A)} \rightleftharpoons \text{Insulin–Antibody (I–A) Complex}$$

When increasing amounts of unlabeled insulin are added to a fixed amount of labeled insulin and anti-insulin antibody, the ratio of bound to free radioactive insulin decreases as expected from the equilibrium expression. If the concentration of the unlabeled insulin is known, then the resulting curve serves as a calibration against which other unknown samples can be compared (**Figure 15–2**).

You have three samples of insulin whose concentrations are unknown. When mixed with the same amount of radioactive insulin and anti-insulin antibody used in Figure 15–2, the three samples gave the following ratios of bound to free insulin:

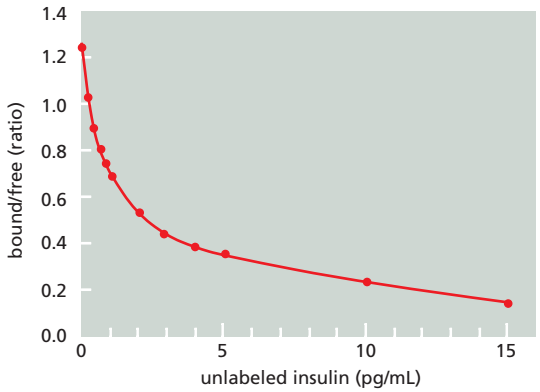


Figure 15–2 Calibration curve for radioimmunoassay of insulin (Problem 15–27).

Sample 1	0.67
Sample 2	0.31
Sample 3	0.46

A. What is the concentration of insulin in each of these unknown samples?
B. What portion of the standard curve is the most accurate, and why?
C. If the antibodies were raised against pig insulin, which is similar but not identical to human insulin, would the assay still be valid for measuring human insulin concentrations?

15–28 Two intracellular molecules, A and B, are normally synthesized at a constant rate of 1000 molecules per second per cell. Each molecule of A survives an average of 100 seconds, while each molecule of B survives an average of 10 seconds.

A. How many molecules of A and B will a cell contain?
B. If the rates of synthesis of both A and B were suddenly increased 10-fold to 10,000 molecules per second—without any change in their average lifetimes—how many molecules of A and B would be present after 1 second?
C. Which molecule would be preferred for rapid signaling? Explain your answer.

DATA HANDLING

15–29 The cellular slime mold *Dictyostelium discoideum* is a eukaryote that lives on the forest floor as independent motile cells called amoebae, which feed on bacteria and yeast. When their food supply is exhausted, the amoebae stop dividing and gather together to form tiny, multicellular, wormlike structures, which crawl about as slugs. How do individual amoebae know when to stop dividing and how to find their way into a common aggregate? A set of classic experiments investigated this phenomenon more than half a century ago.

Amoebae aggregate when placed on a glass coverslip under water, provided that simple salts are present. The center of the aggregation pattern can be removed with a pipette and placed in a field of fresh amoebae, which immediately start streaming toward it. Thus, the center is emitting some sort of attractive signal. Four experiments were designed to determine the nature of the signal. In each, an existing center of aggregation was used as the source of the signal and previously unexposed amoebae served as the target cells. The arrangements of aggregation centers and test amoebae at the beginning and end of the experiments are shown in **Figure 15–3**.

Do these results show that *Dictyostelium discoideum* aggregates through the action of a secreted chemical signal? Explain your reasoning.

15–30 The nicotinic acetylcholine receptor is a neurotransmitter-dependent ion channel, which is composed of four types of subunit. Phosphorylation of

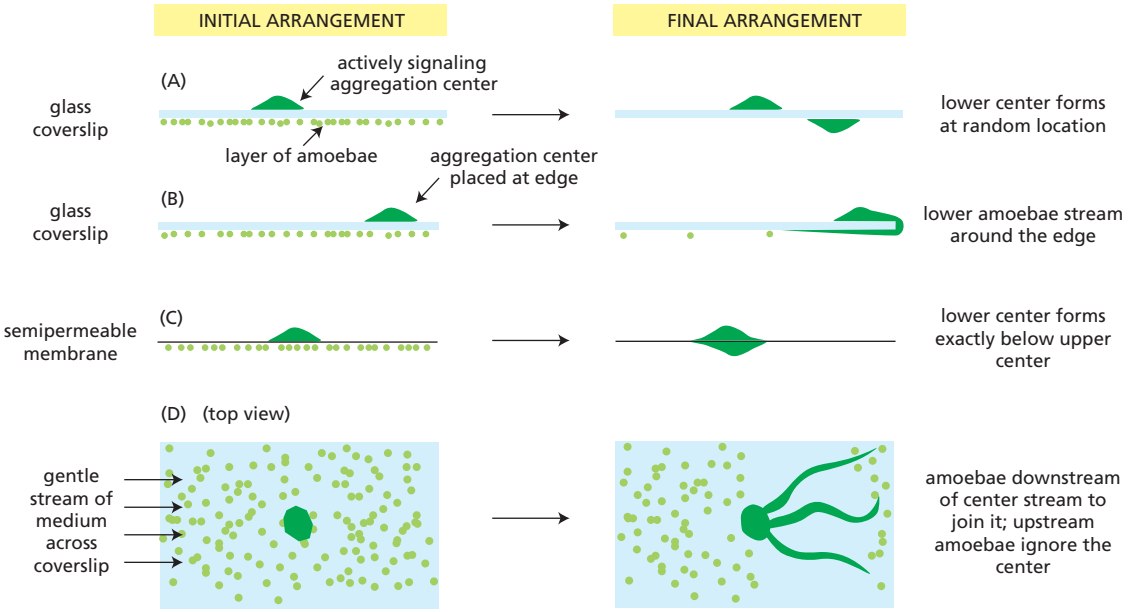


Figure 15–3 Four experiments to study the nature of the attractive signal generated by aggregation centers (Problem 15–29).

the receptor by protein kinase A attaches one phosphate to the γ subunit and one phosphate to the δ subunit. Fully phosphorylated receptors desensitize much more rapidly than unmodified receptors. To study this process in detail, you phosphorylate two preparations of receptor to different extents (0.8 mole phosphate/mole receptor and 1.2 mole phosphate/mole receptor) and measure desensitization over several seconds (**Figure 15–4**). Both preparations behave as if they contain a mixture of receptors; one form that is rapidly desensitized (the initial steep portion of the curves) and another form that is desensitized at the same rate as the untreated receptor.

A. Assuming that the γ and δ subunits are independently phosphorylated at equal rates, calculate the percentage of receptors that carry zero, one, and two phosphates per receptor at the two extents of phosphorylation.

B. Do these data suggest that desensitization requires one phosphate or two phosphates per receptor? If you decide that desensitization requires only one phosphate, indicate whether the phosphate has to be on one specific subunit or can be on either of the subunits.

MEDICAL LINKS

15–31 Surgeons use succinylcholine, which is an acetylcholine analog, as a muscle relaxant. Care must be taken because some individuals recover abnormally slowly from this paralysis, with life-threatening consequences. Such individuals are deficient in an enzyme called pseudocholinesterase, which is normally present in the blood, where it slowly inactivates succinylcholine by hydrolysis to succinate and choline.

If succinylcholine is an analog of acetylcholine, why do you think it causes muscles to relax and not contract as they do in the presence of acetylcholine?

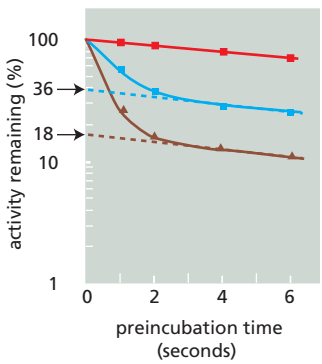


Figure 15–4 Desensitization rates for untreated acetylcholine receptor and two preparations of phosphorylated receptor (Problem 15–30). *Red squares* represent untreated receptors; *blue squares* represent receptors with 0.8 mole phosphate/mole receptor; and *brown triangles* represent receptors with 1.2 mole phosphate/mole receptor. *Arrows* indicate the fractions of the phosphorylated preparations that behaved like the untreated receptor.

SIGNALING THROUGH G-PROTEIN-COUPLED RECEPTORS

TERMS TO LEARN

adenylyl cyclase
arrestin
Ca^{2+}/calmodulin-dependent kinase (CaM-kinase)
calmodulin
CaM-kinase II
cone photoreceptor
CRE-binding (CREB) protein
cyclic AMP (cAMP)
cyclic-AMP-dependent protein kinase (PKA)
cyclic AMP phosphodiesterase
cyclic GMP
cyclic GMP phosphodiesterase
diacylglycerol
G_q
G-protein-coupled receptor (GPCR)
GPCR kinase (GRK)
inhibitory G protein (G_i)
inositol phospholipid signaling pathway
inositol 1,4,5-trisphosphate (IP_3)
IP_3 receptor
nitric oxide (NO)
NO synthase
olfactory receptor
phospholipase C-β (PLCβ)
phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)
protein kinase C (PKC)
regulator of G protein signaling (RGS)
rhodopsin
rod photoreceptor (rod)
ryanodine receptor
stimulatory G protein (G_s)
trimeric GTP-binding protein (G protein)

DEFINITIONS

Match each definition below with its term from the list above.

15–32 G protein that activates adenylyl cyclase and thereby increases cyclic AMP concentration.

15–33 Protein composed of three subunits, one of which is activated by the binding of GTP.

15–34 Ubiquitous calcium-binding protein whose interactions with other proteins are governed by changes in intracellular Ca^{2+} concentration.

15–35 Enzyme that hydrolyzes cyclic AMP to adenosine 5′-monophosphate (5′-AMP).

15–36 Cell-surface receptor that associates with an intracellular G protein upon activation by an extracellular ligand.

15–37 Enzyme that participates in desensitization of GPCRs by phosphorylating them after they have been activated by ligand binding.

15–38 Ca^{2+}-release channel in the ER membrane that is activated by Ca^{2+} binding in the absence of IP_3.

15–39 Enzyme bound to the cytoplasmic surface of the plasma membrane that converts membrane $PI(4,5)P_2$ to diacylglycerol and IP_3.

15–40 Protein that is an α-subunit-specific GTPase-activating protein (GAP).

15–41 Second messenger that is released from a phospholipid in the plasma membrane and diffuses to the ER, where it opens Ca^{2+}-release channels.

15–42 Enzyme that phosphorylates target proteins in response to a rise in intracellular cyclic AMP.

15–43 A Ca^{2+}-dependent protein kinase that is activated by diacylglycerol.

15–44 Light-sensitive GPCR in rod photoreceptor cells of the retina.

15–45 Protein kinase whose activity is regulated by the binding of Ca^{2+}-activated

calmodulin, and which indirectly mediates the effects of Ca^{2+} by phosphorylation of other proteins.

15–46 Protein that binds to the cyclic AMP response elements found in the regulatory region of many genes activated by cyclic AMP.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

15–47 Different isoforms of protein kinase A in different cell types explain why the effects of cyclic AMP vary depending on the target cell.

15–48 The activity of any protein regulated by phosphorylation depends on the balance at any instant between the activities of the kinases that phosphorylate it and the phosphatases that dephosphorylate it.

15–49 Most intracellular signaling pathways provide multiple opportunities for amplifying a response to an extracellular signal.

THOUGHT PROBLEMS

15–50 GPCRs activate G proteins by reducing the strength of GDP binding, allowing GDP to dissociate and GTP, which is present at much higher concentrations, to bind. How do you suppose the activity of a G protein would be affected by a mutation that caused its affinity for GDP to be reduced without significantly changing its affinity for GTP?

15–51 When adrenaline (epinephrine) binds to adrenergic receptors on the surface of a muscle cell, it activates a G protein, initiating a signaling pathway that results in breakdown of muscle glycogen. How would you expect glycogen breakdown to be affected if muscle cells were injected with a nonhydrolyzable analog of GTP, which can't be converted to GDP? Consider what would happen in the absence of adrenaline and after a brief exposure to it.

15–52 Should RGS (regulator of G protein signaling) proteins be classified as GEFs (guanine nucleotide exchange factors) or GAPs (GTPase-activating proteins)? Explain what role this activity plays in modulating G-protein-mediated responses in animals and yeasts.

15–53 What is "cyclic" about cyclic AMP?

15–54 Explain why cyclic AMP must be broken down rapidly in a cell to allow rapid signaling.

15–55 You are trying to purify adenylyl cyclase from brain. The assay is based on the conversion of α-^{32}P-ATP to cAMP. You can easily detect activity in crude brain homogenates stimulated by isoproterenol, which binds to β-adrenergic receptors, but the enzyme loses activity when low-molecular-weight cofactors are removed by dialysis. What single molecule do you think you could add back to the dialyzed homogenate to restore activity?

15–56 Propose specific types of mutations in the gene for the regulatory subunit of cyclic-AMP-dependent protein kinase (PKA) that could lead to either a permanently active PKA, or to a permanently inactive PKA.

15–57 Why do you suppose cells use Ca^{2+} (intracellular concentration 10^{-7} M) for signaling rather than the more abundant Na^{+} (intracellular concentration 10^{-3} M)?

15–58 EGTA chelates Ca^{2+} with high affinity and specificity. How would microinjection of EGTA affect glucagon-triggered breakdown of glycogen in

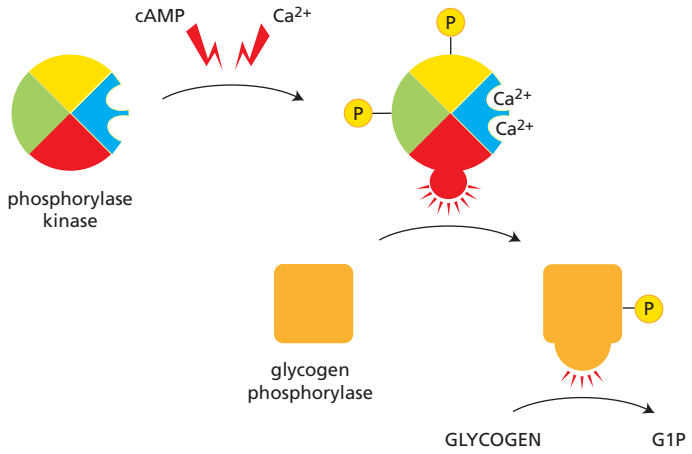


Figure 15–5 Integration of cyclic-AMP-dependent and Ca^{2+}-dependent signaling pathways by phosphorylase kinase in liver and muscle cells (Problem 15–59). G1P is glucose 1-phosphate, the product of phosphorylase kinase, which uses phosphate to cleave glucose units from glycogen.

liver? How would it affect vasopressin-triggered breakdown of glycogen in liver?

15–59 Phosphorylase kinase integrates signals from the cyclic-AMP-dependent and Ca^{2+}-dependent signaling pathways that control glycogen breakdown in liver and muscle cells (**Figure 15–5**). Phosphorylase kinase is composed of four subunits. One is the protein kinase that catalyzes the addition of phosphate to glycogen phosphorylase to activate it for glycogen breakdown. The other three subunits are regulatory proteins that control the activity of the catalytic subunit. Two contain sites for phosphorylation by PKA, which is activated by cyclic AMP. The remaining subunit is calmodulin, which binds Ca^{2+} when the cytosolic Ca^{2+} concentration rises. The regulatory subunits control the equilibrium between the active and inactive conformations of the catalytic subunit, with each phosphate and Ca^{2+} nudging the equilibrium toward the active formation. How does this arrangement allow phosphorylase kinase to serve its role as an integrator protein for the multiple pathways that stimulate glycogen breakdown?

15–60 CaM-kinase II is a remarkable molecular memory device. How does CaM-kinase II "remember" its exposure to Ca^{2+}/calmodulin and why does it eventually "forget"?

15–61 The outer segments of rod photoreceptor cells can be broken off, isolated, and used to study the effects of small molecules on visual transduction because the broken end of each segment remains unsealed. How would you expect the visual response to be affected by the following additions?

A. An inhibitor of cyclic GMP phosphodiesterase.
B. A nonhydrolyzable analog of GTP.
C. An inhibitor of rhodopsin-specific kinase.

15–62 A rise in the cyclic GMP levels in smooth muscle cells causes relaxation of the blood vessels in the penis, resulting in an erection. Explain how the natural signal molecule NO and the drug Viagra® produce an increase in cyclic GMP.

15–63 In muscle cells, adrenaline binds to the β-adrenergic receptor to initiate a signaling cascade that leads to the breakdown of glycogen (**Figure 15–6**). At what points in this pathway is the signal amplified?

15–64 A critical feature of all signaling cascades is that they must be turned off rapidly when the extracellular signal is removed. Examine the signaling cascade in Figure 15–6. Describe how each component of this signaling pathway is returned to its inactive state when adrenaline is removed.

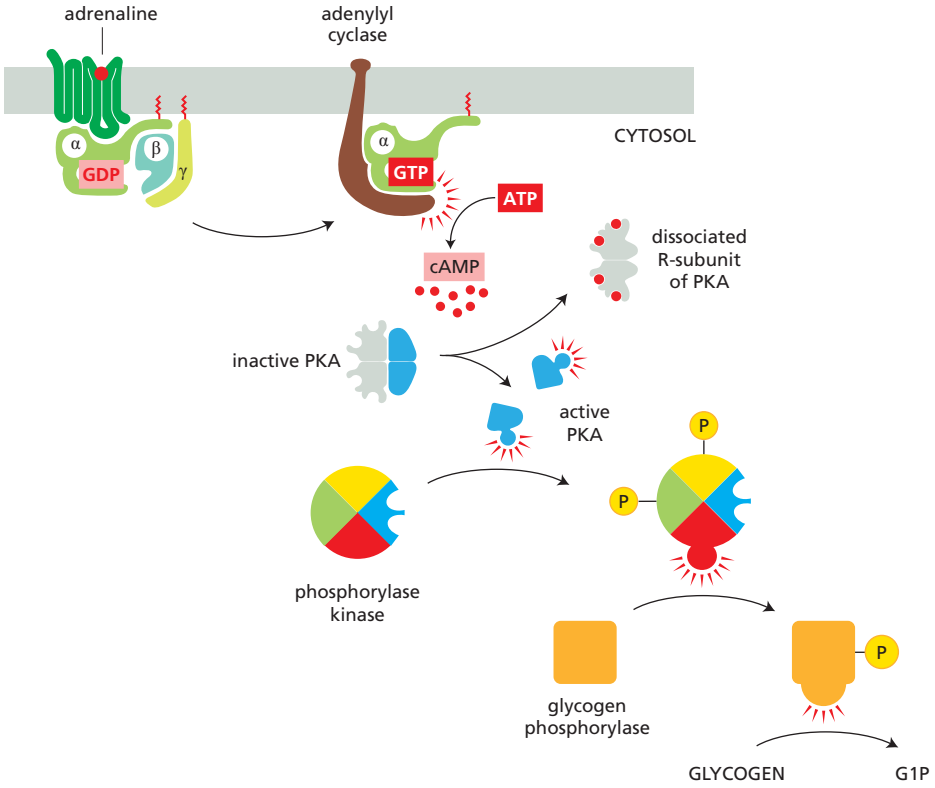


Figure 15–6 Signaling cascade for activation of glycogen breakdown by adrenaline in muscle cells (Problem 15–63). G1P is glucose 1-phosphate; cAMP bound to the regulatory subunits of PKA is shown as *red balls*.

CALCULATIONS

15–65 In a classic paper, the number of β-adrenergic receptors on the membranes of frog erythrocytes was determined by using a competitive inhibitor of adrenaline, alprenolol, which binds to the receptors 500 times more tightly than adrenaline. These receptors normally bind adrenaline and stimulate adenylyl cyclase activity. Labeled alprenolol was mixed with erythrocyte membranes, left for 10 minutes at 37°C, and then the membranes were pelleted by centrifugation and the radioactivity in the pellet was measured. The experiment was done in two ways. The binding of increasing amounts of ^{3}H-alprenolol to a fixed amount of erythrocyte membranes was measured to determine total binding. The experiment was repeated in the presence of a vast excess of unlabeled alprenolol to measure nonspecific binding. The results are shown in **Figure 15–7**.

A. On Figure 15–7, sketch the curve for specific binding of alprenolol to β-adrenergic receptors. Has alprenolol binding to the receptors reached saturation?

B. Assuming that one molecule of alprenolol binds per receptor, calculate the number of β-adrenergic receptors on the membrane of a frog erythrocyte. The specific activity of the labeled alprenolol is 1×10^{13} cpm/mmol, and there are 8×10^8 frog erythrocytes per milligram of membrane protein.

15–66 In visual transduction, one activated rhodopsin molecule leads to the hydrolysis of 5×10^5 cyclic GMP molecules per second. One stage in this enormous signal amplification is achieved by cyclic GMP phosphodiesterase, which hydrolyzes 1000 molecules of cyclic GMP per second. The additional factor of 500 could arise because one activated rhodopsin activates 500 transducin (G_t) molecules, or because one activated transducin activates 500 cyclic GMP phosphodiesterases, or through a combination of both effects. One experiment to address this question measured the

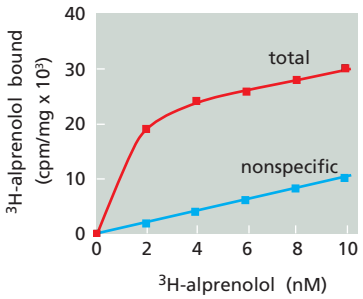


Figure 15–7 Binding of ^{3}H-alprenolol to frog erythrocyte membranes (Problem 15–65).

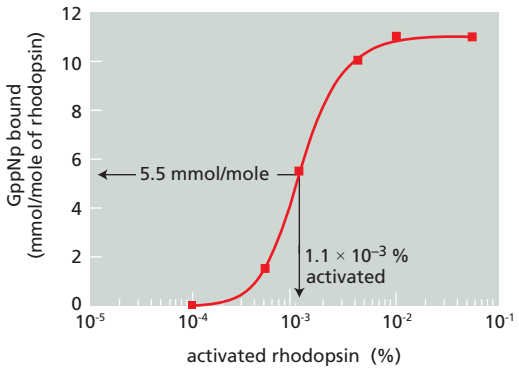


Figure 15–8 Binding of GppNp to rod-cell membranes as a function of the fraction of activated rhodopsin (Problem 15–66). Background binding of GppNp to rod-cell membranes in the dark has been subtracted from the values shown.

amount of GppNp (a nonhydrolyzable analog of GTP) that is bound by transducin in the presence of different amounts of activated rhodopsin. As indicated in **Figure 15–8**, 5.5 mmol of GppNp were bound per mole of total rhodopsin when 0.0011% of the rhodopsin was activated.

A. Assuming that each transducin molecule binds one molecule of GppNp, calculate the number of transducin molecules that are activated by each activated rhodopsin molecule. Which mechanism of amplification does this measurement support?

B. Binding studies have shown that transducin-GDP has a high affinity for activated rhodopsin and that transducin-GTP has a low affinity; conversely, transducin-GTP has a high affinity and transducin-GDP has a low affinity for cyclic GMP phosphodiesterase. Are these affinities consistent with the mechanism of amplification you deduced from the above experiment? Explain your reasoning.

DATA HANDLING

15–67 The mating behavior of yeast depends on signaling peptides termed pheromones that bind to pheromone GPCRs (**Figure 15–9**). When the α-factor pheromone binds to a wild-type yeast cell, it blocks cell-cycle progression, arresting proliferation until a mating partner is found. Yeast mutants with defects in one or more of the components of the G protein have characteristic phenotypes in the absence and in the presence of the α-factor pheromone (**Table 15–1**). Strains with defects in any of these genes cannot undergo the mating response and are therefore termed sterile.

A. Based on genetic analysis of the yeast mutants, decide which component of the G protein normally transmits the mating signal to the downstream effector molecules.

B. Predict the proliferation and mating phenotypes in the absence and presence of the α-factor pheromone of strains with the following mutant G protein α subunits:

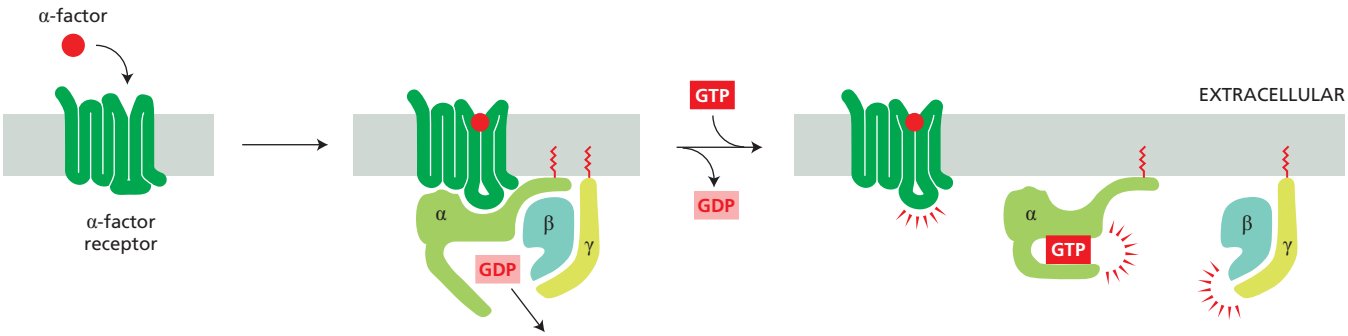


Figure 15–9 α-Factor pheromone signaling via α-factor GPCR and G protein (Problem 15–67).

TABLE 15–1 Mating phenotypes of various mutant and nonmutant strains of yeast (Problem 15–67).

Mutation	Phenotype	
	Minus α factor	Plus α factor
None (wild type)	Normal proliferation	Arrested proliferation, mating response
α subunit deleted	Arrested proliferation	Arrested proliferation, sterile
β subunit deleted	Normal proliferation	Normal proliferation, sterile
γ subunit deleted	Normal proliferation	Normal proliferation, sterile
α and β deleted	Normal proliferation	Normal proliferation, sterile
α and γ deleted	Normal proliferation	Normal proliferation, sterile
β and γ deleted	Normal proliferation	Normal proliferation, sterile

1. An α subunit that can bind GTP but cannot hydrolyze it.
2. An α subunit with an altered N-terminus to which the fatty acid myristoylate cannot be added, thereby preventing its localization to the plasma membrane.
3. An α subunit that cannot bind to the activated pheromone receptor.

15–68 A particularly graphic illustration of the subtle, yet important, role of cyclic AMP in the whole organism comes from studies of the fruit fly *Drosophila melanogaster*. In search of the gene for cyclic AMP phosphodiesterase, one laboratory measured enzyme levels in flies with chromosomal duplications or deletions and found consistent alterations in flies with mutations involving bands 3D3 and 3D4 on the X chromosome. Duplications in this region have about 1.5 times the normal activity of the enzyme; deletions have about half the normal activity.

An independent laboratory at the same institution was led to the same chromosomal region through work on behavioral mutants of fruit flies. The researchers had developed a learning test in which flies were presented with two metallic grids, one of which was electrified. If the electrified grid was painted with a strong-smelling chemical, normal flies quickly learned to avoid it, even when it was no longer electrified. The mutant flies, on the other hand, never learned to avoid the smelly grid; they were aptly called *Dunce* mutants. The *Dunce* mutation was mapped genetically to bands 3D3 and 3D4.

Is the learning defect really due to lack of cyclic AMP phosphodiesterase or are the responsible genes simply closely linked? Further experiments showed that the level of cyclic AMP in *Dunce* flies was 1.6 times higher than in normal flies. Furthermore, sucrose-gradient analysis of homogenates of *Dunce* and normal flies revealed two cyclic AMP phosphodiesterase activities, one of which was missing in *Dunce* flies (**Figure 15–10**).

A. Why do *Dunce* flies have higher levels of cyclic AMP than normal flies?
B. Explain why homozygous (both chromosomes affected) duplications of the nonmutant *Dunce* gene cause cyclic AMP phosphodiesterase levels to be elevated 1.5-fold and why homozygous deletions of the gene reduce enzyme activity to half the normal value.
C. What would you predict would be the effect of caffeine, a phosphodiesterase inhibitor, on the learning performance of normal flies?

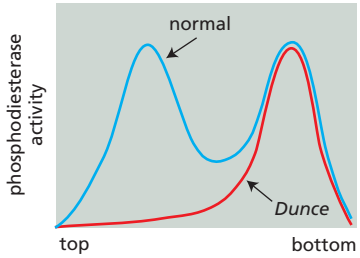


Figure 15–10 Sucrose-gradient analysis of cyclic AMP phosphodiesterase activity in homogenates of normal and *Dunce* flies (Problem 15–68).

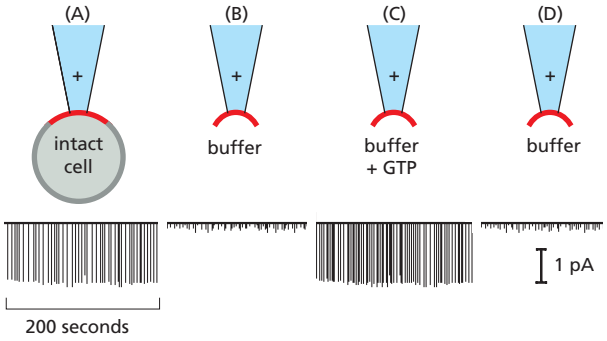


Figure 15–11 Experimental set-up and typical results of patch-clamp analysis of K^+-channel activation by acetylcholine (Problem 15–69). The buffer is a salts solution that does not contain nucleotides or Ca^{2+}. In all these experiments, acetylcholine is present inside the pipet, as indicated by the *plus* sign. The current through the membrane is measured in picoamps (pA). In (C), the GTP is added to the buffer.

15–69 Acetylcholine acts on muscarinic GPCRs in the heart to open K^+ channels, thereby slowing the heart rate. This process can be directly studied using the inside-out membrane patch-clamp technique. The external surface of the membrane is in contact with the solution in the bore of the pipet, and the cytoplasmic surface faces outward and can be exposed readily to a variety of solutions (**Figure 15–11**). Receptors, G proteins, and K^+ channels remain associated with the membrane patch.

When acetylcholine is added to a pipet with a whole cell attached, K^+ channels open as indicated by the flow of current (Figure 15–11A). Under similar circumstances with a patch of membrane inserted into a buffered salts solution, no current flows (Figure 15–11B). When GTP is added to the buffer, current resumes (Figure 15–11C). Subsequent removal of GTP stops the current (Figure 15–11D). The results of several similar experiments to test the effects of different combinations of components are summarized in **Table 15–2**.

A. Why do you think it is that Gβγ activated the channel when the complete G protein did not? Is the active component of the G protein in this system the same as the one that activates adenylyl cyclase in other cells?

B. Addition of GppNp (a nonhydrolyzable analog of GTP) causes the K^+ channel to open in the absence of acetylcholine (Table 15–2, line 4). The flow of current, however, rose very slowly and reached its maximum only after a minute (compare with the immediate rise in Figure 15–11A and C). How do you suppose GppNp causes the channels to open slowly in the absence of acetylcholine?

C. To the extent that these experiments allow, draw a scheme for the activation of K^+ channels in heart cells in response to acetylcholine.

TABLE 15–2 Responses of K^+ channel to various experimental manipulations (Problem 15–69).

	Additions			
	Acetylcholine	Small molecules	G-protein components	K^+ channel
1	+	none	none	closed
2	+	GTP	none	open
3	–	GTP	none	closed
4	–	GppNp	none	open
5	–	none	G protein	closed
6	–	none	G_α	closed
7	–	none	$G_{\beta\gamma}$	open
8	–	none	boiled G protein	closed

MEDICAL LINKS

15–70 During a marathon, runners draw heavily on their internal reserves of glycogen (carbohydrate) and triglycerides (fat) to fuel muscle contraction. Initially, energy is derived mostly from carbohydrates, with increasing amounts of fat being used as the race progresses. If runners use up their muscle glycogen reserves before they finish the race, they hit what is known as "the wall," a point of diminished performance that arises because fatty acids from triglyceride breakdown cannot be delivered to the muscles quickly enough to sustain maximum effort. One trick that marathon runners use to avoid the wall is to drink a cup of strong black coffee an hour or so before the race begins. Coffee contains caffeine, which is an inhibitor of cyclic AMP phosphodiesterase. How do you suppose inhibition of this enzyme helps them avoid the wall?

15–71 Patients with Oguchi's disease have an inherited form of night blindness. After a flash of bright light, these individuals recover their night vision (become dark adapted) very slowly. Night vision depends almost entirely on the visual responses of rod photoreceptor cells. What aspect of the visual response in these patients' rod cells do you suppose is defective? What genes, when defective, might give rise to Oguchi's disease?

15–72 The primary role of platelets is to control blood clotting. When they encounter the exposed basement membrane (collagen fibers) of a damaged blood vessel or a newly forming fibrin clot, they change their shape from round to spiky and stick to the damaged area. At the same time, they begin to secrete serotonin and ATP, which accelerate similar changes in newly arriving platelets, leading to the rapid formation of a clot. The platelet response is regulated by protein phosphorylation. Significantly, platelets contain high levels of two protein kinases: PKC, which initiates serotonin release, and myosin light-chain kinase, which mediates the change in shape.

When platelets are stimulated with thrombin, the light chain of myosin and an unknown protein of 40,000 daltons are phosphorylated. When platelets are treated with a calcium ionophore, which increases membrane permeability to Ca^{2+}, only the myosin light chain is phosphorylated; when they are treated with diacylglycerol, only the 40-kD protein is phosphorylated. Experiments using a range of concentrations of diacylglycerol in the presence or absence of calcium ionophore show that the extent of phosphorylation of the 40-kD protein depends only on the concentration of diacylglycerol (**Figure 15–12A**). Serotonin release, however, depends on diacylglycerol and the calcium ionophore (**Figure 15–12B**).

A. Based on these experimental observations, describe the normal sequence of molecular events that leads to phosphorylation of the myosin light

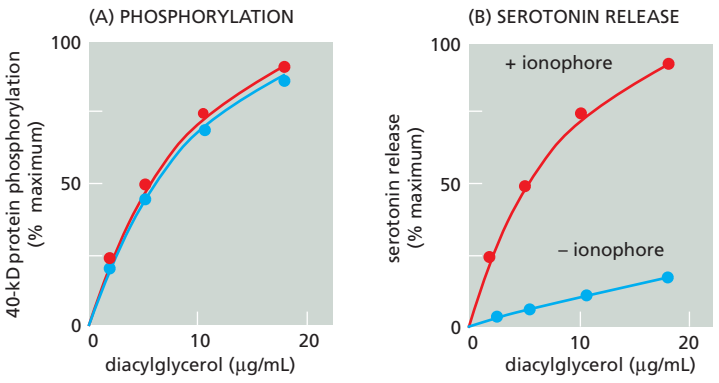


Figure 15–12 Treatment of platelets with calcium ionophore and diacylglycerol (Problem 15–72). (A) Effects on phosphorylation of the 40-kD protein. (B) Effects on serotonin release. *Red circles* indicate the presence of calcium ionophore and *blue circles* indicate its absence.

chain and the 40-kD protein. Indicate how the calcium ionophore and diacylglycerol treatments interact with the normal sequence of events.

B. Why do you think serotonin release requires both calcium ionophore and diacylglycerol?

SIGNALING THROUGH ENZYME-COUPLED RECEPTORS

TERMS TO LEARN

Akt
Cdc42
cytokine receptor
cytoplasmic tyrosine kinase
enzyme-coupled receptor
ephrins
focal adhesion kinase (FAK)
JAK–STAT signaling pathway
Janus kinase (JAK)
MAP kinase module
mTOR
phosphoinositide
phosphoinositide 3-kinase (PI 3-kinase)
phospholipase C-γ (PLCγ)
PI-3-kinase–Akt pathway
pleckstrin homology (PH) domain
protein tyrosine phosphatase
Rac
Ras
Ras-GAP
Ras-GEF
Ras–MAP-kinase signaling pathway
Ras superfamily
receptor serine/threonine kinase
receptor tyrosine kinase (RTK)
Rheb
Rho
Rho family
SH2 domain
Smad family
Src family
STATs
TOR
transforming growth factor-β (TGFβ) superfamily
tyrosine-kinase-associated receptor

DEFINITIONS

Match each definition below with its term from the list above.

15–73 The largest class of cell-surface-bound extracellular signal proteins.

15–74 Large family of structurally related, secreted, dimeric proteins that act as hormones and local mediators to control a wide range of biological functions in all animals.

15–75 Cell-surface receptor that when activated by ligand binding adds phosphates from ATP to tyrosine side chains in its own cytoplasmic domain.

15–76 The founding member of a superfamily of monomeric GTPases that help to relay signals from cell-surface receptors to the nucleus.

15–77 A group of monomeric GTPases that regulate both the actin and microtubule cytoskeletons.

15–78 Cytoplasmic tyrosine kinase present at cell–matrix junctions in association with the cytoplasmic tails of integrins.

15–79 A kinase that is involved in intracellular signaling pathways activated by cell-surface receptors and that phosphorylates inositol phospholipids at the 3 position of the inositol ring.

15–80 Cell-surface receptor in which the cytoplasmic domain either has enzymatic activity itself or is associated with an intracellular enzyme.

15–81 Cell-surface receptor that activates a tyrosine kinase that is noncovalently bound to the receptor.

15–82 A three-component signaling module used in various signaling pathways in eukaryotic cells.

15–83 One of several intracellular signaling pathways that leads from cell-surface receptors to the nucleus, it is distinguished by providing one of the more direct routes.

15–84 Protein domain found in intracellular signaling proteins by which they bind to inositol phospholipids phosphorylated by PI 3-kinase.

15–85 A protein domain that is homologous to a region in Src, is present in many proteins, and binds to a short amino acid sequence containing a phosphotyrosine.

15–86 A crucial signaling protein in the PI-3-kinase–Akt signaling pathway, so named because it is the target of rapamycin.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

15–87 Binding of extracellular ligands to receptor tyrosine kinases (RTKs) activates the intracellular catalytic domain by propagating a conformational change across the lipid bilayer through a single transmembrane α helix.

15–88 PI 3-kinase phosphorylates the inositol head groups of phospholipids at the 3 position of the ring so that they can be cleaved by phospholipase C to produce IP_3.

15–89 Protein tyrosine phosphatases display exquisite specificity for their substrates, unlike most serine/threonine protein phosphatases, which have rather broad specificity.

THOUGHT PROBLEMS

15–90 Antibodies are Y-shaped molecules that carry two identical binding sites. Imagine that you have obtained an antibody that is specific for the extracellular domain of a receptor tyrosine kinase. If cells were exposed to the antibody, would you expect the receptor tyrosine kinase to be activated, inactivated, or unaffected? Explain your reasoning.

15–91 Genes encoding mutant forms of a receptor tyrosine kinase can be introduced into cells that express the normal receptor from their own genes. If the mutant genes are expressed at considerably higher levels than the normal genes, what will be the consequences for receptor-mediated signaling of introducing genes for the following mutant receptors?

A. A mutant receptor tyrosine kinase that lacks its extracellular domain.
B. A mutant receptor tyrosine kinase that lacks its intracellular domain.

15–92 The SH3 domain, which comprises about 60 amino acids, recognizes and binds to structural motifs in other proteins. The motif recognized by SH3 domains was found by constructing a fusion protein between an SH3 domain and glutathione-*S*-transferase (GST). GST fusions allow for easy purification using a glutathione affinity column, which binds GST specifically. After tagging the purified GST–SH3 protein with biotin to make it easy to detect, it was used to screen filters containing *E. coli* colonies expressing a cDNA library. Two different clones were identified that bound to the SH3 domain: in both cases, binding was shown to occur at short proline-rich sequences.

A. Could you use biotin-tagged GST–SH2 proteins in the same way to find cDNAs for proteins that bind to SH2 domains? Why or why not?
B. Many proteins bind to short strings of amino acids in other proteins. How do you think these kinds of interactions differ from the kinds of interactions found between the protein subunits of multisubunit enzymes?

15–93 The Ras protein functions as a molecular switch that is turned on by a guanine nucleotide exchange factor (GEF) that causes it to bind GTP. A GTPase-activating protein (GAP) turns the switch off by inducing Ras to hydrolyze its bound GTP to GDP much more rapidly than in the absence of the GAP. Thus, Ras works like a light switch that one person turns on and another turns off. In a cell line that lacks the Ras-specific GAP, what abnormalities in Ras activity, if any, would you expect to find in the absence of extracellular signals, and in their presence?

15–94 What are the similarities and differences between the reactions that lead to the activation of G proteins and those that lead to the activation of Ras?

15–95 In principle, the activated, GTP-bound form of Ras could be increased by activating a guanine nucleotide exchange factor (GEF) or by inactivating a GTPase-activating protein (GAP). Why do you suppose that Ras-mediated signaling pathways always increase Ras-GTP by activating a GEF rather than inactivating a GAP?

15–96 A single amino acid change in Ras eliminates its ability to hydrolyze GTP, even in the presence of a GTPase-activating protein (GAP). Roughly 30% of human cancers have this change in Ras. You have just identified a small molecule that prevents the dimerization of a receptor tyrosine kinase that signals via Ras. Would you expect this molecule to be effective in the treatment of cancers that express this common, mutant form of Ras? Why or why not?

DATA HANDLING

15–97 What does autophosphorylation mean? When a receptor tyrosine kinase binds its ligand and forms a dimer, do the individual receptor molecules phosphorylate themselves or does one receptor cross-phosphorylate the other, and vice versa? To investigate this question, you've constructed genes for three forms of a receptor tyrosine kinase: the normal form with an active kinase domain and three sites of phosphorylation; a large form that carries an inactivating point mutation in the kinase domain but retains the three phosphorylation sites; and a short version that has an active kinase domain but is lacking the sites of phosphorylation (**Figure 15–13A**). You express the genes singly and in combination in a cell line that lacks this receptor tyrosine kinase, and then break open the cells and add the ligand for the receptor in the presence of radioactive ATP. You immunoprecipitate the receptors and analyze them for expression levels by staining for protein (**Figure 15–13B**) and for phosphorylation by autoradiography (**Figure 15–13C**).

A. What results would you expect on the autoradiograph if individual receptors only phosphorylated themselves?
B. What would you expect if receptors cross-phosphorylated each other?
C. Which model for autophosphorylation do your data support?

15–98 When activated, the platelet-derived growth factor (PDGF) receptor phosphorylates itself on multiple tyrosines. These phosphorylated tyrosines serve as assembly sites for several SH2-domain-containing proteins that include phospholipase C-γ (PLCγ), a Ras-specific GTPase-activating protein (GAP), a subunit of phosphoinositide 3-kinase (PI3K), and a phosphotyrosine phosphatase (PTP) (**Figure 15–14**). PDGF binding stimulates several changes in the target cell, one of which is an increase in DNA synthesis, as measured by incorporation of radioactive thymidine or bromodeoxyuridine into DNA.

To determine which of the bound proteins is responsible for activation of DNA synthesis, you construct several mutant genes for the PDGF receptor that retain individual or combinations of tyrosine

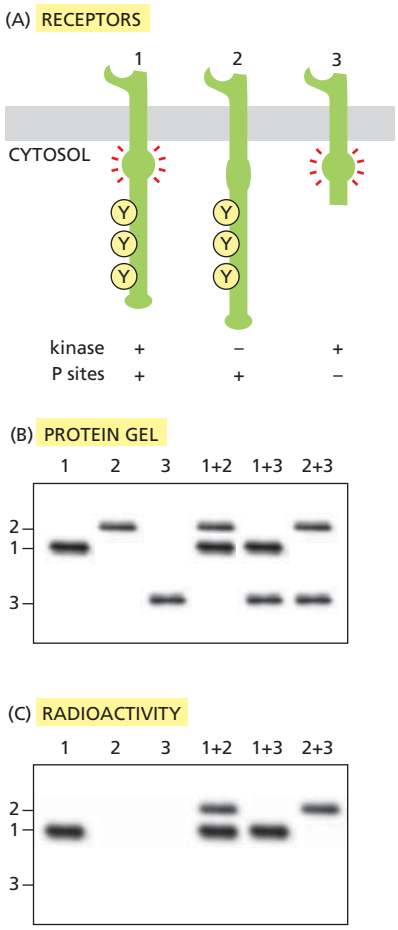


Figure 15–13 Analysis of autophosphorylation (Problem 15–97). (A) Normal and mutant receptor tyrosine kinases. P sites refers to the sites of phosphorylation. (B) Expression of receptor tyrosine kinases. (C) Phosphorylation of receptor tyrosine kinases.

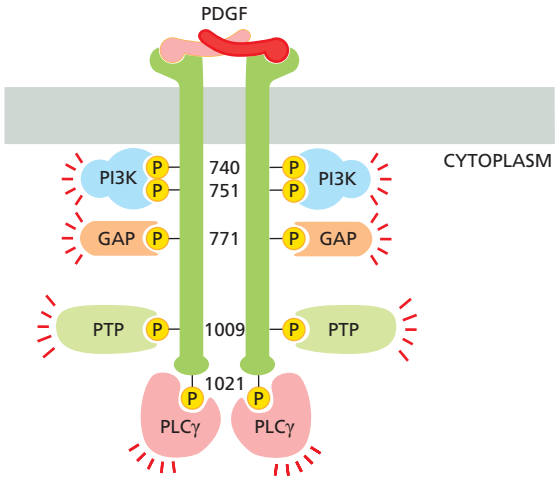
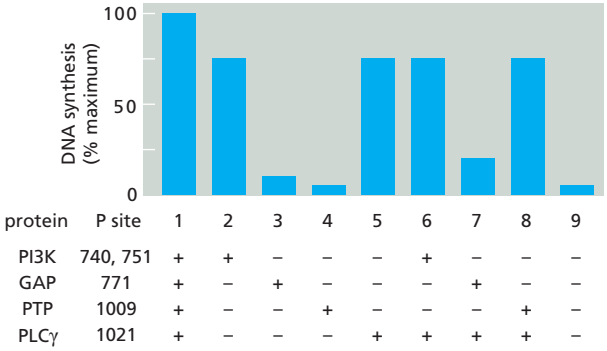


Figure 15–14 The signaling complex assembled on the PDGF receptor (Problem 15–98). Numbers refer to the positions of the phosphorylated amino acids in the sequence of the PDGF receptor.

phosphorylation sites. When expressed in cells that do not make a PDGF receptor of their own, each of the receptors is phosphorylated at its tyrosines upon binding of PDGF. As shown in **Figure 15–15**, DNA synthesis is stimulated to different extents in cells expressing the mutant receptors.

What roles do PI3K, GAP, PTP, and PLCγ play in the stimulation of DNA synthesis by PDGF?



protein	P site	1	2	3	4	5	6	7	8	9
PI3K	740, 751	+	+	–	–	–	+	–	–	–
GAP	771	+	–	+	–	–	–	+	–	–
PTP	1009	+	–	–	+	–	–	–	+	–
PLCγ	1021	+	–	–	–	+	+	+	+	–

Figure 15–15 Stimulation of DNA synthesis by the normal PDGF receptor and by receptors missing some phosphorylation sites (Problem 15–98). Stimulation by the normal receptor is set arbitrarily at 100%. The presence of a phosphorylation site (P site) is indicated by +; absence of a site by –.

15–99 MAP kinase kinase kinase (MAPKKK) activates MAP kinase kinase (MAPKK) by phosphorylation of two serine side chains. Doubly phosphorylated (active) MAPKK, in turn, activates MAP kinase (MAPK) by the phosphorylation of a threonine and a tyrosine. The doubly phosphorylated MAPK then phosphorylates a variety of target proteins to bring about complex changes in cell behavior. It is possible to write down all of the rate equations for the individual steps in this activation cascade, as well as for the removal of the phosphates (inactivation) by protein phosphatase, and to solve them by making reasonable assumptions about the concentrations of the proteins. The calculated plot of activation of the kinases versus input stimulus is shown in **Figure 15–16**. Why is the very steep response curve for MAPK a good thing for this signaling pathway?

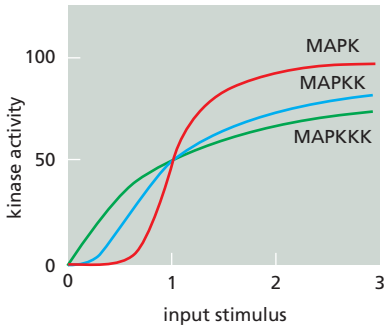


Figure 15–16 Stimulus–response curves for the components of the MAPK cascade (Problem 15–99). For ease of comparison, the curves have been normalized so that an input stimulus of 1 gives 50% activation of the kinases.

15–100 An explicit assumption in the analysis in Problem 15–99 is that the components of the MAP kinase module operate independently of one another, so that the dual phosphorylation events that activate MAPKK and MAPK occur one at a time as molecules collide in solution. How do you suppose the curves in Figure 15–16 would change if a scaffold protein held the kinases of the MAP kinase cascade together? Most MAP kinase modules are scaffolded. What is the advantage of linking these kinases onto scaffold proteins?

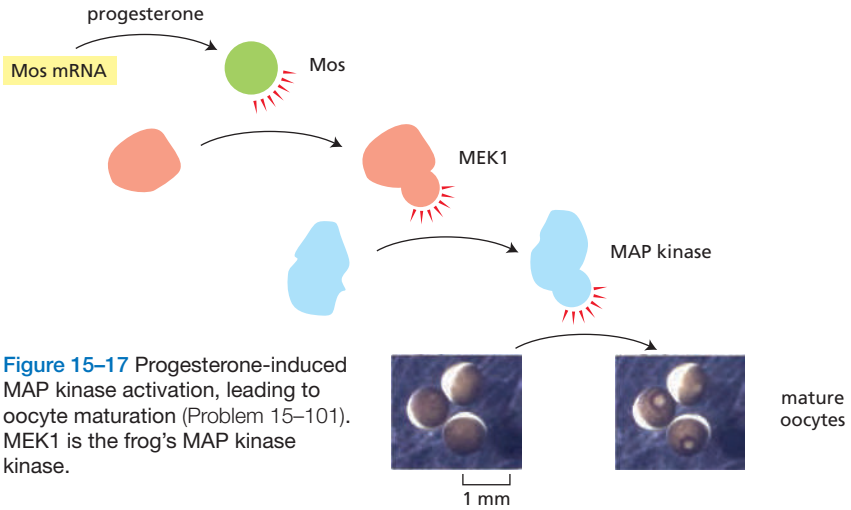


Figure 15–17 Progesterone-induced MAP kinase activation, leading to oocyte maturation (Problem 15–101). MEK1 is the frog's MAP kinase kinase.

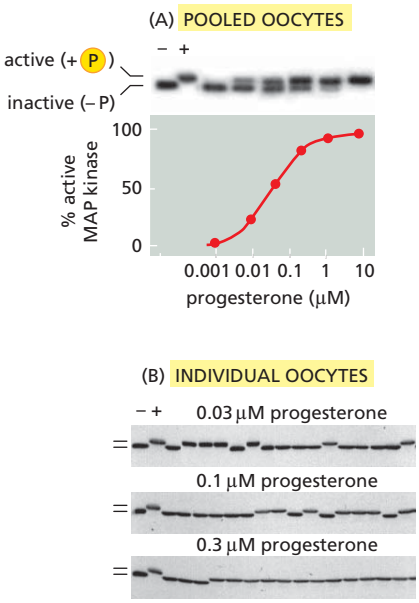


Figure 15–18 Activation of frog oocytes (Problem 15–101). (A) Phosphorylation of MAP kinase in pooled oocytes. (B) Phosphorylation of MAP kinase in individual oocytes. MAP kinase was detected by immunoblotting using a MAP-kinase-specific antibody. The first two lanes in each gel contain nonphosphorylated, inactive MAP kinase (–) and phosphorylated, active MAP kinase (+).

15–101 Activation ("maturation") of frog oocytes is signaled through a MAP kinase signaling module. An increase in the hormone progesterone triggers the module by stimulating the translation of the mRNA for Mos, which is the frog's MAP kinase kinase kinase (**Figure 15–17**). Maturation is easy to score visually by the presence of a white spot in the middle of the brown surface of the oocyte (Figure 15–17). To determine the dose–response curve for progesterone-induced activation of MAP kinase, you place 16 oocytes in each of six plastic dishes and add various concentrations of progesterone. After an overnight incubation, you crush the oocytes, prepare an extract, and determine the state of MAP kinase phosphorylation (hence, activation) by SDS polyacrylamide-gel electrophoresis (**Figure 15–18A**). This analysis shows a graded response of MAP kinase to increasing concentrations of progesterone.

Before you crushed the oocytes, you noticed that not all oocytes in individual dishes had white spots. Had some oocytes undergone partial activation and not yet reached the white-spot stage? To answer this question, you repeat the experiment, but this time you analyze MAP kinase activation in individual oocytes. You are surprised to find that each oocyte has either a fully activated or a completely inactive MAP kinase (**Figure 15–18B**). How can an all-or-none response in individual oocytes give rise to a graded response in the population?

15–102 Akt is a key protein kinase in the signaling pathway that leads to cell growth. Akt is activated by a phosphatidylinositol-dependent protein kinase (PDK1), which phosphorylates threonine 308. At the same time, serine 473 is phosphorylated. Your advisor has been unsuccessful in purifying the protein kinase responsible for the phosphorylation of serine 473, but you think you know what is going on. You construct genes encoding two mutant forms of Akt: one carries a point mutation in the kinase domain, Akt-K179M, which renders it kinase-dead, and the other carries a point mutation in the domain required to bind to PDK1 (Akt-T308A), which cannot be activated by PDK1. You transfect each of these constructs, and a construct for wild-type Akt, into cells that do not express their own Akt. You treat a portion of the cells with an insulin-like growth factor (IGF1), which activates PDK1, and analyze the phosphorylation state of the various forms of Akt using antibodies specific for Akt or for particular phosphorylated amino acids (**Figure 15–19**).

What is the identity of the enzyme that phosphorylates serine 473 on Akt?

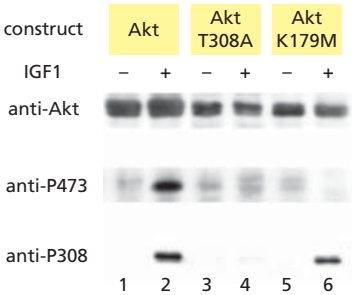


Figure 15–19 Expression levels of various forms of Akt and their degree of phosphorylation in the presence and absence of IGF1 (Problem 15–102). Anti-Akt recognizes all three forms of Akt regardless of their phosphorylation state; anti-P473 specifically recognizes the phosphorylated serine at position 473; anti-P308 specifically recognizes the phosphorylated threonine at position 308.

Figure 15–20 Sequence elements in the transcription factor that responds to IFNγ (Problem 15–103).

15–103 Interferon-γ (IFNγ) is a cytokine produced by activated T lymphocytes. It binds to surface receptors on macrophages and stimulates their efficient scavenging of invading viruses and bacteria via a JAK–STAT signaling pathway. A number of genes are activated in response to IFNγ binding, all of which contain a DNA sequence element with partial dyad symmetry (TTCCXGTAA) that is required for the IFNγ response.

You have cloned the gene for the STAT transcription factor that is activated in response to IFNγ binding. The sequence of the gene indicates that the protein contains several heptad repeat sequences near its N-terminus—a common dimerization domain in many transcription factors—and SH2 and SH3 domains adjacent to a site for tyrosine phosphorylation near the C-terminus (**Figure 15–20**). By making antibodies to the protein, you show that it is normally located in the cytosol. After 15 minutes exposure to IFNγ, the protein becomes phosphorylated on a tyrosine and moves to the nucleus.

Suspecting that tyrosine phosphorylation is the key to the regulation of this transcription factor, you assay its ability to bind the DNA sequence element in the presence of high concentrations of free phosphotyrosine or when mixed with anti-phosphotyrosine antibodies. Both treatments inhibit binding of the protein to DNA, as does treatment with a protein phosphatase. Finally, you measure the molecular weight of the cytosolic and nuclear forms of the protein, which suggest that the cytosolic form is a monomer and the nuclear form is a dimer.

A. Do you think that phosphorylation of the transcription factor is necessary for the factor to bind to DNA, or do you think phosphorylation is required to create an acidic activation domain to promote transcription?
B. Bearing in mind that SH2 domains bind phosphotyrosine, how do you suppose free phosphotyrosine might interfere with the activity of the transcription factor?
C. How might tyrosine phosphorylation of the protein promote its dimerization? How do you think dimerization enhances its binding to DNA?

ALTERNATIVE SIGNALING ROUTES IN GENE REGULATION

TERMS TO LEARN

β-catenin
circadian clock
Cubitus interruptus (Ci)
Delta
Dishevelled
Frizzled
Hedgehog protein
iHog
IκB
LDL-receptor-related protein (LRP)
NFκB proteins
Notch
nuclear receptor superfamily
Patched
Smoothened
steroid hormone
Wnt/β-catenin pathway
Wnt proteins

DEFINITIONS

Match each definition below with its term from the list above.

15–104 Receptor protein involved in what may be the most widely used signaling pathway in animal development; its ligands are cell-surface proteins such as Delta.

15–105 A family of secreted signal molecules that act as local mediators and morphogens during development; they were initially discovered as the products of the *Wingless* gene in flies and the *Int1* gene in mice.

15–106 A signaling pathway activated by Wnt binding to both the Frizzled receptor and the LRP co-receptor.

15–107 A group of secreted signal molecules that act as local mediators and morphogens during development and whose effects are mediated through the cell-surface receptor Patched and its binding partner Smoothened.

15–108 A target of Hedgehog signaling, this gene regulatory molecule is a full-length gene activator in the presence of Hedgehog and a partially proteolyzed gene repressor in its absence.

15–109 Latent gene regulatory proteins that are present in most cells in both animals and plants and are central to many stress, inflammatory, and innate immune responses.

15–110 Hydrophobic signaling molecule with a characteristic four-ringed structure derived from cholesterol.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

15–111 Signaling pathways that activate latent gene regulatory proteins depend on regulated proteolysis to control activity and location.

15–112 Notch is both a cell-surface receptor and a latent gene regulatory protein.

15–113 Because one of the targets of NFκB activation is the gene for IκBα, the cytoplasmic inhibitor of NFκB, a negative feedback loop is established that limits the duration of the NFκB response.

THOUGHT PROBLEMS

15–114 Why do signaling responses that involve changes in proteins already present in the cell occur in milliseconds to seconds, whereas responses that require changes in gene expression require minutes to hours?

15–115 Like Notch, the β-amyloid precursor protein (APP) is cleaved near its transmembrane segment to release an extracellular and an intracellular component. Explain how the fragments of APP relate to the amyloid plaques that are characteristic of Alzheimer's disease.

15–116 The Wnt planar polarity signaling pathway normally ensures that each wing cell in *Drosophila* has a single hair. Overexpression of the *Frizzled* gene from a heat-shock promoter (hs-*Fz*) causes multiple hairs to grow from many cells (**Figure 15–21A**). This phenotype is suppressed if hs-*Fz* is combined with a heterozygous deletion (Dsh^{Δ}/+) of the *Dishevelled* gene (**Figure 15–21B**). Do these results allow you to order the action of Frizzled and Dishevelled in the signaling pathway? If so, what is the order? Explain your reasoning.

15–117 There are two common mutational routes to the uncontrolled cell proliferation and invasiveness that characterize cancer cells. The first is to make a stimulatory gene (a proto-oncogene) hyperactive: this type of mutation has a dominant effect so that only one of the cell's two gene copies needs to undergo change. The second is to make an inhibitory gene (a tumor suppressor gene) inactive: this type of mutation usually is recessive so that both the cell's gene copies must be inactivated.

Mutations of the *Apc* (adenomatous polyposis coli) gene occur in 80%

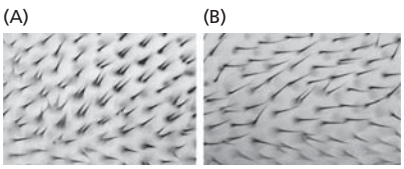


Figure 15–21 Pattern of hair growth on wing cells in genetically different *Drosophila* (Problem 15–116).

of human colon cancers. Normal APC increases the affinity of the degradation complex for β-catenin, which in excess can enter the nucleus and promote transcription of key target genes for cell proliferation. Given this information, which category—oncogene or tumor suppressor—would you expect the *Apc* gene to belong to? Why?

15–118 Latent gene regulatory proteins are prevented from entering the nucleus until the cell receives an appropriate signal. List four ways by which cells keep gene regulatory proteins out of the nucleus, and give an example of a latent gene regulatory protein that is controlled by each mechanism.

15–119 The steroid hormones cortisol, estradiol, and testosterone are all derived from cholesterol by modifications that introduce polar groups such as –OH and =O (**Figure 15–22**). If cholesterol itself was not normally found in cell membranes, do you suppose it could be used effectively as a hormone, provided that an appropriate intracellular receptor was available?

15–120 Most people who are completely blind have circadian rhythms that are 'free-running;' that is, their circadian rhythms are not synchronized to environmental time cues and they oscillate on a cycle of about 24.5 hours. Why do you suppose the circadian clocks of blind people are not entrained to the same 24-hour clock as the majority of the population? Can you guess what symptoms might be associated with a free-running circadian clock? Do you suppose that blind people have trouble sleeping?

DATA HANDLING

15–121 β-Catenin can be phosphorylated by glycogen synthase kinase 3 (GSK3) and it can be degraded in proteasomes. β-Catenin could be sensitized for degradation by phosphorylation, it could be protected from degradation by phosphorylation, or its phosphorylation status could be irrelevant for degradation. To distinguish among these possibilities, you generate cell lines that express either a mutant GSK3 that cannot carry out phosphorylation, or a mutant β-catenin that is missing its site of phosphorylation. In the presence and absence of the proteasome inhibitor, ALLN, both cell lines yield β-catenin that migrates as a single band, with no slower migrating bands visible. In contrast, nonmutant β-catenin and GSK3 display several slower migrating bands in the presence of ALLN, but no slower migrating bands in its absence. What is the relationship between β-catenin phosphorylation and its degradation in proteasomes? Explain your answer.

15–122 The *Hedgehog* gene encodes the Hedgehog precursor protein, which is 471 amino acids long. The precursor protein (**Figure 15–23A**) is normally cleaved between glycine 257 (G257) and cysteine 258 (C258) to generate a fragment that is active in local and long-range signaling. Cleavage is essential for signaling. You clone a segment of the *Hedgehog* gene encoding a portion of the protein that includes the cleavage site and the entire C-terminus. When you purify this protein and incubate it in buffer, you observe cleavage over the course of several hours, as shown in **Figure 15–23B**. If you vary its concentration over a 256-fold range and assay cleavage after 4 hours of incubation, you observe the results shown in **Figure 15–23C**.

A. Explain how these data support the idea that the Hedgehog precursor protein cleaves itself. How do they rule out the possibility that the purified protein is contaminated with a bacterial protease, for example?

B. Does a molecule of precursor protein cleave itself, or does it cleave another molecule of the precursor; that is, is the reaction intramolecular or intermolecular?

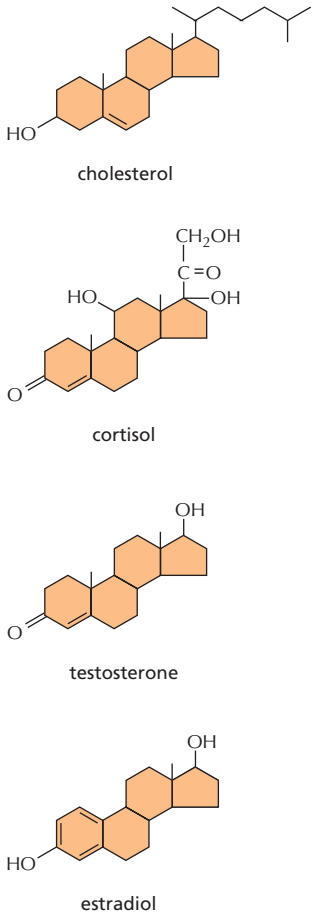


Figure 15–22 Steroid hormones and their parent molecule, cholesterol (Problem 15–119).

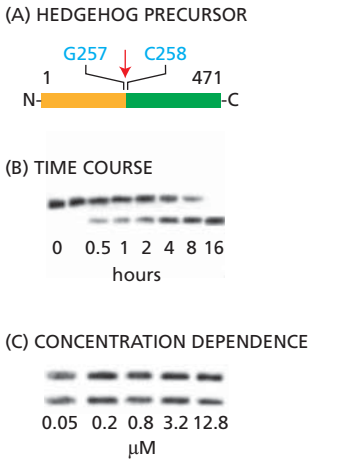


Figure 15–23 Mechanism of cleavage of the Hedgehog precursor protein (Problem 15–122). (A) Site of cleavage *(red arrow)* in the Hedgehog precursor protein. (B) Time course of cleavage of the fragment of the precursor protein. (C) Dependence of cleavage on concentration of the precursor protein fragment.

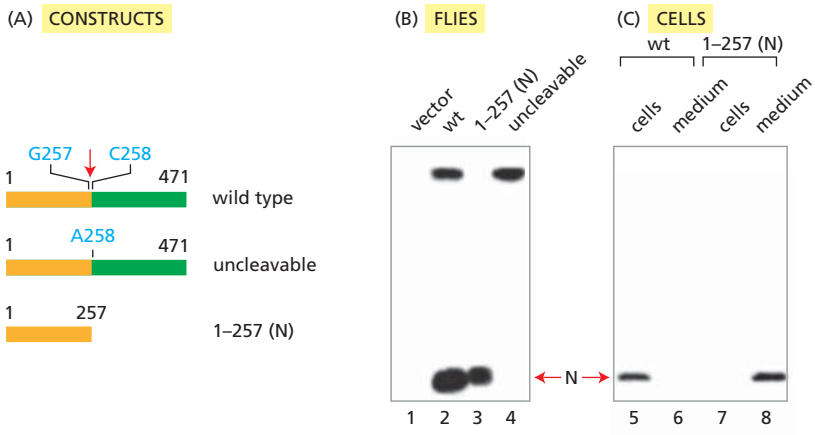


Figure 15–24 Fate of the fragments of Hedgehog after cleavage (Problem 15–123). (A) Constructs encoding different forms of the Hedgehog precursor protein. (B) Results of expression in *Drosophila* embryos. (C) Results of expression in insect cells. Hedgehog fragments were detected using antibodies specific for the N-terminal segment.

15–123 To find out what happens to the fragments of Hedgehog after cleavage, you express three versions: wild-type Hedgehog precursor, an uncleavable form, and the N-terminal cleavage product (**Figure 15–24A**). In fly embryos, the constructs behave as expected: wild-type Hedgehog is cleaved, the uncleavable version is not, and the N-terminal segment is expressed (**Figure 15–24B**). When wild-type Hedgehog and the N-terminal segment are expressed in insect cells, however, the N-terminal segment from wild-type Hedgehog remains associated with the cells, while the synthesized N-terminal segment is secreted into the medium (**Figure 15–24C**). Can you suggest possible explanations for the difference in localization of the N-terminal segment?

15–124 If you overexpress various Hedgehog constructs (see Figure 15–24A) in normal fly embyros and examine the pattern of Wnt expression (a well-characterized target of Hedgehog signaling), you observe a striped pattern of expression in all cases, but some constructs lead to thicker stripes than normal (**Figure 15–25**).

A. Which part of the Hedgehog molecule is responsible for signaling?
B. All the cells in the embryo are overexpressing the various Hedgehog constructs. Why is it, do you suppose, that you observe the same basic striped *pattern* of Wnt expression in all of them?
C. Why do you see stripes of Wnt expression even in the absence of Hedgehog overexpression?

15–125 Studies with the fruit fly *Drosophila* provided initial clues to the complex changes in patterns of gene expression that a simple hormone can trigger. *Drosophila* larvae molt in response to an increase in the concentration of the steroid hormone ecdysone. The polytene chromosomes of the *Drosophila* salivary glands are an excellent experimental system in which to study the pattern of gene activity initiated by the hormone because active genes enlarge into puffs that are visible in the light microscope. Furthermore, the size of a puff is proportional to the rate at which the gene is transcribed. Prior to addition of ecdysone, a few puffs—termed intermolt puffs—are already active. Upon exposure of dissected salivary glands to ecdysone, these intermolt puffs regress, and two additional sets

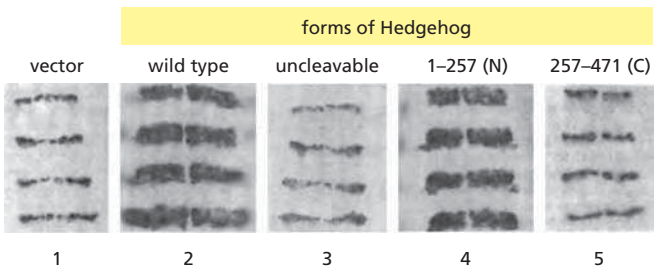


Figure 15–25 Patterns of Wnt expression in *Drosophila* embryos that are overexpressing various Hedgehog constructs (Problem 15–124). Wnt expression was detected by *in situ* hybridization.

of puffs appear. The early puffs arise within a few minutes after addition of ecdysone; the late puffs arise within 4–10 hours. The concentration of ecdysone does not change during this time period. The pattern of puff appearance and disappearance is illustrated for a typical puff in each category in **Figure 15–26A**.

Two critical experiments helped to define the relationships between the different classes of puff. In the first, cycloheximide, which blocks protein synthesis, was added at the same time as ecdysone. As illustrated in **Figure 15–26B**, under these conditions the early puffs did not regress and the late puffs were not induced. In the second experiment, ecdysone was washed out after a 2-hour exposure. As illustrated in **Figure 15–26C**, this treatment caused an immediate regression of the early puffs and a *premature induction* of the late puffs.

A. Why do you think the early puffs didn't regress and the late puffs weren't induced in the presence of cycloheximide? Why do you think the intermolt puffs were unaffected?

B. Why do you think the early puffs regressed immediately when ecdysone was removed? Why do you think the late puffs arose prematurely under these conditions?

C. Outline a model for ecdysone-mediated regulation of the puffing pattern.

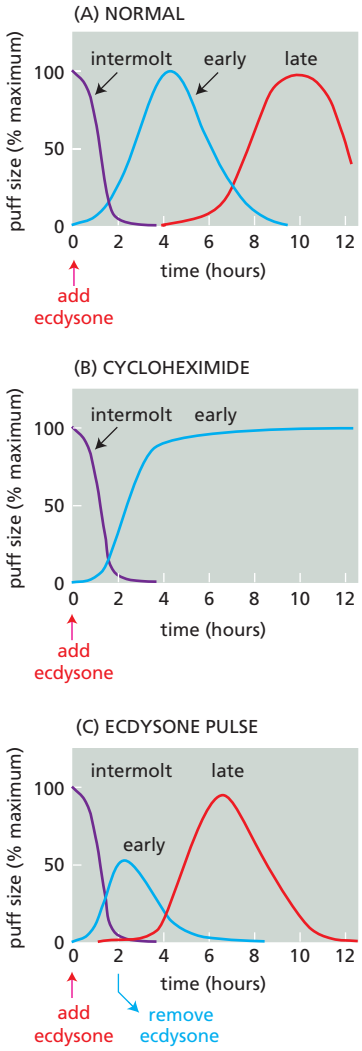


Figure 15–26 Puffing patterns in *Drosophila* salivary gland giant chromosomes (Problem 15–125). (A) Normal puffing pattern. (B) Puffing pattern in the presence of cycloheximide. (C) Puffing pattern after removal of ecdysone.

SIGNALING IN PLANTS

TERMS TO LEARN

auxin	leucine-rich repeat (LRR) receptor kinase
brassinosteroids	phototropin
cryptochrome	phytochrome
ethylene	plant growth regulator (plant hormone)

DEFINITIONS

Match each definition below with its term from the list above.

15–126 A cytoplasmic serine/threonine kinase in plants that is activated by red light and inactivated by far-red light.

15–127 Small gas molecule influential in various aspects of plant development, including fruit ripening and leaf abscission.

15–128 General term for a signal molecule that helps coordinate growth and development in plants.

15–129 Flavoprotein responsive to blue light, found in both plants and animals; in animals it is involved in circadian rhythms.

15–130 A growth regulator that helps plants grow toward light, grow upward rather than branch out, and extend their roots downward.

15–131 Common type of receptor serine/threonine kinase in plants, characterized by an extracellular portion rich in repeated segments containing a high proportion of leucine.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

15–132 Even though plants and animals independently evolved multicellularity, they use virtually all the same signaling proteins and second messengers for cell–cell communication.

15–133 Remarkably, the auxin efflux transporters in the cap cells of the root quickly redistribute themselves in response to a change in the direction

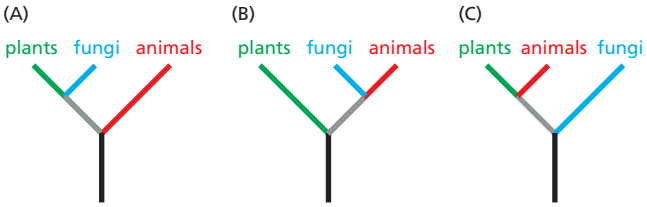


Figure 15–27 Three possible phylogenetic relationships among plants, animals, and fungi (Problem 15–134).

of the gravity vector, so that they pump auxin toward the side of the root pointing downward.

THOUGHT PROBLEMS

15–134 The last common ancestor to plants and animals was a unicellular eukaryote. Thus, it is thought that multicellularity and the attendant demands for cell communication arose independently in these two lineages. This evolutionary viewpoint accounts nicely for the vastly different mechanisms that plants and animals use for cell communication. Fungi use signaling mechanisms and components that are very similar to those used in animals. Which of the phylogenetic trees shown in **Figure 15–27** does this observation support?

15–135 If signaling arose as a solution to the demands of multicellularity, how then do you account for the very similar mechanisms of signaling that are used in animals and the unicellular fungus *Saccharomyces cerevisiae*?

15–136 How is it that plant growth regulators can be present throughout a plant and yet have specific effects on particular cells and tissues?

DATA HANDLING

15–137 The ripening of fruit is a complicated process of development, differentiation, and death (except for the seeds, of course). The process is triggered by minute amounts of ethylene gas. (This was discovered by accident many years ago; the paraffin stoves used to heat greenhouses in the olden days gave off enough ethylene to initiate the process.) The ethylene is normally produced by the fruits themselves in a biochemical pathway, the rate-limiting step of which is controlled by ACC synthase, which converts *S*-adenosylmethionine to a cyclopropane compound that is the immediate precursor of ethylene. Ethylene initiates a program of sequential gene expression that includes the production of several new enzymes, including polygalacturonase, which probably contributes to softening the cell wall.

Your company, Agribucks, is trying to make mutant tomatoes that cannot synthesize their own ethylene. Such fruit could be allowed to stay longer on the vine, developing their flavor while remaining green and firm. They could be shipped in this robust unripe state and exposed to ethylene just before arrival at market. This should allow them to be sold at the peak of perfection, and the procedure involves no artificial additives of any kind.

You decide to use an antisense approach, which works especially well in plants. You place an ACC synthase cDNA into a plant expression vector so that the gene will be transcribed in reverse, introduce it into tomato cells, and regenerate whole tomato plants. Sure enough, ethylene production is inhibited by 99.5% in these transgenic tomato plants, and their fruit fails to ripen. But when placed in air containing a small amount of ethylene, they turn into beautiful, tasty, ripe red fruit in about 2 weeks.

A. How do you imagine that transcribing the ACC synthase gene in reverse blocks the production of ethylene?
B. Will you be a millionaire before you are 30?

MCAT STYLE

Passage 1 (Questions 15–138 to 15–140)

The Ras GTPase was first discovered as a gene that plays an important role in transforming normal cells into cancer cells. Although Ras is normally activated by a receptor tyrosine kinase (RTK), in many kinds of cancer the *Ras* gene has sustained a mutation that makes the Ras protein hyperactive. This mutant Ras sends unregulated signals that drive cell proliferation and contribute to tumor formation. Activated Ras binds and activates a MAP kinase kinase kinase (MAPKKK) called Raf, which activates a MAP kinase kinase (MAPKK) called Mek, which then activates a MAP kinase (MAPK) called Erk.

15–138 What kinds of mutations in the *Ras* gene could lead to hyperactive Ras?

I. Mutations that stimulate Ras to bind the Ras GTPase-activating protein
II. Mutations that decrease the ability of Ras to hydrolyze GTP
III. Mutations that block Ras binding to Ras-GEF

A. I
B. II
C. I and III
D. II and III

15–139 Mutant forms of Raf have also been found to play an important role in cancer. A mutant called Raf-V600E causes Raf to become hyperactive independently of signals from Ras. Drugs that inhibit Raf-V600E cause rapid regression of tumors that express Raf-V600E. It was recently discovered that treatment of cancer cells with these drugs increases Ras activity. Which of the following hypotheses best explains this observation?

A. Erk normally phosphorylates and inhibits Raf to restrict the duration of RTK signaling. Inhibition of Erk therefore leads to increased Ras activity.
B. Erk normally phosphorylates the RTK and inhibits its signaling. Inhibition of Raf-V600E decreases Erk activity, which leads to increased RTK signaling.
C. Erk normally phosphorylates the RTK and stimulates its signaling. Inhibition of Raf-V600E increases RTK signaling, which leads to increased Ras activity.
D. Raf normally phosphorylates the RTK and stimulates its signaling. Inhibition of Raf-V600E therefore increases RTK signaling and increases Ras activity.

15–140 Imagine you are working in a cancer clinic and encounter a patient with a cancer that has the Raf-V600E mutation. You treat with a Raf inhibitor, but the cancer does not respond. You are working on developing a new treatment plan. Which of the following drugs would make the most sense?

A. An inhibitor of Ras-GEF
B. An inhibitor of Erk
C. An inhibitor of Ras
D. An inhibitor of the RTK

Passage 2 (Questions 15–141 to 15–143)

Scaffold proteins are thought to constrain signaling specificity by bringing multiple kinases into close proximity to ensure that they signal to each other, rather than to other proteins in the cell. The role of scaffolds in signaling was elucidated in studies aimed at understanding the specificity of MAP kinase cascades in yeast. In the MAP kinase cascade that prepares the cell for mating, an extracellular mating pheromone activates a G protein, which then activates a MAP kinase cascade that includes Ste11 (MAPKKK), Ste7 (MAPKK), and Fus3 (MAPK) (**Figure 15–28**). Activation of this cascade occurs over a time scale of 5–10 minutes. The MAP kinase cascade that controls the response to starvation is activated by the Ras

GTPase and also includes Ste11 and Ste7; however, the pathway works through a MAPK called Kss1, rather than Fus3. This cascade is activated over a time scale of several hours. How can activation of the same kinases—Ste11 and Ste7—lead to completely different outputs? The discovery that Ste5 binds to the G protein and to all of the MAP kinases in the mating-response pathway led to the idea that Ste5 acts as a scaffold to sequester the MAP kinases and link their activation to the activation of the G protein. Recent work suggests that scaffolds may play even more complex and interesting roles.

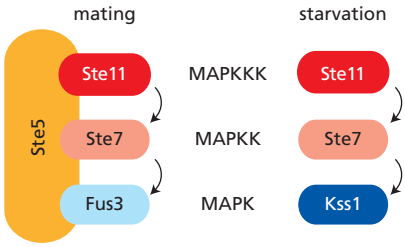


Figure 15–28 Mating-pheromone activation of the MAP kinase cascade in yeast (Problems 15–141 to 15–143).

15–141 Which of the following observations would make you question the sequestration model for scaffolds?

A. Ste7 activates Fus3 and Kss1 *in vitro* in the absence of Ste5.
B. Ste7 dissociates from Ste5 with a half-life of 5 seconds.
C. The Ste5 scaffold binds to Fus3, but does not bind to Kss1.
D. When activated in the mating pathway, Fus3 inactivates Kss1.

15–142 In one series of experiments, purified proteins were used to measure the ability of Ste7 to phosphorylate Fus3 and Kss1. Kinase reactions were carried out in the presence or absence of a domain of Ste5 that was found to play an important role in Fus3 activation. Ste7 robustly phosphorylated Kss1 by itself, and addition of the Ste5 domain had no effect on the K_m or K_{cat} of the reaction. In contrast, the Ste5 domain gave a 5000-fold increase in the rate of phosphorylation of Fus3 by Ste7, with little effect on the K_m. Which one of the following hypotheses could explain these experimental results?

A. Fus3 induces a conformational change in Ste5 that activates Ste7.
B. Ste5 alters the conformation of Fus3 to allow phosphorylation by Ste7.
C. The binding of the Ste5 domain to Ste7 activates its kinase activity.
D. The Ste5 domain positions Ste7 so that it binds more tightly to Fus3.

15–143 In another series of *in vitro* experiments, activation of Fus3 by Ste7 was measured in the presence of either full-length Ste5 or the Ste5 domain that activates Fus3. The rate of activation of Fus3 in the presence of full-length Ste5 was 10-fold lower than in the presence of the Ste5 domain. In addition, it was found that cells expressing the Ste5 domain, instead of full-length Ste5, inappropriately activated Fus3 in response to starvation. Which of the following hypotheses would explain these observations?

I. Full-length Ste5 inhibits Fus3 activation in the absence of mating pheromone, ensuring that starvation signals relayed by Ste7 cannot activate Fus3.
II. Full-length Ste5 contains a domain that promotes feedback activation of Ste7 by Fus3.
III. In the absence of mating pheromone, full-length Ste5 is in a conformation that inhibits its ability to facilitate Fus3 activation. Mating-pheromone signaling triggers a conformational change in Ste5 that relieves the inhibition.

A. I
B. II
C. I and III
D. II and III

CHAPTER 16

The Cytoskeleton

FUNCTION AND ORIGIN OF THE CYTOSKELETON

TERMS TO LEARN

cytoskeleton motor protein protofilament

DEFINITIONS

Match each definition below with its term from the list above.

16–1 A linear chain of protein subunits joined end to end, which associates laterally with other such chains to form cytoskeletal components.

16–2 System of protein filaments in the cytoplasm of a eukaryotic cell that gives the cell its shape and the capacity for directed movement.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

16–3 Microtubules determine the shape of the cell's surface and are necessary for whole-cell locomotion, and drive the pinching of one cell into two.

16–4 Even though the actin bundles at the cores of stereocilia on the hair cells of the inner ear maintain their stable organization for the entire lifetime of the animal, they are continuously remodeled and replaced on average every 48 hours.

16–5 Because bacteria lack the elaborate networks of intracellular membrane-enclosed organelles typical of eukaryotic cells, they do not require cytoskeletal filaments.

THOUGHT PROBLEMS

16–6 In general terms, what are the cellular functions of intermediate filaments, microtubules, and actin filaments?

16–7 If each type of cytoskeletal filament is made up of subunits that are held together by weak noncovalent bonds, how is it possible for a human being to lift heavy objects?

16–8 List differences between bacteria and animal cells that could have depended on the appearance during evolution of some or all of the components of the present eukaryotic cytoskeleton. Why do you suppose a cytoskeleton might have been crucial for each of these differences to evolve?

16–9 The amino acid sequences of actins and tubulins from all eukaryotes are remarkably well conserved, yet the large numbers of proteins that interact with these filaments are no more conserved than most other proteins

IN THIS CHAPTER

in different species. How can it be that the filament proteins themselves are highly conserved, while the proteins that interact with them are not?

CALCULATIONS

16–10 The average time it takes particles to diffuse a distance of x cm is

$$t = x^2/2D$$

where t is the time in seconds and D is the diffusion coefficient, which is a constant that depends on the size and shape of the particle.

A. How long would it take for a small molecule, a protein molecule, and a membrane-enclosed vesicle to diffuse across a cell 10 μm in diameter? A typical diffusion coefficient for a small molecule is 5×10^{-6} cm^2/sec, for a protein molecule 5×10^{-7} cm^2/sec, and for a membrane vesicle 5×10^{-8} cm^2/sec.

B. Why do you suppose a cell relies on the strategy of polymerizing and depolymerizing cytoskeletal filaments, rather than on diffusion of the filaments themselves, to accomplish its cytoskeletal rearrangements?

DATA HANDLING

16–11 One of the most striking examples of a purely actin-based cellular movement is the extension of the acrosomal process of a sea cucumber sperm. The sperm contains a store of unpolymerized actin in its head. When a sperm makes contact with a sea cucumber egg, the actin polymerizes rapidly to form a long spearlike extension. The tip of the acrosomal process penetrates the egg, and it is probably used to pull the sperm inside.

Are actin monomers added to the base or to the tip of the acrosomal bundle of actin filaments during extension of the acrosomal process? If the supply of monomers to the site of assembly depends on diffusion, it should be possible to distinguish between these alternatives by measuring the length of the acrosomal process with increasing time. If actin monomers are added to the base of the process, which is inside the head, the rate of growth should be linear because the distance between the site of assembly and the pool of monomers does not change with time. On the other hand, if the subunits are added to the tip, the rate of growth should decline progressively as the acrosomal process gets longer because the monomers must diffuse all the way down the shaft of the process. In this case, the rate of extension should be proportional to the square root of time. Plots of the length of the acrosomal process versus time and the square root of time are shown in **Figure 16–1**.

A. Are the ascending portions of the plots in Figure 16–1 more consistent with the addition of actin monomers to the base or to the tip of the acrosomal process?

B. Why do you suppose the process grows so slowly at the beginning and at the end of the acrosomal reaction?

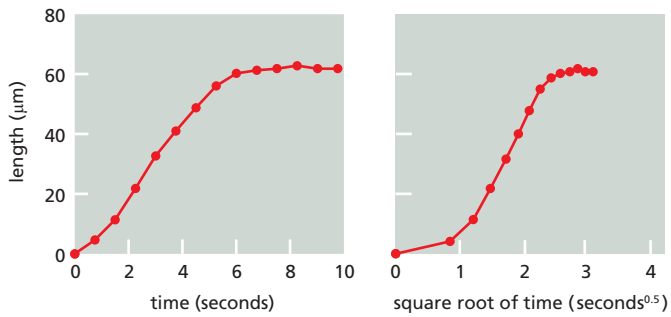


Figure 16–1 Plots of the length of the acrosome versus time and the square root of time (Problem 16–11).

ACTIN AND ACTIN-BINDING PROTEINS

TERMS TO LEARN

Arp2/3 complex
cell cortex
formin
treadmilling

DEFINITIONS

Match each definition below with its term from the list above.

16–12 The process by which a polymeric protein filament is maintained at constant length by addition of protein subunits at one end and loss of subunits at the other.

16–13 Specialized layer of cytoplasm on the inner face of the plasma membrane, rich in actin filaments.

16–14 Protein assembly that nucleates actin filament growth from the minus end, allowing rapid growth at the plus end and forming a treelike web of filaments.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

16–15 In the treelike web of actin filaments that form the cell cortex, an Arp2/3 complex anchors each actin filament branch to the side of another actin filament.

16–16 All the proteins that bind to the ends of actin filaments cap the ends to prevent further polymerization.

THOUGHT PROBLEMS

16–17 A typical time course of polymerization of actin filaments from actin subunits is shown in **Figure 16–2**.

A. Explain the properties of actin polymerization that account for each of the three phases of the polymerization curve.

B. How would the curve change if you doubled the concentration of actin? Would the concentration of free actin at equilibrium be higher or lower than in the original experiment, or would it be the same in both?

16–18 **Figure 16–3** shows the equilibrium distribution of actin in free subunits (monomers) and in filaments, as a function of actin concentration. Indicate the critical concentration of actin on this diagram.

16–19 Imagine that the polymer in **Figure 16–4A** can add subunits at either end, just like actin filaments (and microtubules). Imagine also three hypothetical types of free subunit, as shown in **Figure 16–4B**. Each type

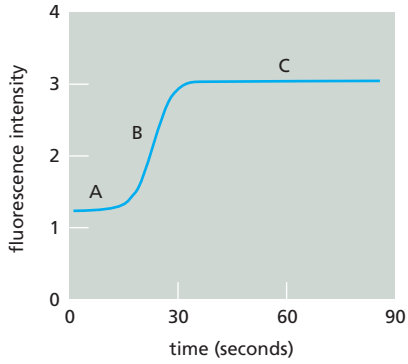


Figure 16–2 Formation of actin filaments over time, starting with purified actin monomers that are labeled with a fluorescent probe (Problem 16–17). Upon polymerization, the fluorescence of the probe increases, which allows polymerization to be measured. The intensity of fluorescence at zero seconds is due to the background fluorescence of the actin monomers. The three phases of polymerization are indicated as A, B, and C. Fluorescence intensity is measured in arbitrary units.

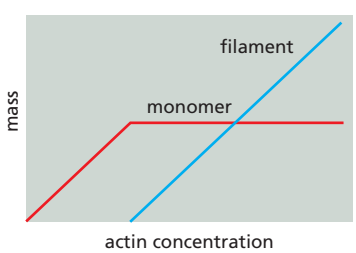


Figure 16–3 Mass of actin monomers and filaments as a function of actin concentration (Problem 16–18).

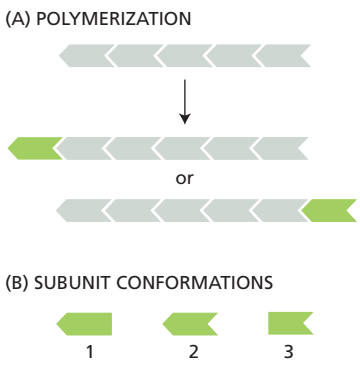


Figure 16–4 Polymerization of a polymer (Problem 16–19). (A) Addition of a subunit to a polymer. (B) Conformations of three hypothetical subunits.

of subunit can add to the polymer and, once added, it adopts the conformation of the other subunits in the polymer (Figure 16–4A). For each of these subunits, decide which end of the polymer, if either, will grow at the faster rate when the concentration of that subunit is higher than the critical concentration required for polymerization. Explain your reasoning. For any of the subunits, will there be a concentration at which one end will preferentially grow while the other shrinks? Why or why not?

16–20 Some actin-binding proteins significantly increase the rate at which the formation of actin filaments is initiated in the cytosol. How might such proteins do this? What must they *not* do when binding the actin monomers?

16–21 The concentration of actin in cells is 50–100 times greater than the critical concentration observed for pure actin in a test tube. How is this possible? What prevents the actin subunits in cells from polymerizing into filaments? Why is it advantageous to the cell to maintain such a large pool of actin subunits?

16–22 Cofilin preferentially binds to older actin filaments and promotes their disassembly. How does cofilin distinguish old filaments from new ones?

DATA HANDLING

16–23 If you add short actin filaments marked by bound myosin heads (myosin-decorated filaments) to a solution with an excess of actin monomers, wait for a few minutes, and then examine the filaments by electron microscopy, you see the picture shown in **Figure 16–5**.

A. Which is the plus end of the myosin-decorated filament and which is the minus end? Which is the "barbed" end and which is the "pointed" end? How can you tell?

B. If you diluted the mixture so that the actin concentration was below the critical concentration, which end would depolymerize more rapidly?

C. When the actin filament depolymerizes, why are subunits removed exclusively from the ends and not from the middle of the filament?

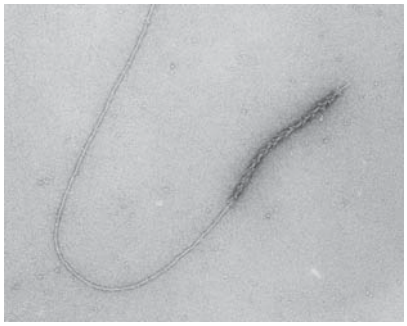


Figure 16–5 Myosin-decorated actin filament after a few minutes in a solution with excess actin monomers (Problem 16–23). The shorter, thicker segment is the myosin-decorated actin filament.

16–24 The growth rates at the plus and minus ends of actin filaments as a function of actin concentration are shown in **Figure 16–6A** and, on an expanded scale, in **Figure 16–6B**.

A. The data in Figure 16–6A were gathered by measuring initial growth rates at each actin concentration. Similar data gathered for any Michaelis–Menten enzyme would generate a hyperbolic plot, instead of the linear plots shown here. Why does the growth rate of actin filaments continue to increase linearly with increasing actin concentration, whereas an enzyme-catalyzed reaction reaches a plateau with increasing substrate concentration?

B. Figure 16–6B shows the filament growth rates at low actin concentration on an expanded scale. Imagine that you could add actin filaments to a solution of actin subunits at the concentrations indicated as A, B, C, D, and E. For each of these concentrations, decide whether the added actin filament would grow or shrink at its plus and minus ends. What is the critical concentration for the plus end? What is the critical concentration for the minus end? Would treadmilling occur at any of these concentrations?

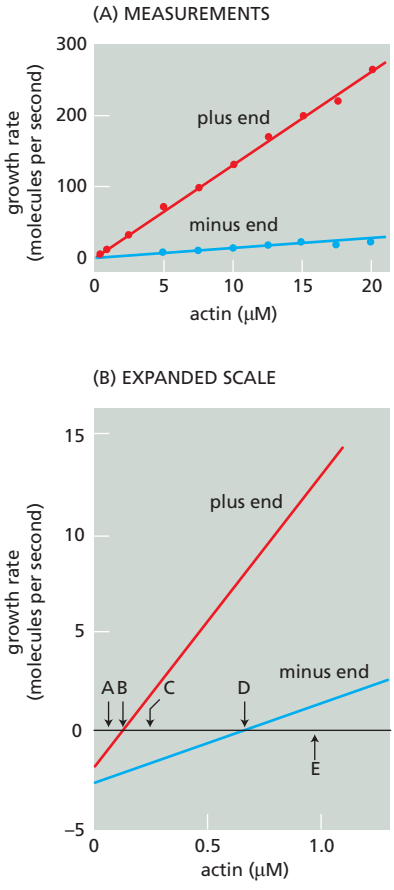


Figure 16–6 Growth rates at the plus and minus ends of actin filaments as a function of actin concentration (Problem 16–24). (A) Measurements of growth rates over a broad range of actin concentrations. (B) Growth rates at low actin concentrations, shown on an expanded scale.

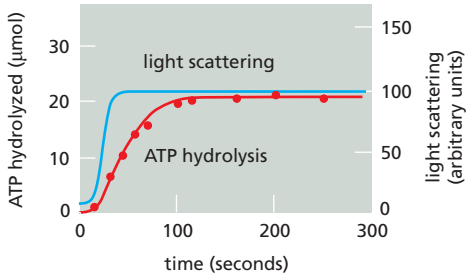


Figure 16–7 The kinetics of actin polymerization and ATP hydrolysis (Problem 16–25).

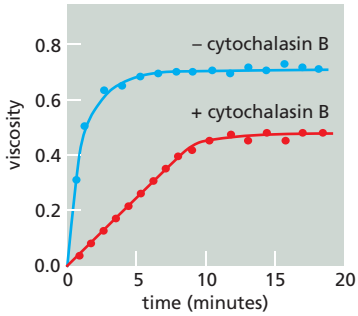


Figure 16–8 Increase in the viscosity of actin solutions in the presence and absence of cytochalasin B (Problem 16–26).

16–25 Your ultimate goal is to understand human consciousness, but your advisor wants you to understand some basic facts about actin assembly first. He tells you that ATP binds to actin monomers and is required for assembly. But, ATP hydrolysis is not necessary for polymerization since ADP can, under certain circumstances, substitute for the ATP requirement. ADP filaments, however, are much less stable than ATP filaments, supporting your secret suspicion that the free energy of ATP hydrolysis really is used to drive actin assembly.

Your advisor suggests that you make careful measurements of the quantitative relationship between the number of ATP molecules hydrolyzed and the number of actin monomers linked into polymer. The experiments are straightforward. To measure ATP hydrolysis, you add γ^{32}P-ATP to a solution of polymerizing actin, take samples at intervals, and determine how much radioactive phosphate has been produced. To assay polymerization, you measure the increase in light scattering that is caused by formation of the actin filaments. Your results are shown in **Figure 16–7**. Your light-scattering measurements indicate that 20 μmoles of actin monomers were polymerized. Since the number of polymerized actin monomers matches exactly the number of ATP molecules hydrolyzed, you conclude that one ATP is hydrolyzed as each new monomer is added to an actin filament.

When you show your advisor the data and tell him your conclusions, he smiles and very gently tells you to look more closely at the graph. He says your data prove that actin can polymerize without ATP hydrolysis.

A. What does your advisor see in the data that you have overlooked?
B. What do your data imply about the distribution of ATP and ADP in polymerizing actin filaments?

16–26 Cytochalasin B strongly inhibits certain forms of cell motility, such as cytokinesis and the ruffling of growth cones, and it dramatically decreases the viscosity of gels formed with mixtures of actin and a wide variety of actin-binding proteins. These observations suggest that cytochalasin B interferes with the assembly of actin filaments. In the classic experiment that defined its mechanism, short lengths of actin filaments were decorated with myosin heads and then mixed with actin subunits in the presence or absence of cytochalasin B. Assembly of actin filaments was measured by assaying the viscosity of the solution (**Figure 16–8**) and by examining samples by electron microscopy (**Figure 16–9**).

A. Suggest a plausible mechanism to explain how cytochalasin B inhibits actin filament assembly. Account for the appearance of the filaments in the electron micrographs and the viscosity measurements (both the altered rate and extent).
B. The normal growth characteristics of an actin filament and the actin-binding properties of cytochalasin B argue that actin monomers undergo a conformational change upon addition to an actin filament. How so?

16–27 Phalloidin, which is a toxic peptide from the mushroom *Amanita phalloides*, binds to actin filaments. Phalloidin tagged with a fluorescent probe is commonly used to stain actin filament assemblies in cells

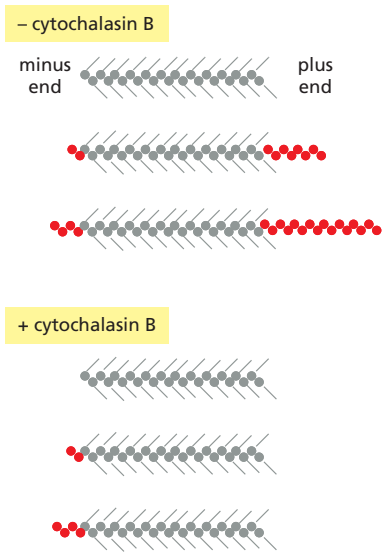


Figure 16–9 Appearance of typical actin filaments formed in the presence and absence of cytochalasin B (Problem 16–26). The decorated actin filaments present before the addition of actin monomers are shown at the *top* of each set of three. Filaments present after increasing times of incubation with actin monomers (*red circles*) are shown *below*.

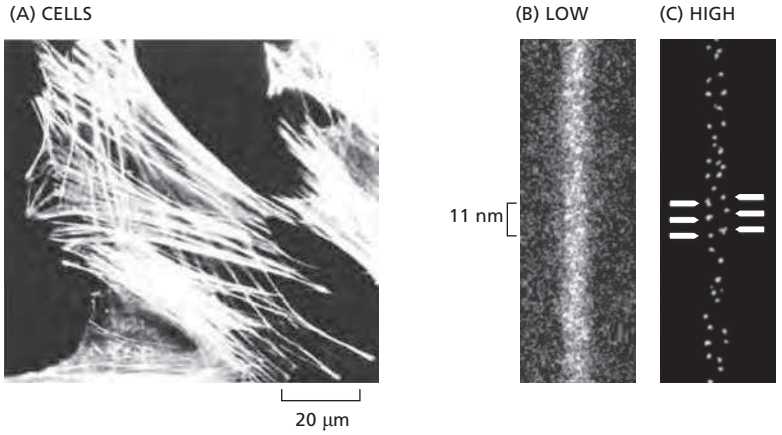


Figure 16–10 Binding of phalloidin to actin filaments (Problem 16–27). (A) The actin cytoskeleton stained with fluorescent phalloidin. (B) An actin filament bound by gold-tagged phalloidin at low contrast. (C) The same actin filament as in (B), but at high contrast. *Bright bands* mark the positions of six gold particles.

(**Figure 16–10A**). If phalloidin is attached to a gold particle instead, its binding to actin filaments can be examined at high resolution by scanning transmission electron microscopy. **Figure 16–10B** shows a micrograph of an actin filament with bound phalloidin and **Figure 16–10C** shows the same picture with the contrast adjusted so that only the points of highest intensity (the gold particles) are visible. Does phalloidin bind to every actin subunit? How can you tell?

16–28 Isolated bundles of actin filaments from the acrosomal processes of *Limulus polyphemus* (horseshoe crab) sperm have readily distinguishable plus ends (tapered) and minus ends (blunt). Assembly at the ends of such bundles was used to determine the mechanism of action of phalloidin, which has a marked effect on actin assembly. When phalloidin is mixed with actin in a molar ratio of at least 1:1, the growth rate increases at both ends, as shown for minus ends in **Figure 16–11A**. Because growth rate = $k_{on}[\text{actin}]_{initial} - k_{off}$, these plots have the form $y = mx + b$, so that the slope of the line equals k_{on} and the y intercept equals $-k_{off}$.

A. By analyzing the on and off rates, decide how phalloidin increases the growth rate of actin filaments. Explain your reasoning.
B. In **Figure 16–11B**, actin filaments grown in the presence or absence of phalloidin were diluted in the absence of actin monomers and their disassembly was assayed. Do these results confirm or contradict your conclusions from part A? Explain your answer.
C. What is the critical concentration for actin assembly at the minus end in the absence of phalloidin? What is the critical concentration for actin assembly at the minus end in the presence of phalloidin?
D. Propose a molecular mechanism for the effects of phalloidin on actin assembly.

16–29 Swinholide A is a member of a class of lipophilic compounds termed macrolides, which include a number of useful antibiotics such as erythromycin, that are synthesized by Actinomycetes. Swinholide A is a "twin" molecule, composed of two identical halves (**Figure 16–12A**). When added to cells growing in culture, swinholide A disrupts the actin cytoskeleton. Your advisor has shown conclusively that swinholide A binds a pair of actin monomers. She suspects that swinholide A causes actin filaments to depolymerize by sequestering actin subunits in a nonfunctional dimeric form and thus accelerating depolymerization through mass-action effects. She wants you to test this hypothesis.

You prepare actin filaments tagged with a probe that fluoresces intensely in the filament but much less so in the free subunits (or swinholide-bound subunits). This allows you to follow depolymerization readily and rapidly as a loss of fluorescence. Just as your advisor predicted,

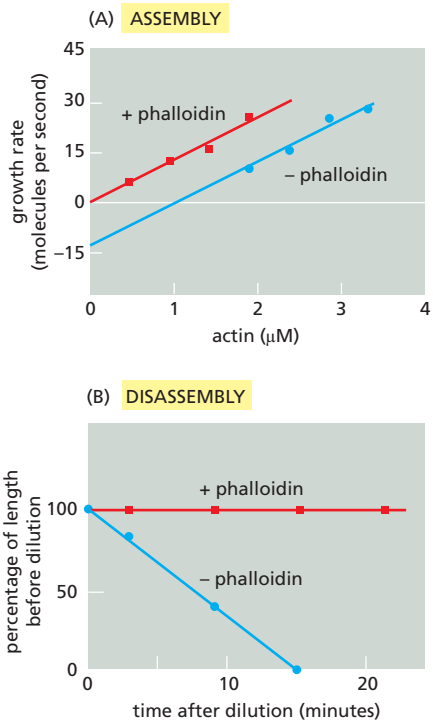


Figure 16–11 Effects of phalloidin on actin filaments (Problem 16–28). (A) Growth rates at the minus ends of acrosomal bundles in the presence and absence of phalloidin. (B) Disassembly of actin filaments upon dilution in the presence and absence of phalloidin.

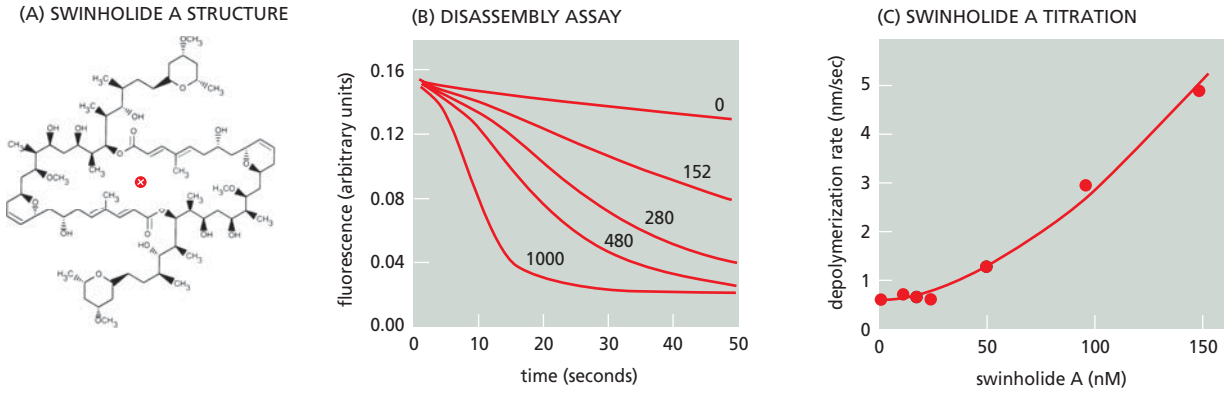


Figure 16–12 Effects of swinholide A on actin filaments (Problem 16–29). (A) Structure of swinholide A. The identical halves of swinholide A are arranged head to tail, so that if the molecule were rotated 180° about the indicated axis (*circle* with an X in it), it would superimpose on itself. For this reason it is said to have a twofold axis of symmetry. (B) Time course of actin filament depolymerization in the presence and absence of swinholide A. *Numbers* indicate the concentration of swinholide A (nM) used in each depolymerization assay. (C) Initial rates of depolymerization as a function of swinholide A concentration.

depolymerization increases in the presence of increasing concentrations of swinholide A (**Figure 16–12B**). But you notice two features of these curves that suggest to you that swinholide A may actually sever actin filaments. One of these features is illustrated in **Figure 16–12C**, which shows a nonlinear dependence of the initial rate of depolymerization on the concentration of swinholide A. A simple mass-action effect—the sequestering of actin monomers by binding to swinholide A—predicts a linear dependence; however, increasing increments in swinholide A concentration have a progressively greater effect on depolymerization.

A. In Figure 16–12B, why does fluorescence reach a plateau value (at about 0.03) instead of decreasing to zero?

B. The other odd feature you noticed about depolymerization in the presence of swinholide A (Figure 16–12B) is that the lines have a "hump" in them in the first few seconds (when their fluorescence is still above 0.12). Why does this hump suggest that swinholide A severs actin filaments?

C. Assuming that swinholide A does sever actin filaments, is one molecule enough, or are multiple molecules needed? How do you know?

16–30 Accessory proteins that regulate the nucleation of actin filaments promote binding of the Arp2/3 complex to actin filaments so that most new filaments form as branches from existing ones. These proteins could stimulate Arp2/3 binding to the sides of existing filaments or to the plus end of a growing filament in a way that does not interfere with growth. Both possibilities would yield the final characteristic branched network of filaments. To distinguish between these alternatives, you mix the regulatory proteins with the Arp2/3 complex and actin subunits in the presence of actin filaments that are capped at their plus ends. After a short incubation you examine the resulting structures by electron microscopy. How will this experiment distinguish between these alternatives? What structures would you expect to see according to each model for nucleation by the Arp2/3 complex?

16–31 You have two proteins that you suspect cap the ends of actin filaments. To determine whether they do and, if so, which protein caps which end, you measure filament formation as a function of actin concentration in the absence of either protein, in the presence of protein 1, and in the presence of protein 2 (**Figure 16–13**). Which protein caps the plus end and which caps the minus end? How can you tell? Give examples of proteins in the cell that you would expect to behave like protein 1 and protein 2.

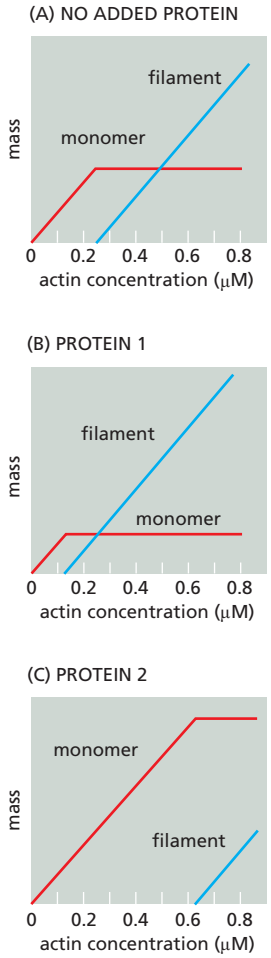


Figure 16–13 Effects of two proteins on actin polymerization (Problem 16–31). (A) Polymerization of pure actin. (B) Actin polymerization in the presence of protein 1. (C) Actin polymerization in the presence of protein 2. The mass of actin, as monomers or filaments, was determined at equilibrium.

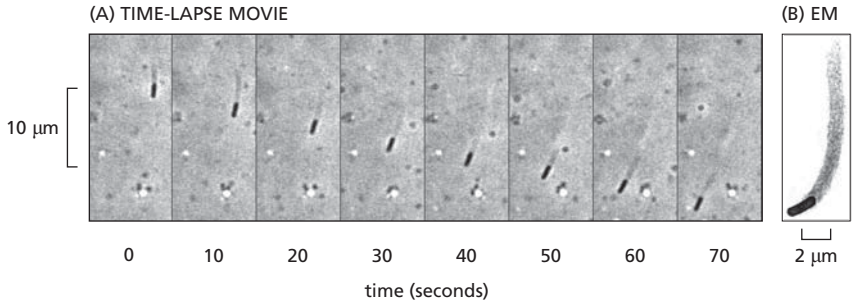


Figure 16–14 Movement of a bacterium through the cytosol on a comet tail of actin filaments (Problem 16–32). (A) Time-lapse movie. (B) Electron micrograph. The bacterium is 2 μm in length.

MEDICAL LINKS

16–32 The intracellular pathogenic bacterium *Listeria monocytogenes* propels itself through the cytosol on a comet tail of actin filaments (**Figure 16–14**). Remarkably, only a single bacterial protein, the transmembrane protein ActA, is required for this motility. ActA is distributed unequally on the surface of the bacterium, with maximum concentrations at the pole in contact with the actin tail. The effects of ActA on actin polymerization in the presence and absence of the Arp2/3 complex (ARP) are shown in **Figure 16–15A**. The first few seconds of the reactions are shown on an expanded scale in **Figure 16–15B**. Polymerization of actin was followed using pyrene-actin, which exhibits much higher fluorescence intensity when actin is polymerized.

A. What are the effects of ActA and the Arp2/3 complex, separately and together, on the rate of nucleation of actin filaments? Explain your answer.

B. How do you suppose that the polymerization of actin by ActA and the Arp2/3 complex propels the bacterium across the cell? In the comet tail of actin filaments, which ends—plus or minus—do you suppose are pointed at the bacterium?

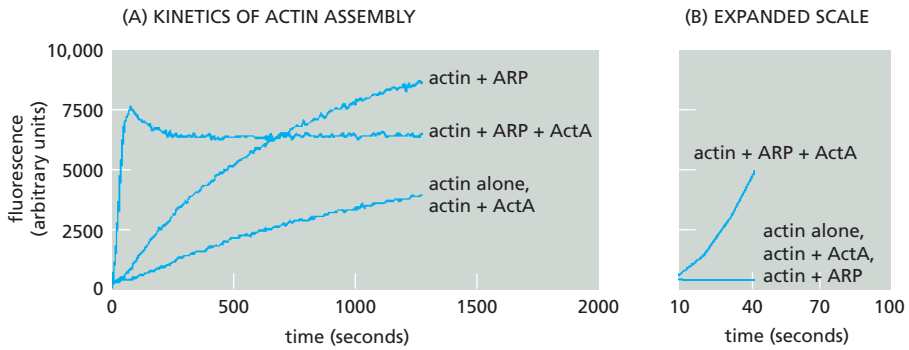


Figure 16–15 Effects of ActA and the Arp2/3 complex (ARP) on actin polymerization (Problem 16–32). (A) Kinetics of polymerization of actin in the presence of ActA and the Arp2/3 complex. (B) Kinetics of polymerization on an expanded scale. In all cases, actin was present at 2 μM, and ActA and the Arp2/3 complex were present at 30 nM.

MYOSIN AND ACTIN

TERMS TO LEARN

myofibril
myosin
stress fiber

DEFINITIONS

Match each definition below with its term from the list above.

16–33 The motor protein in muscle that generates the force for muscle contraction.

16–34 Long, highly organized bundle of actin, myosin, and other proteins in the cytoplasm of muscle cells that contracts by a sliding-filament mechanism.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

16–35 Myosin II molecules have two motor domains and a rodlike tail that allows them to assemble into bipolar filaments, which are crucial for the efficient sliding of oppositely oriented actin filaments past each other.

16–36 Motor neurons trigger action potentials in muscle cell membranes that open voltage-sensitive Ca^{2+} channels in T tubules, allowing extracellular Ca^{2+} to enter the cytosol, bind to troponin C, and initiate rapid muscle contraction.

16–37 When activated by Ca^{2+} binding, troponin C causes troponin I to release its hold on actin, thereby allowing the tropomyosin molecules to shift their positions slightly so that the myosin heads can bind to the actin filaments.

THOUGHT PROBLEMS

16–38 Living systems continually transform chemical free energy into motion. Muscle contraction, ciliary movement, cytoplasmic streaming, cell division, and active transport are examples of the ability of cells to transduce chemical free energy into mechanical work. In all these instances, a protein motor harnesses the free energy released in a chemical reaction to drive an attached molecule (the ligand) in a particular direction. Analysis of free-energy transduction in favorable biological systems suggests that a set of general principles governs the process in cells.

1. A cycle of reactions is used to convert chemical free energy into mechanical work.
2. At some point in the cycle a ligand binds very tightly to the protein motor.
3. At some point in the cycle the motor undergoes a major conformational change that alters the physical position of the ligand.
4. At some point in the cycle the affinity for the ligand markedly decreases, allowing the ligand to detach from the motor.

These principles are illustrated by the two cycles for free-energy transduction shown in **Figure 16–16**: (1) the sliding of actin and myosin filaments against each other and (2) the active transport of Ca^{2+} from

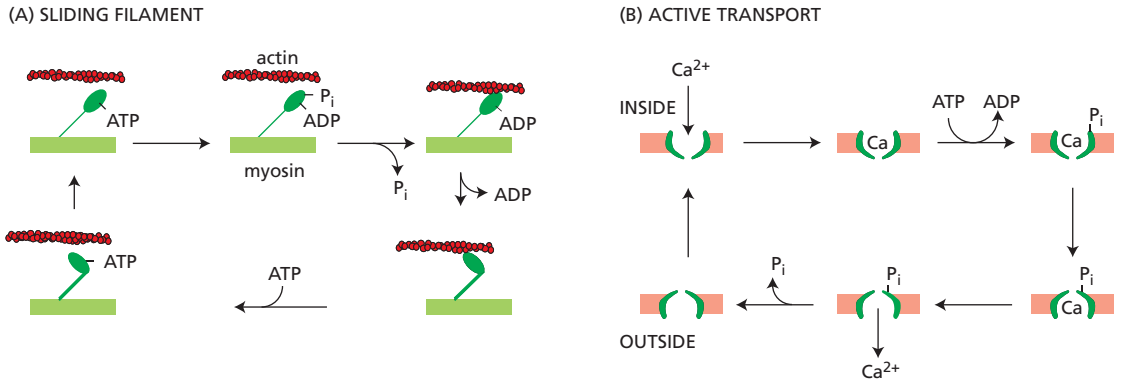


Figure 16–16 Transduction of chemical free energy into mechanical work (Problem 16–38). (A) Sliding of actin filaments relative to myosin filaments. (B) Active transport of Ca^{2+} from the inside to the outside of the cell. In both cycles, *arrows* are drawn in only one direction to emphasize their normal operation. The phosphorylation and dephosphorylation steps in the active transport cycle are catalyzed by enzymes that are not shown in the diagram.

inside the cell, where its concentration is low, to the cell exterior, where its concentration is high. An examination of these cycles underscores the principles of free-energy transduction.

A. What is the source of chemical free energy that powers these cycles, and what is the mechanical work that each cycle accomplishes?
B. What is the ligand that is bound tightly and then released in each of the cycles? Indicate the points in each cycle where the ligand is bound tightly.
C. Identify the conformational changes in the protein motor that constitute the "power stroke" and "return stroke" of each cycle.

16–39 Which one of the following changes takes place when a skeletal muscle contracts?

A. Z discs move farther apart.
B. Actin filaments contract.
C. Myosin filaments contract.
D. Sarcomeres become shorter.

16–40 Two electron micrographs of striated muscle in longitudinal section are shown in **Figure 16–17**. The sarcomeres in these micrographs are in two different states of contraction.

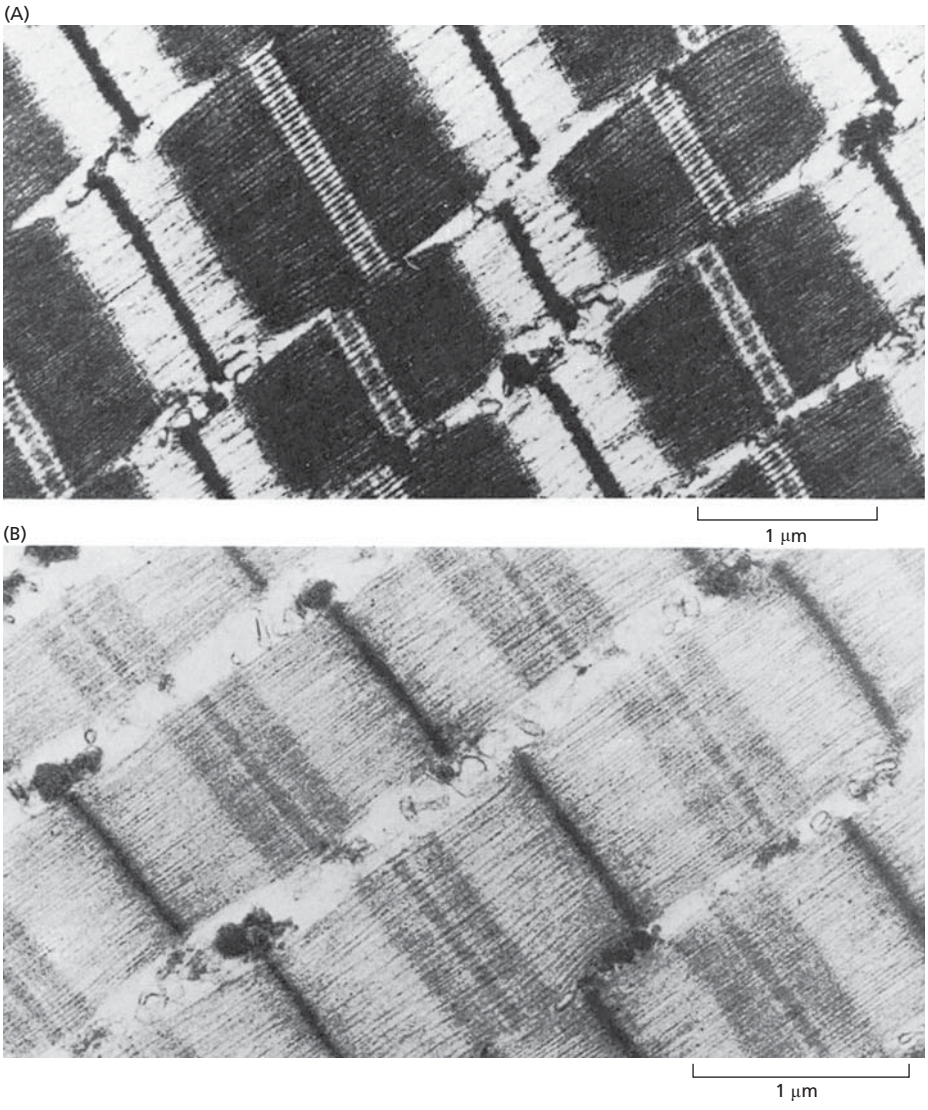


Figure 16–17 Two electron micrographs of striated muscle in longitudinal section (Problem 16–40). The micrograph in (B) is a much lighter exposure than the one in (A). At the same exposure, the entire space between the thin dark lines in (B) would be as dark as the fat dark band in (A).

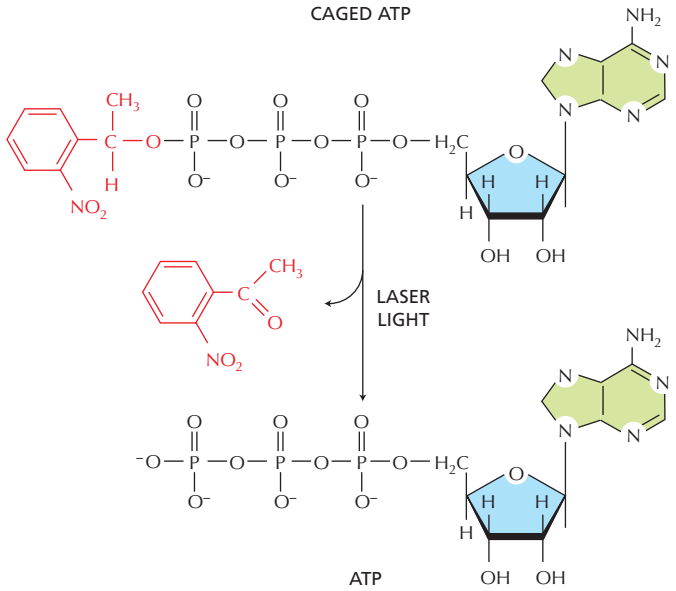


Figure 16–18 Caged ATP (Problem 16–43).

A. Using the micrograph in Figure 16–17A, identify the locations of the following:
   1. Dark band
   2. Light band
   3. Z disc
   4. Myosin II filaments
   5. Actin filaments (show plus and minus ends)
   6. α-Actinin
   7. Nebulin
   8. Titin

B. Locate the same features on the micrograph in Figure 16–17B. Be careful!

16–41 Troponin molecules are evenly spaced along an actin filament with one troponin bound at every seventh actin molecule. How do you suppose troponin molecules can be positioned this regularly?

16–42 What two major roles does ATP hydrolysis play in muscle contraction?

DATA HANDLING

16–43 As a laboratory exercise, you and your classmates are carrying out experiments on isolated muscle fibers using "caged" ATP (**Figure 16–18**). Since caged ATP does not bind to muscle components, it can be added to a muscle fiber without stimulating activity. Then, at some later time it can be split by a flash of laser light to release ATP instantly throughout the muscle fiber.

To begin the experiment, you treat an isolated, striated muscle fiber with glycerol to make it permeable to nucleotides. You then suspend it in a buffer containing ATP in an apparatus that allows you to measure any tension generated by fiber contraction. As illustrated in **Figure 16–19**, you measure the tension generated after several experimental manipulations: removal of ATP by dilution, addition of caged ATP, and activation of caged ATP by laser light. You are somewhat embarrassed because your results are very different from everyone else's. In checking over your experimental protocol, you realize that you forgot to add Ca^{2+} to your buffers. The teaching assistant in charge of your section tells you that your experiment is actually a good control for the class, but you will have to answer the following questions to get full credit.

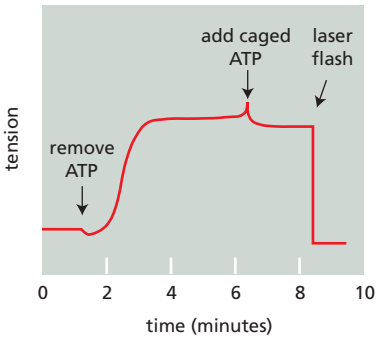


Figure 16–19 Tension in a striated muscle fiber as a result of various experimental manipulations (Problem 16–43).

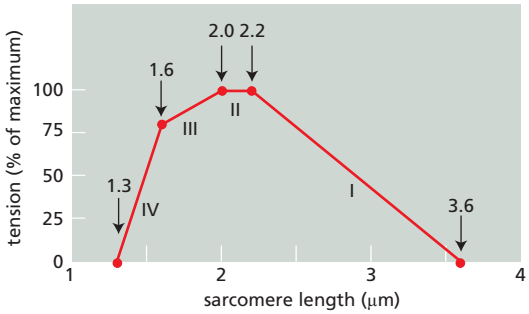


Figure 16–20 Tension as a function of sarcomere length during isometric contraction (Problem 16–44).

A. Why did the ATP in the suspension buffer not cause the muscle fiber to contract?
B. Why did the subsequent removal of ATP generate tension? Why did tension develop so gradually? (If our muscles normally took a full minute to contract, we would move very slowly.)
C. Why did laser illumination of a fiber containing caged ATP lead to relaxation?

16–44 Detailed measurements of sarcomere length and tension during isometric contraction in striated muscle provided crucial early support for the sliding-filament model of muscle contraction. Based on your understanding of the sliding-filament model and the structure of a sarcomere, propose a molecular explanation for the relationship of tension to sarcomere length in the portions of **Figure 16–20** marked I, II, III, and IV. (In this muscle, the length of the myosin filament is 1.6 μm, and the lengths of the actin thin filaments that project from the Z discs are 1.0 μm.)

MICROTUBULES

TERMS TO LEARN

axoneme
centriole
centrosome
cilium
dynamic instability
dynein
flagellum
γ-tubulin ring complex (γ-TuRC)
kinesin
kinesin-1
microtubule-associated protein (MAP)
microtubule-organizing center (MTOC)
tubulin

DEFINITIONS

Match each definition below with its term from the list above.

16–45 The property of sudden conversion from growth to shrinkage, and vice versa, in a protein filament such as a microtubule or an actin filament.

16–46 Centrally located organelle of animal cells that is the primary microtubule-organizing center and acts as the spindle pole during mitosis.

16–47 Protein assembly containing a special form of tubulin, along with other proteins, that is an efficient nucleator of microtubule growth.

16–48 Short cylindrical array of microtubules, a pair of which are embedded in the major microtubule-organizing center of an animal cell.

16–49 A member of the family of motor proteins that move along microtubules by walking toward the minus end.

16–50 A motor protein that moves along microtubules by walking toward the plus end.

16–51 Bundle of microtubules and associated proteins that forms the core of a cilium or flagellum in a eukaryotic cell and is responsible for their movements.

16–52 Long, hairlike protrusion from the surface of a eukaryotic cell whose undulations drive the cell through a liquid medium.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

16–53 The structural polarity of all microtubules is such that α-tubulin is exposed at one end and β-tubulin is exposed at the opposite end.

16–54 The role of GTP hydrolysis in tubulin polymerization is similar to the role of ATP hydrolysis in actin polymerization: both serve to weaken the bonds in the polymer and thereby promote depolymerization.

16–55 All microtubule-organizing centers contain centrioles that help nucleate microtubule polymerization.

16–56 In most animal cells, minus-end directed microtubule motors deliver their cargo to the periphery of the cell, whereas plus-end directed microtubule motors deliver their cargo to the interior of the cell.

THOUGHT PROBLEMS

16–57 Why do you suppose it is much easier to add tubulin to existing microtubules than to start a new microtubule from scratch?

16–58 In a 13-filament microtubule, the majority of lateral interactions are between like subunits, with α-tubulin binding to α-tubulin, and β-tubulin binding to β-tubulin. Between the first and thirteenth protofilaments, however, there is a seam at which α-tubulin interacts with β-tubulin (**Figure 16–21**). Are these heterotypic interactions (α with β) likely to be stronger than, weaker than, or the same strength as homotypic interactions (α with α, or β with β)? Explain your reasoning.

16–59 The microtubules in **Figure 16–22A** were obtained from a population that was growing rapidly, whereas the one in **Figure 16–22B** came from microtubules undergoing catastrophic shrinkage. Comment on any differences between the two images and suggest likely explanations for those you observe.

16–60 Dynamic instability causes microtubules either to grow or to shrink rapidly. Consider an individual microtubule that is in its shrinking phase.

A. What must happen at the end of the microtubule in order for it to stop shrinking and start growing?
B. How would an increase in the tubulin concentration affect this switch from shrinking to growing?
C. What would happen if GDP, but no GTP, were present in the solution?
D. What would happen if the solution contained an analog of GTP that could not be hydrolyzed?

16–61 The β-tubulin subunit of an αβ-tubulin dimer retains its bound GTP for a short time after it has been added to a microtubule, yielding a GTP cap whose size depends on the relative rates of polymerization and GTP hydrolysis. A simple notion about microtubule growth dynamics is that the ends with GTP caps grow, whereas ends without GTP caps shrink. To test this idea, you allow microtubules to form under conditions where you can watch individual microtubules. You then sever one microtubule in the middle using a laser beam. Would you expect the newly exposed plus and minus ends to grow or to shrink? Explain your answer.

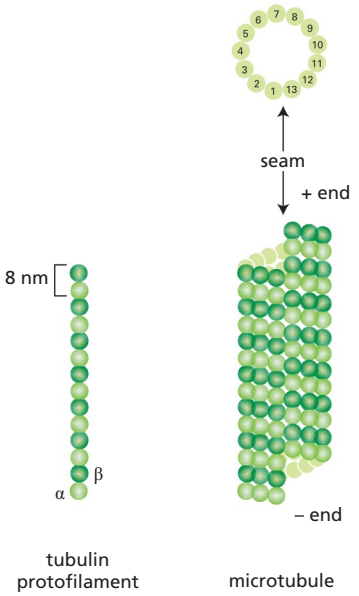


Figure 16–21 Structure of a 13-protofilament microtubule, showing the seam between the first and thirteenth protofilaments (Problem 16–58).

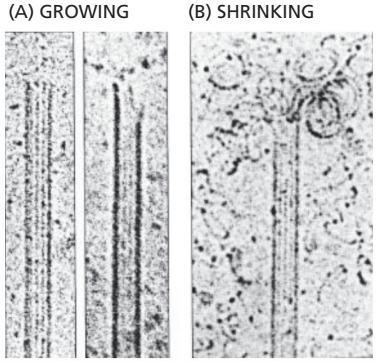


Figure 16–22 Electron microscopic analysis of microtubule dynamics (Problem 16–59). (A) Rapidly growing microtubules. (B) Catastrophically shrinking microtubule.

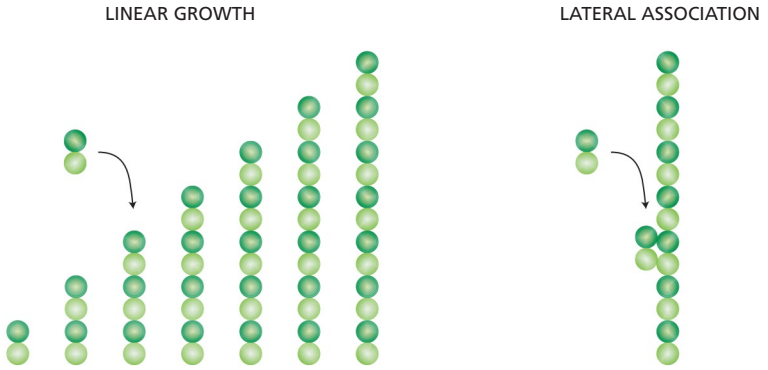


Figure 16–23 Model for microtubule nucleation by pure αβ-tubulin dimers (Problem 16–63).

16–62 The drugs Taxol®, extracted from the bark of yew trees, and colchicine, an alkaloid from autumn crocus, have opposite effects. Taxol binds tightly to microtubules and stabilizes them. When added to cells, it causes much of the free tubulin to assemble into microtubules. In contrast, colchicine prevents microtubule formation. Taxol and colchicine are equally toxic to dividing cells, and both are used as anticancer drugs. Based on your knowledge of microtubule dynamics, explain why these drugs are toxic to dividing cells despite their opposite modes of action.

16–63 A solution of pure αβ-tubulin dimers is thought to nucleate microtubules by forming a linear protofilament about seven dimers in length. At that point, the probabilities that the next αβ-dimer will bind laterally or to the end of the protofilament are about equal. The critical event for microtubule formation is thought to be the first lateral association (**Figure 16–23**). How does lateral association promote the subsequent rapid formation of a microtubule?

16–64 How does a centrosome "know" when it has found the center of the cell?

16–65 How are γ-TuRC and the Arp2/3 complex similar, and how are they different?

16–66 When cells enter mitosis, their existing array of cytoplasmic microtubules has to be rapidly broken down and replaced with the mitotic spindle, which pulls the chromosomes into the daughter cells. The enzyme katanin, named after Japanese samurai swords, is activated during the onset of mitosis and cleaves microtubules into short pieces. What do you suppose is the fate of the microtubule fragments created by katanin?

16–67 Kinesin-1 motors are highly processive, moving long distances on microtubule tracks without dissociating. By contrast, myosin II motors in skeletal muscle do not move processively; they take only one or a few steps before letting go. How are these different degrees of processivity adapted to the biological functions of kinesin-1 and myosin II?

16–68 An electron micrograph of a cross section through a flagellum is shown in **Figure 16–24**.

A. Assign the following components to the indicated positions on the figure.
   A microtubule
   B microtubule
   Outer dynein arm
   Inner dynein arm
   Inner sheath
   Nexin
   Radial spoke
   Singlet microtubule
B. Which of the above structures are composed of tubulin?

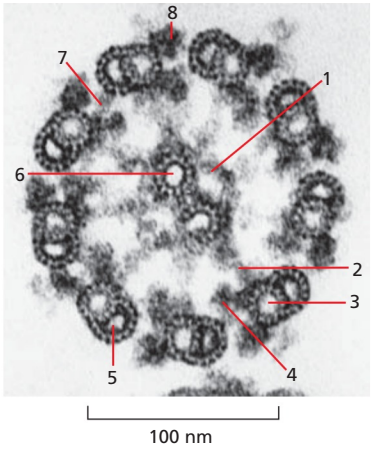


Figure 16–24 Electron micrograph of a cross section through a flagellum of *Chlamydomonas reinhardtii* (Problem 16–68).

16–69 The sliding-microtubule mechanism for ciliary bending is undoubtedly correct. The consequences of sliding are straightforward when a pair of outer doublets is considered in isolation. The dynein arms are arranged so that, when activated, they push their neighboring outer doublet outward toward the tip of the cilium. If the pair of outer doublets is linked together by nexin molecules, they will bend so that the one that has been pushed toward the tip will define the inside of the curve (see Figure 16–28A). It is confusing, however, to think about sliding in the circular array of outer doublets in the axoneme. If all the dynein arms in a circular array were equally active, there could be no significant relative motion. (The situation is equivalent to a circle of strongmen, each trying to lift his neighbor off the ground; if they all succeeded, the group would levitate.)

Devise a pattern of dynein activity (consistent with axoneme structure and the directional pushing of dynein) that could account for bending of the axoneme in one direction. How would this pattern change for bending in the opposite direction?

16–70 In addition to conducting impulses in both directions, nerve axons carry vesicles to and from the cell body along microtubule tracks. Do outbound vesicles move along microtubules that are oriented in one direction and incoming vesicles move along oppositely oriented microtubules? Or are microtubules all oriented in the same direction, with different motor proteins providing the directionality?

To distinguish between these possibilities, you prepare a cross section through a nerve axon and decorate the microtubules with tubulin, which binds to the tubulin subunits of the microtubule to form hooks. The decorated microtubules are illustrated in **Figure 16–25**. Do all the microtubules run in the same direction or not? How can you tell?

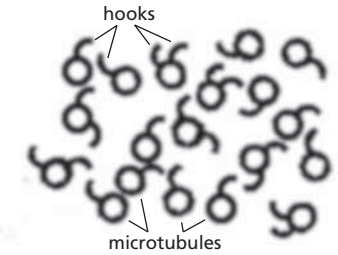


Figure 16–25 Tubulin-decorated microtubules in a cross section through a nerve axon (Problem 16–70). The hooks represent the tubulin decoration.

CALCULATIONS

16–71 At 1.4 mg/mL pure tubulin, microtubules grow at a rate of about 2 μm/min. At this growth rate, how many αβ-tubulin dimers (8 nm in length) are added to the ends of a microtubule each second?

16–72 The function of microtubules depends on their specific spatial organization within the cell. How are specific arrangements created, and what determines the formation and disappearance of individual microtubules?

To address these questions, investigators have studied the *in vitro* assembly of αβ-tubulin dimers into microtubules. Below 15 μM αβ-tubulin, no microtubules are formed; above 15 μM, microtubules form readily (**Figure 16–26A**). If centrosomes are added to the solution of tubulin, microtubules begin to form at less than 5 μM (**Figure 16–26B**). (Different assays were used in the two experiments—total weight of microtubules in Figure 16–26A and the average number of microtubules per centrosome in Figure 16–26B—but the lowering of the critical concentration for microtubule assembly in the presence of centrosomes is independent of the method of assay.)

A. Why do you think that the concentration at which microtubules begin to form (the critical concentration) is different in the two experiments?

B. Why do you think that the plot in Figure 16–26A increases linearly with increasing tubulin concentration above 15 μM, whereas the plot in Figure 16–26B reaches a plateau at about 25 μM?

C. The concentration of αβ-tubulin dimers (the subunits for assembly) in a typical cell is 1 mg/mL and the molecular weight of a tubulin dimer is 110,000. What is the molar concentration of tubulin dimers in cells? How does the cellular concentration compare with the critical concentrations in the two experiments in Figure 16–26? What are the implications for the assembly of microtubules in cells?

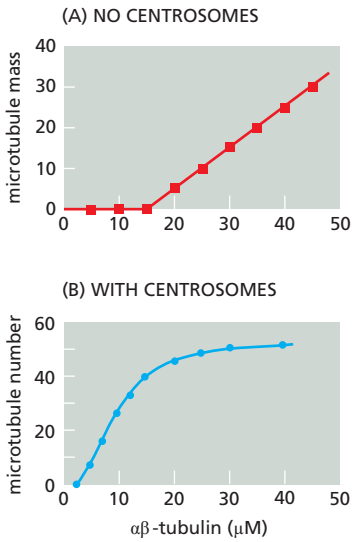


Figure 16–26 Analysis of microtubule assembly (Problem 16–72). (A) Mass of microtubules assembled in the absence of centrosomes as a function of tubulin concentration. Tubulin assembly into microtubules was measured by the increase in solution turbidity. (B) Average number of microtubules per centrosome as a function of tubulin concentration. Concentrations refer to αβ-tubulin dimers, which are the subunits of assembly.

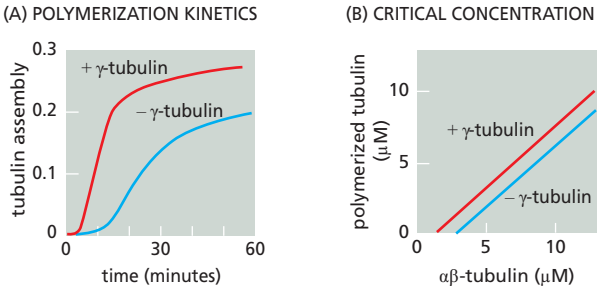


Figure 16–27 Effects of γ-tubulin on microtubule polymerization (Problem 16–73). (A) Kinetics of polymerization in the presence and absence of γ-tubulin. (B) The critical concentration for microtubule assembly in the presence of 0.6 nM γ-tubulin and in its absence.

16–73 The γ-tubulin ring complex (γ-TuRC), which nucleates microtubule assembly in cells, includes γ-tubulin and several accessory proteins. To get at its mechanism of nucleation, you have prepared monomeric γ-tubulin by *in vitro* translation and purification. You measure the effect of adding monomeric γ-tubulin to a solution of αβ-tubulin dimers, as shown in **Figure 16–27**.

A. In the presence of monomeric γ-tubulin, the lag time for assembly of microtubules decreases, and assembly occurs more rapidly (Figure 16–27A). How would you account for these two effects of γ-tubulin?

B. The critical concentration of αβ-tubulin needed for the assembly of microtubules is reduced from about 3.2 μM in the absence of γ-tubulin to about 1.7 μM in its presence (Figure 16–27B). How do you suppose γ-tubulin lowers the critical concentration? How does this account for the greater extent of polymerization in Figure 16–27A? (Think about the end—plus or minus—at which polymerization occurs in the presence of γ-tubulin.)

16–74 Using the equation for diffusion given in Problem 16–10, calculate the average time it would take for a vesicle to diffuse to the end of an axon 10 cm in length. The diffusion coefficient of a typical vesicle is 5×10^{-8} cm^2/sec.

16–75 A mitochondrion 1 μm long can travel the 1 meter length of the axon from the spinal cord to the big toe in a day. The Olympic men's freestyle swimming record for 200 meters is 1.75 minutes. In terms of body lengths per day, who is moving faster: the mitochondrion or the Olympic record holder? (Assume that the swimmer is 2 meters tall.)

16–76 During the flagellar beat cycle in *Chlamydomonas*, the bent segment of the flagellum extends roughly through half the circumference of a circle (**Figure 16–28**).

A. How much sliding of microtubule doublets against one another is required to account for the observed bending of the flagellum into a semicircle? Calculate how much farther the doublet on the inside of the semicircle protrudes beyond the doublet on the outside of the semicircle at the tip of the flagellum (Figure 16–28A). The width of a flagellum is 180 nm.

B. The elastic protein molecule (nexin) that links adjacent outer doublets must stretch to accommodate the bending of a flagellum into a semicircle. If the length of an unstretched nexin molecule at the base of a flagellum is 30 nm, what is the length of a stretched molecule at the tip of a flagellum (Figure 16–28B)? Adjacent doublets are 30 nm apart.

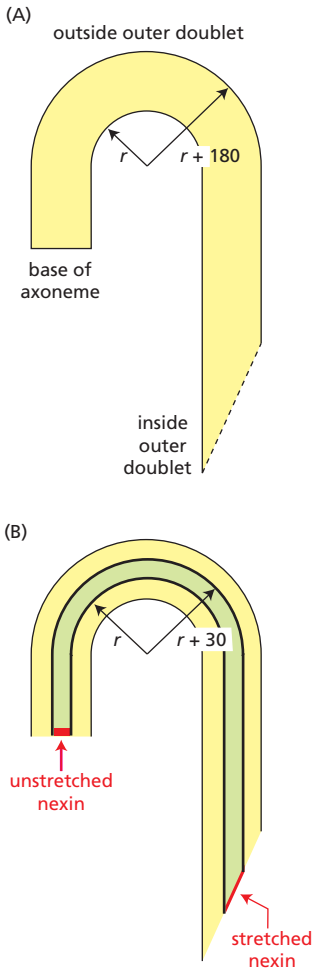


Figure 16–28 Flagella bent into half-circles (Problem 16–76). (A) Representation showing the "inside" and "outside" doublets, which are 180 nm apart. (B) Representation showing adjacent doublets, which are 30 nm apart, and the nexin molecules that link them.

DATA HANDLING

16–77 The orientation of the αβ-tubulin dimer in a microtubule was determined in several ways. GTP-coated fluorescent beads, for example, were found to bind exclusively at the plus ends of microtubules. By contrast, gold beads coated with antibodies specific for a peptide of α-tubulin

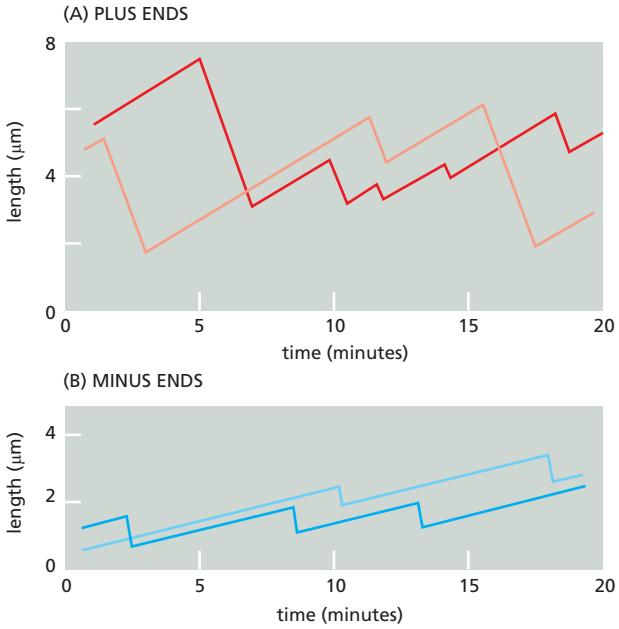


Figure 16–29 Analysis of growth kinetics of individual microtubules (Problem 16–78). (A) Changes in length at the plus ends. Results from two individual microtubules are indicated by different shades of *red.* (B) Changes in length at the minus ends. Results from two individual microtubules are indicated by different shades of *blue*.

bound exclusively at the minus end. How do these observations define the orientation of the αβ-tubulin dimer in the microtubule? Which tubulin subunit, α or β, is at which end? Explain your reasoning.

16–78 The complex kinetics of microtubule assembly make it hard to predict the behavior of individual microtubules. Some microtubules in a population can grow, even as the majority shrink to nothing. One simple hypothesis proposed to explain this behavior is that a growing end is protected from disassembly by a GTP cap and that a faster-growing end has a longer GTP cap. Real-time video observations of changes in length with time are shown for two individual microtubules in **Figure 16–29**. Measurements of their rates of growth and shrinkage show that the plus end of each microtubule grows three times faster than the minus end, and shrinks at half the rate.

A. Are changes in length at the two ends of a microtubule dependent or independent of one another? How can you tell?

B. What does the GTP-cap hypothesis predict about the rate of switching between growing and shrinking states at the fast-growing end relative to the slow-growing end? Does the outcome of this experiment support the GTP-cap hypothesis?

C. What do you suppose would happen if centrosomes were used to nucleate growth? What would happen if microtubule-associated proteins (MAPs) were included?

16–79 Comparisons of microtubule behavior between species point to differences that raise questions about the biological importance of dynamic instability. Notothenioid fish, for example, which live in the Southern Ocean at a constant temperature of –1.8°C, have remarkably stable microtubules compared with warm-blooded vertebrates such as the cow. This is an essential modification for notothenioid fish because normal microtubules disassemble completely into αβ-tubulin dimers at 0°C. Measurements on individual microtubules in solutions of pure tubulin show that notothenioid fish microtubules grow at a much slower rate, shrink at a much slower rate, and only rarely switch from growth to shrinkage (catastrophe) or from shrinkage to growth (rescue) (**Table 16–1**).

A. The amino acid sequences of the α- and β-tubulin subunits from notothenioid fish differ from those of the cow at positions and in ways that might reasonably be expected to stabilize the microtubule, in accord with

TABLE 16–1 Properties of individual microtubules in notothenioid fish and the domestic cow (Problem 16–79).

Microtubules	Growth rate (μm/min)	Shrinkage rate (μm/min)	Catastrophe frequency (min^{-1})	Rescue frequency (min^{-1})
Notothenioid fish	0.27	0.8	0.008	<0.0004
Domestic cow	2.18	61.2	0.52	3.1

Multiple individual microtubules were observed by video microscopy near the body temperature for each species: 5°C for fish and 37°C for cow. Average growth rates were calculated for growing microtubules, and average shrinkage rates were calculated for shrinking microtubules. Changes from growth to shrinkage (catastrophe) and from shrinkage to growth (rescue) were averaged over the observation period and expressed as frequency of events per minute.

the data in Table 16–1. Would you expect these changes to strengthen the interactions between the α- and β-tubulin subunits in the αβ-dimer, between adjacent dimers in the protofilament, or between tubulin subunits in adjacent protofilaments? Explain your reasoning.

B. Dynamic instability is thought to play a fundamental role in the rapid microtubule rearrangements that occur in cells. How do you suppose cells in these notothenioid fishes manage to alter their microtubule architecture quickly enough to accomplish essential cell functions? Or do you suppose that these cells exist with a stable microtubule cytoskeleton that only slowly rearranges itself?

16–80 A standard purification scheme for tubulin is to prepare a cell extract, chill it to 0°C, spin it at high speed, and save the supernatant. The supernatant is then warmed to 37°C and incubated in the presence of GTP. The mixture is then spun at high speed and the pellet is saved and redissolved. Then the cycle is repeated: chill the dissolved pellet, spin at high speed, save the supernatant, incubate with GTP at 37°C, spin at high speed, save the pellet. After a few cycles one obtains a pure preparation of tubulin. Explain how this procedure yields pure tubulin.

16–81 In addition to centrosomes, flagellar axonemes also can serve as nucleation sites for microtubule assembly. The following experiment was designed to determine whether these two structures nucleate microtubule growth by binding to the plus end or to the minus end of the nascent microtubule. Flagellar axonemes were included as a control since their plus and minus ends can be distinguished. Centrosomes and flagellar axonemes were incubated briefly in unlabeled tubulin to nucleate microtubule growth. A high concentration of biotin-labeled tubulin was then added and the incubation was continued for 10 minutes. At that point the preparations were fixed and the biotin-labeled segments were visualized by adding fluorescein-labeled antibodies specific for biotin. The lengths of the biotin-labeled segments were measured and plotted as shown in **Figure 16–30**.

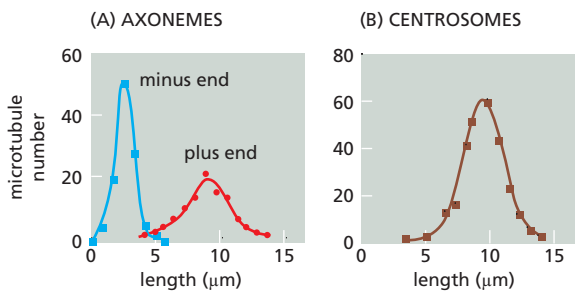


Figure 16–30 Length distributions of microtubules (Problem 16–81). (A) Nucleation by flagellar axonemes, whose plus and minus ends can be distinguished. (B) Nucleation by centrosomes.

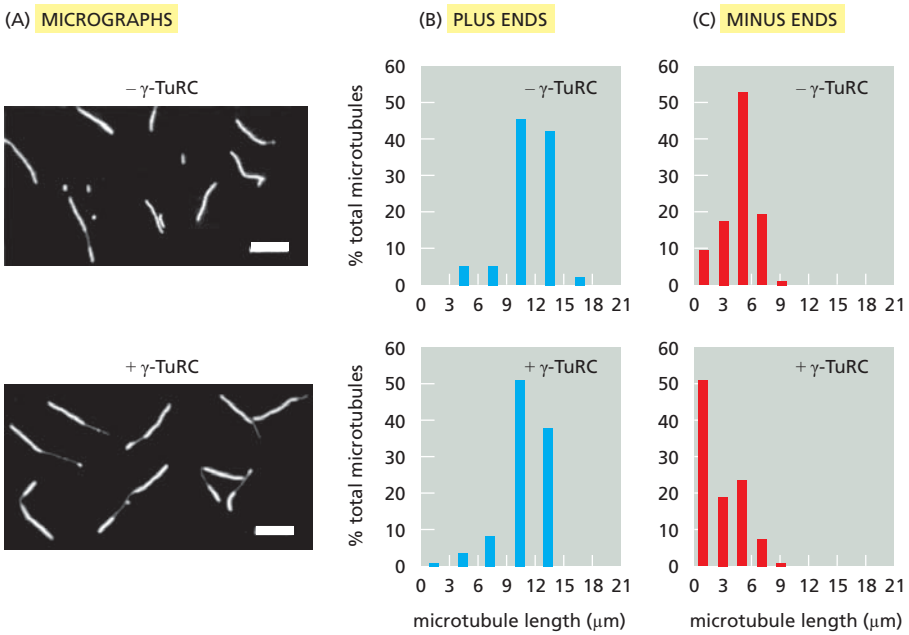


Figure 16–31 Effects of γ-TuRC on microtubule assembly (Problem 16–82). (A) Example of microtubules grown in the presence and absence of γ-TuRC. Scale bar is 10 μm. (B) Distribution of the lengths of bright segments at microtubule plus ends in the presence and absence of γ-TuRC. (C) Distributions of the lengths of bright segments at microtubule minus ends in the presence and absence of γ-TuRC. In (B) and (C), only microtubules with a defined dim segment and one or two bright terminal segments were counted.

A. Which end of a newly assembled microtubule is attached to the plus end of the flagellar axoneme?
B. Which end of a microtubule assembled on a flagellar axoneme grows faster?
C. Which end of an assembled microtubule is attached to a centrosome? Explain your reasoning.

16–82 In the paper that defined the γ-tubulin ring complex (γ-TuRC), the authors purified the complex from *Xenopus* oocytes and showed that it dramatically stimulated the nucleation of microtubules. To determine whether nucleation occurred at plus ends or at minus ends, they polymerized microtubules in two steps. In the first step, microtubules nucleated with or without γ-TuRC were allowed to form in the presence of a small amount of αβ-tubulin containing a low proportion of rhodamine-labeled αβ-tubulin, which makes the microtubules fluoresce dimly. In the second step, these microtubules were allowed to extend at both ends in the presence of a higher proportion of rhodamine-tagged tubulin to label the ends brightly. The longer bright segment identifies the plus end, and the shorter segment the minus end (**Figure 16–31A**). Measurements of the lengths of a large number of bright segments in individual microtubules yielded the data in **Figure 16–31B and C**. At which end of the microtubule is γ-TuRC when it nucleates? Explain your reasoning.

16–83 A useful technique for studying a microtubule motor is to attach the motor proteins by their tails to a glass coverslip (the tails stick avidly to a clean glass surface) and then to allow microtubules to settle onto them. In the light microscope, the microtubules can be seen to move over the surface of the coverslip as the heads of the motors propel them (**Figure 16–32**).

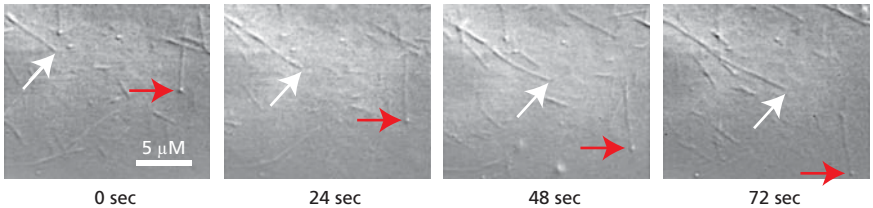


Figure 16–32 Movement of microtubules on a bed of microtubule motor molecules (Problem 16–83). *Red arrows* mark the movement of a microtubule with a gold bead attached via antibodies to the minus end of a microtubule; *white arrows* mark the movement of a microtubule without an attached bead. Pictures were taken using video-enhanced differential-interference-contrast (VE-DIC) microscopy.

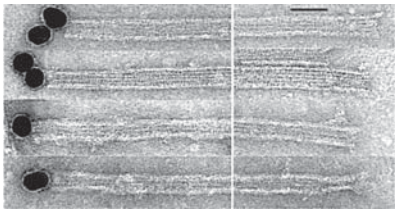


Figure 16–33 Microtubules with α-tubulin antibody-coated gold beads attached to one end (Problem 16–84). At the *vertical line* a section of each microtubule has been removed so that the two ends can be displayed side by side.

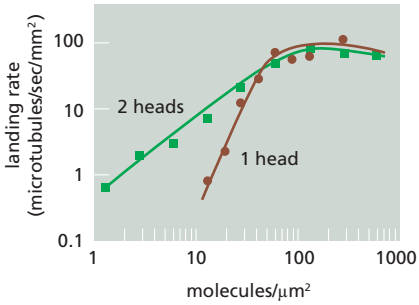


Figure 16–34 Landing rates—binding and moving—of microtubules as a function of motor protein density (Problem 16–85). Results with wild-type kinesin are shown as *green squares*, while those with recombinant kinesin are shown as *brown circles*.

A. Since the motor proteins attach in random orientations to the coverslip, how can they generate coordinated movement of individual microtubules, rather than engaging in a tug-of-war?
B. In which direction will microtubules crawl on a bed of dynein motor molecules (that is, will they move plus-end first or minus-end first)?
C. In the experiment shown in Figure 16–32, some of the microtubules were marked by gold beads that were bound by minus-end-specific antibodies. Is the motor protein on the coverslip a plus-end or minus-end directed motor? How can you tell?

16–84 In Problem 16–77, the orientation of the αβ-tubulin dimer in the microtubule was determined by showing that α-tubulin antibody-coated gold beads bound to the minus end. The electron micrographs, however, just showed microtubules with beads at one end (**Figure 16–33**). How do you suppose the investigators knew which end was which? Design an experiment to determine the orientation of microtubules labeled at one end with a gold bead.

16–85 Kinesin carries vesicles for long distances along microtubule tracks in the cell. Are the two motor domains of a kinesin molecule essential to accomplish this task, or could a one-headed motor protein function just as well? Using recombinant DNA techniques, a version of kinesin was prepared that was identical to normal kinesin except that one motor domain was absent. Wild-type kinesin with two motor domains and recombinant kinesin with one were attached to coverslips at a variety of densities and the rate at which microtubules were bound and moved (collectively, termed the landing rate) was measured (**Figure 16–34**).

A. Why do you suppose that the curves at low motor densities are so different?
B. What do these experiments say about the design of the kinesin motor: are two heads required for vesicle transport, or is only one needed? Explain your reasoning.

16–86 The movements of single motor-protein molecules can be analyzed directly. Using polarized laser light, it is possible to create interference patterns that exert a centrally directed force, ranging from zero at the center to a few piconewtons at the periphery (about 200 nm from the center). Individual molecules that enter the interference pattern are rapidly pushed to the center, allowing them to be captured and moved at the experimenter's discretion.

Using such "optical tweezers," single kinesin molecules can be positioned on a microtubule that is fixed to a coverslip. Although a single kinesin molecule cannot be seen optically, it can be tagged with a silica bead and tracked indirectly by following the bead (**Figure 16–35A**). In the absence of ATP, the kinesin molecule remains at the center of the interference pattern, but with ATP it moves toward the plus end of the microtubule. As kinesin moves along the microtubule, it encounters the force of the interference pattern, which simulates the load kinesin carries during its actual function in the cell. Moreover, the pressure against the silica bead counters the effects of Brownian (thermal) motion, so that the position of the bead more accurately reflects the position of the kinesin molecule on the microtubule.

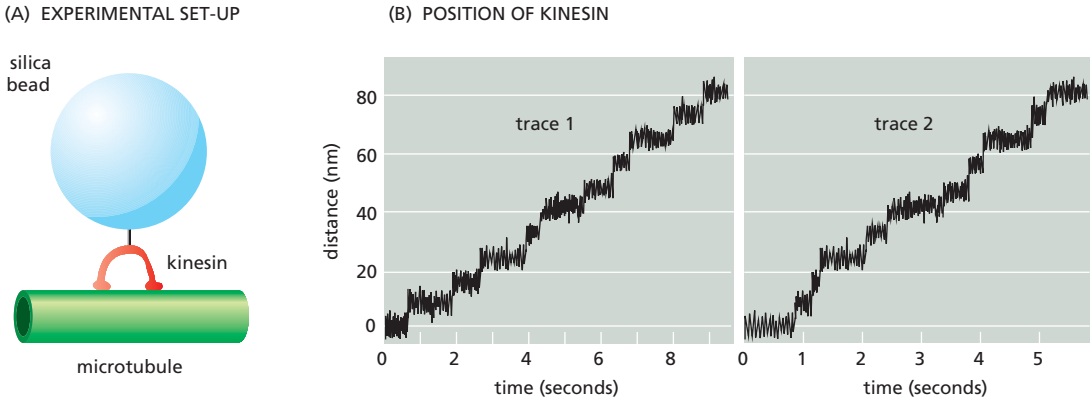


Figure 16–35 Movement of kinesin along a microtubule (Problem 16–86). (A) Experimental set-up, with kinesin linked to a silica bead, moving along a microtubule. (B) Position of kinesin (as visualized by the position of the silica bead) relative to the center of the interference pattern, as a function of time of movement along the microtubule. The jagged nature of the trace results from Brownian motion of the bead. The movements of two different kinesin molecules are shown.

Traces of the movements of two kinesin molecules along a microtubule are shown in **Figure 16–35B**.

A. As shown in Figure 16–35B, all movement of kinesin is in one direction (toward the plus end of the microtubule). What supplies the free energy needed to ensure a unidirectional movement along the microtubule?
B. What is the average rate of movement of each kinesin along the microtubule?
C. What is the length of each step that a kinesin takes as it moves along a microtubule?
D. From other studies it is known that kinesin has two globular domains that can each bind to β-tubulin, and that kinesin moves along a single protofilament in a microtubule. In each protofilament, the β-tubulin subunit repeats at 8-nm intervals. Given the step length and the interval between β-tubulin subunits, how do you suppose a kinesin molecule moves along a microtubule?
E. Is there anything in the data in Figure 16–35B that tells you how many ATP molecules are hydrolyzed per step?

MEDICAL LINKS

16–87 Mice that are homozygous for a knockout of the gene for the kinesin motor protein KIF1B die at birth. Heterozygous knockouts survive, but suffer from a progressive muscle weakness similar to human neuropathies. Humans with Charcot–Marie–Tooth disease type 2A have a mutation in one copy of the gene for KIF1B that prevents the protein from binding to ATP. The heterozygous mice and the human patients have very similar progressive neuropathies. How do you suppose that the loss of one copy of a gene for a kinesin motor can have such profound effects on nerve function?

INTERMEDIATE FILAMENTS AND SEPTINS

TERMS TO LEARN

keratin
neurofilament
septum

DEFINITIONS

Match each definition below with its term from the list above.

16–88 Member of a family of intermediate filaments that are found in high concentrations along the axons of vertebrate neurons.

16–89 The most diverse intermediate filament family, with about 20 types found in human epithelial cells and 10 more specific to hair and nails.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

16–90 Like actin filaments and microtubules, cytoplasmic intermediate filaments are found in all eukaryotes.

16–91 The cytoplasmic cytoskeleton and the nuclear lamina are connected by proteins that extend from one side to the other of the nuclear pore.

THOUGHT PROBLEMS

16–92 Why is it that intermediate filaments have identical ends and lack polarity, whereas actin filaments and microtubules have two distinct ends with a defined polarity?

16–93 Which of the following types of cell would you expect to contain a high density of cytoplasmic intermediate filaments? Explain your answers.

A. *Amoeba proteus* (a free-living amoeba).
B. Human skin epithelial cell.
C. Smooth muscle cell in the digestive tract of a vertebrate.
D. Nerve cell in the spinal cord of a mouse.
E. Human sperm cell.
F. Plant cell.

16–94 Disulfide bonds do not form in the cytosol of eukaryotic cells (see Problem 3–37). Yet keratin intermediate filaments in the skin are cross-linked by disulfide bonds. How can that be?

16–95 Although knockouts of genes for some intermediate filaments have detectable phenotypes in mice, gene knockouts for vimentin or another vimentin family member, glial fibrillary acid protein (GFAP), appear normal. Mice with knockouts for both vimentin and GFAP, however, exhibit impaired function of their astrocytes, which are accessory cells in the central nervous system. Why do you suppose that individual knockouts for vimentin and GFAP are normal, while the combined knockout has a demonstrable deficiency?

16–96 There are no known motor proteins that move on intermediate filaments. Suggest an explanation for this observation.

16–97 Compare the structure of intermediate filaments with that of the myosin II filaments in skeletal muscle cells. What are the major similarities? What are the major differences? How do the differences in structure relate to their function?

DATA HANDLING

16–98 The intermediate filament networks in cells must be dealt with in some way when a cell divides. **Figure 16–36** shows the vimentin networks [tagged with green fluorescent protein (vimentin–GFP)] in kidney cells from baby hamster (BHK-21) and rat kangaroo (PtK2) that are undergoing division. By examining these photographs, decide how each of these cell types handles its vimentin network.

16–99 Although the mechanism of disassembly of most intermediate filaments is unclear, it is well defined for their ancestors, the nuclear lamins. The nuclear envelope is strengthened by a fibrous meshwork of lamins (the nuclear lamina), which supports the membrane on the nuclear side. When cells enter mitosis, the nuclear envelope breaks down and the nuclear lamina disassembles. Assembly and disassembly of the nuclear

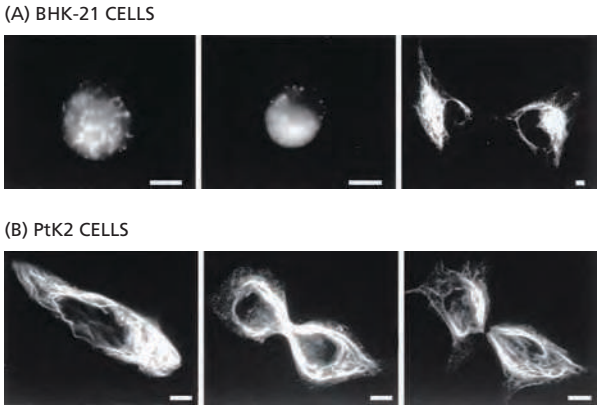


Figure 16–36 Vimentin networks during cell division (Problem 16–98). (A) BHK-21 cells. The three images from *left* to *right* correspond to prometaphase and anaphase of mitosis and to the daughter cells. Note that the first two images are magnified relative to the third. (B) PtK2 cells. The images from *left* to *right* correspond to prometaphase, telophase, and late cytokinesis. Note that in late cytokinesis, the cells are still connected by a bridge of cytoplasm. The scale bar in each picture is 5 μm. *Bright areas* represent vimentin-GFP fluorescence.

lamina may be controlled by reversible phosphorylation of lamins A, B, and C, since the lamins from cells that are in mitosis carry significantly more phosphate than do the lamins from cells that are in interphase.

To investigate the role of phosphorylation, you label cells with ^{35}S-methionine and purify lamins A, B, and C from mitotic cells and from interphase cells. You then analyze the purified lamins from each source, along with a mixture of them, by two-dimensional gel electrophoresis (**Figure 16–37A**). You also treat the samples with alkaline phosphatase, which removes phosphates from proteins, and analyze them in the same way (**Figure 16–37B**).

A. Why does treatment with alkaline phosphatase reduce the number of lamin spots to three, regardless of the number seen in the absence of phosphatase treatment?
B. How many phosphate groups are attached to lamins A, B, and C during interphase? How many are attached during mitosis? How can you tell?
C. Why was ^{35}S-methionine rather than ^{32}P-phosphate used to label lamins in experiments designed to measure phosphorylation differences? How would the autoradiograms have differed if ^{32}P-phosphate had been used instead?
D. Do you think these results prove that lamin disassembly during mitosis is caused by their reversible phosphorylation? Why or why not?

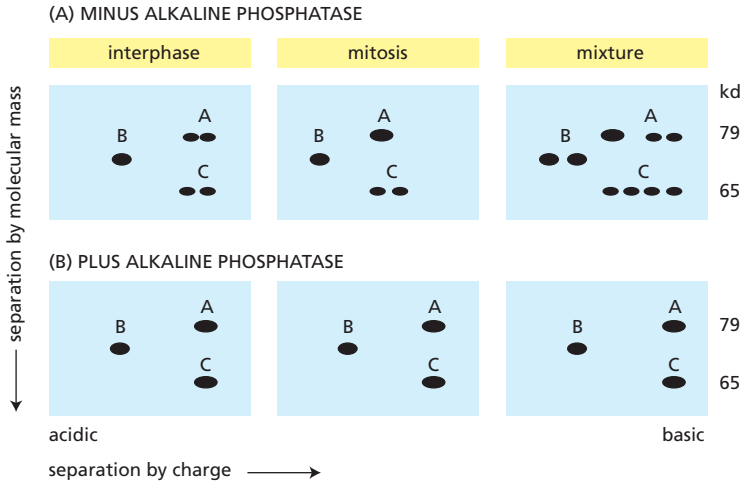


Figure 16–37 Two-dimensional separation of nuclear lamins from cells in interphase and mitosis (Problem 16–99). (A) No treatment with alkaline phosphatase. (B) Treatment with alkaline phosphatase. *Letters* identify the positions of lamins A, B, and C. The purified lamins from interphase and mitotic cells were added together to create the mixture. Acidic proteins are more negatively charged; basic proteins are more positively charged. Each *rectangle* represents a distinct two-dimensional separation of nuclear lamins.

CELL POLARIZATION AND MIGRATION

TERMS TO LEARN

blebbing
chemotaxis
filopodium
invadopodium
lamellipodium
Rho family protein
WASp protein

DEFINITIONS

Match each definition below with its term from the list above.

16–100 A group of closely related monomeric GTPases that includes Cdc42, Rac, and Rho.

16–101 Flattened, two-dimensional protrusion of membrane, supported by a meshwork of actin filaments, that is extended from the leading edge of crawling epithelial cells, fibroblasts, and some neurons.

16–102 Essentially a one-dimensional structure that protrudes from a cell and contains a core of long, bundled actin filaments.

16–103 A distinct form of membrane protrusion that is often observed when cells are cultured on a pliable extracellular matrix substrate.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

16–104 When fragments of the lamellipodium of cultured keratocytes are sliced off, they continue to crawl normally, looking like tiny keratocytes.

16–105 Neutrophils move toward a source of bacterial infection by chemotaxis, using receptors on their surface to respond to a gradient of *N*-formylated peptides derived from bacterial proteins.

THOUGHT PROBLEMS

16–106 Distinguish among the three processes—protrusion, attachment, and traction—that make up the crawling movements of cells.

16–107 Actin filaments are said to "push" on the cell membrane to cause it to form a protrusion. But there are problems with a pushing mechanism at both ends of the filaments. When a plus end reaches the membrane and abuts it, how are new subunits added to extend the filament (allowing it to push)? And how is the minus end of the filament anchored so that the filament isn't simply pushed back into the cell's interior? What do you suppose might be the answers to these questions?

16–108 The characteristic actin staining in a quiescent cell is shown in **Figure 16–38A**. When such cells are injected with a constitutively activated form of Rac, Rho, or Cdc42 monomeric GTPases, they dramatically alter their actin cytoskeletons. Which GTPase is associated with formation of stress fibers (**Figure 16–38B**), lamellipodia (**Figure 16–38C**), and filopodia (**Figure 16–38D**)?

16–109 How is the unidirectional motion of a lamellipodium maintained?

DATA HANDLING

16–110 Kinesin motors transport oligomers of neurofilament proteins down axonal microtubules to sites where they are used in the construction or repair of neurofilaments. Classic studies that followed pulse-labeled neurofilament proteins during axonal transport agree that the peak of radioactivity broadens markedly during transport. More recent studies demonstrated that unphosphorylated neurofilament proteins bind

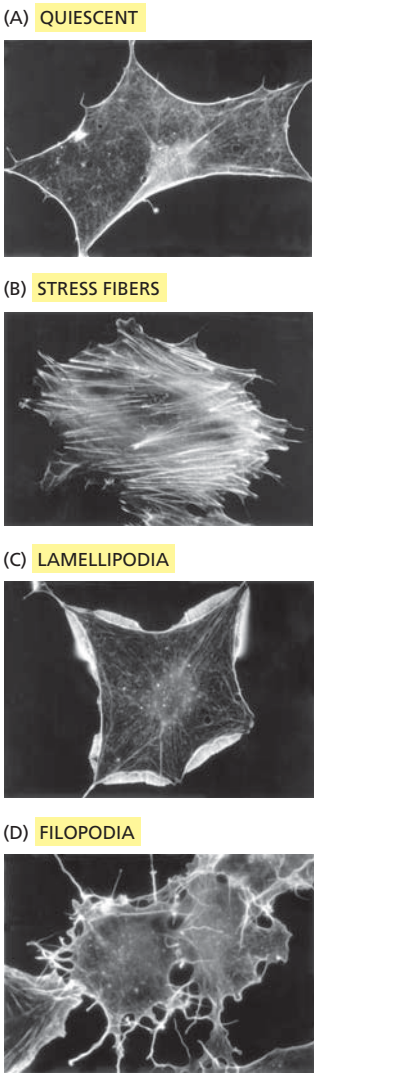


Figure 16–38 Actin cytoskeleton in different cells (Problem 16–108). (A) Quiescent cell. (B) Cell with prominent stress fibers. (C) Cell with multiple lamellipodia. (D) Cell with many long filopodia. Cells in B, C, and D were injected with an activated form of a monomeric GTPase.

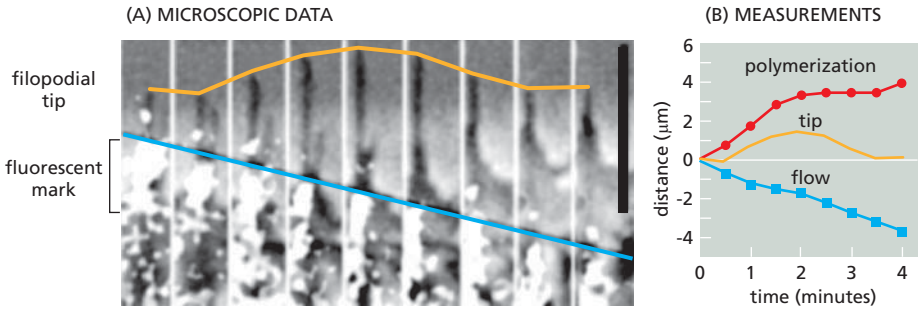


Figure 16–39 Regulation of filopodial extension and retraction (Problem 16–111). (A) Time-lapse observations of a single filopodium. The *yellow line* identifies the position of the tip of the filopodium; the *blue line* marks the position of the fluorescent segment. The black scale bar at the right is 5 μm. (B) Summary of the data. For the data summary, the positions of the tip and the fluorescent segment were arbitrarily set at zero at zero minutes, as was the difference between the tip and the fluorescent mark. The position of the filopodium is labeled "tip"; the position of the fluorescent mark is labeled "flow" for retrograde flow; and the difference is labeled "polymerization" for actin polymerization.

strongly to kinesin motors and weakly to existing neurofilaments. By contrast, the phosphorylated forms bind weakly to kinesin motors and strongly to neurofilaments.

A. How might the phosphorylation-dependence of oligomer binding to kinesin motors and neurofilaments account for the broadening of the transport wave?

B. If you could track the movement of single oligomers down an axon, how would you expect them to move? Consider an oligomer at the leading edge of the transport wave and one at the trailing edge.

16–111 Nerve growth cones navigate along stereotyped pathways during development by continually extending and retracting slender filopodia to sense directional cues in the environment. The bundled actin cytoskeleton in such a filopodium grows at the tip by actin polymerization, and is pulled back into the cell over time, a phenomenon known as retrograde flow. In principle, extension and retraction of filopodia could be controlled by regulating the rate of actin polymerization or the rate of retrograde flow. The experiments below were carried out to determine which rate—polymerization or retrograde flow—is regulated.

Actin monomers tagged with caged rhodamine were injected into cells and allowed to incorporate into actin filaments. The rhodamine in a narrow segment of the actin bundles near the tip of a single filopodium was uncaged by brief irradiation, yielding a fluorescent mark that allowed retrograde flow to be observed directly over time (**Figure 16–39A**). Extension and retraction of the tip of the filopodium was followed microscopically (Figure 16–39A). Actin polymerization was taken as the distance between the fluorescent mark and the tip of the filopodium (Figure 16–39A). A summary of the data for this single filopodium is shown in **Figure 16–39B**. Are extension and contraction of this filopodium regulated by the rate of actin polymerization or by the rate of retrograde flow? Explain your reasoning.

16–112 Activation of Cdc42, a monomeric GTPase, triggers actin polymerization and bundling to form either filopodia or shorter cell protrusions called microspikes. These effects of Cdc42 could be mediated by N-WASp, which is a multifunctional protein. As shown in **Figure 16–40A**, N-WASp

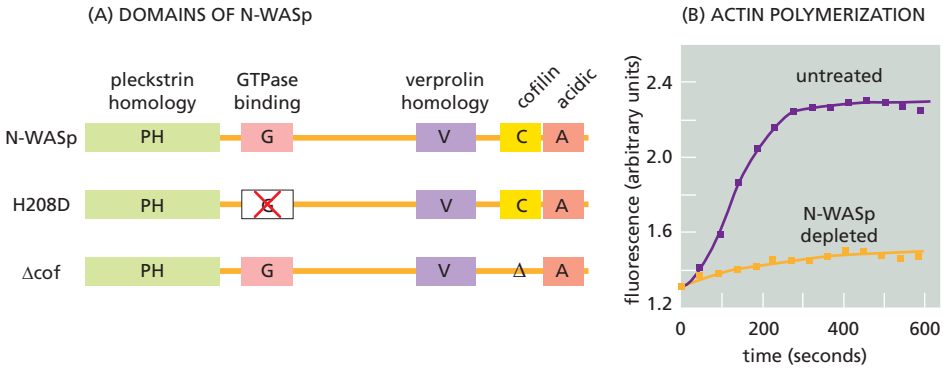


Figure 16–40 The role of N-WASp in Cdc42-triggered actin polymerization and bundling (Problem 16–112). (A) Domain structure of N-WASp and two mutants defective in individual domains. H208D carries a mutation in the G domain so that it cannot bind Cdc42. Δcof is deleted for the cofilin domain. (B) Actin polymerization in untreated and N-WASp-depleted egg extracts. In the polymerization assays, the extracts were supplemented with pyrene-actin, which fluoresces much more highly in the filament than it does on the subunit. Cdc42 with bound GTPγS was present in both reactions.

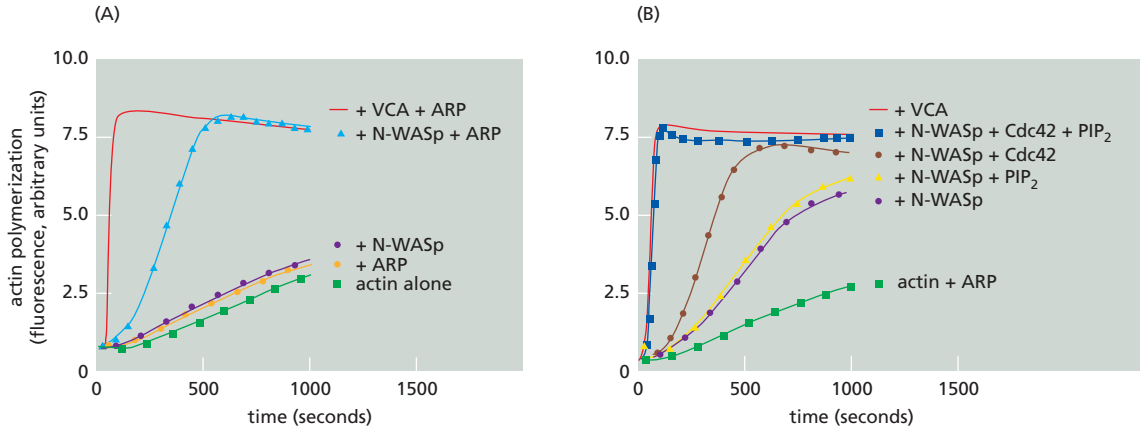


Figure 16–41 Polymerization of fluorescent actin in the presence of various purified components (Problem 16–113). (A) Mixtures of actin, N-WASp, the Arp2/3 complex (ARP), and the C-terminal segment of N-WASp (VCA). (B) Mixtures of actin, N-WASp, ARP, Cdc42-GTPγS, and PIP_2-containing vesicles. Vesicles without PIP_2 do not stimulate in any combination with the other components.

contains a pleckstrin homology (PH) domain, which binds to PIP_2 (phosphatidylinositol bisphosphate, $PI(4,5)P_2$); a Cdc42 GTPase-binding domain (G); a verprolin homology domain (V), which binds to actin; a cofilin homology domain (C), which can bind to actin filaments; and a C-terminal acidic domain (A), which binds the Arp2/3 complex.

In *Xenopus* egg extracts, a convenient source of cytoskeletal components, the addition of Cdc42 charged with GTPγS, a nonhydrolyzable analog of GTP, stimulates actin polymerization (**Figure 16–40B**). If the extract is depleted of N-WASp using N-WASp-specific antibodies, no actin polymerization is observed when Cdc42-GTPγS is added (see Figure 16–40B). Actin polymerization can be restored by the addition of purified N-WASp, but not by the addition of either of two mutant forms of N-WASp: one (H208D) that cannot bind to Cdc42, and a second (Δcof) that eliminates the function of the cofilin domain (see Figure 16–40A).

Do these experiments support a role for N-WASp in the rearrangement of actin filaments in response to Cdc42 activation? Explain your reasoning. Include a discussion of why the two mutant forms of N-WASp do not restore actin polymerization.

16–113 To determine the mechanism by which N-WASp mediates activation by Cdc42, actin polymerization was measured in the presence of purified components. In the presence of the Arp2/3 complex (ARP), N-WASp stimulates actin polymerization substantially over ARP or N-WASp alone, but not nearly so dramatically as the C-terminal segment of N-WASp that contains just the verprolin (V), cofilin (C), and acidic (A) domains (**Figure 16–41A**). To account for the difference between N-WASp and its C-terminal VCA segment, N-WASp and ARP were mixed with combinations of Cdc42-GTPγS and vesicles containing PIP_2 [$PI(4,5)P_2$], as shown in **Figure 16–41B**.

A. What is required for full-length N-WASp to stimulate actin polymerization as efficiently as its C-terminal VCA segment? Explain your reasoning.

B. Based on these results, propose a model for the activation of N-WASp and its stimulation of actin polymerization.

MCAT STYLE

Passage 1 (Questions 16–114 to 16–116)

Studying the motility of the pathogenic bacteria *Listeria* and *Shigella*, which cause food poisoning and dysentery, respectively, significantly advanced our understanding of actin polymerization. These bacteria escape immune surveillance by entering one cell's cytoplasm and then spreading from cell to cell without exposing themselves to the outside environment. They harness actin polymerization

to push themselves against the plasma membrane, generating a membrane protrusion that is engulfed by a neighboring cell, giving them direct access to that cell's cytoplasm. The comet tail of actin filaments in the wake of a moving *Listeria* bacterium initially suggested that motility was actin based. Biochemical studies identified host-cell surface proteins required for polymerization of actin, which then led to the discovery of the Arp2/3 complex and the full *in vitro* reconstitution of bacterial motility.

16–114 Early analysis in *Listeria* pointed to the ActA protein, an integral membrane protein expressed on the cell surface, as the sole *Listeria* protein required for motility. Which combination of the following observations provides evidence that ActA is *necessary* and *sufficient* for motility?

I. ActA binds to the Arp2/3 complex.
II. *E. coli* expressing ActA can move in host-cell cytoplasm.
III. *Listeria* lacking the ActA gene fail to move in host-cell cytoplasm.

A. I and II
B. I and III
C. II and III
D. I, II, and III

16–115 The mechanism of bacterial motility was initially mysterious and controversial: Was actin polymerization sufficient or did motility require actin-based motor proteins, as well? Which one of the following observations suggests that actin polymerization by itself generates the crucial force that drives motility?

A. Bacteria that lack ActA do not show motility.
B. Drugs that depolymerize actin block motility.
C. Inhibiting myosins does not inhibit motility.
D. The Arp2/3 complex is required for motility.

16–116 Efficient movement of both *Shigella* and *Listeria* in a reconstituted system requires the protein cofilin. Which one of the following actions describes the correct role for cofilin in bacterial motility?

A. Blocks hydrolysis of ATP by actin to preserve the ATP cap.
B. Bundles actin filaments to maintain a tight comet tail of actin.
C. Depolymerizes actin filaments to allow subunits to recycle.
D. Promotes actin polymerization to propel bacteria forward.

Passage 2 (Questions 16–117 to 16–119)

Early analyses of microtubule dynamics were carried out before development of methods for imaging microtubule growth and shrinkage in real time by video microscopy. These early studies used electron microscopy to measure the number and length of microtubules at fixed time points after experimental manipulations. One important experiment compared microtubule growth in the presence and absence of centrosomes. In the absence of centrosomes, microtubule growth was only observed at tubulin concentrations above 14 μM. In the presence of centrosomes, microtubule growth was observed at tubulin concentrations of 4 μM and above.

16–117 Which of the following hypotheses best accounts for how centrosomes lower the concentration of tubulin required to support microtubule growth?

A. Centrosomes decrease the frequency of changes from normal growth to rapid shrinkage—catastrophes—at microtubule plus ends.
B. Centrosomes increase the rate of microtubule growth at both ends, making it easier to grow large numbers of longer microtubules.
C. Centrosomes lower the critical concentration for growth at both ends, allowing microtubules to grow at lower tubulin concentration.
D. Centrosomes nucleate microtubules by capping the minus end, allowing growth at the lower critical concentration typical of plus ends.

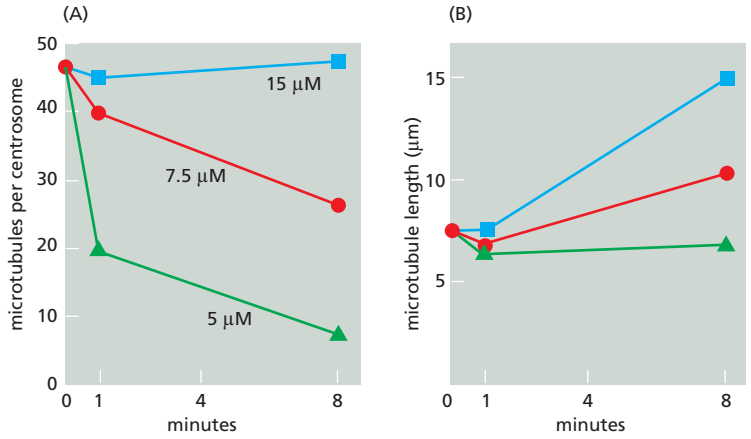


Figure 16–42 Dilution of centrosome-nucleated microtubule arrays, pre-formed at 25 μM tubulin, into solutions with tubulin at 15 μM (*blue*), 7.5 μM (*red*), or 5 μM (*green*) (Problem 16–118). (A) Number of microtubules per centrosome. (B) Average length of microtubules attached to a centrosome.

16–118 In another experiment, microtubules were grown from centrosomes at a tubulin concentration of 25 μM. The centrosomes were then diluted into lower concentrations of tubulin, and the number and length of microtubules were plotted as a function of time (**Figure 16–42**). This experiment revealed that when diluted to 7.5 μM tubulin, the number of microtubules attached to each centrosome decreased with time, and the average length of the remaining microtubules increased. At neither 15 μM nor 5 μM did *both* these changes occur. Which interpretation best accounts for the decrease in microtubule number and the increase in length upon dilution to 7.5 μM tubulin?

A. After dilution, some microtubules continue to hydrolyze GTP, which allows them to continue to grow from their plus ends.
B. After dilution, the tubulin concentration is below the critical concentration for plus-end growth, so some microtubules shrink.
C. At lower tubulin levels, microtubules with a GTP cap continue to grow, but those with a GDP cap rapidly depolymerize.
D. Dilution releases some microtubules from the centrosome, allowing them to rapidly depolymerize from their minus ends.

16–119 A key experiment analyzed microtubule growth in the absence of centrosomes at 20 μM tubulin. Since microtubules are large structures that scatter light, their polymerization can be assayed by monitoring the turbidity of the solution. During polymerization, the reaction mixture was pulled repeatedly through a narrow-gauge syringe to generate shear forces. This procedure caused a rapid and large decrease in turbidity that lasted for only a short time before turbidity again increased. Which one of the following statements provides the most reasonable explanation for this observation?

A. Shear forces increased the rate of GTP hydrolysis at the plus and minus ends.
B. Shear forces increased the rate of microtubule depolymerization at both ends.
C. Shear forces induced frequent catastrophes at both ends of the microtubule.
D. Shear forces induced microtubule breakage, exposing ends with bound GDP.

CHAPTER 17

The Cell Cycle

OVERVIEW OF THE CELL CYCLE

TERMS TO LEARN

cell cycle
G_1 phase
G_2 phase
interphase
restriction point
sister chromatid
Start

DEFINITIONS

Match the definition below with its term from the list above.

17–1 The long period of the cell cycle between one mitosis and the next.

17–2 The orderly sequence of events by which a cell duplicates its contents and divides into two.

17–3 The checkpoint in the cell cycle that governs the cell's commitment to enter S phase.

17–4 The phase of the eukaryotic cell cycle between the end of cytokinesis and the start of DNA synthesis.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–5 Since there are about 10^{13} cells in an adult human, and about 10^{10} cells die and are replaced each day, we become new people every three years.

17–6 Although the lengths of all phases of the cell cycle are variable to some extent, by far the greatest variation occurs in the duration of G_1.

THOUGHT PROBLEMS

17–7 If the most basic function of the cell cycle is to duplicate accurately the DNA in the chromosomes and then distribute the copies precisely to the daughter cells, why are there gaps between S phase and M phase?

17–8 Many cell-cycle genes from human cells function when expressed in yeast cells. Why do you suppose that is considered remarkable? After all, many human genes encoding enzymes for metabolic reactions also function in yeast, and no one thinks that is remarkable.

17–9 The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* provide facile genetic systems for studying a wide range of eukaryotic cell biological processes. If cell-cycle progression is essential for cell viability, as it is in these yeasts, how is it possible to isolate cells that are defective in cell-cycle genes?

IN THIS CHAPTER

17–10 For many experiments, it is desirable to have a population of cells that are traversing the cell cycle synchronously. One of the first, and still often used, methods for synchronizing cells is the so-called double thymidine block. When high concentrations of thymidine are added to the culture fluid, cells in S phase stop DNA synthesis, though other cells are not affected. The excess thymidine blocks the enzyme ribonucleotide reductase, which is responsible for converting ribonucleotides into deoxyribonucleotides. When this enzyme is inhibited, the supply of deoxyribonucleotides falls and DNA synthesis stops. When the excess thymidine is removed by changing the medium, the supply of deoxyribonucleotides rises and DNA synthesis resumes normally.

For a cell line with a 22-hour cell cycle divided so that M phase = 0.5 hour, G_1 phase = 10.5 hours, S phase = 7 hours, and G_2 phase = 4 hours, a typical protocol for synchronization by a double thymidine block would be as follows:

1. At 0 hours (t = 0 hours) add excess thymidine.
2. After 18 hours (t = 18 hours) remove excess thymidine.
3. After an additional 10 hours (t = 28 hours) add excess thymidine.
4. After an additional 16 hours (t = 44 hours) remove excess thymidine.

A. At what point in the cell cycle is the cell population when the second thymidine block is removed?

B. Explain how the times of addition and removal of excess thymidine synchronize the cell population.

CALCULATIONS

17–11 The fraction of cells in a population that are undergoing mitosis (the mitotic index) is a convenient way to estimate the length of the cell cycle. You have decided to measure the cell cycle in the liver of the adult mouse by measuring the mitotic index. Accordingly, you have prepared liver slices and stained them to make cells in mitosis easy to recognize. After 3 days of counting, you have found only 3 mitoses in 25,000 cells. Assuming that M phase lasts 30 minutes, calculate the average length of the cell cycle in the liver of an adult mouse.

17–12 The overall length of the cell cycle can be measured from the doubling time for a population of exponentially proliferating cells. The doubling time of a population of mouse L cells was determined by counting the number of cells in samples of culture fluid at various times (**Figure 17–1**). What is the overall length of the cell cycle in mouse L cells?

DATA HANDLING

17–13 You have isolated a temperature-sensitive mutant of budding yeast. It proliferates well at 25°C, but at 35°C all the cells develop a large bud and then halt their progression through the cell cycle. The characteristic morphology of the cells at the time they stop cycling is known as the landmark morphology.

It is very difficult to obtain synchronous cultures of this yeast, but you would like to know exactly where in the cell cycle the temperature-sensitive gene product must function—its execution point, in the terminology of the field—in order for the cell to complete the cycle. A clever friend, who has a good microscope with a heated stage and a video camera, suggests that you take movies of a field of cells as they experience the temperature increase, and follow the morphology of the cells as they stop cycling. Since the cells do not move much, it is relatively simple to study individual cells. To make sense of what you see, you arrange a circle of pictures of cells at the start of the experiment in order of the size of their daughter buds. You then find the corresponding pictures of those same

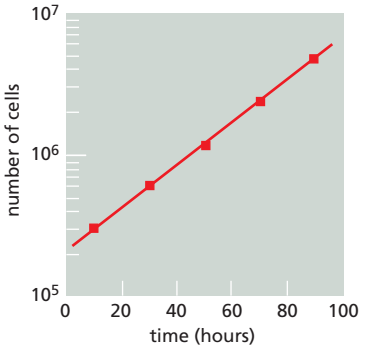


Figure 17–1 Increase in the number of mouse L cells with time (Problem 17–12).

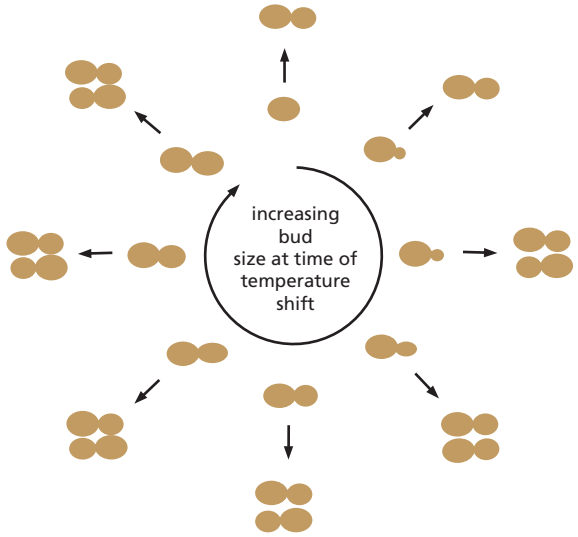


Figure 17–2 Time-lapse photography of a temperature-sensitive mutant of yeast (Problem 17–13). Cells on the *inner ring* are arranged in order of their bud size, which corresponds to their position in the cell cycle. After 6 hours at 37°C, they have given rise to the cells shown on the *outer ring*. No further growth or division occurs.

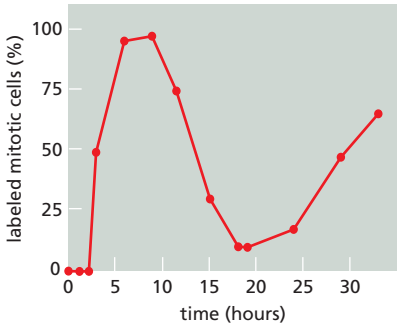


Figure 17–3 Percentage of mitotic cells that were labeled as a function of time after a brief incubation with radioactive thymidine (Problem 17–14).

cells 6 hours later, when growth and division have completely stopped. The results with your mutant are shown in **Figure 17–2**.

A. Indicate on the diagram in Figure 17–2 where the execution point for your mutant lies.
B. Does the execution point correspond to the time at which the cell cycle is arrested in your mutant? How can you tell?

17–14 Cells that grow and divide in medium containing radioactive thymidine incorporate the thymidine into their DNA during S phase. Consider a simple experiment in which cells were labeled by a brief (30 minute) exposure to radioactive thymidine. The medium was then replaced with one containing unlabeled thymidine, and the cells were allowed to grow and divide for some additional time. At different time points after replacement of the medium, cells were examined in a microscope. Cells in mitosis were identified by their condensed chromosomes. The fraction of mitotic cells that had radioactive DNA was determined by autoradiography and plotted as a function of time after the thymidine labeling (**Figure 17–3**).

A. Would all the cells in the population be expected to contain radioactive DNA after the labeling procedure?
B. Initially, there were no mitotic cells that contained radioactive DNA (Figure 17–3). Why is this?
C. Explain the rise and fall of the curve in Figure 17–3.
D. Given that mitosis lasts 30 minutes, estimate the lengths of the G_1, S, and G_2 phases from these data. (Hint: use the points where the curves correspond to 50% labeled mitoses to estimate the lengths of phases in the cell cycle.)

17–15 Hoechst 33342 is a membrane-permeant dye that fluoresces when it binds to DNA. When a population of cells is incubated briefly with Hoechst dye and then sorted in a flow cytometer, which measures the fluorescence of each cell, the cells display various levels of fluorescence as shown in **Figure 17–4**.

A. Which cells in Figure 17–4 are in the G_1, S, G_2, and M phases of the cell cycle? Explain the basis for your answer.

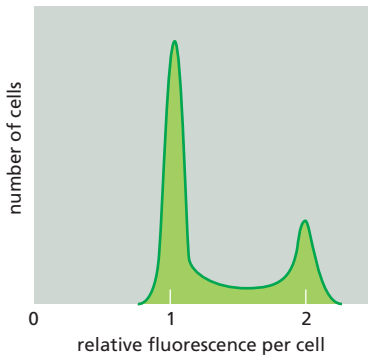


Figure 17–4 Analysis of Hoechst 33342 fluorescence in a population of cells sorted in a flow cytometer (Problem 17–15).

B. Sketch the sorting distributions you would expect for cells that were treated with inhibitors that block the cell cycle in the G_1, S, or M phase. Explain your reasoning.

THE CELL-CYCLE CONTROL SYSTEM

TERMS TO LEARN

anaphase-promoting complex or cyclosome (APC/C)
Cdc20
Cdc25
Cdh1
Cdk-activating kinase (CAK)
Cdk inhibitor protein (CKI)
cell-cycle control system
cyclin
cyclin–Cdk complex
cyclin-dependent kinase (Cdk)
G_1-Cdk
G_1-cyclin
G_1/S-Cdk
G_1/S-cyclin
G_2/M transition
M-Cdk
M-cyclin
metaphase-to-anaphase transition
S-Cdk
SCF
S-cyclin
Wee1

DEFINITIONS

Match the definition below with its term from the list above.

17–16 A member of the family of protein kinases that have to be complexed with a cyclin protein in order to act.

17–17 The ubiquitin ligase that promotes the destruction of a specific set of proteins, thereby promoting the separation of sister chromatids and the completion of M phase.

17–18 The cyclin–Cdk complex responsible for stimulating entry into mitosis at the G_2/M checkpoint.

17–19 One of a family of proteins that rise and fall in concentration in step with the eukaryotic cell cycle, thereby regulating the activity of the crucial protein kinases that control progression through the cell cycle.

17–20 The final major checkpoint in the cell cycle, where the control system stimulates sister-chromatid separation, leading to the completion of mitosis and cytokinesis.

17–21 A timing mechanism that triggers events of the cell cycle in a set sequence, using feedback from the processes it controls to ensure that one stage is complete before the next one begins.

17–22 General term for one of the several protein assemblies that form periodically during the cell cycle as the level of cyclin increases, and partially activate the cyclin-dependent kinase component.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–23 The regulation of cyclin–Cdk complexes depends entirely on phosphorylation and dephosphorylation.

17–24 In order for proliferating cells to maintain a relatively constant size, the length of the cell cycle must match the time it takes for the cell to double in size.

THOUGHT PROBLEMS

17–25 Vertebrate cells use several different Cdks to manage the various transitions in the cell cycle, yet budding yeast is able to get by with a single Cdk. How do budding yeast cells manage that neat trick?

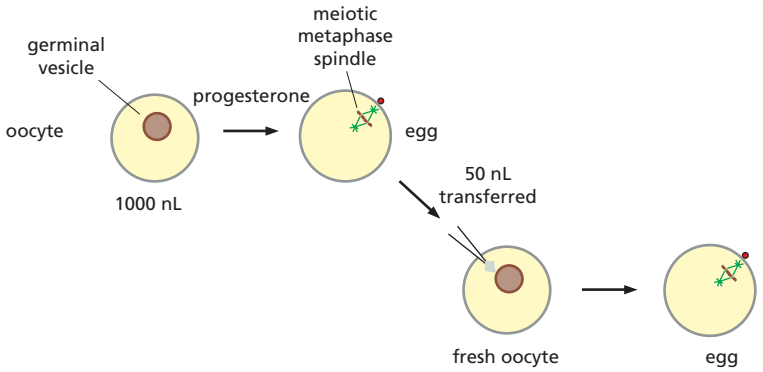


Figure 17–5 Progesterone- and MPF-induced maturation of oocytes (Problem 17–26).

DATA HANDLING

17–26 Frog oocytes mature into eggs when incubated with progesterone. Egg maturation is characterized by disappearance of the nucleus (termed germinal vesicle breakdown) and formation of a meiotic spindle. The requirement for progesterone can be bypassed by microinjecting 50 nL of egg cytoplasm directly into a fresh oocyte (1000 nL), which then matures normally (**Figure 17–5**). Progesterone-independent maturation is triggered by maturation-promoting factor (MPF) activity in the egg cytoplasm—later called mitosis-promoting factor and shown to be M-Cdk.

At early times after progesterone treatment, inhibition of protein synthesis by cycloheximide blocks egg maturation. However, a few hours before oocytes become eggs—a time that corresponds to the appearance of MPF activity—progesterone-induced maturation can no longer be blocked by cycloheximide.

Is synthesis of MPF itself the cycloheximide-sensitive event? To test this possibility, you transfer MPF serially from egg to oocyte to test whether its activity diminishes with dilution. You first microinject 50 nL of cytoplasm from an activated egg into an immature oocyte as shown in Figure 17–5; when the oocyte matures into an egg, you transfer 50 nL of its cytoplasm into another immature oocyte; and so on. Surprisingly, you find that you can continue this process for at least 10 transfers, even when the recipient oocytes are bathed in cycloheximide! Moreover, the apparent MPF activity in the last egg is equal to that in the first egg.

A. What dilution factor is achieved by 10 serial transfers of 50 nL into 1000 nL? Do you consider it likely that a molecule might have an undiminished biological effect over this concentration range?
B. How do you suppose MPF activity can be absent from immature oocytes yet appear in activated eggs, even when protein synthesis has been blocked by cycloheximide?
C. Propose a means by which MPF activity might be maintained through repeated serial transfers.

17–27 You have isolated two temperature-sensitive strains of yeast (which you've named *giant* and *tiny*) that show very different responses to elevated temperature. At high temperature, *giant* cells grow until they become enormous, but no longer divide. By contrast, *tiny* cells have a short cell cycle and divide when they are much smaller than usual. You are amazed to discover that these strains arose by different mutations in the same gene. Based on your understanding of cell cycle regulation by Cdk1, Wee1, and Cdc25, propose an explanation for how two different mutations in one of these genes might have given rise to the *giant* and *tiny* strains.

S PHASE

TERMS TO LEARN

Cdc6	geminin
Cdt1	origin recognition complex (ORC)
cohesin	prereplicative complex (pre-RC)

DEFINITIONS

Match the definition below with its term from the list above.

17–28 Complex of proteins that holds sister chromatids together along their length until they separate at mitosis.

17–29 Large protein complex that is bound throughout the cell cycle at origins of replication in eukaryotic chromosomes.

17–30 Protein that binds to and inhibits a key component of the prereplicative complex.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–31 Initiation of DNA synthesis is permitted only at origins of replication that contain a prereplicative complex.

17–32 While other proteins come and go during the cell cycle, the proteins of the origin recognition complex remain bound to the DNA throughout.

THOUGHT PROBLEMS

17–33 In budding yeast, a prereplicative complex, consisting of ORC, Cdc6, and Mcm proteins, is established at origins of replication during the G_1 phase. S-Cdk then triggers origin firing and helps to prevent re-replication. But not all yeast origins begin replication at the same time: some fire early in S phase, while others fire late. How is it possible for S-Cdk to trigger origin firing at a variety of different times, yet also prevent re-replication at all origins? Propose a scheme that could account for this behavior of S-Cdk.

17–34 The yeast cohesin subunit Scc1, which is essential for sister-chromatid cohesion, can be artificially regulated for expression at any point in the cell cycle. If expression is turned on at the beginning of S phase, all the cells divide satisfactorily and survive. By contrast, if Scc1 expression is turned on only after S phase is completed, the cells fail to divide and they die, even though Scc1 accumulates in the nucleus and interacts efficiently with chromosomes. Why do you suppose that cohesin must be present during S phase for cells to divide normally?

DATA HANDLING

17–35 Early clues about the regulation of S phase came from studies in which human cells at various cell-cycle stages were fused to form single cells with two nuclei. **Figure 17–6** shows the outcome of pairwise fusions between G_1, S, and G_2 cells. Given what we now know about the roles of cyclin–Cdk complexes in progression of the cell cycle, how would you interpret the outcomes of each of these experiments? Do these experiments suggest that there may be a block to re-replication in the cell cycle?

17–36 Using a clever genetic screen, you have identified a temperature-sensitive (ts) mutant in a yeast gene (*Scc1*) that appears to be required for

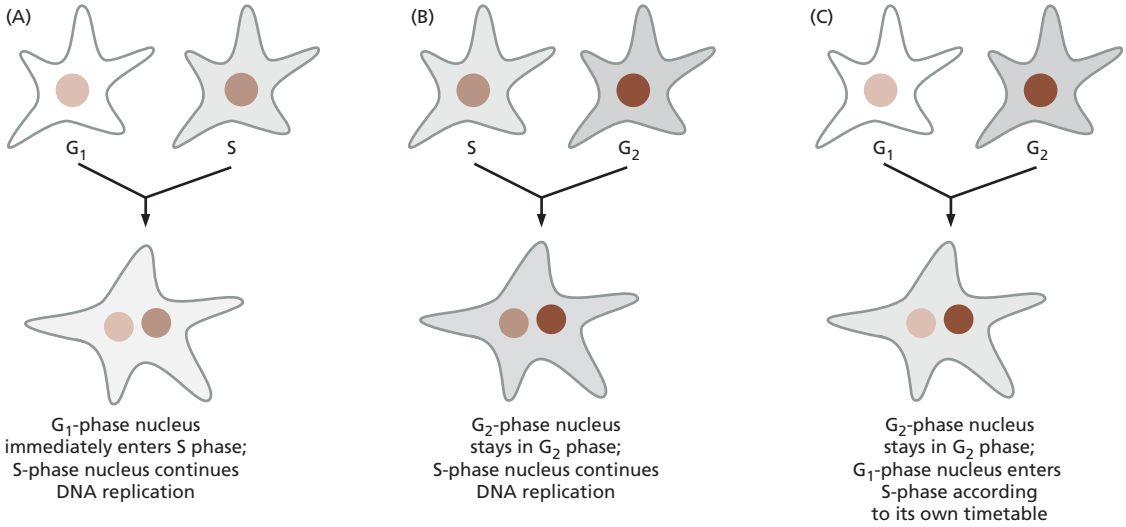


Figure 17–6 Results of cell-fusion experiments using mammalian cells at different stages of the cell cycle (Problem 17–35). (A) Fusion of S and G_1 cells. (B) Fusion of S and G_2 cells. (C) Fusion of G_1 and G_2 cells.

sister-chromatid cohesion. To assay directly for sister-chromatid cohesion, you insert a tandem array of 336 short DNA sequences, to which a bacterial protein can bind tightly, adjacent to the centromere of chromosome V. You then express a fusion of the bacterial protein with green fluorescent protein (GFP) in the same cells. When the GFP fusion protein binds to its recognition sequences, it creates a bright dot of green fluorescence on the chromosome. To test for the effects of mutant Scc1 on sister-chromatid cohesion, you isolate unbudded cells from wild-type and *Scc1*^{ts} cells that were grown at 25°C, and grow them at 37°C for various times. Representative examples of small-budded cells in S phase and large-budded cells that have passed the metaphase-to-anaphase transition are shown for both strains in **Figure 17–7**.

A. Do sister chromatids in wild-type cells adhere to each other normally during S phase, and separate normally during mitosis? How can you tell?
B. Do sister chromatids in *Scc1*^{ts} cells adhere normally in S phase, and separate normally during mitosis? How can you tell?
C. In the large-budded cells from the *Scc1*^{ts} strain, why do both sister chromatids remain in one cell?

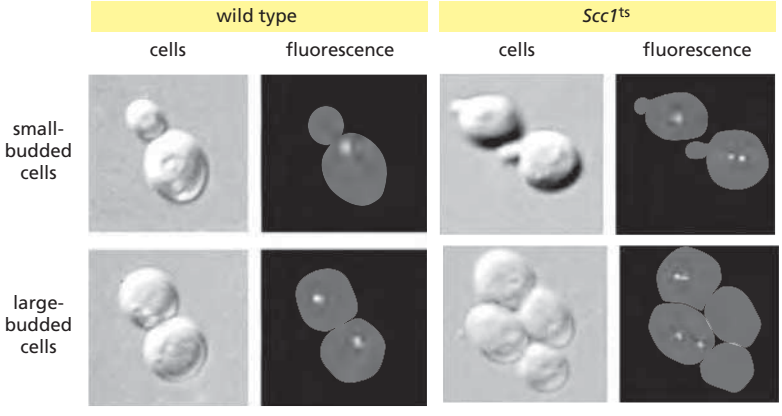


Figure 17–7 Small- and large-budded cells from wild-type and *Scc1*^{ts} cells grown at 37°C (Problem 17–36). For each strain, a matched set of pictures shows the appearance of the cells and the corresponding sites of fluorescence.

MITOSIS

TERMS TO LEARN

anaphase A
anaphase B
astral microtubule
bi-orientation
centrosome
condensin
interpolar microtubule
kinetochore
kinetochore microtubule
metaphase plate
microtubule flux
mitotic spindle
securin
separase
spindle assembly checkpoint
telophase

DEFINITIONS

Match the definition below with its term from the list above.

17–37 Movement of tubulin subunits toward the spindle poles as a result of addition of new subunits at the plus ends of microtubules and their disassembly at minus ends.

17–38 Stage of mitosis in which the spindle poles move apart.

17–39 Mechanism ensuring that cells do not enter anaphase until all chromosomes are correctly bi-oriented on the mitotic spindle.

17–40 Centrally located organelle of animal cells that after duplication organizes each spindle pole during mitosis.

17–41 Imaginary plane midway between the spindle poles in which chromosomes are positioned at metaphase.

17–42 Microtubules that overlap in the spindle midzone and interact via their plus ends, generating an antiparallel array.

17–43 Final stage of mitosis in which the two sets of separated chromosomes decondense and become enclosed by nuclear envelopes.

17–44 Complex of proteins that uses the energy of ATP hydrolysis to promote the compaction and resolution of sister chromatids.

17–45 Protease whose activation at the end of metaphase results in the cleavage of cohesin and the separation of sister chromatids.

17–46 Microtubule that radiates outward from the spindle pole and contacts the cell cortex, helping to position the spindle in the cell.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–47 After the nuclear envelope breaks down, microtubules gain access to the chromosomes and, every so often, a randomly probing microtubule connects with a kinetochore and captures the chromosome.

17–48 Chromosomes are positioned on the metaphase plate by equal and opposite forces that pull them toward the two poles of the spindle.

17–49 Once formed, kinetochore microtubules depolymerize at their plus ends (the ends attached to the kinetochores) throughout mitosis.

17–50 The six stages of M phase—prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis—occur in strict sequential order.

THOUGHT PROBLEMS

17–51 A living cell from the lung epithelium of a newt is shown at different stages in M phase in **Figure 17–8**. Order these light micrographs

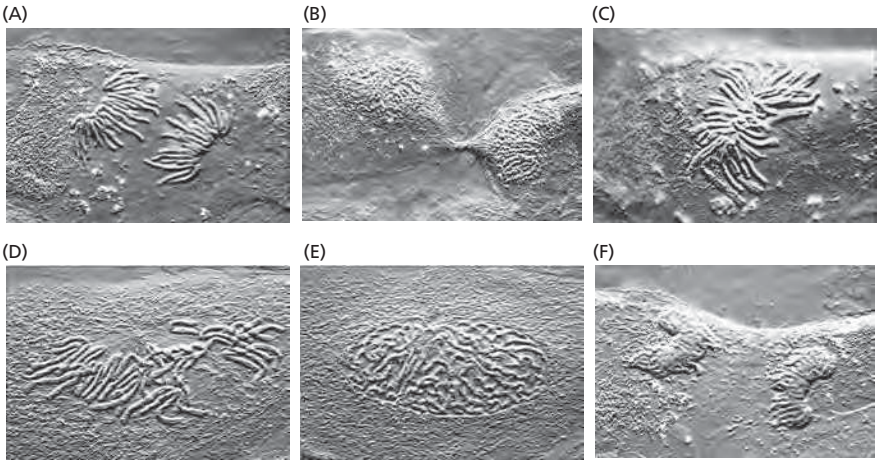


Figure 17–8 Light micrographs of a single cell at different stages of M phase (Problem 17–51). (Courtesy of Conly L. Rieder.)

into the correct sequence, and identify the stage in M phase that each represents.

17–52 It is remarkable that the concentration of M-cyclin in the cleaving clam egg rises very slowly and steadily throughout the cell cycle, whereas M-Cdk activity increases suddenly at mitosis (**Figure 17–9**). How is the activity of M-Cdk so sharply regulated in the presence of a gradual increase in M-cyclin?

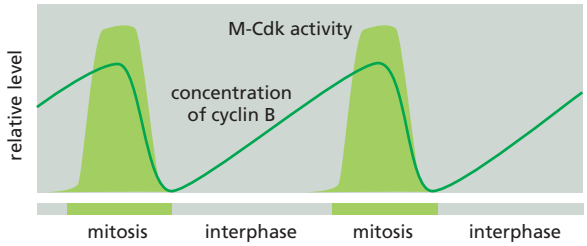


Figure 17–9 The rise and fall of M-Cdk activity and M-cyclin concentration during the cell cycle in a cleaving clam egg (Problem 17–52).

17–53 Describe the three main classes of spindle microtubule in animal cells and their functions during mitosis.

17–54 When kinesin-5 motor proteins, which contain two plus-end directed motor domains, are incubated with microtubules, they will organize the microtubules into an astral array. How do you suppose such an array is generated? Will the plus ends of the microtubules be located in the center of the array or at the periphery? Or will some plus ends be at the center and others at the periphery?

17–55 Examine the schematic representation of centrosome duplication in **Figure 17–10**. By analogy with DNA replication, would you classify centrosome duplication as conservative or semiconservative? Explain your reasoning.

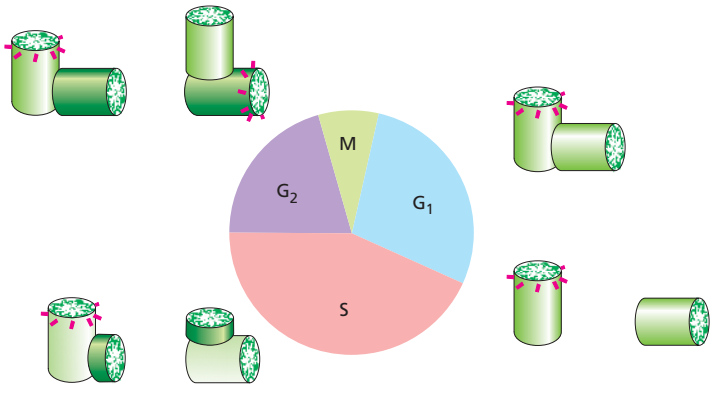


Figure 17–10 The centrosome duplication cycle (Problem 17–55). The individual centrioles that make up the centrosome are shown as *cylinders*.

17–56 How many kinetochores are there in a human cell at mitosis?

17–57 Both sister chromatids of a chromosome occasionally end up in one daughter cell. Suggest some possible causes for such an event. What would be the consequences for the daughter cells if this event occurred in mitosis?

17–58 How can there be a constant poleward flux of tubulin subunits in the absence of any visible change in the appearance of the spindle?

17–59 Nocodazole reversibly inhibits microtubule polymerization, which is essential for formation of the mitotic spindle. By treating a population of mammalian cells with nocodazole for a time and then washing it out of the medium, it is possible to synchronize the cell population. In the presence of nocodazole, where in the cell cycle would you expect the cells to accumulate? What mechanism do you suppose is responsible for stopping cell-cycle progression in the presence of nocodazole?

17–60 Budding yeast cells that are deficient for Mad2, a component of the spindle assembly checkpoint, are killed by treatment with benomyl, which causes microtubules to depolymerize. In the absence of benomyl, however, the cells are perfectly viable. Explain why Mad2-deficient cells live in the absence of benomyl but die in its presence.

17–61 If a fine glass needle is used to manipulate a chromosome inside a living cell during early M phase, it is possible to trick the kinetochores on the two sister chromatids into attaching to the same spindle pole. This arrangement is normally unstable and is rapidly converted to the standard arrangement with sister chromatids attached to opposite poles. The abnormal attachment can be stabilized if the needle is used to gently pull the chromosome so that the microtubules that attach it to the same pole are under tension. What does this suggest to you about the mechanism by which kinetochores normally become attached and stay attached to microtubules from opposite spindle poles during M phase? Explain your answer.

17–62 Discuss the following analogy: "Chromosomes are pulled to the spindle pole like fish on a line."

17–63 Order the following events in animal cell division.

A. Alignment of chromosomes at the spindle equator.
B. Attachment of microtubules to chromosomes.
C. Breakdown of nuclear envelope.
D. Condensation of chromosomes.
E. Decondensation of chromosomes.
F. Duplication of centrosome.
G. Elongation of the spindle.
H. Pinching of cell in two.
I. Re-formation of nuclear envelope.
J. Separation of centrosomes.
K. Separation of sister chromatids.

CALCULATIONS

17–64 High doses of caffeine (**Figure 17–11**) interfere with the DNA damage response in mammalian cells. Why then do you suppose the Surgeon General has not yet issued an appropriate warning to heavy coffee and cola drinkers? A typical cup of coffee (150 mL) contains 100 mg of caffeine (196 g/mole). How many cups of coffee would you have to drink to reach the dose (10 mM) required to interfere with the DNA damage response? (A typical adult contains about 40 liters of water.)

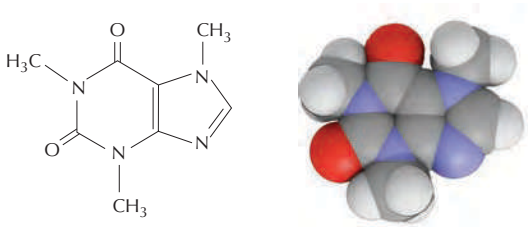


Figure 17–11 Structure of caffeine (Problem 17–64).

17–65 How much DNA does a single microtubule carry in mitosis? From the information in **Table 17–1**, calculate the average length of chromosomes in each organism in base pairs (bp) and in millimeters (1 bp = 0.34 nm), and then calculate how much DNA (in base pairs) each microtubule carries on average in mitosis. Do microtubules carry about the same amount of DNA or does it vary widely in different organisms?

TABLE 17–1 DNA content, haploid number of chromosomes, and microtubules per chromosome in a variety of organisms (Problem 17–65).

Type of organism	Species	DNA content (bp)	Number of chromosomes	Microtubules/ chromosome
Yeast	*S. cerevisiae*	1.4×10^7	16	1
Yeast	*S. pombe*	1.4×10^7	3	3
Protozoan	*Chlamydomonas*	1.1×10^8	19	1
Fly	*Drosophila*	1.7×10^8	4	10
Human	*Homo sapiens*	3.2×10^9	23	25
Plant	*Haemanthus*	1.1×10^{11}	18	120

DATA HANDLING

17–66 The activities of Wee1 kinase and Cdc25 phosphatase determine the state of phosphorylation of tyrosine 15 in the Cdk1 component of M-Cdk. When tyrosine 15 is phosphorylated, M-Cdk is inactive; when tyrosine 15 is not phosphorylated, M-Cdk is active (**Figure 17–12**). Just as the activity of M-Cdk itself is controlled by phosphorylation, so too are the activities of Wee1 kinase and Cdc25 phosphatase.

The regulation of these various activities can be studied in extracts of frog oocytes. In such extracts, Wee1 kinase is active and Cdc25 phosphatase is inactive. As a result, M-Cdk is inactive because its Cdk1 component is phosphorylated on tyrosine 15. M-Cdk in these extracts can be rapidly activated by addition of okadaic acid, which is a specific inhibitor of serine/threonine protein phosphatases. Using antibodies specific for Cdk1, Wee1, and Cdc25, it is possible to examine their phosphorylation

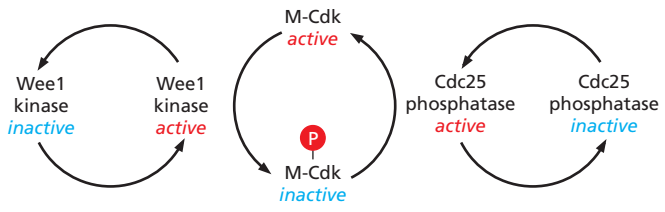


Figure 17–12 Control of M-Cdk activity by Wee1 tyrosine kinase and Cdc25 tyrosine phosphatase (Problem 17–66).

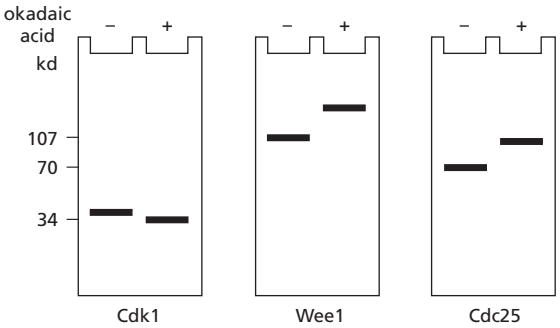


Figure 17–13 Effects of okadaic acid on the phosphorylation states of Cdk1, Wee1, and Cdc25 (Problem 17–66). Molecular mass markers are shown in kilodaltons on the *left*.

states by changes in mobility upon gel electrophoresis (**Figure 17–13**). (Phosphorylated proteins generally migrate more slowly than their nonphosphorylated counterparts.)

A. Based on the results with okadaic acid, decide whether the active forms of Wee1 kinase and Cdc25 phosphatase are phosphorylated or nonphosphorylated. In Figure 17–12, indicate the phosphorylated forms of Wee1 and Cdc25. Also, label the arrows connecting their active and inactive forms to show which transitions are controlled by protein kinases and which are controlled by protein phosphatases.
B. Are the protein kinases and phosphatases that control Wee1 and Cdc25 specific for serine/threonine side chains or for tyrosine side chains? How do you know?
C. How does addition of okadaic acid cause an increase in phosphorylation of Wee1 and Cdc25, but a decrease in phosphorylation of Cdk1?
D. If you assume that Cdc25 and Wee1 are targets for phosphorylation by active M-Cdk, can you explain how the appearance of a small amount of active M-Cdk would lead to its rapid and complete activation?

17–67 Cohesins and condensins are very similar in structure yet carry out quite different biochemical tasks: cohesion of sister chromatids and condensation of chromosomes, respectively. You are skeptical that such similar molecules can perform such distinct functions, and set out to determine if they are truly different using purified components. You incubate pure cohesin or condensin with nicked circular DNA and ATP. You then add topoisomerase II to link duplexes that have been juxtaposed. (Topoisomerase II binds to one duplex and breaks both strands, attaching itself covalently to the ends and holding them together. The topoisomerase II complex can gate the passage of a second duplex through the break and then reseal the original duplex. By linking—or unlinking—duplexes, topoisomerase II can alter their topology in informative ways.)

As shown in **Figure 17–14**, condensin and cohesin yield very different results upon incubation with topoisomerase II and analysis by gel

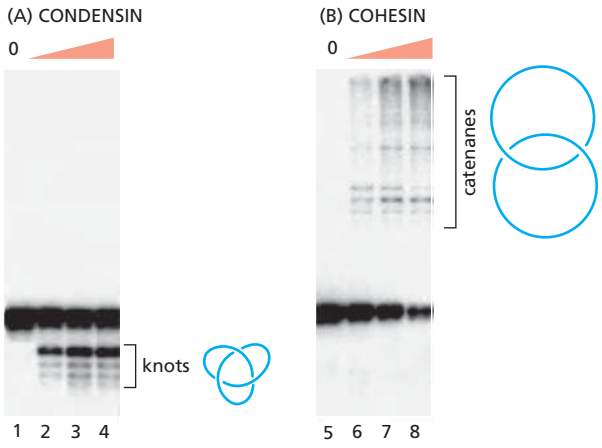


Figure 17–14 Topological analysis of the functions of condensin and cohesin (Problem 17–67). (A) Electrophoretic analysis of migration of circular DNA incubated with condensin and topoisomerase II. The knots formed by incubation are all like the one illustrated. (B) Electrophoretic analysis of migration of circular DNA incubated with cohesin and topoisomerase II. The products of incubation are catenanes; a dimeric catenane is shown. In both gels, the heavy band of material corresponds to the nicked circles that were added to the incubation mixture. *Orange wedges* indicate increasing concentrations of condensin and cohesin.

electrophoresis. Incubation with condensin followed by topoisomerase generates a particular kind of trefoil knot (Figure 17–14A), whereas incubation with cohesin followed by topoisomerase generates a series of catenanes (circles joined like links of a chain, Figure 17–14B).

A. Do these results support the proposed roles of cohesin and condensin? How so?
B. Suggest a plausible mechanism by which binding of cohesin might allow topoisomerase II to link molecules into catenanes.
C. Knots are much more difficult to think about, but often are very revealing of mechanistic details. See if you can figure out a way to use the binding of condensin molecules, coupled with one duplex-crossing event catalyzed by topoisomerase II, to tie a circular molecule into any kind of a knot.

17–68 A classic paper clearly distinguished the properties of astral microtubules from those of kinetochore microtubules. Centrosomes were used to initiate microtubule growth, and then chromosomes were added. The chromosomes bound to the free ends of the microtubules, as illustrated in **Figure 17–15**. The complexes were then diluted to very low tubulin concentration (well below the critical concentration for microtubule assembly) and examined again (Figure 17–15). As is evident, only the kinetochore microtubules were stable to dilution.

A. Why do you think the kinetochore microtubules are stable?
B. How would you explain the disappearance of the astral microtubules after dilution? Do they detach from the centrosome, depolymerize from an end, or disintegrate along their length at random?
C. How would a time course after dilution help to distinguish among these possible mechanisms for disappearance of the astral microtubules?

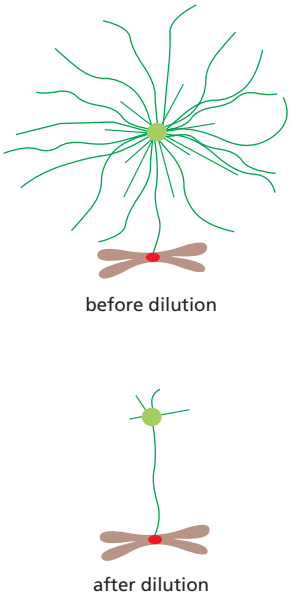


Figure 17–15 Arrangements of centrosomes, chromosomes, kinetochores, and microtubules before and after dilution to low tubulin concentration (Problem 17–68).

17–69 In higher eukaryotes, rare chromosomes containing two centromeres at different locations are highly unstable: they are literally torn apart at anaphase when the chromosomes separate. You wonder whether the same phenomenon occurs in yeast, whose chromosomes are too small to analyze microscopically.

You construct a plasmid with two centromeres, as shown in **Figure 17–16**. Growth of this plasmid in bacteria requires the bacterial origin of replication (*Ori*) and a selectable marker (Amp^R); its growth in yeast requires the yeast origin of replication (*Ars1*) and a selectable marker (*Trp1*). You prepare a plasmid stock by growth in *E. coli*. This dicentric plasmid transforms yeast with about the same efficiency as a plasmid that contains a single centromere. Individual colonies, however, were found to contain plasmids with a single centromere or no centromere, but never a plasmid with two centromeres. By contrast, colonies that arose after transformation with a monocentric plasmid invariably contained intact plasmids.

A. Considering their extreme instability in yeasts, why are dicentric plasmids stable in bacteria?
B. Why do you suppose the dicentric plasmid is unstable in yeasts?
C. Suggest a mechanism for deletion of one of the centromeres from a dicentric plasmid grown in yeast. Can this mechanism account for loss of both centromeric sequences from some of the plasmids?

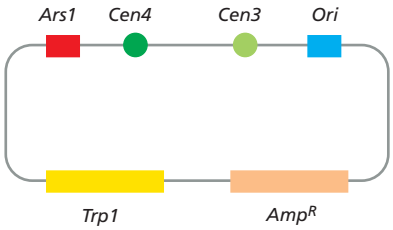


Figure 17–16 Structure of a dicentric plasmid (Problem 17–69). *Cen3* and *Cen4* refer to centromeres from yeast chromosomes 3 and 4, respectively.

17–70 Among the variety of microtubule-dependent motors associated with mitotic spindles are ones that bind to chromosome arms. The role of one such motor protein, Xkid, during spindle assembly was investigated by removing the protein (by immunodepletion) from frog egg extracts, which will form spindles under defined conditions. Extracts that have Xkid, and immunodepleted extracts that are missing Xkid, both assemble normal-looking spindles, as assessed by tubulin staining (**Figure 17–17**). In the presence of Xkid the chromosomes are aligned on the metaphase

plate (Figure 17–17A), whereas in its absence the chromosomes are dispersed throughout the spindle (Figure 17–17B).

A. Suggest a mechanism by which Xkid might function to align chromosomes on the metaphase plate. Include in your description whether you think Xkid is a plus-end or a minus-end directed motor, and why.
B. Is Xkid a plausible candidate for the mediator of the polar ejection force that pushes chromosomes away from the poles?
C. At the transition from metaphase to anaphase, Xkid is normally degraded. What would you expect to happen to the chromosomes if the cell were unable to degrade Xkid?

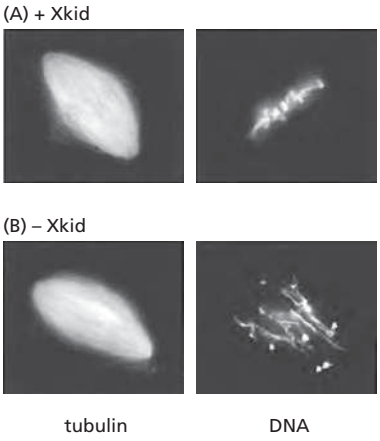


Figure 17–17 Assembly of mitotic spindles in frog egg extracts (Problem 17–70). (A) In the presence of Xkid. (B) In the absence of Xkid. The spindle microtubules were made visible with fluorescent tubulin and the DNA was visualized with a fluorescent stain.

17–71 Bipolar spindles assemble in the absence of centrosomes in *Sciara* when development occurs parthenogenetically. (Normally, the sperm delivers a centrosome to the egg along with a haploid genome.) These spindles look normal except that they lack astral microtubules (**Figure 17–18**). They can also support the rapid, synchronous series of early nuclear divisions that occur in a common cytoplasm in *Sciara* (similar to the early nuclear divisions in *Drosophila*). The products of these early mitotic events, however, are clearly different in normal embryos and parthenogenetic ones. The nuclei in normal embryos are well distributed in the common cytoplasm (Figure 17–18A), but those in parthenogenetic embryos are clustered together (Figure 17–18B). Can you suggest a way in which astral microtubules might function to keep nuclei well distributed in the common cytoplasm?

17–72 At the transition from metaphase to anaphase, M-Cdk is inactivated and chromosomes begin to separate into sister chromatids. M-Cdk is inactivated by the anaphase-promoting complex (APC/C), which destroys the cyclin B component of M-Cdk, eliminating its kinase activity. You want to know how the separation of sister chromatids is related to M-Cdk inactivation. To answer this question, you make cell-free extracts from unfertilized frog eggs. When nuclei are added to the extract, they spontaneously form spindles with condensed chromosomes aligned on the metaphase plate. Anaphase and the separation of sister chromatids can be triggered by addition of Ca^{2+}, which activates APC/C and turns off M-Cdk.

To investigate the control of sister-chromatid separation, you make use of two mutant forms of cyclin B (**Figure 17–19**). Cyclin BΔ90 is missing the destruction box, a sequence of amino acids required for inactivation

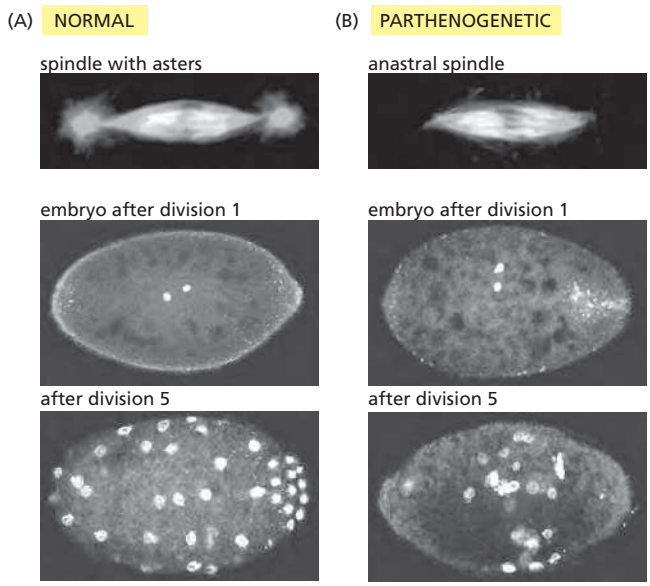


Figure 17–18 Bipolar spindles and nuclear divisions in *Sciara* (Problem 17–71). (A) In normal embryos. (B) In parthenogenetic embryos.

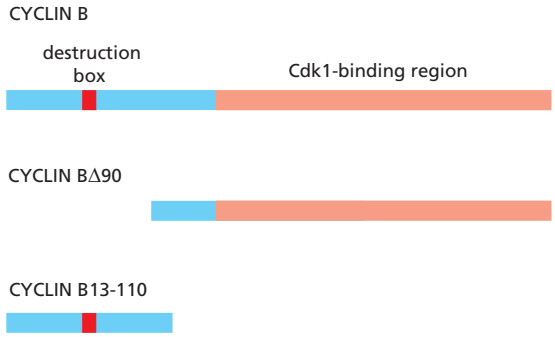


Figure 17–19 Cyclin B and two mutants (Problem 17–72).

by APC/C, but it retains its ability to bind to Cdk1 and make functional M-Cdk. Cyclin B13-110 retains the destruction box, but cannot bind to Cdk1. When either protein is added in excess to the extract, M-Cdk activity remains high after addition of Ca^{2+}. The two proteins differ, however, in their effects on chromatid separation. In the presence of cyclin BΔ90, sister chromatids separate normally; in the presence of cyclin B13-110, sister chromatids remain linked.

A. Why does M-Cdk remain active in the presence of Ca^{2+} when cyclin BΔ90 is added to the extract?
B. Why does M-Cdk remain active when cyclin B13-110 is added to the extract?
C. How is the separation of sister chromatids related to M-Cdk inactivation? Do sister chromatids separate because a linker protein must be phosphorylated by M-Cdk in order for it to hold the chromatids together? Or do chromatids separate because APC/C degrades the linker protein?

17–73 By the turn of the twentieth century, it was clear that chromosomes were the carriers of hereditary information, but it was not clear whether each chromosome carried the total hereditary information or just a portion. According to the first view, multiple chromosomes were required to raise the total quantity of hereditary material above the threshold value needed for proper development. According to the second view, multiple chromosomes were needed so that all portions of the hereditary information would be represented. This question was answered definitively in a classic series of experiments carried out by Theodor Boveri from 1901 to 1905.

To answer this question, Boveri followed the development of sea urchin eggs that had been fertilized by two sperm, a frequent occurrence during artificial fertilization. Instead of a normal bipolar mitotic spindle and division into two cells, these abnormally fertilized eggs form a tetrapolar mitotic spindle and then divide into four cells. The three sets of chromosomes—one from the egg and two from the sperm—are distributed randomly among the four spindles as shown for four chromosomes in **Figure 17–20A**. Sometimes one of the spindle poles fails to form, resulting in a tripolar mitotic spindle followed by division into three cells (**Figure 17–20B**).

The species of sea urchin that Boveri studied has a diploid chromosome number of 18, but will develop normally to a free-swimming pluteus larva with a haploid number of 9. Boveri reasoned that for a tripolar or tetrapolar egg to develop to a normal pluteus, each cell resulting from the initial three-way or four-way division would need to have either 9 total chromosomes or 9 different chromosomes—depending on which view of chromosome inheritance was correct. Boveri followed the development of 695 tripolar eggs and found that 58 developed into a normal pluteus. Among 1170 tetrapolar eggs, none formed a normal pluteus.

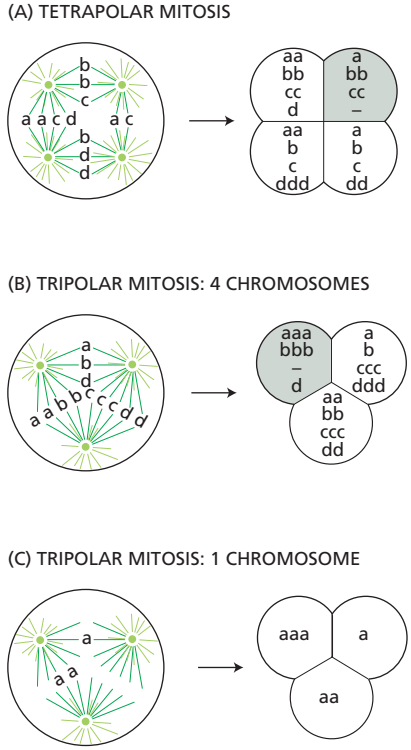


Figure 17–20 Distributions of chromosomes among multiple spindles (Problem 17–73). (A) Example of a random arrangement of three sets of four chromosomes (indicated by letters) among the four mitotic spindles in a tetrapolar egg. Note that in this example, all four cells have at least four chromosomes. If total number were the critical aspect of chromosomes in heredity, these cells should develop into a pluteus. On the other hand, if each of the four cells has to have at least one copy of each different chromosome, these cells would not be expected to form a pluteus because one cell (*shaded*) is missing a chromosome. (B) Example of a random arrangement of three sets of four chromosomes among the three mitotic spindles in a tripolar egg. Once again, if total number of chromosomes were critical, these cells should develop into a pluteus; but if chromosome type is critical, they will not since the *shaded* cell is missing one chromosome. (C) Example of one arrangement—out of 10 possible—of three chromosomes on a tripolar spindle.

A. To set up the expectations for this experiment, it is instructive to consider first a hypothetical case in which the egg and two sperm each contribute a single chromosome. For tripolar spindles, there are 10 different arrangements of three chromosomes on three spindles. Sketch these 10 arrangements. Upon separation of sister chromatids and cell division, how many of these arrangements would be expected to produce three cells that each carry at least one chromosome? One arrangement and its division into three cells is shown in **Figure 17–20C**. (If you want to try your hand at tetrapolar spindles, there are 20 arrangements.)

B. If the total number of chromosomes were the critical factor, the number of tripolar eggs in which each cell gets the minimum number of chromosomes would be the same as that calculated in part A, regardless of the number of chromosomes. By contrast, if the distribution of chromosomes were the critical factor, the number of tripolar eggs that generate three cells, each with at least one copy of each different chromosome, would be expected to decrease with increasing numbers of chromosomes. The number of plutei should decrease according to the fraction calculated in part A raised to the power of 9 (the number of different chromosomes). Which hypothesis—total number of chromosomes or distribution of chromosomes—do Boveri's observations support?

CYTOKINESIS

TERMS TO LEARN

cell plate
contractile ring
cytokinesis
midbody
phragmoplast
preprophase band
syncytium

DEFINITIONS

Match the definition below with its term from the list above.

17–74 Cytoplasm containing many nuclei enclosed by a single plasma membrane.

17–75 Structure formed at the end of cleavage that can persist for some time as a tether between the two daughter cells.

17–76 Division of the cytoplasm of a plant or animal cell into two.

17–77 Structure made of microtubules and actin filaments that forms in the prospective plane of division of a plant cell and guides formation of the cell plate.

17–78 Circular band containing actin and myosin that forms under the surface of animal cells undergoing cell division and contracts to pinch the two daughter cells apart.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–79 Cytokinesis follows mitosis as inevitably as night follows day.

17–80 Whether cells divide symmetrically or asymmetrically, the mitotic spindle positions itself centrally in the cytoplasm.

THOUGHT PROBLEMS

17–81 What are the two distinct cytoskeletal machines that are assembled to carry out the mechanical processes of mitosis and cytokinesis in animal cells?

17–82 You have obtained an antibody to myosin that prevents the movement of myosin molecules along actin filaments. If this antibody were injected into cells, would you expect the movement of chromosomes at anaphase to be affected? How would you expect antibody injection to affect cytokinesis? Explain your answers.

17–83 If a cell just entering mitosis is treated with nocodazole, which destabilizes microtubules, the nuclear envelope breaks down and chromosomes condense, but no spindle forms and mitosis arrests. In contrast, if such a cell is treated with cytochalasin D, which destabilizes actin filaments, mitosis proceeds normally, but a binucleate cell is generated and proceeds into G_1. Explain the basis for the different outcomes of these treatments with cytoskeleton inhibitors. What do these results tell you about cell-cycle checkpoints in M phase?

CALCULATIONS

17–84 When cells divide after mitosis, their surface area increases—a natural consequence of dividing a constant volume into two compartments. The increase in surface requires an increase in the amount of plasma membrane. One can estimate this increase by making certain assumptions about the geometry of cell division. Assuming that the parent cell and the two progeny cells are spherical, one can apply the familiar equations for the volume $[(4/3)\pi r^3]$ and surface area $(4\pi r^2)$ of a sphere.

A. Assuming that the progeny cells are equal in size, calculate the increase in plasma membrane that accompanies cell division. (Although this problem can be solved algebraically, you may find it easier to substitute real numbers. For example, let the volume of the parent cell equal 1.) Do you think that the magnitude of this increase is likely to cause a problem for the cell? Explain your answer.

B. During early development, many fertilized eggs undergo several rounds of cell division without any overall increase in total volume. For example, *Xenopus* eggs undergo 12 rounds of division before growth commences and the total cell volume increases. Assuming once again that all cells are spherical and equal in size, calculate the increase in plasma membrane that accompanies development of the early embryo, going from one large cell (the egg) to 4096 small cells (12 divisions).

DATA HANDLING

17–85 Megakaryocytes, which are the precursor cells of blood platelets, undergo a unique differentiation program, becoming polyploid through repeated cycles of DNA synthesis without concomitant cell division. Such cells contain some 4 to 128 times the normal DNA content in a single large nucleus. Ultimately, mature megakaryocytes begin to bud off platelets as shown in **Figure 17–21**. Careful observations of individual precursor cells that were stimulated to undergo polyploidization show the sequence of events in **Figure 17–22**. How do these events differ from the normal sequence in cell division? At what stage in M phase do these cells deviate from normal cells? What sorts of molecular differences might you expect to find among the components involved in M phase in these cells versus normal cells?

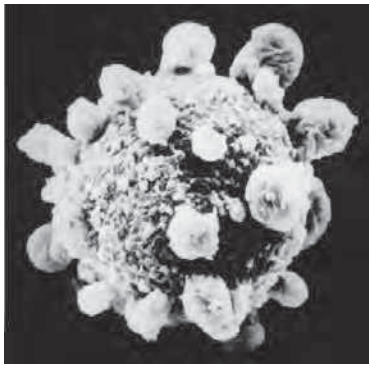


Figure 17–21 A megakaryocyte budding off platelets (Problem 17–85).

MEDICAL LINKS

17–86 Globoid cell leukodystrophy (GLD, also known as Krabbe's disease) is a hereditary metabolic disorder characterized morphologically by distinctive multinucleated globoid cells in the white matter of the brain. Deficiency of an enzyme of sphingolipid catabolism leads to accumulation

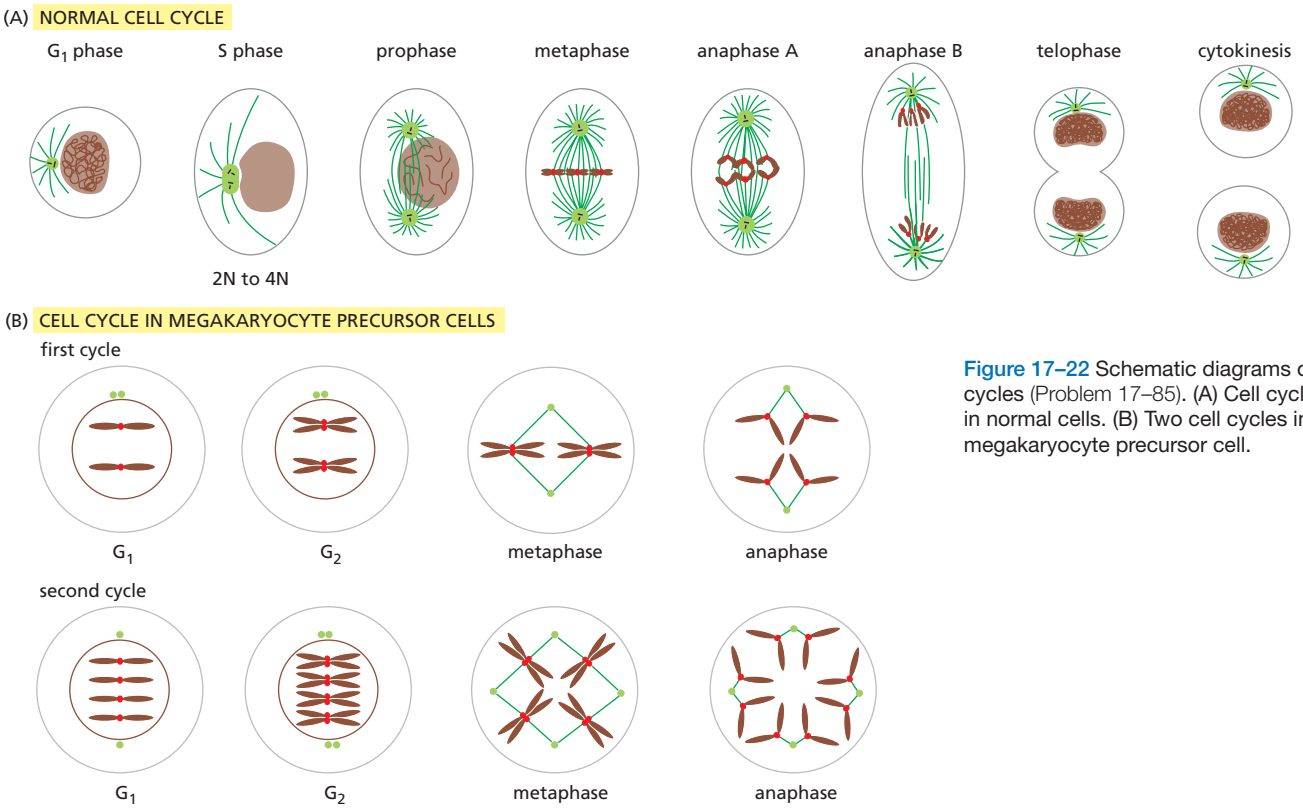


Figure 17–22 Schematic diagrams of cell cycles (Problem 17–85). (A) Cell cycle in normal cells. (B) Two cell cycles in a megakaryocyte precursor cell.

of psychosine in the brain (**Figure 17–23A**). Psychosine binds to a G-protein-coupled receptor that is expressed in only a few cell types. To test whether there might be a relationship between psychosine, its receptor, and multinucleate cells, you express the psychosine receptor in cells that normally lack it, and measure the effects of psychosine treatment by FACS (fluorescence-activated cell sorting) analysis (**Figure 17–23B**).

Do these results support the idea that psychosine acts through its receptor to inhibit cytokinesis? Explain your reasoning.

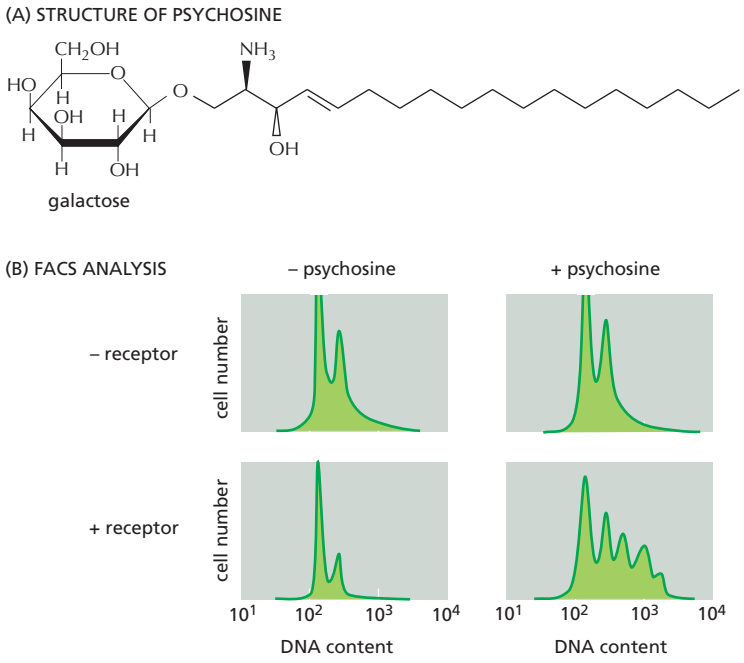


Figure 17–23 Analysis of the role of psychosine in the generation of multinucleate cells (Problem 17–86). (A) Structure of psychosine. (B) Effects of psychosine on cells that do or do not express the psychosine receptor. FACS analysis measures the DNA content of individual cells using a fluorescent DNA dye.

MEIOSIS

TERMS TO LEARN

bivalent
chiasma
meiosis I
meiosis II
nondisjunction
pairing
synaptonemal complex

DEFINITIONS

Match the definition below with its term from the list above.

17–87 The failure of homologs to separate properly.

17–88 The unique form of cell division that segregates homologs.

17–89 An inter-homolog connection that arises from an individual crossover event between nonsister chromatids.

17–90 A four-chromatid structure that arises from the close juxtaposition of homologs as prophase progresses.

17–91 The closely packed array of transverse filaments that links the axial cores of paired homologs.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–92 Meiosis segregates the paternal homologs into sperm and the maternal homologs into eggs.

17–93 During meiosis I, the sister chromatids of each homolog are pulled apart and separated into different daughter nuclei.

17–94 In most species, paired homologs are locked together by homologous recombination events that lead to reciprocal DNA exchanges between nonsister chromatids.

THOUGHT PROBLEMS

17–95 You are studying a mutant of maize called plural abnormalities of meiosis I (*PamI*), which is severely compromised in meiosis. When you visualize paired chromosomes in the pachytene stage of prophase I, you observe many examples of the kind of defects shown in **Figure 17–24** in *PamI* homozygous strains. These abnormal structures are rarely if ever present in wild-type cells that are undergoing meiosis. What is abnormal about the chromosomes in Figure 17–24?

17–96 Down syndrome (trisomy 21) and Edwards syndrome (trisomy 18) are the most common autosomal trisomies seen in human infants. Does this fact mean that these chromosomes are the most difficult to segregate properly during meiosis?

CALCULATIONS

17–97 The human genome consists of 23 pairs of chromosomes (22 pairs of autosomes and one pair of sex chromosomes). During meiosis, the maternal and paternal sets of homologs pair, and then are separated into gametes, so that each contains 23 chromosomes. If you assume that the chromosomes in the paired homologs are randomly assorted to daughter cells, how many potential combinations of paternal and maternal homologs can be generated during meiosis? (For the purposes of this calculation, assume that no recombination occurs.)

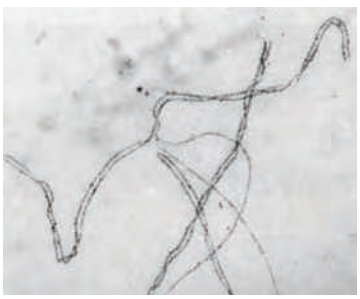


Figure 17–24 Electron micrograph of chromosomes in *PamI* cells undergoing meiosis (Problem 17–95). A region of the *PamI* nucleus containing abnormal structures is shown.

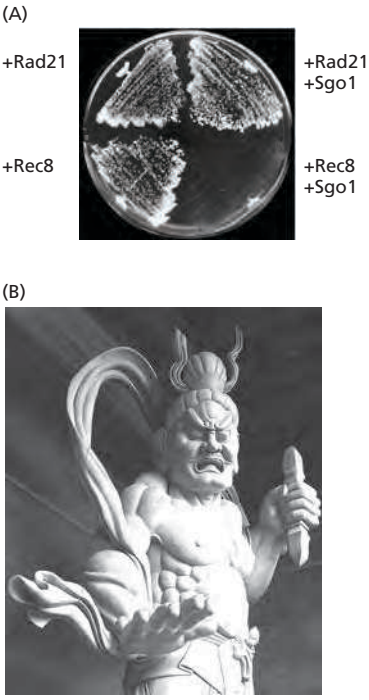


Figure 17–25 Shugoshin (Problem 17–98). (A) Expression of various combinations of cohesins and Sgo1 in *S. pombe*. Rad21 is the normal mitotic cohesin. Absence of growth is apparent when Rec8 and Sgo1 are coexpressed. (B) Shugoshin, a guardian spirit of Japanese temples.

DATA HANDLING

17–98 In mitosis, sister chromatids are held together by cohesins, which are cleaved at the metaphase-to-anaphase transition, allowing the sister chromatids to separate at anaphase. The same is true for meiosis, except that sister chromatids stay together during meiosis I and then separate during meiosis II. How is cohesion of sisters maintained during meiosis I? In the fission yeast *S. pombe*, a special cohesin subunit called Rec8 is expressed only during meiosis, and found to be concentrated at centromeres, where meiotic homologs are stuck together until meiosis II. Rec8 is essential for meiosis and cannot be replaced by the mitotic version known as Rec21. If Rec8 is deliberately expressed during mitosis, when it is not normally present, it does not inhibit mitosis because it is degraded. Thus, you suspect that another protein stops Rec8 degradation during meiosis I. To find this putative inhibitor protein, you devise a clever search strategy. You search for other proteins that when coexpressed with Rec8 during mitosis cause cell death. As shown in **Figure 17–25A**, you find one protein, which you name shugoshin (Sgo1) after the guardian spirits of Japanese temples (**Figure 17–25B**), that is lethal when coexpressed with Rec8 during mitosis.

A. Explain why coexpression of Rec8 and Sgo1 in mitosis is lethal.
B. How might Sgo1 act to prevent sister-chromatid separation during meiosis I?
C. What do you suppose would happen if Sgo1 were not expressed during meiosis I?

CONTROL OF CELL DIVISION AND CELL GROWTH

TERMS TO LEARN

ATM	mitogen	replicative cell senescence
ATR	Myc	retinoblastoma protein (Rb)
E2F protein	p53	telomerase
G_0	Ras	telomere
growth factor		

DEFINITIONS

Match the definition below with its term from the list above.

17–99 A specialized, nondividing state that cells enter by partly disassembling their cell-cycle control system and exiting from the cell cycle.

17–100 Extracellular substance that stimulates cell growth.

17–101 End of a chromosome, associated with a characteristic DNA sequence that is replicated in a special way.

17–102 Phenomenon observed in primary cell cultures as they age, in which cell proliferation slows down and finally halts.

17–103 Extracellular substance that stimulates cell division.

17–104 Gene regulatory factor that is activated by G_1-Cdk complexes in animal cells and binds to specific DNA sequences in the promoters of genes that encode proteins required for S-phase entry.

17–105 The protein kinase that is initially activated in response to x-ray-induced DNA damage and is defective in patients with ataxia telangiectasia.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

17–106 Serum deprivation causes proliferating cells to stop where they are in the cell cycle and enter G_0.

17–107 Budding yeast and mammalian cells respond to DNA damage in the same way: they transiently arrest their cell cycles to repair the damage and if repair cannot be completed, they resume their cycles despite the damage.

17–108 If we could turn on telomerase activity in all our cells, we could prevent aging.

THOUGHT PROBLEMS

17–109 How do mitogens, growth factors, and survival factors differ from one another?

17–110 For each of the following, decide whether such cells exist in humans and, if they do, give examples.

A. Cells that do not grow and do not divide.
B. Cells that grow, but do not divide.
C. Cells that divide, but do not grow.
D. Cells that grow and divide.

17–111 Why do you suppose cells have evolved a special G_0 state to exit the cell cycle, rather than just stopping in G_1 at a G_1 checkpoint?

17–112 Platelet-derived growth factor (PDGF) is encoded by a gene that can cause cancer when expressed inappropriately. Why then do cancers not arise at wounds when PDGF is released from platelets?

17–113 One important biological effect of a large dose of ionizing radiation is to halt cell division.

A. How does a large dose of ionizing radiation stop cell division?
B. What happens if a cell has a mutation that prevents it from halting cell division after being irradiated?
C. What might be the effects of such a mutation if the cell was not irradiated?
D. An adult human who has reached maturity will die within a few days of receiving a radiation dose large enough to stop cell division. What does this tell you (other than that one should avoid large doses of radiation)?

17–114 What do you suppose happens in mutant cells with the following defects?

A. Cannot degrade M-phase cyclins.
B. Always express high levels of p21.
C. Cannot phosphorylate Rb.

17–115 Replicative cell senescence occurs at a characteristic number of population doublings, typically about 40 for cells taken from normal human tissue. This observation suggests that in some way individual cells can "count" the number of times they have divided. How does the structure of telomeres figure into a cell's calculations?

17–116 Liver cells proliferate both in patients with alcoholism and in patients with liver tumors. What do you suppose are the differences in the mechanisms by which cell proliferation is induced in these diseases?

TABLE 17–2 Effects of extracellular growth factors on the timing of entry into S phase (Problem 17–117).

	Order of addition			
Experiment	1	2	3	S phase entry
1	EGF	PDGF	IGF1	12 hours
2	EGF	IGF1	PDGF	12 hours
3	PDGF	EGF	IGF1	1 hour
4	PDGF	IGF1	EGF	6 hours
5	IGF1	EGF	PDGF	12 hours
6	IGF1	PDGF	EGF	6 hours

Cells were treated for 6 hours with the indicated growth factors in the order listed. They were thoroughly washed to remove one factor before the next one was added. After the regimen of factor pretreatment, complete medium with ^{3}H-thymidine was added and the time when labeled nuclei appeared was determined.

DATA HANDLING

17–117 Vertebrate cells pause in the G_1 phase of the cell cycle until conditions are appropriate for their entry into S phase. Some of the requirements for the passage through G_1 have been defined using mouse 3T3 cells. In the absence of serum, these cells do not enter S phase. When serum is added to a culture of such arrested cells, they progress through G_1 and begin to enter S phase 12 hours later. The serum requirement can be met by supplying three extracellular growth factors: PDGF (platelet-derived growth factor), EGF (epidermal growth factor), and IGF1 (insulin-like growth factor 1). When these factors are mixed with appropriate nutrients and added to quiescent cells, the cells begin to enter S phase 12 hours later. If any one of the factors is left out, the cells do not enter S phase.

Do all three growth factors have to be present at the same time? Is their stimulation of cells independent of one another? Or do they stimulate cells in an ordered sequence? To address these questions, you pretreat cells with the growth factors in a defined order and then add complete medium (containing serum and nutrients) in the presence of ^{3}H-thymidine. At various times thereafter, you fix cells and subject them to autoradiography. You define the time of appearance of the first labeled nuclei as the time of entry into S phase. The results of these experiments are given in **Table 17–2**.

Do the cells require these growth factors simultaneously, independently, or in an ordered sequence? Explain your answer.

17–118 When cells in G_0 are exposed to mitogenic growth factors, they enter S phase about 20 hours after stimulation, as can be detected by incorporation of the nucleoside analog BrdU. If antibodies to cyclin D are microinjected into cells up to 12 hours after adding mitogenic growth factors, very few of the injected cells incorporate BrdU. By contrast, cyclin D-specific antibodies have little effect on BrdU incorporation when injected more than 14 hours after exposure to growth factors (**Figure 17–26**). What critical event in G_1 do antibodies against cyclin D block? Why do antibodies against cyclin D have no effect after 14 hours?

17–119 You have found a new way to study radiation-sensitive yeast mutants. By culturing cells on a thin layer of agar on a microscope slide, you can record the fate of individual cells. When you irradiate wild-type cells with

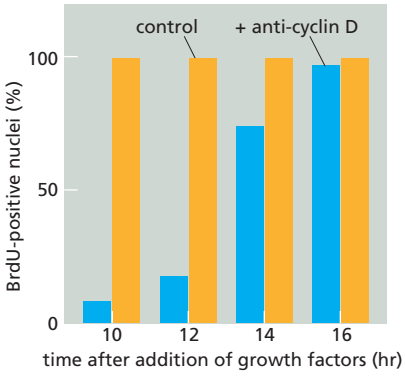


Figure 17–26 Percentage of control cells and cells injected with cyclin D antibodies that had entered S phase as detected by incorporation of BrdU (Problem 17–118). After addition of mitogenic growth factors, cells were injected with cyclin D antibodies (or not) at the times indicated, and then were assayed for BrdU incorporation 26 hours after addition of the mitogen, a time when uninhibited cells should have been well into S phase. *Orange bars* indicate results with control cells that were not injected with cyclin D antibodies. *Blue bars* indicate results with cells that were injected with cyclin D antibodies.

TABLE 17–3 Fractions of arrested and nonviable cells after x-ray treatment (Problem 17–119).

Strain	Arrested at 10 hours (%)	Nonviable (%)
Wild type	50	50
Rad52	90	95
Rad9	20	70

x-rays, which cause chromosome breaks, and follow their growth for the next 10 hours, you find that most of the cells arrest temporarily at the large-bud (dumbbell) stage, but about half the cells eventually recover and form small viable colonies after 10 hours (**Figure 17–27**). The fraction that is still arrested at the dumbbell stage after 10 hours is equal to the fraction of nonviable cells (**Table 17–3**).

You repeat these experiments with seven different radiation-sensitive (*Rad*) mutants. For six of the mutants, you observe a similar equality of 10-hour arrested cells and nonviable cells, as shown in Table 17–3 for the *Rad52* mutant. Relative to wild-type cells, however, a higher fraction of *Rad* cells are nonviable (Table 17–3).

One of the seven *Rad* mutants has a strikingly different phenotype. Many fewer *Rad9* cells arrest even temporarily at the dumbbell stage, and after 10 hours only 20% are still arrested (Table 17–3). Although many of the cells divide once or twice, they mostly form nonviable microcolonies (Figure 17–27, Table 17–3).

A. For the wild-type cells, decide which cells in the population appear most likely to remain arrested at the dumbbell stage after 10 hours (Figure 17–27). Given that the cells used in these experiments are haploid and that x-ray-induced breaks are repaired by homologous recombination, decide which stage of the cell cycle the sensitive cells are in.
B. By staining with DNA-binding reagents and tubulin-specific antibodies, you show that cells in the dumbbell stage have a single nucleus and no visible spindle. Given what you know about cell-cycle checkpoints, in what stage of the cell cycle do you think the cells are arrested?
C. Why do half of the wild-type cells temporarily arrest at the dumbbell stage but then go on to form viable colonies after 10 hours?
D. Why do you suppose that so many more *Rad52* mutant cells (relative to wild-type cells) are arrested at the dumbbell stage after 10 hours?
E. Why do you suppose that so few *Rad9* mutant cells arrest even temporarily at the dumbbell stage? Why are so many of the *Rad9* cells nonviable?
F. Would you expect the fraction of nonviable *Rad9* cells to increase, decrease, or stay the same, if the cells were artificially delayed for a couple of hours, using a microtubule inhibitor that reversibly prevents spindle formation? Explain your reasoning.

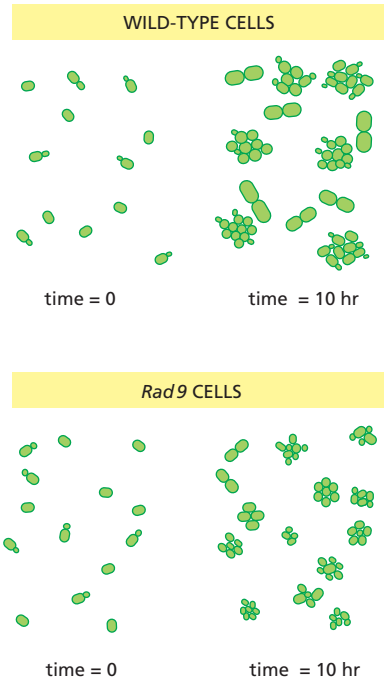


Figure 17–27 Time-lapse pictures of wild-type and *Rad9* cells at zero and 10 hours after x-ray irradiation (Problem 17–119).

17–120 Fission yeast respond to damaged DNA and unreplicated DNA by delaying entry into mitosis. You want to know how the signals from damaged and unreplicated DNA interact with the mitotic entry checkpoint. You screen a large number of mutant yeast strains and find six that do not delay mitosis in response to DNA damage, unreplicated DNA, or both, as shown in **Table 17–4**. Which of the signaling pathways shown in **Figure 17–28** is supported by your data? On the pathway you choose, indicate where each of the mutant genes acts.

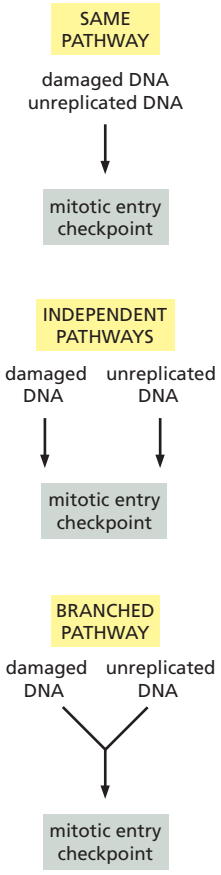


Figure 17–28 Possible pathways by which signals from damaged DNA and unreplicated DNA might interact with the mitotic entry checkpoint (Problem 17–120).

TABLE 17–4 Mutant strains that affect the mitotic entry checkpoint (Problem 17–120).

Mutant strains	Mitotic delay in response to	
	Damaged DNA	Unreplicated DNA
Rad24	No	Yes
Cdc2-3w	Yes	No
Hus1	No	No
Hus2	No	No
Rad1	No	No
Cdc2-F15	Yes	No

17–121 In the cell cycle of the fission yeast *S. pombe*, G_1 is very short and G_2 is very long (just the opposite of the situation in budding yeast). When a temperature-sensitive (ts) *Wee1* mutant is grown continuously at 25°C, it has a normal cell cycle and a normal size (**Figure 17–29A**). When shifted to 37°C, the mutant cells undergo a shortened first cell cycle because inactivation of Wee1 kinase reduces the threshold size for mitosis, generating smaller-than-normal cells (**Figure 17–29B**). Surprisingly, when these ts *Wee1* cells are grown continuously at 37°C, the cell cycle is of normal length, but now with a long G_1 and a short G_2; nevertheless, small cells are still generated (**Figure 17-29C**). What do you suppose would happen to ts *Wee1* cells grown continuously at 37°C if the G_1 phase did not increase in length? Propose an explanation for how G_1 might be lengthened in ts *Wee1* cells grown at 37°C.

17–122 To try to decide whether the effect of overexpression of cyclin D and Cdk4 in flies results primarily from an effect on growth rate or, alternatively, primarily from an effect on cell cycle, you examine terminally differentiated cells that are no longer dividing. You find that in *Drosophila* eyes,

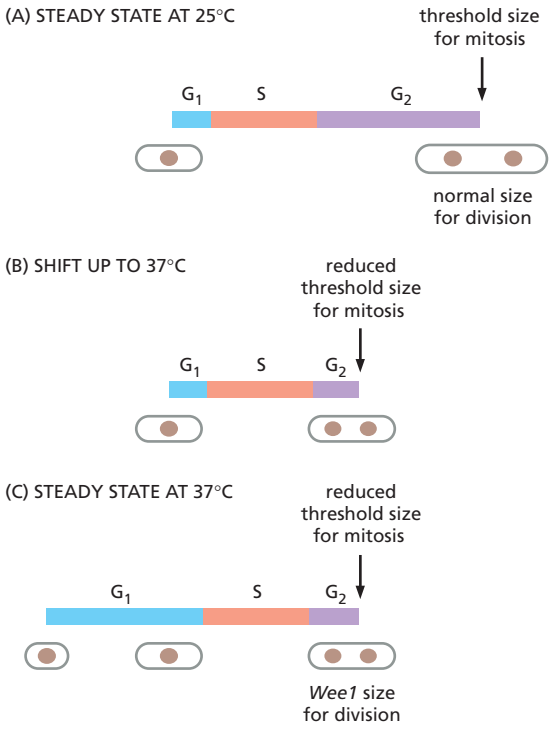


Figure 17–29 Lengths of the cell cycle and sizes of ts *Wee1* cells grown at 25°C and at 37°C (Problem 17–121). (A) The cell cycle in ts *Wee1* cells grown continuously at 25°C. (B) The cell cycle in ts *Wee1* cells immediately after the shift from 25°C to 37°C. (C) The cell cycle in ts *Wee1* cells grown continuously at 37°C.

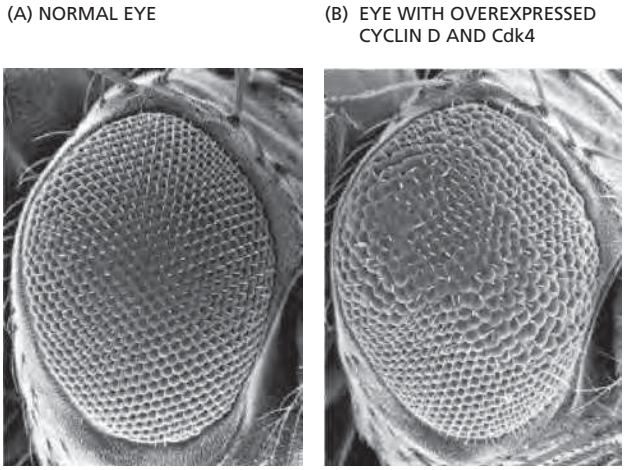


Figure 17–30 Effects of overexpression of cyclin D and Cdk4 (Problem 17–122). (A) A normal eye. (B) An eye with a patch of cells overexpressing cyclin D and Cdk4.

overexpression of cyclin D and Cdk4 causes cell enlargement in postmitotic (nondividing) cells (**Figure 17–30**).

Do these results support a primary role of cyclin D and Cdk4 on growth, or do they support a primary effect on cell cycle? Explain your reasoning.

MCAT STYLE

Passage 1 (Questions 17–123 to 17–126)

In the membrane trafficking pathway, membrane-bound vesicles bud from the Golgi apparatus and are transported to the cell surface, where they fuse with the plasma membrane. Imagine that you have discovered a new compound that blocks fusion of vesicles with the plasma membrane. You treat cells in early G_2 phase with the compound and discover that they fail to enter mitosis. You hypothesize that you have identified a new cell-cycle checkpoint that monitors membrane growth to ensure that cells only enter mitosis when sufficient membrane growth has occurred.

17–123 How might you test whether cells have entered mitosis?

I. Determine whether chromosome condensation has occurred
II. Measure the levels of M-Cdk activity
III. Test whether cells have replicated their DNA

A. I
B. I and II
C. I and III
D. II and III

17–124 You hypothesize that blocking membrane growth triggers a checkpoint signaling cascade that blocks entry into mitosis. Given the key role of M-Cdk in driving entry into mitosis, what do you suppose is the most effective way that a checkpoint signal might block entry into mitosis? The scheme for M-Cdk activation is shown in **Figure 17–31**.

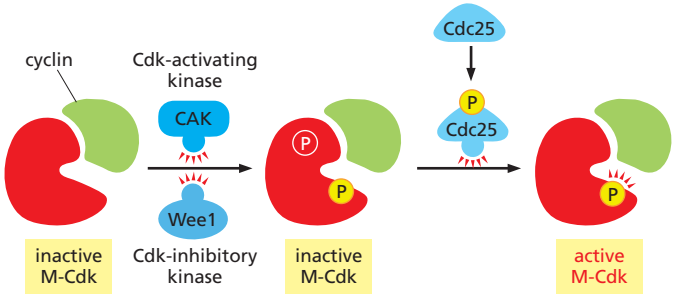


Figure 17–31 Scheme for M-Cdk activation (Problem 17–124). Inhibitory phosphate is shown in *red*; activating phosphates are shown in *yellow*.

A. Activate CAK
B. Increase cyclin synthesis
C. Inhibit Cdc25
D. Inhibit Wee1

17–125 To explain how a checkpoint signal is generated, you hypothesize that blocking fusion of vesicles with the plasma membrane causes accumulation of vesicles in the cytoplasm. The unfused vesicles send a repressive signal that blocks entry into mitosis. Which known checkpoint signaling mechanism would this be analogous to?
A. Blocking chromosome cohesion leads to free chromosomes that block S phase.
B. Duplicated chromosomes send a signal that triggers entry into mitosis.
C. Kinetochores unattached to microtubules send a signal that blocks anaphase.
D. Uncondensed chromosomes send a signal that blocks entry into S phase.

17–126 When you treat cells with the compound in early G_1, you find that they arrest before S phase. You hypothesize that a similar checkpoint acts during G_1 phase to ensure that sufficient growth has occurred before cells enter S phase. Which of the following would be the most plausible mechanism of this arrest?
A. Inhibition of DNA polymerase
B. Inhibition of G_1 cyclin transcription
C. Inhibition of Rb activity
D. Overproduction of E2F

CHAPTER 18

Cell Death

TERMS TO LEARN

anti-apoptotic Bcl2 protein
anti-IAP
Apaf1
apoptosis
apoptosome
Bak
Bax
Bcl2
$BclX_L$
Bcl family
BH3-only protein
caspase
cytochrome *c*
death-inducing signaling complex (DISC)
death receptor
effector Bcl2 protein
executioner caspase
extrinsic pathway
Fas
Fas ligand
IAP (inhibitor of apoptosis)
initiator caspase
intrinsic pathway (mitochondrial pathway)
p53
programmed cell death
survival factor

DEFINITIONS

Match the definition below with its term from the list above.

18–1 Protease that has a cysteine at its active site and cleaves its target proteins at specific aspartic acids.

18–2 Wheel-like assembly composed of seven copies of the Apaf1/cytochrome *c* complex.

18–3 Form of cell death that leads to fragmentation of the DNA, shrinkage of the cytoplasm, membrane changes, and cell death, without lysis or damage to neighboring cells.

18–4 An assembly of several proteins, including initiator caspases, on the cytosolic portion of the Fas death receptor.

18–5 Apoptotic program triggered by the binding of an extracellular signal protein.

18–6 Extracellular signal molecule that inhibits apoptosis.

18–7 Apoptotic program that depends on the release into the cytosol of proteins from the mitochondrial intermembrane space.

18–8 Cell-surface molecule that triggers apoptosis when bound by an extracellular signal protein.

18–9 When cleaved by an initiator caspase, this protease is activated and participates in the widespread cleavage events that kill the cell.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

18–10 In normal adult tissues, cell death usually balances cell division.

18–11 Mammalian cells that do not have cytochrome *c* should be resistant to apoptosis induced by DNA damage.

THOUGHT PROBLEMS

18–12 In apoptosis, the cell destroys itself from within and avoids leakage of the cell contents into the extracellular space. What might be the consequences if apoptosis were not achieved in so neat and orderly a fashion?

18–13 Compare the rules of cell proliferation and apoptosis in an animal to the rules that govern human behavior in society. What would happen to an animal if its cells behaved like people normally behave in our society? Could the rules that govern cell proliferation be applied to human societies?

18–14 Look carefully at the electron micrographs in **Figure 18–1**. Describe the differences between the cell that died by necrosis and the one that died by apoptosis. How do the pictures confirm the differences between the two processes? Explain your answer.

18–15 Development of the nematode *Caenorhabditis elegans* generates exactly 959 somatic cells; it also produces an additional 131 cells that are later eliminated by apoptosis. Classical genetic experiments in *C. elegans* isolated mutants that led to the identification of the first genes involved in apoptosis. Of the many mutations affecting apoptosis in the nematode, none have ever been found in the gene for cytochrome *c*. Why do you suppose that such a central effector molecule in apoptosis was not found in the many genetic screens for "death" genes that have been carried out in *C. elegans*?

18–16 Imagine that you could microinject cytochrome *c* into the cytosol of wild-type mammalian cells and of cells that were doubly defective for Bax and Bak. Would you expect one, both, or neither type of cell to undergo apoptosis? Explain your reasoning.

18–17 Mice that are defective for Apaf1 ($Apaf1^{-/-}$) or for caspase-9 ($Casp9^{-/-}$) die around the time of birth and exhibit a characteristic set of abnormalities, including brain overgrowth and cranial protrusions. Why do you suppose such abnormalities arise in these deficient mice?

18–18 In contrast to their similar brain abnormalities, newborn mice deficient in Apaf1 or caspase-9 have distinctive abnormalities in their paws. Apaf1-deficient mice fail to eliminate the webs between their developing digits, whereas caspase-9-deficient mice have normally formed digits (**Figure 18–2**). If Apaf1 and caspase-9 function in the same apoptotic pathway, how is it possible for these deficient mice to differ in web-cell apoptosis?

CALCULATIONS

18–19 Fas ligand is a trimeric, extracellular protein that binds to its receptor, Fas, which is composed of three identical transmembrane subunits (**Figure 18–3**). The binding of Fas ligand alters the conformation of Fas so that it binds an adaptor protein, which then recruits and activates caspase-8, triggering a caspase cascade that leads to cell death. In humans, the autoimmune lymphoproliferative syndrome (ALPS) is associated with dominant mutations in Fas that include point mutations and C-terminal truncations. In individuals that are heterozygous for such mutations, lymphocytes do not die at their normal rate and accumulate in abnormally large numbers, causing a variety of clinical problems. In contrast to these patients, individuals that are heterozygous for mutations that eliminate Fas expression entirely have no clinical symptoms.

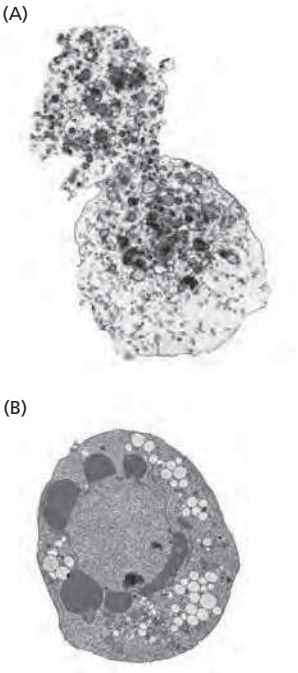


Figure 18–1 Cell death (Problem 18–14). (A) By necrosis. (B) By apoptosis.

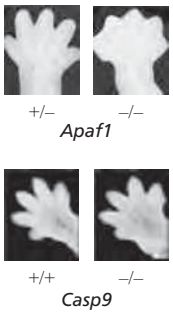


Figure 18–2 Appearance of paws in $Apaf1^{-/-}$ and $Casp9^{-/-}$ newborn mice relative to normal newborn mice (Problem 18–18).

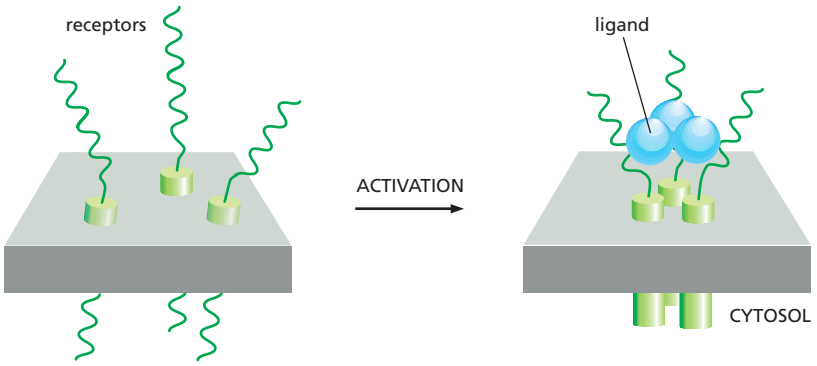


Figure 18–3 The binding of trimeric Fas ligand to Fas (Problem 18–19).

A. Assuming that the normal and dominant forms of Fas are expressed to the same level and bind Fas ligand equally, what fraction of Fas–Fas ligand complexes on a lymphocyte from a heterozygous ALPS patient would be expected to be composed entirely of normal Fas subunits?
B. In an individual heterozygous for a mutation that eliminates Fas expression, what fraction of Fas–Fas ligand complexes would be expected to be composed entirely of normal Fas subunits?
C. Why are the Fas mutations that are associated with ALPS dominant, while those that eliminate expression of Fas are recessive?

DATA HANDLING

18–20 When human cancer cells are exposed to ultraviolet (UV) light at 90 mJ/cm^2, most of the cells undergo apoptosis within 24 hours. Release of cytochrome *c* from mitochondria can be detected as early as 6 hours after exposure of a population of cells to UV light, and it continues to increase for more than 10 hours thereafter. Does this mean that individual cells slowly release their cytochrome *c* over this time period? Or, alternatively, do individual cells release their cytochrome *c* rapidly, but with different cells being triggered over the longer time period?

To answer this fundamental question, you have fused the gene for green fluorescent protein (GFP) to the gene for cytochrome *c*, so that you can observe the behavior of individual cells by confocal fluorescence microscopy. In cells that are expressing the cytochrome *c*–GFP fusion, fluorescence shows the punctate pattern typical of mitochondrial proteins. You then irradiate these cells with UV light and observe individual cells for changes in the punctate pattern. Two such cells (outlined in white) are shown in **Figure 18–4A and B**. Release of cytochrome *c*–GFP is detected as a change from a punctate to a diffuse pattern of fluorescence. Times after UV exposure are indicated as hours:minutes below the individual panels.

Which model for cytochrome *c* release do these observations support? Explain your reasoning.

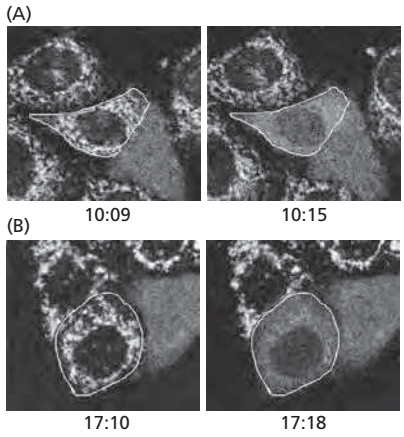


Figure 18–4 Time-lapse video fluorescence microscopic analysis of cytochrome *c*–GFP release from mitochondria of individual cells (Problem 18–20). (A) Cells observed for 6 minutes, 10 hours after UV irradiation. (B) Cells observed for 8 minutes, 17 hours after UV irradiation. One cell in (A) and one in (B), each *outlined in white*, have released their cytochrome *c*–GFP during the time frame of the observation, which is shown as hours:minutes below each panel.

18–21 One common cell strategy for generating a rapid response is to use a positive feedback loop. The rapid release of cytochrome *c* from mitochondria in response to cell damage could result from such a positive feedback loop. For example, the caspases activated by cytochrome *c* could act on mitochondria to release additional cytochrome *c*. To test this possibility, cells expressing a cytochrome *c*–GFP fusion were incubated in the presence or absence of the broad-spectrum caspase inhibitor zVAD prior to exposure to a variety of apoptosis-inducing agents. The average time for release of cytochrome *c*–GFP from the mitochondria in individual cells was then compared, as shown in **Figure 18–5**.

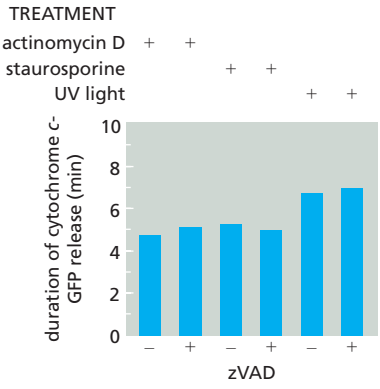


Figure 18–5 Duration of cytochrome *c*–GFP release from mitochondria in cells treated with various apoptosis-inducing agents in the presence and absence of a caspase inhibitor (Problem 18–21). Bars represent the average of measurements on multiple individual cells.

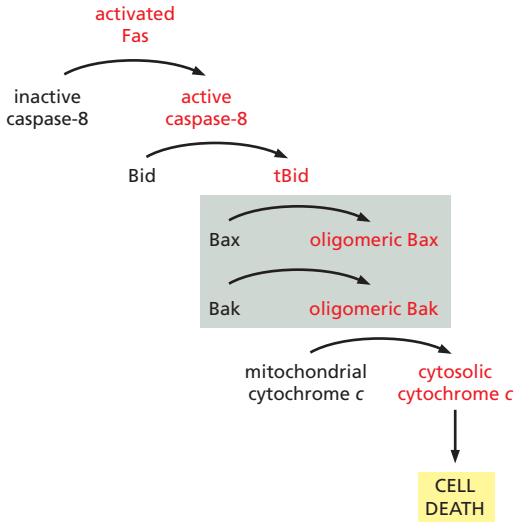


Figure 18–6 Fas-triggered activation of the intrinsic pathway of apoptosis in MEFs (Problem 18–22). Bax and Bak are *shaded* to indicate that oligomerization of both depends on tBid.

If a caspase-mediated positive feedback loop were involved in the rapid release of cytochrome *c*–GFP from mitochondria, what results would you have expected for cells that were incubated in the presence of the caspase inhibitor? Do your expectations match the observed results? Is a caspase-mediated positive feedback loop involved in cytochrome *c* release?

18–22 Activation of Fas activates caspase-8, which triggers the extrinsic pathway of apoptosis. In some cells, the activation of Fas also engages the intrinsic pathway of apoptosis. In these cells, caspase-8 cleaves the protein Bid to produce an active fragment, tBid, that binds to the mitochondrial membrane. tBid promotes oligomerization of Bax and of Bak, which stimulates the release of cytochrome *c* into the cytosol to trigger events leading to apoptosis (**Figure 18–6**). To study this pathway in more detail, you've generated mouse embryo fibroblasts (MEFs) that are $Bax^{-/-}$, $Bak^{-/-}$, or $Bax^{-/-}Bak^{-/-}$. You have also constructed a vector that expresses tBid so that you can study the process independent of Fas.

In untreated MEFs and in MEFs treated with the empty vector, which doesn't carry tBid, very few cells undergo apoptosis (**Figure 18–7**). By contrast, when MEFs are treated with the vector that expresses tBid, the wild-type cells and the cells individually defective for Bax or Bak show a dramatic increase in apoptotic cells. MEFs that are defective for both Bax and Bak, however, show no increase in the number of apoptotic cells (Figure 18–7). Among the various mutant cells, cytochrome *c* is retained in the mitochondria only in the $Bax^{-/-}Bak^{-/-}$ MEFs treated with tBid. What do these results tell you about the requirements for Bax and Bak in cytochrome *c*-induced apoptosis? Explain your reasoning.

18–23 A variety of treatments can cause cells to undergo apoptosis. You wish to know which of these treatments induce signals that are processed through Bid, Bax, and Bak (see Figure 18–6). You generate $Bid^{-/-}$ and $Bax^{-/-}Bak^{-/-}$ mouse embryo fibroblasts (MEFs) and test them for apoptosis in response to several treatments, with the results shown in **Table 18–1**.

A. Based on these results, indicate where each of the signals enters the apoptotic pathway relative to Bid, Bax, and Bak.

B. How do you suppose that Fas ligand, which binds to Fas, manages to cause apoptosis in Bid-deficient cells and in Bax- and Bak-deficient cells?

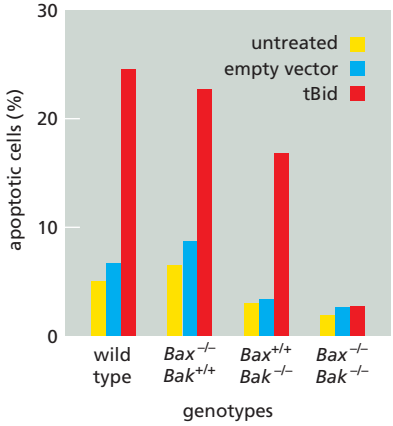


Figure 18–7 Apoptosis induced by tBid expression in wild-type, Bax-deficient, Bak-deficient, and doubly deficient MEFs (Problem 18–22).

TABLE 18–1 Results in *Bid*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs of various treatments that cause apoptosis in wild-type cells (Problem 18–23).

		Apoptosis	
Treatment	Effect	*Bid*$^{-/-}$	*Bax*$^{-/-}$*Bak*$^{-/-}$
Fas ligand	Activates Fas	Yes	Yes
Staurosporine	Inhibits protein kinases	Yes	No
UV light	Damages DNA	Yes	No
Etoposide	Inhibits topoisomerase II	Yes	No
Tunicamycin	Blocks *N*-linked glycosylation	Yes	No
Thapsigargin	Inhibits Ca^{2+} pump in endoplasmic reticulum	Yes	No

MEDICAL LINKS

18–24 One important role of Fas and Fas ligand is to mediate the elimination of tumor cells by killer lymphocytes. In a study of 35 primary lung and colon tumors, half the tumors were found to have amplified and overexpressed a gene for a secreted protein that binds to Fas ligand. How do you suppose that overexpression of this protein might contribute to the survival of these tumor cells? Explain your reasoning.

18–25 Alkylating agents are commonly used for cancer chemotherapy because they are highly cytotoxic, inducing death by apoptosis. Agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) alkylate a variety of cellular targets including DNA, RNA, proteins, and lipids. Which of these targets generates the signal for apoptosis? The most common mutagenic lesion to DNA, O^6-methylguanine, can be removed by the enzyme O^6-methylguanine methyl transferase (MGMT). To test the possibility that alkylation of DNA is responsible for the apoptotic signal, you compare MNNG-induced apoptosis in cells that are deficient for MGMT with apoptosis in cells that overexpress MGMT (**Figure 18–8A**). As a control, you compare apoptosis mediated by γ-irradiation (**Figure 18–8B**). Do these results support the idea that alkylation of DNA leads to the apoptotic signal? Why or why not?

MCAT STYLE

Passage 1 (Questions 18–26 to 18–28)

Early studies of apoptosis discovered that addition of dATP to a protein extract made from human cells triggers the initial events of apoptosis, including activation of a key caspase. To determine how the apoptotic events were initiated, the scientists fractionated the extract on an ion-exchange column, which separated proteins that bound to the column from those that flowed through it. Neither the bound nor the free fraction alone was able to activate caspase in response to dATP; however, when the fractions were combined in the presence of dATP, they initiated apoptotic events. Purification of the activity in the bound fraction yielded a single protein that was found to be cytochrome *c*, providing the first evidence that cytochrome *c* controls apoptosis. Purification of the activity in the unbound fraction also yielded a single protein. The two purified proteins in the presence of dATP were sufficient to initiate apoptotic events.

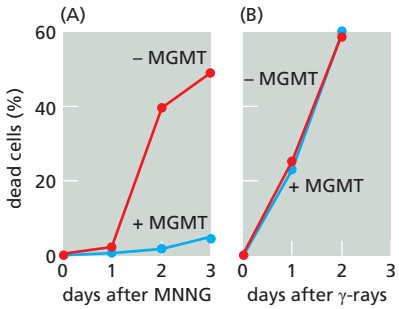


Figure 18–8 Apoptosis induced in MGMT-deficient and MGMT-overexpressing cells (Problem 18–25). (A) Apoptosis induced by MNNG. (B) Apoptosis induced by γ-irradiation. *Red circles* indicate cells deficient for MGMT; *blue circles* indicate cells that overexpress MGMT.

18–26 Which one of the following hypotheses best explains the role of the protein in the unbound fraction?

A. It activates executioner caspases, which trigger apoptotic events.
B. It forms a complex with cytochrome *c* that initiates apoptotic events.
C. It inhibits apoptosis, and cytochrome *c* inactivates it to initiate apoptotic events.
D. It promotes release of cytochrome *c* from mitochondria to initiate apoptosis.

18–27 The discovery that cytochrome *c* promotes apoptosis focused attention on how cytochrome *c* is released into the cytosol. It was initially unclear whether the cytochrome *c* that causes apoptosis comes from mitochondria, or instead was expressed from a nuclear gene and synthesized in the cytosol. Which one of the following observations would have provided the best evidence that it is the cytochrome *c* released from mitochondria that causes apoptosis?

A. Active Fas death receptors trigger release of cytochrome *c* from mitochondria.
B. Bcl2 blocks release of cytochrome *c* from mitochondria and also inhibits apoptosis.
C. Caspase inhibitors block release of cytochrome *c* from mitochondria.
D. Extracellular signals that block apoptosis do not produce cytosolic cytochrome *c*.

18–28 Which of the following would block release of cytochrome *c* from mitochondria?

I. Bcl2 protein
II. Survival factors
III. Fas ligand

A. I
B. II
C. I and II
D. I, II, and III

Cell Junctions and the Extracellular Matrix

CHAPTER 19

IN THIS CHAPTER

CELL–CELL JUNCTIONS

TERMS TO LEARN

adherens junction
adhesion belt
anchoring junction
apical
basal
cadherin
cadherin superfamily
classical cadherin
connective tissue
connexin
connexon
desmosome
epithelia
epithelial tissue
gap junction
homophilic
immunoglobulin (Ig) superfamily
integrin
nonclassical cadherin
PDZ domain
plasmodesmata
polarized
scaffold protein
selectin
tight junction
transmembrane adhesion protein

DEFINITIONS

Match each definition below with its term from the list above.

19–1 Member of a family of cell-surface carbohydrate-binding proteins that mediate transient, Ca^{2+}-dependent cell–cell adhesion in the bloodstream, for example, between white blood cells and the endothelium of the blood vessel wall.

19–2 Like-to-like protein interactions in which a molecule on one cell binds to an identical, or closely related, molecule on an adjacent cell.

19–3 Type of anchoring junction that links the intermediate filaments in two adjoining cells.

19–4 Describes a structural property of epithelial sheets, and their individual cells, which have one surface attached to the basal lamina below and the opposite surface exposed to the medium above.

19–5 A protein structure that creates direct channels from the cytoplasm of one cell to the cytoplasm of an adjacent cell.

19–6 Anchoring junction that connects actin filaments in one cell to those in the next cell.

19–7 A member of a family of proteins that mediate Ca^{2+}-dependent cell–cell adhesion in animal tissues.

19–8 Communicating cell–cell junctions in plants in which a channel of cytoplasm lined by plasma membrane connects two adjacent cells through a small pore in their cell walls.

19–9 Beltlike anchoring junction that encircles the apical end of an epithelial cell and attaches it to the adjoining cells.

19–10 Main type of occluding junction in vertebrates; it seals adjacent epithelial cells together, preventing the passage of most dissolved molecules from one side of the epithelial sheet to the other.

19–11 Describes the tip of a cell; for an epithelial cell, it is the exposed free surface, opposite to the surface attached to the basal lamina.

19–12 Water-filled pore in the plasma membrane formed by a ring of six protein subunits, which link to an identical assembly in an adjoining cell to form a continuous channel between the two cells.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

19–13 Given the numerous processes inside cells that are regulated by changes in Ca^{2+} concentration, it seems likely that Ca^{2+}-dependent cell–cell adhesions are also regulated by changes in Ca^{2+} concentration.

19–14 Cadherins promote cell–cell interactions by binding to cadherin molecules of the same or closely related subtype on adjacent cells.

19–15 The selectins on one cell promote transient cell–cell interactions by binding to carbohydrate moieties on the surface of another cell.

19–16 Although cadherins and Ig family members are frequently expressed on the same cells, the adhesions mediated by Ig molecules are much stronger and, thus, are largely responsible for holding cells together.

19–17 Virtually all epithelia are anchored to other tissues on their basal side and free of such attachment on their apical side.

19–18 Tight junctions perform two distinct functions: they seal the space between cells to restrict paracellular flow and they fence off plasma membrane domains to prevent the mixing of apical and basolateral membrane proteins.

19–19 Like the corresponding transport processes through the plasma membrane, paracellular transport can be either active or passive.

19–20 Unlike conventional ion channels, individual gap-junction channels remain open continuously once they are formed.

19–21 The cells in a plant can be viewed as forming a syncytium, in which many cell nuclei share a common cytoplasm.

THOUGHT PROBLEMS

19–22 Comment on the following (1922) quote from Warren Lewis, who was one of the pioneers of cell biology. "Were the various types of cells to lose their stickiness for one another and for the supporting extracellular matrix, our bodies would at once disintegrate and flow off into the ground in a mixed stream of cells."

19–23 Cell adhesion molecules were originally identified using antibodies raised against cell-surface components to block cell aggregation. In the adhesion-blocking assays, the researchers found it necessary to use antibody fragments, each with a single binding site (so-called Fab fragments), rather than intact IgG antibodies, which are Y-shaped molecules with two identical binding sites. The Fab fragments were generated by digesting the IgG antibodies with papain—a protease—to separate the two binding sites (**Figure 19–1**). Why do you suppose it was necessary to use Fab fragments to block cell aggregation?

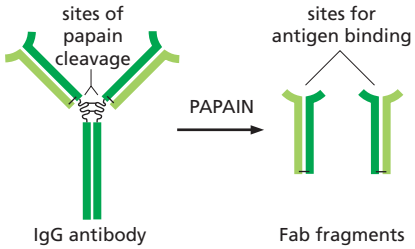


Figure 19–1 Production of Fab fragments from IgG antibodies by digestion with papain (Problem 19–23).

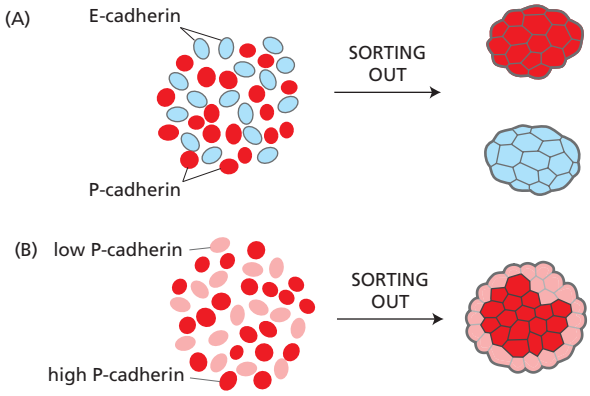


Figure 19–2 Cadherin-dependent cell sorting (Problem 19–24). (A) Sorting of a mixture of cells that express two different cadherins. (B) Sorting of a mixture of cells that express two different levels of the same cadherin.

19–24 Mouse L cells have proven to be an extremely useful model system for investigating the properties of cadherins because they do not normally express any cadherins. If different populations of L cells are transfected with vectors expressing one or the other of two different cadherins, and are then dissociated and mixed together, they segregate into two separate balls of cells, each held together by a different cadherin. If two populations of cells expressing different levels of the same cadherin are mixed, they segregate into a single ball of cells, with the low-expressing population on the outside (**Figure 19–2**).

A. Why do you suppose populations of cells expressing different levels of the same cadherin segregate with this characteristic layered structure? Why don't they segregate into two separate balls? Or a mixed ball? Or a ball with the low-expressing population on the inside?

B. What sort of final architecture might you expect if you were to mix together two populations of cells that expressed P-cadherin in common, but, in addition, one population expressed E-cadherin and the other expressed N-cadherin?

19–25 Although we have accumulated a great deal of information about the components and morphological appearance of tight junctions, we still know relatively little about how molecules pass through them. One elegant study used a graded series of polyethylene glycol (PEG) molecules with radii ranging from 0.35 nm to 0.74 nm. By placing the PEG molecules on one side of an epithelial sheet of intestinal cells, and measuring their rate of appearance on the other side, the researchers determined the permeability of the tight junction as a function of PEG radius (**Figure 19–3**). They interpret these results in terms of a common restrictive pore with a radius of about 0.43 nm and a much rarer, nonrestrictive passageway.

A. The size of the restrictive pore is defined by the sharp cutoff of the initial part of the curve. Why do you suppose that the permeability of PEG molecules that are smaller than the pore—the three smallest molecules—declines markedly with increasing size? Why don't all of the PEG molecules that can fit through the pore permeate it at the same rate?

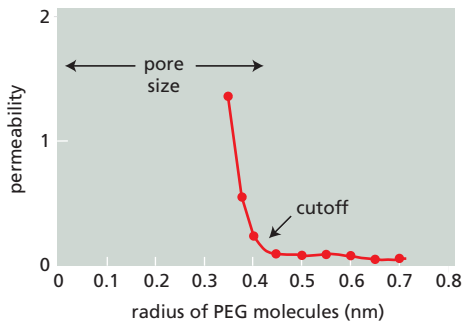


Figure 19–3 Paracellular permeability of a graded series of PEG molecules (Problem 19–25).

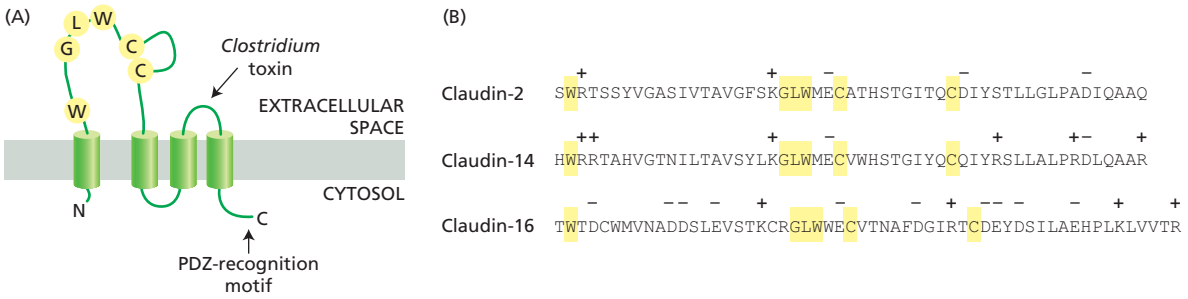


Figure 19–4 Claudin structure (Problem 19–26). (A) Model depicting the conserved structural and functional features of claudins. *Highlighted* amino acids in the first extracellular loop indicate the signature residues characteristic of all claudins. Although there has been no direct demonstration of a disulfide bond between the conserved cysteines, it seems likely given the oxidizing extracellular environment and their conservation between claudins. PDZ-recognition motif indicates the binding site for proteins that contain PDZ domains, which can bind the C-terminal tails of specific transmembrane molecules. The binding site for the *Clostridium* toxin is also indicated (see Problem 19–39). (B) Amino acid sequences of the first extracellular loop in three different claudins. *Highlighted* residues indicate the signature amino acids. Charged residues are indicated with + and – signs.

B. The identity of the larger, but much rarer, paracellular passageway is not known. Can you suggest some possibilities?

19–26 Claudin molecules in one cell bind to those in an adjacent cell to form a tight junction. Interactions between the extracellular domains in the paired claudin molecules form the pores that restrict the paracellular transport of small molecules and ions. The first extracellular loop of claudin molecules (**Figure 19–4A**) is thought to form the pore itself. **Figure 19–4B** shows the sequences of the first extracellular loop in three claudin molecules. Based on these sequences, which one of the claudins do you suppose might form a cation pore? Explain your reasoning.

19–27 The permeability of gap junctions is regulated by Ca^{2+}. Would you expect gap junctions to open or to close when the intracellular concentration of Ca^{2+} rises above its normal levels? Why is this response advantageous?

19–28 If the cells in a plant are all connected to one another by plasmodesmata, why doesn't the cytoplasm from the entire plant leak out onto the ground when you cut the stem?

19–29 It requires a force of about 20 piconewtons (pN) to pull a transmembrane protein like P-selectin out of a pure lipid bilayer. To pull a P-selectin molecule out of the plasma membrane requires more than 110 pN. Why do you suppose it takes so much more force to pull P-selectin out of the plasma membrane than out of a lipid bilayer?

CALCULATIONS

19–30 Atomic force microscopy can be used to measure the strength of individual interactions. This technique showed that the binding strength between individual adhesion molecules in a marine sponge averaged 125 piconewtons (pN) under physiological conditions. Just how strong is such an interaction? Since the purpose of these bonds is to hold cells together, this question can be rephrased in terms of the number of cells one interaction could support against the force of gravity in seawater; that is, what number of cells weighs 125 pN?

The weight (W) of one cell in seawater is given by the Archimedes equation

$$W = gV(\rho_{cell} - \rho_{seawater})$$

where

g is the gravitational constant (9.81 N/kg)
V is the volume of the cell $[(4/3)\pi r^3]$
ρ_{cell} is the density of the cell (1100 kg/m^3)
$\rho_{seawater}$ is the density of seawater (1018 kg/m^3)

Assuming that the cells are spheres with a radius of 5 μm, calculate the weight of one cell in seawater. How many cells could be supported by a bond with a strength of 125 pN?

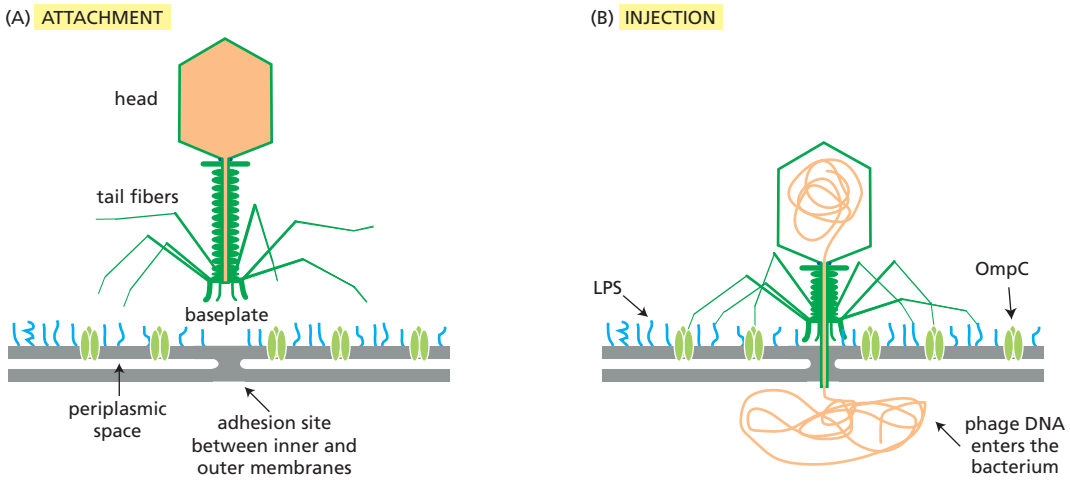


Figure 19–5 Infection by bacteriophage T4 (Problem 19–31). (A) Attachment to bacterial surface. (B) Injection of its DNA.

19–31 The attachment of bacteriophage T4 to *E. coli* K illustrates the value of multiple weak interactions, which allow relative motion until fixed connections are made (**Figure 19–5**). During infection, T4 first attaches to the surface of *E. coli* by the tips of its six tail fibers. It then wanders around the surface until it finds an appropriate place for the attachment of its baseplate. When the baseplate is securely fastened, the tail sheath contracts, injecting the phage DNA into the bacterium (Figure 19–5). The initial tail-fiber cell-surface interaction is critical for infection: phages that lack tail fibers are totally noninfectious.

Analysis of T4 attachment is greatly simplified by the ease with which resistant bacteria and defective phages can be obtained. Bacterial mutants resistant to T4 infection fall into two classes: one lacks a major outer membrane protein called OmpC (outer membrane protein C); the other contains alterations in the long polysaccharide chain normally associated with bacterial lipopolysaccharide (LPS). The infectivity of T4 on wild-type and mutant cells is indicated in **Table 19–1**. These results suggest that each T4 tail fiber has two binding sites: one for LPS and one for OmpC. Electron micrographs showing the interaction between isolated tail fibers and LPS suggest that individual associations are not very strong, since only about 50% of the fibers appear to be bound to LPS.

A. Assume that at any instant each of the six tail fibers has a 0.5 probability of being bound to LPS and the same probability of being bound to OmpC. With this assumption, the fraction of the phage population on the bacterial surface that will have none of its six tail fibers attached in a given instant is $(0.5)^{12}$ (which is the probability of a given binding site being unbound, 0.5, raised to the number of binding sites, two on each of six tail fibers). In light of these considerations, what fraction of the phage population will be attached at any one instant by at least one tail

TABLE 19–1 Infectivity of phage T4 on various bacterial mutants (Problem 19–31).

Bacterial strain	Phage T4 infectivity relative to nonmutant
ompC$^+$ LPS$^+$	1
ompC$^-$ LPS$^+$	10^{-3}
ompC$^+$ LPS$^-$	10^{-3}
ompC$^-$ LPS$^-$	10^{-7}

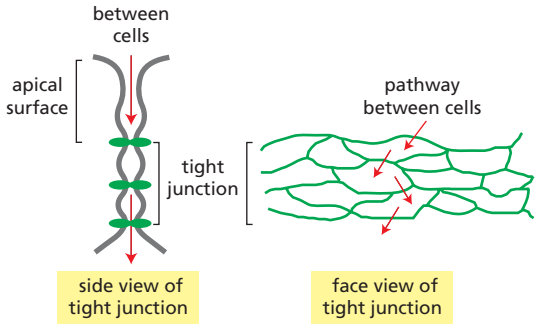


Figure 19–6 Two views of a tight junction that links cells in an epithelium (Problem 19–32).

fiber? (The attached fraction is equal to one minus the unattached fraction.) Suppose that the bacteria were missing OmpC. What fraction of the phage population would now be attached by at least one tail fiber at any one instant?

B. Surprisingly, the above comparison of wild-type and *ompC*$^-$ bacteria suggests only a very small difference in the attached fraction of the phage population at any one instant. As shown in Table 19–1, phage infectivities on these two strains differ by a factor of 1000. Can you suggest an explanation that might resolve this apparent paradox?

19–32 You and your advisor noted some time ago that there is a rough correlation between the electrical resistance of an epithelium and the number of sealing strands in the tight junctions. This makes intuitive sense because current across an epithelium is carried by small ions that must penetrate the tight junction (**Figure 19–6**). You can imagine two ways that resistance might depend on the number of sealing strands. If each sealing strand provided a given resistance, then the overall resistance of a tight junction would be linearly related to the number of sealing strands (like electrical resistors in series). On the other hand, if each sealing strand could exist in two states—a closed, high-resistance state and an open, low-resistance state—the resistance of the tight junction would be related to the probability that all strands in a given pathway through the junction would be open at the same time. In that case, the overall resistance would be logarithmically related to the number of sealing strands.

To put the idea on a quantitative basis so that you can distinguish between these two possibilities, you measure the resistance of four different epithelia from a rabbit: the very leaky proximal tubule of the kidney, the less leaky gall bladder, the tight distal tubule of the kidney, and the very tight bladder epithelium. In addition, you prepare freeze-fracture electron micrographs, from which you determine the average number of sealing strands in the tight junctions that surround each cell in these epithelia. The results are shown in **Table 19–2**. Which of the two

TABLE 19–2 Correlation between electrical resistance and the number of sealing strands in the tight junctions from various epithelia (Problem 19–32).

Rabbit epithelium	Strands in tight junction (mean number)	Electrical resistance (relative)
Proximal tubule	1.2	1.0
Gall bladder	3.3	4.7
Distal tubule	5.3	52
Urinary bladder	8.0	470

proposed interpretations of the correlation between electrical resistance of the epithelium and the number of sealing strands in a tight junction is supported by your measurements?

19–33 Plasmodesmata perform critical functions in plant cells, yet the protein components that presumably confer these functions have proven very difficult to characterize. Part of the problem arises because plasmodesmata are only a small fraction of the cell's total cytoplasm; thus, they are difficult to purify. What fraction of a plant cell's cytoplasm is contained within its plasmodesmata? As the basis for a rough estimate, assume that a plant cell with 1000 μm^3 of cytoplasm has 100 plasmodesmata, each of which is a cylinder 30 nm in diameter and 100 nm in length.

DATA HANDLING

19–34 You suspect that the cadherin you've cloned is attached to the cytoskeleton, presumably by its cytoplasmic tail. To identify the proteins to which it binds, you express your cadherin in a mouse cell line that doesn't express any other cadherins. You label the cells with ^{35}S methionine for 16 hours, homogenize them in detergent, and precipitate the cadherin and any associated proteins using antibodies against the extracellular domain of the cadherin. You then analyze the immune precipitates by electrophoresis on SDS polyacrylamide gels, followed by autoradiography to make the labeled proteins visible. As shown in lane 1 in **Figure 19–7A**, the antibody precipitates three major proteins in cells transfected with a plasmid expressing the full-length intact cadherin. These proteins are not precipitated from untreated control cells (Figure 19–7A, lane 7), verifying the specificity of your antibody.

To identify the portion of the cadherin molecule that is required for binding the other two proteins, you make a series of constructs with deletions in the cytoplasmic tail, and carry out the same sort of analysis (Figure 19–7A and B).

A. In the autoradiograph in Figure 19–7A, identify the bands corresponding to your cadherin and the bands corresponding to proteins that bind to your cadherin. Give your reasoning.
B. Which segment of the cadherin is required for binding to the other proteins?
C. How might you check to see whether the segment you've identified is all that is needed to bind to the other proteins?

19–35 Freeze-fracture electron micrographs provide beautiful images of tight junctions. You are curious to see whether the junctional strands are really as static as those images suggest. By fusing the gene for green fluorescent protein (GFP) to the *claudin-1* gene, you can express a fusion protein in which the cytoplasmic tail of claudin-1 is tagged with GFP. When you transfect this fusion gene into mouse L cells, which express none of their own claudins, fluorescent strands appear on the surface of the cells where they are in contact with one another. It is difficult to maintain

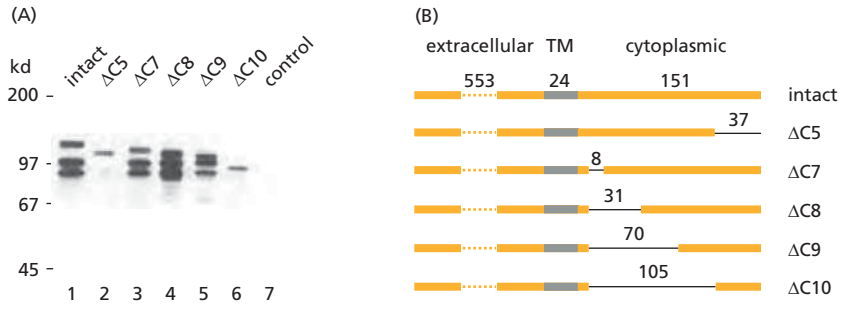


Figure 19–7 Analysis of cadherin interactions with cellular proteins (Problem 19–34). (A) Immunoprecipitation experiments using anti-cadherin antibodies. (B) Schematic illustration of the various cDNA constructs transfected into cells. The numbers of amino acids in the extracellular, transmembrane (TM), and cytoplasmic domains are indicated. The deleted segments are shown as single lines with the number of deleted amino acids indicated. The left end of the deletion in ΔC5 and the right end of the deletion in ΔC10 are at the same site in the protein.

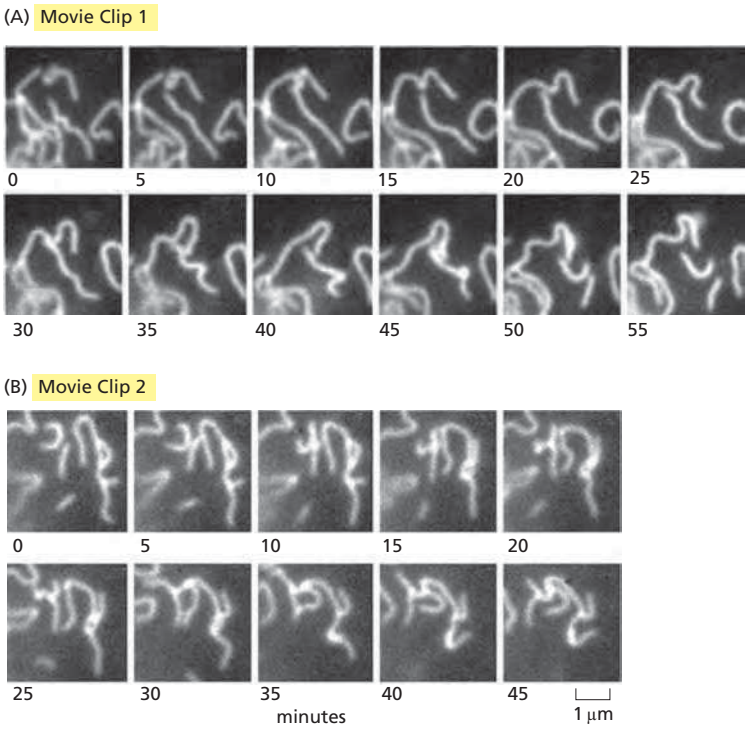


Figure 19–8 Dynamic behavior of paired claudin strands within apposing plasma membranes (Problem 19–35). (A) Frames from movie clip 1. (B) Frames from movie clip 2.

focus on these moving cells for extended times, but occasionally you can get 40–50 minutes of clear observation (**Figure 19–8**).

A. From what you can see in Figure 19–8, does it look like individual strands rapidly elongate? Or rapidly shorten? If your answer to either is yes, show examples on the figure.
B. Do individual strands join together end-to-end? Or break apart? If your answer to either is yes, show examples on the figure.
C. Does the end of an individual strand join to the side of another strand, forming a T-junction? If so, indicate such sites on the figure.
D. A reviewer of your manuscript objects that the plasticity of the strands you observe may be artificial. The reviewer suggests that the presence of GFP at the C-terminus prevents claudin-1 from binding to scaffolding proteins through their PDZ domains. How would you respond?

19–36 Cells in a developing embryo make and break gap-junction connections in specific and interesting patterns, suggesting that gap junctions play an important role in the signaling processes that occur between these cells. At the eight-cell stage, mouse embryos undergo compaction, changing from a clump of loosely associated cells to a tightly sealed ball (**Figure 19–9**). You wish to know whether gap junctions are present before or after this change in adhesion.

Using very fine glass micropipettes, you can measure electrical events and at the same time microinject the enzyme horseradish peroxidase (HRP), 40,000 daltons, or the fluorescent dye fluorescein, 330 daltons. Fluorescein glows bright green under ultraviolet illumination, and HRP can be detected by fixing the cells and incubating them with appropriate substrates. You obtain different results at both the two-cell and eight-cell

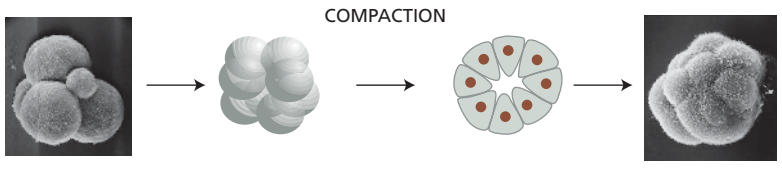


Figure 19–9 Compaction of the eight-cell mouse embryo (Problem 19–36).

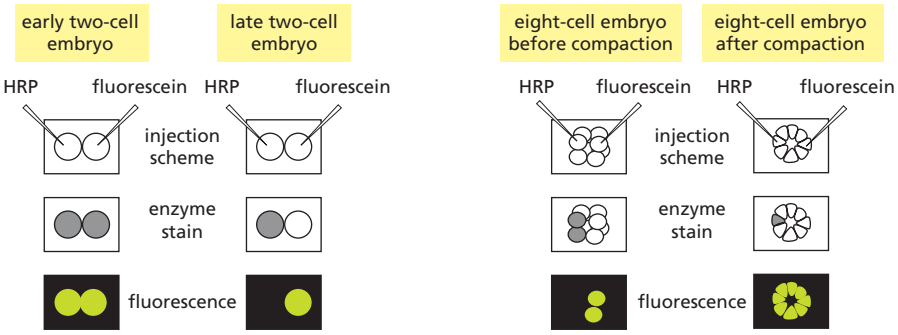


Figure 19–10 Microinjection of HRP and fluorescein in two-cell and eight-cell mouse embryos (Problem 19–36).

stages, depending on whether the injections are made immediately after cell division or later (Figure 19–10). Immediately after cell division, cytoplasmic bridges linger for a while before cytokinesis is completed.

A. Why do both HRP and fluorescein enter neighboring cells early, but not late, at the two-cell stage?
B. At what stage of embryo development do gap junctions form? Explain your reasoning.
C. In which of the four stages of development diagrammed in Figure 19–10 would you detect electrical coupling if you injected current from the HRP injection electrode and recorded voltage changes in the fluorescein electrode?

19–37 Tobacco mosaic virus (TMV), like many other plant viruses, spreads through its host by moving from cell to cell via plasmodesmata. For many years this observation presented a puzzle. The normal size-exclusion limit for diffusion through plasmodesmata is around 800 daltons—sufficient for small molecules—yet the TMV particle, by comparison, is huge: a rigid rod 18 nm in diameter and 300 nm in length, formed by many copies of a coat protein arranged helically around a central, single-stranded RNA genome. Two early clues to the solution of this puzzle were: (1) the coat protein is not required for the cell-to-cell spread of infection; and (2) TMV encodes a 30-kd "movement protein" (MP) that is essential for the spread of infection.

To investigate the effects of the MP, the properties of plasmodesmata in normal tobacco cells (MP^-) were compared with those in transgenic cells that expressed the MP (MP^+). Fluorescence probes of known size were injected into one cell and the appearance of fluorescence was monitored in an adjacent cell, as shown in Figure 19–11. The results for all probes are summarized in Table 19–3. Based on these results, can you suggest a mechanism for how a TMV infection spreads from cell to cell?

TABLE 19–3 Movement of fluorescent probes from one cell to another (Problem 19–37).

Fluorescent probe		Tobacco cells[a]	
Size (daltons)	Radius (nm)	MP^-	MP^+
457	0.6	100%	100%
749	0.7	50%	100%
3900	1.6	14%	100%
9400	2.4	0%	93%
17,200	3.1	0%	0%

[a]The percentage of injections in which the probe was observed to move from the injected cell to the adjacent cell.

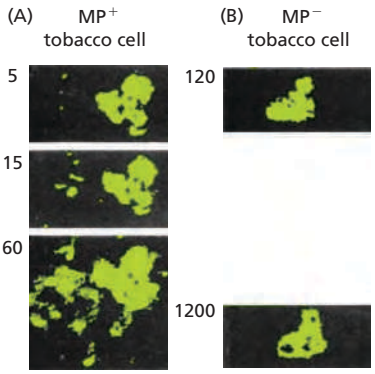


Figure 19–11 Movement of fluorescent probe after injection into a tobacco cell (Problem 19–37). (A) Injection of the 9400-dalton probe into an MP^+ tobacco cell. Fluorescence appears in the adjacent cell over the period of 60 seconds. (B) Injection of the 9400-dalton probe into an MP^- tobacco cell. No fluorescence appears in the adjacent cell within 20 minutes (1200 seconds).

19–38 You want to understand how the density of P-selectin in blood vessel walls affects the rolling interactions of neutrophils when they are subjected to hydrodynamic drag forces in the blood. You introduce P-selectin into a synthetic lipid bilayer and attach it to a glass slide mounted in a flow chamber. This arrangement allows you to measure neutrophil attachment at different densities of P-selectin and at different flow rates.

At high densities of P-selectin, from 40 to 400 molecules per μm^2, the neutrophils attached to the membrane and rolled jerkily in the direction of the flow. At densities from 1 to 15 molecules per μm^2, the cells behaved differently: they either moved at the flow rate of the medium or were transiently tethered before moving again. When the bilayer surface was treated with an antibody against P-selectin, or when EDTA was added to the medium, no tethering occurred.

At the lower densities of P-selectin, the number of tethering events was directly proportional to the density of P-selectin. By recording the results under a video microscope, you measured how long each cell remained attached during a tethering event. A plot of the log of the number of cells remaining bound at increasing times shows that the rate of dissociation of the cells followed a simple exponential decay curve (e^{-kt}) with a "cellular off rate" (k_{off}) of about 1 per second. Moreover, the off rate was unaffected by the density of P-selectin in the range of 1 to 15 molecules per μm^2.

A. What is the point of doing the experiments with antibody or EDTA?
B. Is a single interaction between P-selectin and the glycoprotein ligand on the surface of the neutrophil sufficient to tether the cell to the surface transiently? Explain your reasoning.
C. If you increase the flow rate (hence, the shear force exerted on the cells) by a factor of three—equivalent to about 112 piconewtons—the cellular off rate increases to about 3.5 per second. How do you imagine that force might alter a dissociation rate?

MEDICAL LINKS

19–39 The food-poisoning bacterium *Clostridium perfringens* makes a toxin that binds to various claudins. When the C-terminus of the toxin is bound to a claudin, the N-terminus can insert into the adjacent cell membrane, forming holes that kill the cell. The portion of the toxin that binds to the claudins has proven to be a valuable reagent for investigating the properties of tight junctions. MDCK cells are a common choice for studies of tight junctions because they can form an intact epithelial sheet with high transepithelial resistance. MDCK cells express two claudins: claudin-1, which is not bound by the toxin, and claudin-4, which is.

When an intact MDCK epithelial sheet is incubated with the C-terminal toxin fragment, claudin-4 disappears, becoming undetectable within 24 hours. In the absence of claudin-4, the cells remain healthy and the epithelial sheet appears intact. The mean number of strands in the tight junctions that link the cells also decreases over 24 hours from about four to about two, and they are less highly branched. A functional assay for the integrity of the tight junctions shows that transepithelial resistance decreases dramatically in the presence of the toxin, but the resistance can be restored by washing out the toxin (**Figure 19–12A**). Curiously, the toxin produces these effects only when it is added to the basolateral side of the sheet; it has no effect when added to the apical surface (**Figure 19–12B**).

A. How can it be that two tight-junction strands remain, even though all of the claudin-4 has disappeared?
B. Why do you suppose the toxin works when it is added to the basolateral side of the epithelial sheet, but not when added to the apical side?

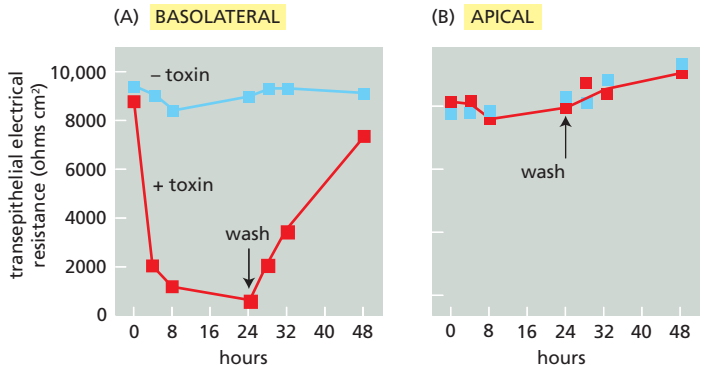


Figure 19–12 Effects of *Clostridium* toxin on the barrier function of the tight junctions in MDCK cells (Problem 19–39). (A) Addition of toxin from the basolateral side of the epithelial sheet. (B) Addition of toxin from the apical side of the epithelial sheet. Higher resistance (ohms cm^2) gives less paracellular current.

THE EXTRACELLULAR MATRIX OF ANIMALS

TERMS TO LEARN

basal lamina (basement membrane)
collagen
collagen fibril
elastic fiber
elastin
fibril-associated collagen
fibrillar collagen
fibroblast
fibronectin
glycosaminoglycan (GAG)
hyaluronan
laminin
matrix metalloprotease
proteoglycan
RGD sequence
serine protease
type III fibronectin repeat
type IV collagen

DEFINITIONS

Match each definition below with its term from the list above.

19–40 Fibrous protein rich in glycine and proline that, in its many forms, is a major component of the extracellular matrix and connective tissues.

19–41 An extracellular proteolytic enzyme that degrades proteins such as collagen, laminin, and fibronectin in a Ca^{2+}- or Zn^{2+}-dependent reaction.

19–42 Extracellular matrix protein found in basal laminae, where it forms a sheetlike network.

19–43 General name for a long, linear, highly charged polysaccharide—composed of a repeating pair of sugars, one of which is always an amino sugar—that is found covalently linked to a protein core in the extracellular matrix.

19–44 Extracellular matrix protein that binds to cell-surface integrins to promote adhesion of cells to the matrix and to provide guidance to migrating cells in developing tissues.

19–45 Hydrophobic protein that forms extracellular extensible fibers that give tissues their stretchability and resilience.

19–46 Common cell type in connective tissue that secretes an extracellular matrix rich in collagen and other extracellular matrix macromolecules.

19–47 Thin mat of extracellular matrix that separates epithelial sheets, and many other types of cells such as muscle or fat cells, from connective tissue.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

19–48 The extracellular matrix is a relatively inert scaffolding that stabilizes the structure of tissues.

19–49 The elasticity of elastin derives from its high content of α helices, which act as molecular springs.

19–50 In humans, all forms of fibronectin are produced from one large gene by alternative splicing.

19–51 A sheet of basal lamina underlies all epithelia.

19–52 A proteoglycan in the basal lamina of the kidney glomerulus plays a critical role in filtering the molecules that pass from the bloodsteam into the urine.

19–53 Several forms of muscular dystrophy (muscle-wasting diseases) arise from defective components in the basal lamina that surrounds muscle fibers.

THOUGHT PROBLEMS

19–54 Carboxymethyl Sephadex, which is commonly used for purifying proteins, is a negatively charged cross-linked dextran with properties similar to glycosaminoglycans. Sephadex comes in the form of dry beads that swell tremendously when added to water. You packed a chromatography column with the swollen gel. When you start equilibrating the column with a buffer that contains 50 mM NaCl at neutral pH, you are alarmed to see a massive shrinkage in gel volume. Why does the dry Sephadex swell so dramatically when it is placed in water? Why does the swollen gel shrink so much when a salt solution is added?

19–55 At body temperature, L-aspartate in proteins racemizes to D-aspartate at an appreciable rate. Most proteins in the body have very low levels of D-aspartate, if it can be detected at all. Elastin, however, has a fairly high level of D-aspartate. Moreover, the amount of D-aspartate increases in direct proportion to the age of the person from whom the sample was taken. Why do you suppose that most proteins have little if any D-aspartate, while elastin has levels of D-aspartate that increase steadily with age?

19–56 Some have speculated that Abraham Lincoln had Marfan's syndrome, mainly due to his height (6'4" when the average was 5'6") and his thin physique. What protein is mutated in patients who suffer from Marfan's syndrome?

19–57 The glycosaminoglycan polysaccharide chains that are linked to specific core proteins to form the proteoglycan components of the extracellular matrix are highly negatively charged. How do you suppose these negatively charged polysaccharide chains help to establish a hydrated gel-like environment around the cell? How would the properties of these molecules differ if the polysaccharide chains were uncharged?

19–58 The unusually thick basal lamina of the kidney glomerulus is a key component of the complex molecular filter that controls passage of solutes into the urine. Typically, 180 L of fluid are filtered through the kidney each day, but most is reabsorbed, with only about 1.5 L being released as urine. The initial filtration is size, shape, and charge selective.

A. The effective pore size is smaller for negatively charged solutes than it is for positively charged ones of the same size. What features of the basal

lamina do you suppose might contribute to this difference in filtration of charged molecules?

B. For neutral solutes of the same molecular weight, the effective pore size is smaller for spherical molecules than for elongated molecules. What do you suppose is the basis for this shape selectivity?

19–59 Discuss the following statement: "The basal lamina of muscle fibers serves as a molecular bulletin board, in which adjoining cells can post messages that direct the differentiation and function of the underlying cells."

19-60 Certain bacteria secrete enzymes that can digest protein or carbohydrate components of the basal lamina. Why do you suppose they do so?

DATA HANDLING

19–61 Hyaluronan, a polysaccharide composed of strictly alternating *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) residues, is spun out directly from the cell surface by hyaluronan synthase, an enzyme embedded in the plasma membrane. Studies with different hyaluronan synthases have failed to agree on such basic questions as whether new sugars are added one at a time or as disaccharide units, and whether they are added to the reducing or nonreducing end of the polysaccharide chain.

You are studying the enzyme from *Pasteurella multocida*. When this enzyme is expressed in *E. coli*, the bacteria spin out a capsule of hyaluronan. To study the reaction mechanism, you use purified membrane fragments containing the recombinant hyaluronan synthase and add a radio-labeled tetrasaccharide acceptor (**Figure 19–13A**), along with the activated forms of the sugar subunits: UDP-*N*-acetylglucosamine and UDP-glucuronic acid. In short incubations with the complete mixture, the tetrasaccharide primer elongates by several units, although it appears that only odd-numbered chains are produced (**Figure 19–13B**, lane 2). If you leave out one or both activated sugars, you get the results shown in Figure 19–13B, lanes 3–5.

A. Based on these data, would you say that the enzyme normally adds residues one at a time or as disaccharide units? Explain your reasoning.

B. Why do you suppose that only odd-number chains are visible in your assay?

C. Does the enzyme add sugar units to the reducing end or to the nonreducing end of the tetrasaccharide acceptor?

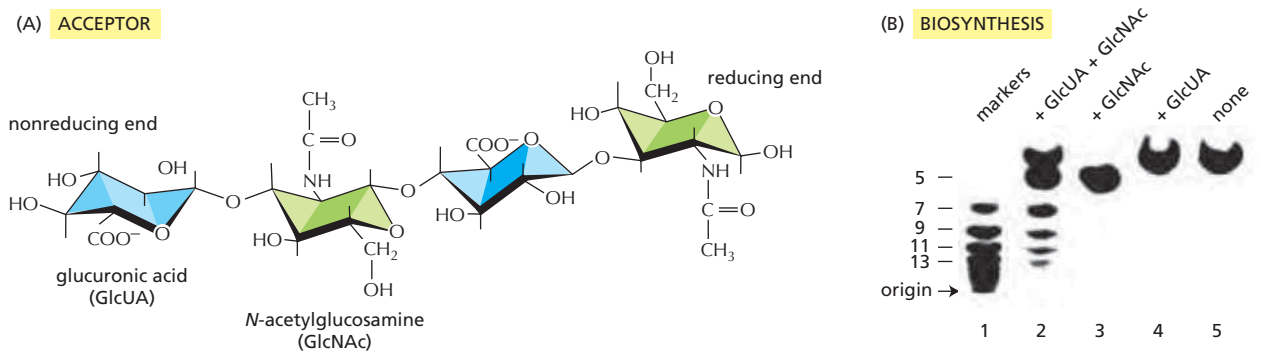


Figure 19–13 Synthesis of hyaluronan by hyaluronan synthase (Problem 19–61). (A) Tetrasaccharide acceptor. The reducing and nonreducing ends of the oligosaccharide are indicated. (B) Synthesis of polysaccharide chains by hyaluronan synthase. After incubation of the ^{3}H-labeled tetrasaccharide primer with hyaluronan synthase and one or both of the activated sugar monomers, the different-length products were separated by thin-layer chromatography and visualized by fluorography. Numbers on the left indicate the length, in sugar residues, of the polysaccharides in the marker lane. GlcNAc is *N*-acetylglucosamine, GlcUA is glucuronic acid.

TABLE 19–4 Fibronectin-related peptides tested for their ability to promote cell sticking (Problem 19–62).

Peptide	Sequence	Concentration required for 50% cell attachment (nM)
Fibronectin		0.10
Peptide 1	YAVTGRGDSPASSKPISINYRTEIDKPSQM(C)*	0.25
Peptide 2	VTGRGDSPASSKPI(C)	1.6
Peptide 3	SINYRTEIDKPSQM(C)	>100
Peptide 4	VTGRGDSPA(C)	2.5
Peptide 5	SPASSKPIS(C)	>100
Peptide 6	VTGRGD(C)	10
Peptide 7	GRGDS(C)	3.0
Peptide 8	RGDSPA(C)	6.0
Peptide 9	RVDSPA(C)	>100

*The (C) at the C-terminus indicates the cysteine linkage to the carrier protein.

19–62 Binding of fragments and competition for binding can be used to identify the portion of a larger ligand that is critical for binding. Fibronectin, which is a large glycoprotein component of the extracellular matrix, binds to fibronectin receptors on cell surfaces. Fibronectin can stick cells to the surface of a plastic dish, to which they would otherwise not bind, forming the basis of a simple binding assay. By attaching small fragments of fibronectin to dishes, researchers identified the cell-binding domain as a 108-amino-acid segment about three-quarters of the way from the N-terminus.

Synthetic peptides corresponding to different portions of the 108-amino-acid segment were then tested in the cell-binding assay to localize the active region precisely. Two experiments were conducted. In the first, peptides were linked covalently to plastic dishes via a disulfide bond to an attached carrier protein, and then tested for their ability to promote cell sticking (**Table 19–4**). In the second experiment, plastic dishes were coated with native fibronectin, and cells that stuck to the dishes in the presence of the synthetic peptides were counted (**Table 19–5**).

A. The two experiments used different assays to detect the cell-binding segment of fibronectin. Does the sticking of cells to the dishes mean the same thing in both assays? Explain the difference between the assays.
B. From the results in Tables 19–4 and 19–5, deduce the amino acid sequence in fibronectin that is recognized by the fibronectin receptor.
C. How might you make use of these results to design a method for isolating the fibronectin receptor?

TABLE 19–5 Fibronectin-related peptides tested for their ability to block cell sticking (Problem 19–62).

Peptide	Percent of input cells sticking
GRGDSPC	2.0
GRGDAPC	1.9
GKGDSPC	48
GRADSPC	49
GRGESPC	44
None	47

19–63 It is not an easy matter to assign particular functions to specific components of the basal lamina, since the overall structure is a complicated composite material with both mechanical and signaling properties. Nidogen, for example, cross-links two central components of the basal lamina by binding to the laminin γ-1 chain and to type IV collagen. Given such a key role, it was surprising that mice with a homozygous knockout of the gene for nidogen-1 were entirely healthy, with no abnormal

TABLE 19–6 Phenotypes of mice with genetic defects in components of the basal lamina (Problem 19–63).

Protein	Genetic defect	Phenotype
Nidogen-1	Gene knockout (–/–)	None
Nidogen-2	Gene knockout (–/–)	None
Laminin γ-1	Nidogen binding-site deletion (+/–)	None
Laminin γ-1	Nidogen binding-site deletion (–/–)	Dead at birth

+/– stands for heterozygous, –/– stands for homozygous.

phenotype. Similarly, mice homozygous for a knockout of the gene for nidogen-2 also appeared completely normal. By contrast, mice that were homozygous for a defined mutation in the gene for laminin γ-1, which eliminated just the binding site for nidogen, died at birth with severe defects in lung and kidney formation. The mutant portion of the laminin γ-1 chain is thought to have no other function than to bind nidogen, and does not affect laminin structure or its ability to assemble into the basal lamina. How would you explain these genetic observations, which are summarized in **Table 19–6**? What would you predict would be the phenotype of a mouse that was homozygous for knockouts of both nidogen genes?

19–64 The basal lamina normally provides an impenetrable barrier to cells, but cells such as lymphocytes and macrophages are able to cross the barrier by digesting the components of the lamina using matrix metalloproteases (MMPs). This family of proteases is implicated in normal bodily functions, as well as many diseases. For example, in order for cancer cells to metastasize they have to penetrate the basal lamina. Because of their fundamental importance to basic science and clinical medicine, MMPs have been studied extensively, and many mouse knockouts have been made.

Thus far, these studies have mainly highlighted the complexity of MMP functions. Take the case of MT1-MMP, which is anchored to the cell membrane. MT1-MMP null mice show skeletal abnormalities, grow slowly at birth, and usually die within a few weeks. Cells derived from these animals cannot penetrate collagen gels (unlike their normal counterparts). One consequence is a complete lack of white adipose tissue (**Figure 19–14**). The adipocytes in the mutant mice are very small compared to those in wild-type mice, and they appear to be trapped in a tangle of collagen fibers. In addition, DNA microarray analysis of their mRNAs shows that they have not fully differentiated. The link between MT1-MMP deficiency and failure of adipocytes to differentiate is not understood. Suggest some possible explanations for how a lack of MT1-MMP might block adipocyte differentiation.

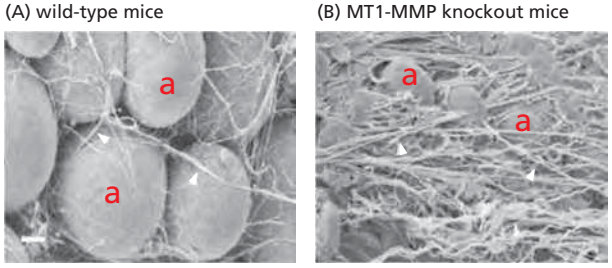


Figure 19–14 Adipocytes in normal and MT1-MMP knockout mice (Problem 19–64). (A) Wild-type mice. (B) MT1-MMP knockout mice. A few adipocytes are labeled with "a." *White arrowheads* point to collagen fibers. Scale bar is 10 μm. Both micrographs are shown at the same magnification.

MEDICAL LINKS

19–65 Defects in collagen genes are responsible for several inherited diseases, including osteogenesis imperfecta, a disease characterized by brittle bones, and Ehlers–Danlos syndrome, which can lead to sudden death due to ruptured internal organs or blood vessels. In both diseases, the medical problems arise because the defective gene in some way compromises the function of collagen fibrils. For example, homozygous deletions of the type I collagen *α1(I)* gene eliminates α1(I) collagen entirely, thereby preventing formation of any type I collagen fibrils. Such homozygous mutations are usually lethal in early development. The more common situation is for an individual to be heterozygous for the mutant gene, having one normal gene and one defective gene. Here the consequences are less severe.

A. Type I collagen molecules are composed of two copies of the α1(I) chain and one copy of the α2(I) chain. Calculate the fraction of type I collagen molecules, $[\alpha1(I)]_2\alpha2(I)$, that will be normal in an individual who is heterozygous for a deletion of the entire *α1(I)* gene. Repeat the calculation for an individual who is heterozygous for a point mutation in the *α1(I)* gene.

B. Type III collagen molecules are composed of three copies of the α1(III) chain. Calculate the fraction of type III collagen molecules, $[\alpha1(III)]_3$, that will be normal in an individual who is heterozygous for a deletion of the entire *α1(III)* gene. Repeat the calculation for an individual who is heterozygous for a point mutation in the *α1(III)* gene.

C. Which kind of collagen gene defect—deletion or point mutation—is more likely to be dominant (that is, to cause the heterozygote to display a mutant phenotype)?

CELL–MATRIX JUNCTIONS

TERMS TO LEARN

anchorage dependence
focal adhesion kinase (FAK)

DEFINITIONS

Match each definition below with its term from the list above.

19–66 Cytoplasmic protein tyrosine kinase present at cell–matrix junctions in association with the cytoplasmic tails of integrins.

19–67 Dependence of cell growth, proliferation, and survival on attachment to a substratum.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

19–68 Integrins can convert mechanical signals into intracellular molecular signals.

19–69 Various types of integrins connect extracellular binding sites to all the different kinds of cytoskeletal elements, including actin, microtubules, and intermediate filaments.

19–70 Integrins are thought to be rigid rods that span the membrane and link binding sites outside the cell to those inside the cell.

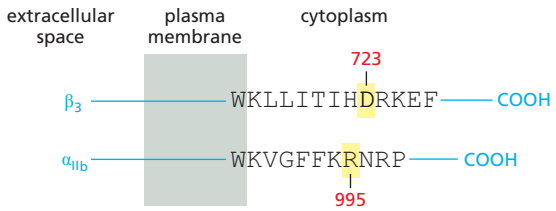


Figure 19–15 Schematic representation of $\alpha_{IIb}\beta_3$ integrin (Problem 19–71). The D723 and R995 residues are indicated.

THOUGHT PROBLEMS

19–71 The affinity of integrins for matrix components can be modulated by changes to their cytoplasmic domains: a process known as inside-out signaling. You have identified a key region in the cytoplasmic domains of $\alpha_{IIb}\beta_3$ integrin that seems to be required for inside-out signaling (**Figure 19–15**). Substitution of alanine for either D723 in the β chain or R995 in the α chain leads to a high level of spontaneous activation, under conditions where the wild-type chains are inactive. Your advisor suggests that you convert the aspartate in the β chain to an arginine (D723R) and the arginine in the α chain to an aspartate (R995D). You compare all three α chains (R995, R995A, and R995D) against all three β chains (D723, D723A, and D723R). You find that all pairs have a high level of spontaneous activation, except D723 vs R995 (the wild type) and D723R vs R995D, which have low levels. Based on these results, how do you think the $\alpha_{IIb}\beta_3$ integrin is held in its inactive state?

CALCULATIONS

19–72 Platelets are flat, disclike cells about 2 μm in diameter. Estimates of the number of integrin molecules on their surface vary around a mean of about 80,000. If the integrins themselves are about 10 nm in diameter, how tightly packed are they? (Assume that the total membrane area is $2\pi r^2$.)

DATA HANDLING

19–73 The ability of a cell to control integrin–ligand interactions from within is termed inside-out signaling. The major surface protein of blood platelets, $\alpha_{IIb}\beta_3$ integrin, binds to fibrinogen when platelets are stimulated with clotting factors such as thrombin. By binding to a receptor on the cell surface, thrombin triggers an intracellular signaling pathway that activates $\alpha_{IIb}\beta_3$ integrin, allowing platelets to aggregate to form blood clots. Platelets do not bind fibrinogen or aggregate until stimulated, although $\alpha_{IIb}\beta_3$ integrin is always present on their surface. What regulates the activity of this all-important integrin?

If the genes for the subunits of $\alpha_{IIb}\beta_3$ are expressed in Chinese hamster ovary (CHO) cells, the cells fail to aggregate when incubated with fibrinogen in the presence or absence of thrombin. If the cells are first incubated with MAb 62 antibodies, which bind to $\alpha_{IIb}\beta_3$ integrin and activate it, the cells aggregate within minutes of adding fibrinogen. CHO cells without $\alpha_{IIb}\beta_3$ do not aggregate when treated this way.

By deleting the short cytoplasmic domains of α_{IIb} and β_3, various combinations of truncated and wild-type α_{IIb} and β_3 chains can be tested in CHO cells. All combinations of the α_{IIb} and β_3 chains allow cells to aggregate in the presence of fibrinogen and MAb 62; however, the truncated α_{IIb} chain allows aggregation even in the absence of MAb 62 (**Table 19–7**).

A. Why do you suppose that truncating the cytoplasmic domain of the α_{IIb} subunit increases the affinity of the integrin for fibrinogen and allows the cells to aggregate?

TABLE 19–7 Fibrinogen-dependent aggregation of CHO cells expressing various wild-type and mutant α_{IIb} and β_3 subunits (Problem 19–73).

		Aggregation	
α_{IIb} chain	β_3 chain	Without MAb 62	With MAb 62
Normal	Normal	–	+++
Truncated	Normal	+++	+++
Normal	Truncated	–	+++
Truncated	Truncated	+++	+++

B. The $\alpha_{IIb}\beta_3$ integrin is accessible on the surface of the CHO cells, as revealed by the various aggregation studies. Why, then, does thrombin not stimulate the cells to aggregate?
C. There are two genes for α_{IIb} in diploid human cells. If one of the two genes suffered a truncation of the kind described in this problem, do you think the individual would show any blood-clotting problems?

THE PLANT CELL WALL

TERMS TO LEARN

cellulose
cellulose microfibril
cross-linking glycan
lignin
pectin
primary cell wall
secondary cell wall
turgor pressure

DEFINITIONS

Match each definition below with its term from the list above.

19–74 Thin and extensible cell-covering on new plant cells that can accommodate their growth.

19–75 Bundle of about 40 long, linear chains of covalently linked glucose residues, all with the same polarity, organized in an overlapping parallel array.

19–76 The large internal hydrostatic pressure that develops in plant cells due to the osmotic imbalance between the cell interior and the fluid in the plant cell wall.

19–77 A complex network of phenolic compounds that is an abundant polymer in secondary cell walls.

19–78 Rigid cell-covering laid down in layers inside the initial covering once cell growth has stopped.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

19–79 Each cell wall consists of a thin, semirigid primary cell wall adjacent to the cell membrane and a thicker, more rigid secondary cell wall outside the primary wall.

19–80 Turgor pressure is the main driving force for cell expansion during growth, and it provides much of the mechanical rigidity of living plant tissues.

19–81 Unlike the extracellular matrix of animal cells, which contains a large amount of protein, plant cell walls are composed entirely of polysaccharides.

19–82 If the entire cortical array of microtubules were disassembled by drug treatment, new cellulose microfibrils would be laid down in random orientations.

THOUGHT PROBLEMS

19–83 Your boss is coming to dinner! All you have for a salad is some wilted, day-old lettuce. You vaguely recall that there is a trick to rejuvenating wilted lettuce, but you can't remember what it is. Should you soak the lettuce in salt water, soak it in tap water, or soak it in sugar water, or maybe just shine a bright light on it and hope that photosynthesis will perk it up?

19–84 In plant cells, the cortical array of microtubules determines the orientation of cellulose microfibrils, which in turn fixes the direction of cell expansion. Cells elongate perpendicular to the orientation of the cellulose microfibrils. The plant growth factors ethylene and gibberellic acid have opposite effects on the orientation of microtubule arrays in epidermal cells of young pea shoots. Gibberellic acid promotes an orientation of the cortical microtubule array that is perpendicular to the long axis of the cell, whereas ethylene treatment causes the microtubule arrays to orient parallel to the long axis of the cell (**Figure 19–16**).

Which treatment do you think would produce short, fat shoots, and which would produce long, thin shoots?

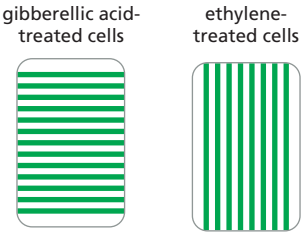


Figure 19–16 Effects of gibberellic acid and ethylene on the orientation of cortical arrays of microtubules (Problem 19–84).

CALCULATIONS

19–85 A plant must be able to respond to changes in the water status of its surroundings. It does so by the flow of water molecules through water channels called aquaporins. The hydraulic conductivity of a single aquaporin is 4.4×10^{-22} m^3 per second per MPa (megapascal) of pressure. What does this correspond to in terms of water molecules per second at atmospheric pressure? [Atmospheric pressure is 0.1 MPa (1 bar) and the concentration of water is 55.5 M.]

DATA HANDLING

19–86 The synthesis of cellulose is simple from a chemical standpoint: UDP-glucose polymerizes to form cellulose, with release of UDP, which is recycled to form more UDP-glucose. Although cellulose is the most abundant macromolecule on Earth, purifying cellulose synthase from plants proved impossible for many years. Success came from studying bacteria such as *Acetobacter xylinum* that make large amounts of pure cellulose under the right conditions. Curiously, this bacterium requires the signaling dinucleotide, cyclic di-GMP, for full activation of cellulose synthesis (**Figure 19–17A**). Active cellulose synthase can be isolated from detergent-extracted bacterial cell walls and readily purified since it gets trapped in its own insoluble cellulose product, rather like a silkworm in its cocoon. To identify the cellulose synthase, you use the affinity label ^{32}P-azido-UDP-glucose. When exposed to ultraviolet (UV) light, the azido moiety forms a cross-link to any protein that binds the affinity label.

You test the affinity label with the detergent-solubilized cell walls and with the purified enzyme, with the results shown in **Figure 19–17B**. Two major bands show labeling: a 57-kd band in the soluble fraction and an 83-kd band in the purified fraction. Both correspond to visible bands on

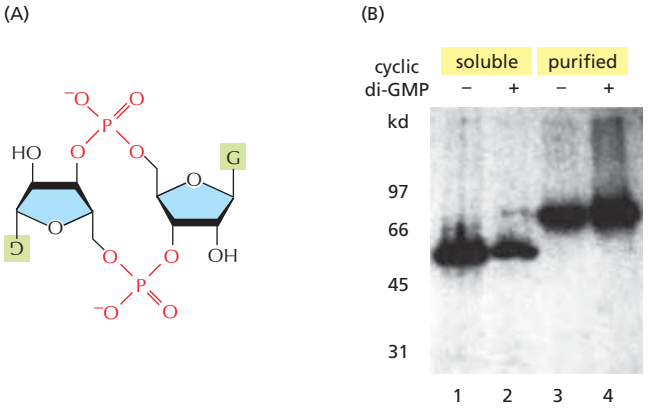


Figure 19–17 Cellulose synthase (Problem 19–86). (A) The structure of cyclic di-GMP, the activator of cellulose synthase. (B) Photoaffinity labeling of cellulose synthase using ^{32}P-azido-UDP-glucose activated by UV light. Lanes 1 and 2 are detergent-solubilized cell walls; lanes 3 and 4 are a fraction purified from the cellulose product. Positions of markers of known molecular mass are indicated on the left in kilodaltons. The presence or absence of added cyclic di-GMP is indicated at the top by plus and minus symbols.

the stained gel. If you omit UV light, the 57-kd band still gets labeled, but the 83-kd band does not. Addition of large amounts of the unlabeled natural substrate, UDP-glucose, blocked labeling of the 83-kd band but not labeling of the 57-kd band.

Which band most likely corresponds to the cellulose synthase? In the explanation of your answer, include the response to cyclic di-GMP, the results in the presence and absence of UV light, and the effects of excess UDP-glucose.

MCAT STYLE

Passage 1 (Questions 19–87 to 19–88)

Metastasis occurs when cells from a primary tumor invade and colonize other tissues. Metastasis is a complex, multistep process. Tumor cells must lose adhesion with other tumor cells, invade local tissues and vessels, move through the circulation, leave the vessels, and establish new colonies at distant sites. Tumor cells gain the ability to cross epithelial layers and migrate through tissues by mutations, although the nature of the mutations that drive metastasis is poorly understood.

Mutations that block expression of the *E-cadherin* gene are thought to be an important step in metastasis. To better understand how loss of E-cadherin contributes to metastasis, scientists created two cell lines that differed in their expression of E-cadherin. One cell line expressed normal E-cadherin, but at 10% of the usual levels. The other cell line expressed normal E-cadherin at the usual levels, and also, at high levels a mutant form that included the cytoplasmic domain but lacked the rest of the protein. Both cell lines exhibited strongly reduced cell adhesion in culture. However, only the cell line with reduced expression of normal E-cadherin metastasized when introduced into mice.

19–87 Which of the following hypotheses is most consistent with the observations on cell adhesion and metastasis in these cell lines?

A. Loss of E-cadherin releases signaling proteins that normally bind to its cytoplasmic domain, but promote metastasis when free.
B. The cytoplasmic domain of E-cadherin binds proteins required for cell adhesion, but those proteins are not involved in metastasis.
C. The E-cadherin transmembrane domain by itself is sufficient to promote the cell–cell adhesion that prevents metastasis.
D. The loss of adhesion caused by inactivation of E-cadherin is sufficient to explain how E-cadherin mutations promote metastasis.

19–88 The promotion of metastasis by the loss of E-cadherin suggests that E-cadherin inhibits metastasis. Which one of the following types of proteins are most likely involved in the inhibition of metastasis by E-cadherin?

A. Catenins
B. Laminins

C. Metalloproteases
D. Talins

Passage 2 (Questions 19–89 to 19–91)

Metastasis requires that cancer cells gain the ability to colonize and survive in a completely new tissue environment. To better understand metastasis, one study characterized three different mouse mammary carcinoma cell lines, which were engineered to express a fluorescent marker. Investigators injected the cell lines into the mouse tail vein and followed their ability to colonize the lung. When cells from lines A and B were injected, they were found in lung tissues—indicating that they were able to invade the tissue—but they did not proliferate. In contrast, cells from line C were able to invade lung tissue and proliferate, and they formed metastases efficiently.

To investigate the basis for the different metastatic abilities of the cell lines, the investigators cultured the three cell lines in two ways: on standard tissue-culture plates, which provide a surface that can bind to a cell's adhesion proteins; and in Matrigel®, which is a commonly used extracellular matrix preparation. Plates are often referred to as a two-dimensional, or 2D, culture condition, whereas Matrigel, which provides a matrix similar to that in tissues, is referred to as a three-dimensional, or 3D, culture condition.

In 2D cultures, all three cell lines proliferated. In 3D culture conditions, however, only cell line C proliferated. In addition, the three cell lines showed different morphologies when grown under 3D culture conditions (**Figure 19–18**). The A cells were rounded and poorly adhesive. The B cells were flattened and elongated. The C cells were more elongated and flattened, and they made multiple attachments to the 3D matrix via extensions of the plasma membrane called filopodia.

19–89 Which of the following molecules would you expect to be present in Matrigel, but not in standard tissue culture medium?

I. Collagen
II. Integrin
III. Laminin

A. I and II
B. I and III
C. II and III
D. I, II, and III

19–90 Which one of the following hypotheses is most consistent with the data?

A. All three cell lines secrete an extracellular matrix component that is necessary for metastasis.
B. Only cell line C secretes an extracellular matrix component that is necessary for metastasis.
C. Metastasis of cell line C is due to a difference in interactions with the extracellular matrix.
D. Metastasis of cell line C is due to an inability to interact with the extracellular matrix.

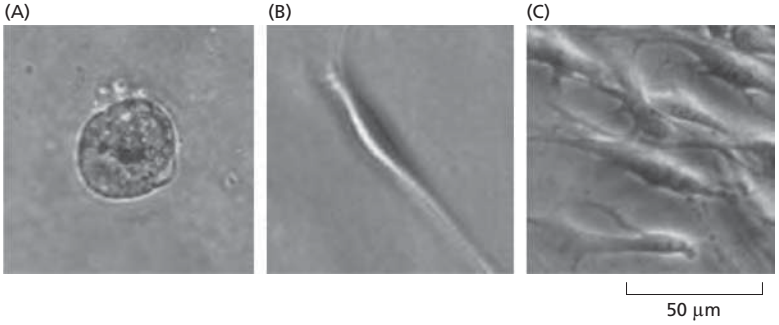


Figure 19–18 Morphologies of three cell lines grown in Matrigel (Problems 19–89 to 19–91). Cell lines A, B, and C are displayed in panels (A), (B), and (C), respectively.

19–91 The investigators searched for adhesion molecules that might generate the signals controlling the different behaviors of the cells. Which of the following proteins do you suppose would be the best candidate for the relevant adhesion molecule?

A. Collagen
B. Fibronectin
C. Integrin
D. Laminin

CHAPTER
20

Cancer

IN THIS CHAPTER

CANCER AS A MICROEVOLUTIONARY PROCESS

TERMS TO LEARN

benign
carcinogenesis
carcinoma
chemical carcinogen
genetically unstable
leukemia
lymphoma
malignant
metastases
primary tumor
replicative cell senescence
sarcoma
somatic mutation
stroma
transformed
tumor progression

DEFINITIONS

Match each definition below with its term from the list above.

20–1 The generation of a cancer.

20–2 Describes a tumor or tumor cell that can invade surrounding tissue or form tumors at other sites in the body.

20–3 A cancer arising from connective tissue or muscle cells.

20–4 The neoplasm from which metastases were originally derived.

20–5 The process by which an initially mild disorder of cell behavior gradually evolves into a full-blown cancer.

20–6 Describes cells that accumulate genetic changes at an abnormally rapid rate.

20–7 Describes a tumor that is self-limiting in its growth and noninvasive.

20–8 Phenomenon observed in primary cell cultures as they age, in which cell proliferation slows down and finally halts.

20–9 A cancer arising from epithelial cells.

20–10 A change in DNA sequence that distinguishes a cell from its normal neighbors in a tissue.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

20–11 All the various cell types in a typical carcinoma, including fibroblasts, inflammatory cells, and blood vessels, evolve from the cancer cell population.

20–12 Genetic instability in the form of point mutations, chromosome rearrangements, and epigenetic changes needs to be maximal to allow the development of cancer.

20–13 Cancer cells consume glucose more rapidly than their normal neighbors because they need much more energy (ATP) to drive their high rates of proliferation.

THOUGHT PROBLEMS

20–14 The incidence of colon cancer increases with age, as shown in **Figure 20–1**, where the number of newly diagnosed cases in women in 1 year is plotted as a function of age at diagnosis. Studies of many other types of cancer show the same sort of age dependence. Assuming that the rate of mutation is constant throughout life, why do you suppose the incidence of cancer increases so dramatically with age?

20–15 In contrast to colon cancer, whose incidence increases dramatically with age, incidence of osteosarcoma—a tumor that occurs most commonly in the long bones—peaks during adolescence. Osteosarcomas are relatively rare in young children (up to age 9) and in adults (over 20). Why do you suppose that the incidence of osteosarcoma does not show the same sort of age-dependence as colon cancer?

20–16 As shown in **Figure 20–2**, plots of deaths due to breast cancer and cervical cancer in women differ dramatically from the same plot for colon cancer. At around age 50, the age-dependent increase in death rates for breast and cervical cancer slows markedly, whereas death rates due to colon cancer (and most other cancers) continue to increase. Why do you suppose that the age-dependent increases in death rates for breast and cervical cancer slow after age 50?

CALCULATIONS

20–17 The progressive shortening of telomeres in proliferating human somatic cells limits the number of cell divisions to about 50. It has been suggested that this limitation restricts the maximum size of tumors, thus affording some protection against cancer. Assuming that 10^8 cells have a mass of 1 gram, calculate the mass of a tumor that originated from 50 doublings of a single cancerous cell.

20–18 Tumor progression—the gradual accumulation of mutations in five or six different genes—provides a natural explanation for the rapid rise in cancer incidence with increasing age. Although this idea is well accepted, it is not the only possible explanation. More than 50 years ago, an entirely different idea was proposed. The central hypothesis was that five or six cancer cells had to be in contact with one another before they could begin to proliferate. (Framed in modern terms, you might imagine that an autocrine growth factor produced by the tumor cells was needed for their growth. Individual cells produced too little to be effective, whereas a small clump of cells secreted enough to trigger their own proliferation.)

If the probability of any cell mutating to a cancer cell is x, the probability of it being surrounded by n similarly mutated cells is x^n. In a tissue of N cells, the probability of having a critical cluster of cancer cells is

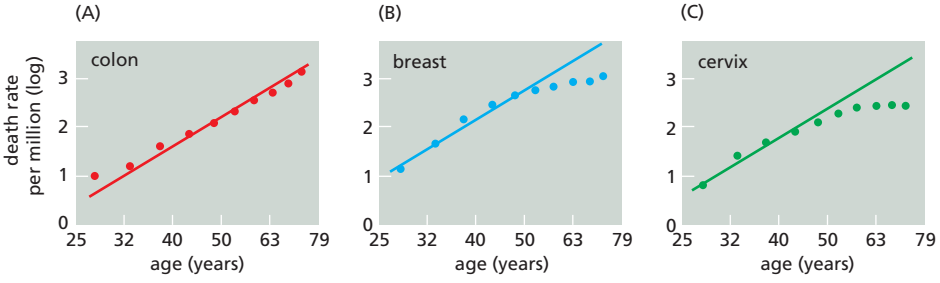


Figure 20–2 Cancer death rates as a function of age (Problem 20–16). (A) Death rates for colon cancer in females. (B) Death rates for breast cancer in females. (C) Death rates for cervical cancer. The data in all cases are plotted as log of the death rate versus the patient age (on a log scale) at death. The data for colon cancer, displayed on a linear–linear plot, would give the same shape of curve as shown in Figure 20–1. The straight lines in (B) and (C) are fit to the data for the earlier age groups, whereas the line in (A) is fit to all the data points.

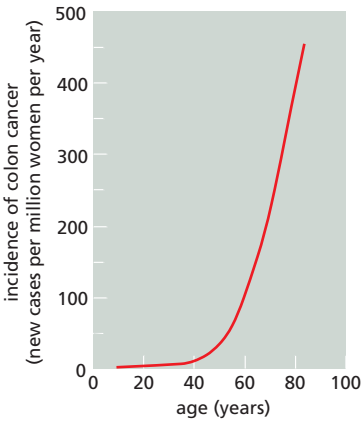


Figure 20–1 Colon cancer incidence as a function of age (Problem 20–14).

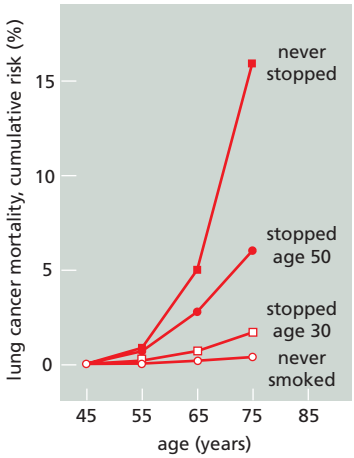


Figure 20–3 Cumulative risk of lung cancer mortality for nonsmokers, smokers, and former smokers (Problem 20–19). Cumulative risk is the running total of deaths, as a percentage, for each group. Thus, for continuing smokers, 1% died of lung cancer between ages 45 and 55; an additional 4% died between 55 and 65 (giving a cumulative risk of 5%); and 11% more died between 65 and 75 (for a cumulative risk of 16%).

Nx^n. Suppose, for the sake of argument, that 1% of the cells in a tissue are mutated, that five of these cells must be in contact to initiate a cancer, and that there are 10^9 cells in the tissue. Given these parameters, there would be 0.1 critically sized cancer colony $[10^9 \times (10^{-2})^5 = 0.1]$. After a doubling in age (hence doubling the number of mutations) there would be 3.2 cancer colonies $[10^9 \times (2 \times 10^{-2})^5 = 3.2]$. This equation predicts that the incidence of a cancer will increase rapidly with age, in much the same way as it would in the tumor-progression model.

In its simplest form, this hypothesis is ruled out by the following experimental observation: an applied chemical carcinogen induces cancers in direct proportion to its concentration. This means that increasing the concentration of carcinogen by a factor of two doubles the number of cancers; increasing it by a factor of four quadruples the number of cancers.

A. How does the linear dependence of cancer on the concentration of chemical carcinogen rule out the cell-cluster model for cancer formation?

B. How is the linear relationship between cancer and chemical carcinogen concentration explained in the tumor-progression model?

DATA HANDLING

20–19 Mortality due to lung cancer was followed in groups of males in the United Kingdom for 50 years. **Figure 20–3** shows the cumulative risk of dying from lung cancer as a function of age and smoking habits for four groups of males: those who never smoked, those who stopped at age 30, those who stopped at age 50, and those who continued to smoke. These data show clearly that individuals can substantially reduce their cumulative risk of dying from lung cancer by stopping smoking. What do you suppose is the biological basis for this observation?

MEDICAL LINKS

20–20 By 1950 it was clear that patients with lung cancer included more heavy smokers than nonsmokers, an association that was not apparent in other diseases. At the time, some considered that the only reasonable interpretation was that smoking is a factor in the disease; others were not prepared to deduce causation from the association. To resolve the issue of causation, a prospective study was carried out to determine the frequency with which lung cancer appeared, in the future, among people whose smoking habits were already known.

A simple questionnaire was sent to about 60,000 doctors in the United Kingdom; about 40,000 responded. Roughly 16,000 were not used because they were from women or men under 35 years old, who were only rarely affected by lung cancer. A preliminary report was published in 1954, 29 months after the questionnaire was sent out. During that time 789 deaths had occurred among the test group, with 36 deaths attributable to a certified diagnosis of lung cancer. Deaths in each of several diseases were analyzed for four groups: (1) nonsmokers; (2) smokers of 1–14 cigarettes per day; (3) smokers of 15–24 cigarettes per day; and (4) smokers of more than 25 cigarettes per day. The number of deaths in each group was compared with the expected number based on the percentage of all respondents in that group (**Figure 20–4**).

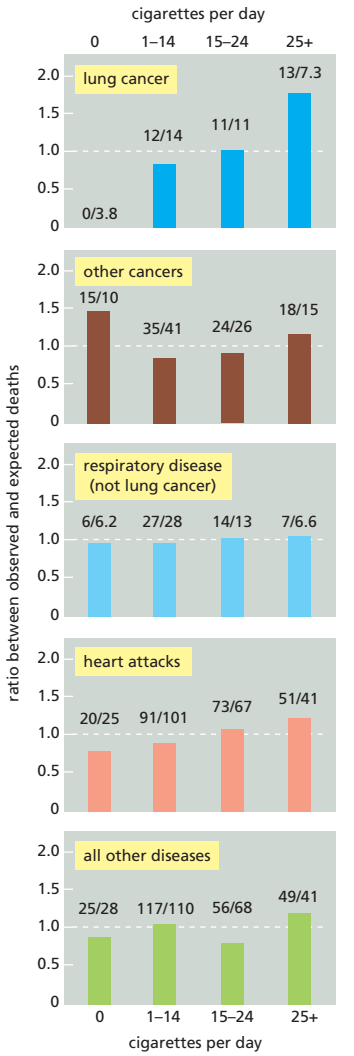


Figure 20–4 Variation in mortality with amount smoked (Problem 20–20). *Numbers* above the bars give the *observed* deaths/*expected* deaths.

Among the diseases examined in this preliminary study, which one(s) appear to correlate with amount of tobacco smoked?

CANCER-CRITICAL GENES: HOW THEY ARE FOUND AND WHAT THEY DO

TERMS TO LEARN

cancer-critical gene	*p53*	retinoblastoma
cancer stem cell	proto-oncogene	retrovirus
colorectal cancer	*Ras*	tumor suppressor gene
driver	*Rb* gene	tumor virus
oncogene	Rb protein	*v-Ras*
passenger		

DEFINITIONS

Match each definition below with its term from the list above.

20–21 General term for a mutant gene whose overactive form causes cancer.

20–22 General term for a normal gene in which a gain-of-function mutation can drive a cell toward cancer.

20–23 Tumor suppressor gene—found mutated in about half of human cancers—that encodes a transcription regulator that is activated by DNA damage.

20–24 The rare cell associated with a cancer that is capable of indefinite self-renewal and is responsible for maintaining the cancer.

20–25 Rare type of human cancer in which cells of the retina are converted to a cancerous state by an unusually small number of mutations.

20–26 Common carcinoma of the epithelium lining the colon and rectum.

20–27 Any one of a number of genes, in which mutation frequently contributes to the causation or evolution of cancer.

20–28 General term for a normal gene in which a loss-of-function mutation can contribute to cancer.

20–29 A mutation that happens to have occurred in cells that become cancer cells, but is irrelevant to the development of the disease.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

20–30 Oncogenes and tumor suppressor genes can both be detected by introducing fragmented DNA from cancer cells into suitable cell lines and isolating colonies that display cancerous properties.

20–31 The chemical carcinogen dimethylbenz[a]anthracene (DMBA) must be an extraordinarily specific mutagen since 90% of the skin tumors it causes have an A-to-T alteration at exactly the same site in the mutant *Ras* gene.

20–32 Individuals who inherit one normal and one inactive copy of a tumor suppressor gene are more likely to develop cancer than individuals with two normal copies.

20–33 In the cellular regulatory pathways that control cell growth and proliferation, the products of oncogenes are stimulatory components and the products of tumor suppressor genes are inhibitory components.

20–34 When cancer cells from individual patients are analyzed, they are often found to have mutations in multiple components of the Rb pathway (*Rb* itself, along with genes that directly regulate *Rb*), which governs initiation of the cell-division cycle.

20–35 The loss of p53 protein makes some cancer cells much less sensitive to irradiation and to many anticancer drugs, which would otherwise destroy tumors by inducing proliferating cells to either stop dividing or undergo apoptosis.

20–36 Cancers commonly display genome instability; however, instability can appear as chromosome breaks, translocations, and deletions in cancers from one tissue, but mainly as point mutations in cancers from another tissue.

20–37 It is clear from studies in mice that mutagenic activation of a single oncogene is sufficient to convert a normal cell into a cancer cell.

20–38 Cancer therapies directed solely at killing the rapidly dividing cells that make up the bulk of a tumor are unlikely to eliminate the cancer from many patients.

THOUGHT PROBLEMS

20–39 By analogy with automobiles, defects in cancer-critical genes have been likened to broken brakes and stuck accelerators, which are caused in some cases through faulty service by bad mechanics. Using this analogy, decide how oncogenes, tumor suppressor genes, and genome maintenance genes relate to broken brakes, stuck accelerators, and bad mechanics. Explain the basis for each of your choices.

20–40 The *Rb* gene is one example of a category of antiproliferative genes in humans. Typically, when both copies of such genes are lost, cancers develop. Do you suppose that cancer could be eradicated if tumor suppressor genes such as *Rb* could be expressed at high levels in all human cells? What would be the effect on the human? Explain your answers.

20–41 Overexpression of the Myc protein is a common feature of many types of cancer cells, contributing to their excessive cell growth and proliferation. By contrast, when Myc is overexpressed in most normal cells, the result is not excessive proliferation, but cell-cycle arrest or apoptosis. How do you suppose that overexpression of Myc can have such different outcomes in normal cells and in cancer cells?

20–42 About 20% of colorectal cancers have mutations in the *B-Raf* gene. B-Raf is a serine/threonine protein kinase that functions in the Ras–Raf–Mek–Erk–MAP-kinase cascade, which mediates cellular responses to growth signals. When the pathway is stimulated, Ras activates B-Raf by causing a protein kinase to add phosphates to threonine 598 and serine 601. Activated B-Raf then adds phosphates to key residues in Mek to trigger the rest of the pathway and stimulate cell growth. The mutations of *B-Raf* found in cancer cells give rise to a constantly active form of B-Raf that does not need to be phosphorylated by Ras. In one sample of colorectal cancers, 95% of the mutant *B-Raf* genes had glutamate in place of valine at position 599. Why do you suppose that B-Raf with glutamate at position 599 is active?

20–43 Mouse mammary tumor virus (MMTV) is an oncogenic retrovirus that causes breast cancer in mice when it integrates into the genome. You want to know whether it carries its own oncogene into the cell or generates an oncogene upon integration. You isolate 26 different breast cancers from mice that were exposed to MMTV and determine the sites at

TABLE 20–1 Genotypes of progeny mice from crosses between doubly heterozygous *p53*$^{+/-}$ *Mdm2*$^{+/-}$ mice (Problem 20–44).

Genotype	Progeny mice (number)	Progeny mice (expected)
p53$^{+/+}$ *Mdm2*$^{+/+}$	3	
p53$^{+/+}$ *Mdm2*$^{+/-}$	5	
p53$^{+/+}$ *Mdm2*$^{-/-}$	0	
p53$^{+/-}$ *Mdm2*$^{+/+}$	7	
p53$^{+/-}$ *Mdm2*$^{+/-}$	11	
p53$^{+/-}$ *Mdm2*$^{-/-}$	0	
p53$^{-/-}$ *Mdm2*$^{+/+}$	1	
p53$^{-/-}$ *Mdm2*$^{+/-}$	7	
p53$^{-/-}$ *Mdm2*$^{-/-}$	2	

which the retroviruses are integrated. In 18 of 26 tumors, the viruses are found at a variety of sites that are all located in a 20-kb segment of the mouse genome. Upon closer examination of these 18 tumors, you find that an RNA is expressed from the region of the mouse genome near the integrated virus, but not from the corresponding region in normal mouse breast cells. Do these observations argue for MMTV carrying an oncogene or for it generating an oncogene upon integration? Explain your reasoning.

CALCULATIONS

20–44 The *p53* gene encodes a key regulatory protein that can arrest cell growth, induce cell death, or promote cell senescence in response to DNA damage or other types of cell stress. Its central role in governing a cell's response to stress is highlighted by the finding that it is inactivated by mutation in half of all human cancers. Somewhat surprisingly, mice that lack p53 are fine in all respects—except that they develop tumors by 10 months of age. The product of a second gene, *Mdm2*, negatively regulates p53, targeting it for destruction by attaching ubiquitin to it. Your lab is investigating these genes using mouse knockouts. You can generate *Mdm2*$^{+/-}$ mice perfectly well, but when these mice are mated together, no viable *Mdm2*$^{-/-}$ offspring are born. To investigate the genetic interactions between *p53* and *Mdm2*, you generate doubly heterozygous *p53*$^{+/-}$ *Mdm2*$^{+/-}$ mice and mate them together. The genotypes of the progeny mice are shown in **Table 20–1**.

A. The *p53* and *Mdm2* genes are on different chromosomes and thus assort independently during meiosis. Assuming that *p53*$^{+}$*Mdm2*$^{+}$, *p53*$^{+}$*Mdm2*$^{-}$, *p53*$^{-}$*Mdm2*$^{+}$, and *p53*$^{-}$*Mdm2*$^{-}$ haploid gametes are produced at equal frequencies by the male and female parents, calculate how frequently each of the progeny genotypes would be generated by random assortment. Which, if any, of the genotypes appear to be significantly underrepresented among the progeny?

B. How would you interpret the differences in number of progeny expected and actually generated for *p53*$^{+/+}$ *Mdm2*$^{-/-}$, *p53*$^{+/-}$ *Mdm2*$^{-/-}$, and *p53*$^{-/-}$ *Mdm2*$^{-/-}$ mice?

DATA HANDLING

20–45 Now that DNA sequencing is so inexpensive, reliable, and fast, your mentor has set up a consortium of investigators to pursue the ambitious

goal of tracking down *all* the mutations in a set of human tumors. He has decided to focus on breast cancer and colorectal cancer because they cause 14% of all cancer deaths. For each of 11 breast cancers and 11 colorectal cancers, you design primers to amplify 120,839 exons in 14,661 transcripts from 13,023 genes. As controls, you amplify the same regions from DNA samples taken from two normal individuals. You sequence the PCR products and use analytical software to compare the 456 Mb of tumor sequence with the published human genome sequence. You are astounded to find 816,986 putative mutations. This represents more than 37,000 mutations per tumor! Surely that can't be right.

Once you think about it for a while, you realize the computer sometimes makes mistakes in calling bases. To test for that source of error, you visually inspect every sequencing read and find that you can exclude 353,738 changes, leaving you with 463,248, or about 21,000 mutations per tumor. Still a lot!

A. Can you suggest at least three other sources of apparent mutations that do not actually contribute to the tumor?
B. After applying a number of criteria to filter out irrelevant sequence changes, you find a total of 1307 mutations in the 22 breast and colorectal cancers, or about 59 mutations per tumor. How might you go about deciding which of these sequence changes are likely to be driver mutations and which are probably passenger mutations that occurred in genes with nothing to do with cancer (but were found in the tumors because they happened to occur in the same cells with true cancer mutations)?
C. Will your comprehensive sequencing strategy detect all possible genetic changes that affect the targeted genes in the cancer cells?

20–46 Remarkably, the *Ink4A-ARF* locus encodes two different tumor suppressor proteins, Ink4A and ARF, which share a common exon but are translated in different reading frames (**Figure 20–5A**). Mutations in human tumors that were initially thought to affect Ink4A were later shown to affect a novel protein encoded in a different reading frame; hence, the name of the gene: *ARF*, for alternative reading frame. One of the principal functions of ARF is to inhibit Mdm2, which in turn inhibits p53 (see Problem 20–44). The relationship among ARF, Mdm2, and p53 is commonly represented as shown in **Figure 20–5B**. The sort of "double negative" implied in this relationship can be confusing: ARF is an inhibitor of an inhibitor of p53.

A. Would you expect *ARF* knockout mice to be more prone, or less prone, to getting tumors than a wild-type mouse? Explain your reasoning.
B. Do you suppose that a $p53^{+/+}$ $Mdm2^{-/-}$ mouse, which will die in early embryogenesis, would be rescued by knockout of the *ARF* gene? That is, would you expect a $p53^{+/+}$ $Mdm2^{-/-}$ $ARF^{-/-}$ mouse to be viable or dead? Explain your reasoning.
C. The *Myc* oncogene, in addition to stimulating cell-proliferation pathways, also activates ARF, thereby indirectly influencing the activity of p53. How would you account for the observation that mice expressing the *Myc* oncogene get tumors more quickly in $ARF^{+/-}$ mice than in $ARF^{+/+}$ mice (**Figure 20–5C**)?

20–47 The formation of tumors is a multistep process that involves the activation of several oncogenes, the inactivation of several tumor suppressors, or a combination of both. This notion is strongly supported by experiments in transgenic mice. The *Ras* and *Myc* oncogenes, both under control of the MMTV (mouse mammary tumor virus) promoter, were introduced separately into the mouse germ line, and the resulting mice were bred to generate mice that carried both oncogenes.

Mice with *Ras*, *Myc*, or both oncogenes developed tumors at a higher frequency than normal animals. Female mice were most rapidly affected

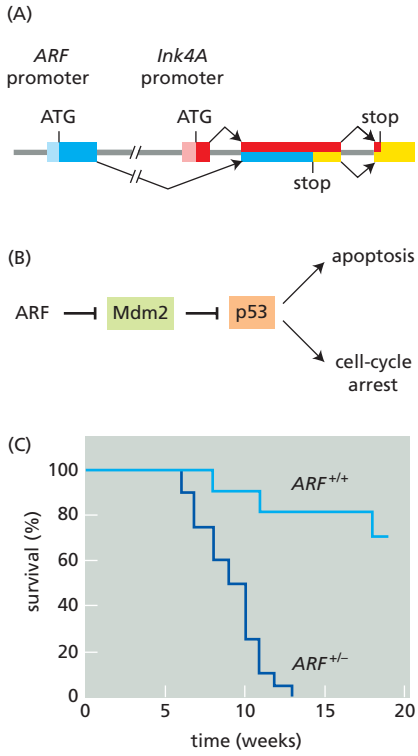


Figure 20–5 Gene structure and function of ARF (Problem 20–46). (A) Gene structures of *ARF* and *Ink4A*. The ATG in the initial exon of each gene indicates the start site of translation, which corresponds to AUG in the RNA transcript. The reading frame for *Ink4A* is shown in *red*, and the one for *ARF* is shown in *blue*. Note that the amino acid sequences of Ink4A and ARF are completely different. (B) Functional relationship between ARF, Mdm2, and p53. The *on-side T* symbol indicates inhibition; for example, ARF inhibits Mdm2. (C) Survival of mice expressing the *Myc* oncogene on a genetic background that is either $ARF^{+/+}$ or $ARF^{+/-}$. Mice that have survived for a given length of time are expressed as a percentage of the initial population. All the dead mice died of cancer.

because the MMTV promoter, which is responsive to steroid hormones, turns on the transferred oncogenes in response to the hormonal changes at puberty. In **Figure 20–6**, the rate of appearance of tumors is plotted as the percentage of tumor-free females at different times after puberty.

A. Assume that the lines drawn through the data points are an accurate representation of the data. How many events in addition to expression of the oncogenes are required to generate a tumor in each of the three kinds of mice? (You may wish to read Problem 20–51.)
B. Is activation of the cellular *Ras* gene the event required to trigger tumor formation in mice that are already expressing the MMTV-regulated *Myc* gene (or vice versa)?
C. Why do you think the rate of tumor production is so high in the mice containing both oncogenes?

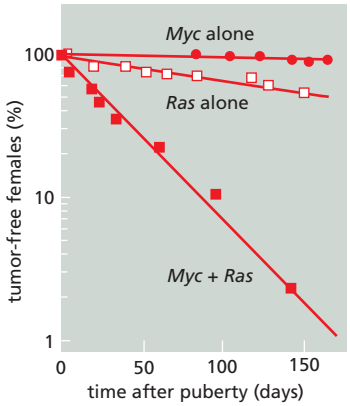


Figure 20–6 Fraction of tumor-free female mice as a function of time after puberty (Problem 20–47).

20–48 In a classic set of experiments, Barbara McClintock described chromosome rearrangements in corn that resemble similar rearrangements present in many cancer cells. In studies of the genetics of color variegation in corn, she generated speckled kernels by crossing a strain that contains an x-ray-induced rearrangement of chromosome 9 (**Figure 20–7**). This chromosome carries a color marker (*C*, colored; recessive form *c*, colorless), which allowed her to follow its inheritance in individual kernels. When strains carrying the rearranged chromosome 9 bearing the dominant *C* allele were crossed with wild-type corn bearing the recessive *c* allele, a small number of kernels in the progeny ears of corn had a speckled appearance.

This color variegation arises by a complex mechanism. In meiosis, recombination within the rearranged segment generates a chromosome with two centromeres as shown in **Figure 20–8**. In a fraction of these meioses, the recombined chromosome with two centromeres gets strung out between the two poles at the first meiotic anaphase, forming a bridge between the two meiotic poles. Some time in anaphase to telophase the strained chromosome breaks at a random position between the duplicated centromeres. The broken ends of the chromosome tend to fuse together after the next S phase, which generates a new dicentric chromosome whose structure depends on where the previous break occurred (Figure 20–8). The forces acting during the subsequent mitosis in turn will break this chromosome, and the bridge–breakage–fusion cycle will repeat itself in the next cell cycle, unless a repair mechanism adds a telomere to the broken end.

Figure 20–8 shows the chromosomal location of another genetic marker on chromosome 9, which can cause a "waxy" alteration to the starch deposited in the kernels. The waxy allele can be detected by staining with iodine. Waxy (*wx*) is recessive to the normal, nonwaxy (*Wx*) allele. By following the inheritance of the *C* and *Wx* markers in the kernels, McClintock gained an understanding of the behavior of broken chromosomes. She observed three types of patches within the otherwise colored, nonwaxy (*C*-*Wx*) kernels: colorless, nonwaxy (*c*-*Wx*) patches;

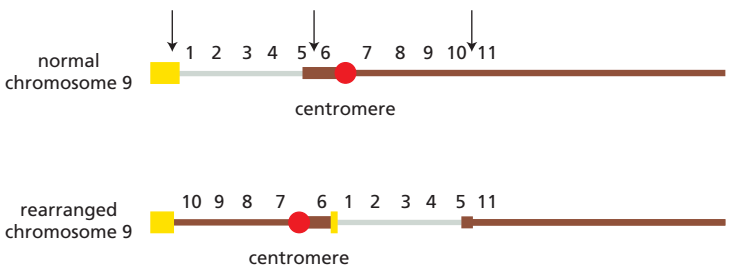


Figure 20–7 Normal and rearranged chromosomes 9 in corn (Problem 20–48). *Arrows* above the normal chromosome indicate sites of breakage that gave rise to the rearranged chromosome.

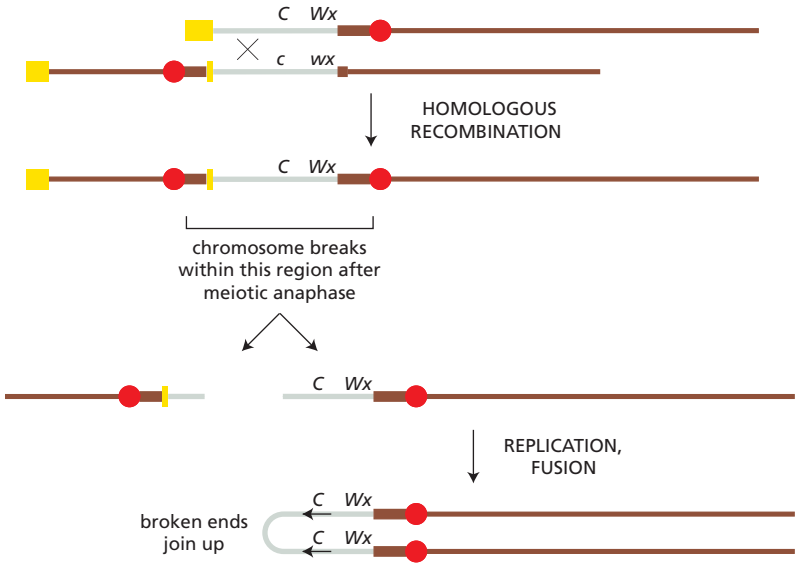


Figure 20–8 Recombination between a normal and a rearranged chromosome 9 to give a dicentric chromosome (Problem 20–48). Homologous recombination occurs at the X. Only the product with two centromeres is shown; the other product, which has no centromere, will be lost because it cannot attach to the spindle. Breakage of the original dicentric chromosome followed by replication and fusion of the ends gives rise to a second dicentric chromosome.

colorless, nonwaxy (*c-Wx*) patches containing one or more colorless, waxy (*c-wx*) spots; and intensely colored, nonwaxy (*?-Wx*) patches (**Figure 20–9**).

A. Patches arise because cells with a different genetic constitution divide to give identical neighbors that remain together in a cluster. Starting with the dicentric chromosome shown at the bottom of Figure 20–8, show how bridge–breakage–fusion cycles might account for the three types of patches shown in Figure 20–9. What is the genetic constitution of the intensely colored patches? (In these crosses, the dominant alleles—*C* and *Wx*—are carried on the rearranged dicentric chromosome at the bottom of Figure 20–8, and the recessive alleles—*c* and *wx*—are carried on the normal, unrearranged chromosome 9.)

B. Would you ever expect to see colored spots within colorless patches? Why or why not?

C. Would you ever expect to see colorless spots within the intensely colored patches? Why or why not?

20–49 A small fraction—2–3%—of all cancers, across many subtypes, displays a quite remarkable phenomenon: tens to hundreds of rearrangements that primarily involve a single chromosome, or chromosomal region. The breakpoints can be tightly clustered, with several in a few kilobases; the junctions of the rearrangements often involve segments of DNA that were not originally close together on the chromosome. The copy number of various segments within the rearranged chromosome was found to be either zero, indicating deletion, or one, indicating retention.

You can imagine two ways in which such multiple, localized rearrangements might happen: a progressive rearrangements model with ongoing inversions, deletions, and duplications involving a localized area, or a catastrophic model in which the chromosome is shattered into fragments that are stitched back together in random order by nonhomologous end joining (**Figure 20–10**).

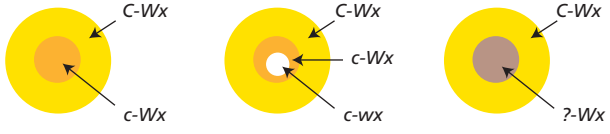


Figure 20–9 Three types of patches observed in speckled kernels (Problem 20–48).

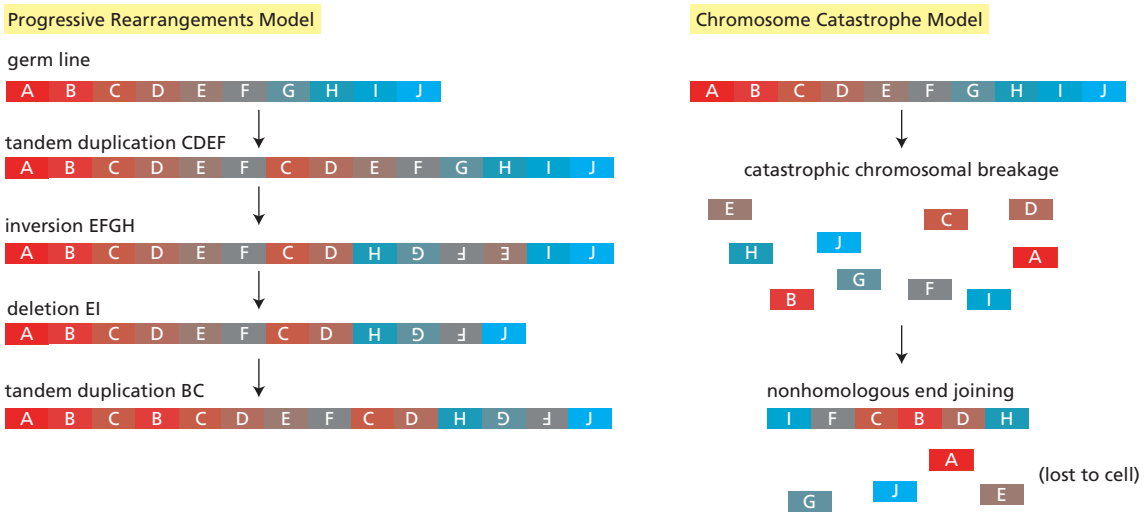


Figure 20–10 Two models to explain the multiple, localized chromosome rearrangements found in some cancers (Problem 20–49). The progressive rearrangements model shows a sequence of rearrangements that disrupts the chromosome, generating increasingly complex chromosomal configurations. The chromosome catastrophe model shows the chromosome being fragmented and then reassembled randomly, with some pieces left out.

A. Which of the two models in Figure 20–10 accounts more readily for the features of these highly rearranged chromosomes? Explain your reasoning.
B. For whichever model you choose, suggest how such multiple rearrangements might arise. (The true mechanism is not known.)
C. Do you suppose such rearrangements are likely to be causative events in the cancers in which they are found, or are they probably just passenger events that are unrelated to the cancer? If you think they could be driver events, suggest how such rearrangements might activate an oncogene or inactivate a tumor suppressor gene.

MEDICAL LINKS

20–50 The clinical trial of a gene therapy protocol to cure the human genetic disease, severe combined immunodeficiency syndrome (SCID), used retroviruses to carry in the missing *Il2rg* gene. The trial ended in disaster. Nearly 3 years after retroviral gene therapy was completed, two of the treated children developed T-cell leukemia. In both cases, the therapeutic retrovirus had integrated near the *Lmo2* gene, a known human T cell proto-oncogene, causing it to be aberrantly expressed. It had generally been assumed that insertional mutagenesis by replication-defective retroviruses (the kind used in the trial) would be so rare as to be of negligible consequence. Finding two such insertions among 10 treated infants raised serious concerns about the future of retroviral gene therapy.

To gather information on the basis for this effect, another group examined their collection of retrovirally induced blood-cell tumors in mice. In a survey of 600 tumors, they found two leukemias with integrations at the *Lmo2* gene and two with integrations at the *Il2rg* gene. Surprisingly, one of these leukemias had one retrovirus integrated at *Lmo2* and a second integrated at *Il2rg*. This observation raised the possibility that the two integrations were co-selected because they cooperate to induce leukemia. The implication is that the gene therapy trial led to cancers because of the leukemia-promoting *combination* of retroviral expression of *Il2rg* and retroviral integration near *Lmo2*. Retroviral expression of other kinds of genes might not cause any problems.

This intriguing explanation for the gene therapy results rests on the assumption that finding a leukemia with integrations at *Lmo2* and *Il2rg* by random chance is exceedingly small. Just what is the probability of finding such a dual integration by random chance in a survey of 600 tumors? One way to approach this question is to begin by calculating the chance of finding a random integration at *Il2rg* in 600 tumors that all

have a retroviral integration at *Lmo2*. Assume that integration in a 100 kb target around the *Il2rg* gene would be necessary to alter expression of the *Il2rg* gene. Also, assume that there are exactly two integration events in each of the 600 tumors: one at *Lmo2* and one that is random.

A. Given that the mouse genome is 2×10^6 kb, what fraction of random integration events (f_i) will be inside the 100-kb target? What fraction (f_o) will be outside the target?
B. What is the probability (P_N) that in 600 tumors you will not see a second integration in the target? [$P_N = (f_o)^{600}$]
C. What is the probability (P_Y) that in 600 tumors you will find a second integration event in the target? ($P_Y = 1 - P_N$)
D. Given that only 2 out of 600 tumors actually had a retroviral integration at *Lmo2*, what is the chance of getting a dual retroviral integration at *Lmo2* and *Il2rg*?
E. Indicate in a general way how each specific assumption affects your calculations. If the *Il2rg* target were 10 kb instead of 100 kb, would the probability calculated in part D be increased or decreased? If there were, on average, fewer than two retroviral integration events per tumor, would the probability in part D be increased or decreased? If there were more than two integration events per tumor, how would the probability in part D be affected? If retroviral integration were not random, how would the calculation in part D be affected?

20–51 Retinoblastoma is an extremely rare cancer of the retina in the eye. The disease mainly affects children up to the age of 5 years because it can only occur while the nerve precursor cells are still dividing. In some cases tumors occur in only one eye, but in other cases tumors develop in both eyes. The bilateral cases all show a familial history of the disease; most of the cases affecting only one eye arise in families with no previous disease history.

An informative difference between unilateral and bilateral cases becomes apparent when the fraction of still undiagnosed cases is plotted against the age at which diagnosis is made (**Figure 20–11**). The regular decrease with time shown by the bilateral cases suggests that a single chance event is sufficient to trigger the onset of bilateral retinoblastoma. By contrast, the presence of a "shoulder" on the unilateral curve suggests that multiple events in one cell are required to trigger unilateral retinoblastoma. (A shoulder arises because the events accumulate over time. For example, if two events are required, most affected cells at early times will have suffered only a single event and will not generate a tumor. With time the probability increases that a second event will occur in an already affected cell and therefore cause a tumor.)

One possible explanation for these observations is that tumors develop when both copies of the critical gene (the retinoblastoma, *Rb*, gene) are lost or mutated. In the inherited (bilateral) form of the disease, a child receives a defective *Rb* gene from one parent: tumors develop in an eye when the other copy of the gene is lost through somatic mutation. In fact, the loss of a copy of the gene is frequent enough that tumors usually occur in both eyes. If a person starts with two good copies of the *Rb* gene, tumors arise in an eye only if both copies are lost *in the same cell*. Since such double loss is very rare, it is usually confined to one eye.

To test this hypothesis, you use a cDNA clone of the *Rb* gene to probe the structure of the gene in cells from normal individuals and from patients with unilateral or bilateral retinoblastoma. As illustrated in **Figure 20–12**, normal individuals have four restriction fragments that hybridize to the cDNA probe (which means each of these restriction fragments contains at least one exon). Fibroblasts (nontumor cells) from the two patients also show the same four fragments, although three of the fragments from the child with bilateral retinoblastoma are present

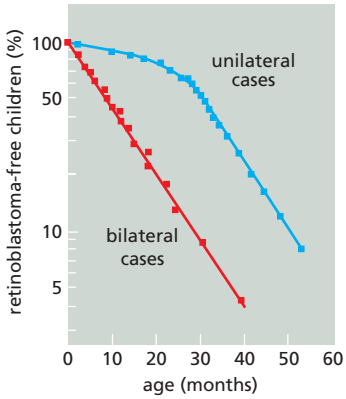


Figure 20–11 Time of onset of unilateral and bilateral cases of retinoblastoma (Problem 20–51). A population of children, all of whom ultimately developed retinoblastoma, is represented in this graph. The fraction of the population that is still tumor-free is plotted against the time after birth.

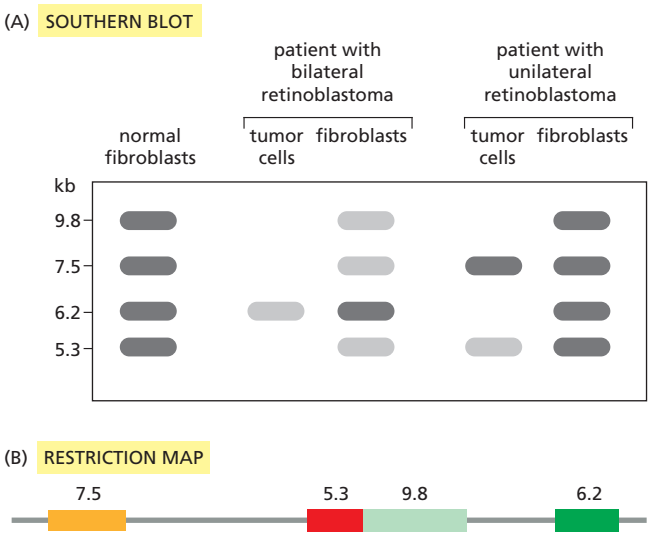


Figure 20–12 Patterns of blot hybridization of restriction fragments from the retinoblastoma gene (Problem 20–51). (A) Southern blot for normal individuals and for patients with unilateral and bilateral retinoblastoma. *Lighter shading* of some bands indicates half the normal number of copies. (B) The order of the restriction fragments in the *Rb* gene. Fragments that contain exons (*rectangles*) hybridize to the cDNA clone that was used as a probe in these experiments.

in only half the normal amount. Tumor cells from the two patients are missing some of the restriction fragments.

A. Explain why fibroblasts and tumor cells from the same patient show different band patterns.
B. What are the structures of the *Rb* genes in the fibroblasts from the two patients? What are their structures in the tumor cells from the two patients?
C. Are these results consistent with the hypothesis that retinoblastoma is due to the loss of the *Rb* gene?

CANCER PREVENTION AND TREATMENT: PRESENT AND FUTURE

TERMS TO LEARN

DNA tumor virus
multidrug resistance
papillomavirus (HPV)

DEFINITIONS

Match each definition below with its term from the list above.

20–52 The cause of human warts and a causative factor in carcinomas of the uterine cervix.

20–53 Phenomenon in which a cell becomes insensitive not only to a drug it has been treated with, but also to others to which it has never been exposed.

TRUE/FALSE

Decide whether each of these statements is true or false, and then explain why.

20–54 Many of the most potent carcinogens are chemically inert until after they have been modified by cytochrome P-450 oxidases in the liver.

20–55 Viruses and other infectious agents play no role in human cancers.

20–56 The main environmental causes of cancer are the products of our highly industrialized way of life such as pollution and food additives.

20–57 Anticancer therapies take advantage of some molecular abnormality of cancer cells that distinguishes them from normal cells.

20–58 Oncogene dependence describes the phenomenon in which a cancer cell suffers additional mutations or epigenetic changes that make it dependent on the hyperactivity of an oncogene, just as addicts become reliant on high doses of their drug.

20–59 The hypermutable nature of tumor cells means that treatments with single anticancer drugs or even combinations of such drugs are unlikely to eradicate all the cancer cells.

THOUGHT PROBLEMS

20–60 Epidemiological studies can provide suggestive links between environmental factors and cancer. For example, as shown in **Figure 20–13**, the curve for deaths due to lung cancer in the United States parallels the curve for per capita cigarette consumption. However, the curve for lung cancer is displaced by some 25 years from that for cigarette smoking. What do you suppose is the basis for this delay? What would you say to your uncle, who insists that people who smoke are inherently more cancer-prone and that lung cancer really has nothing to do with cigarettes?

20–61 Virtually all cancer treatments are designed to kill cancer cells, usually by inducing apoptosis. However, one particular cancer—acute promyelocytic leukemia (APL)—has been successfully treated with all-*trans*-retinoic acid, which causes the promyelocytes to differentiate into neutrophils. How might a change in the state of differentiation of APL cancer cells help the patient?

20–62 One major goal of modern cancer therapy is to identify small molecules—anticancer drugs—that can be used to inhibit the products of specific cancer-critical genes. If you were searching for such molecules, would you design inhibitors for the products of oncogenes or the products of tumor suppressor genes? Explain why you would (or would not) select each type of gene.

20–63 You've just read about this really cool technique for high-throughput screens of protein kinase inhibitors. The trouble is, you don't understand it. It is clearly important since it allows one to rapidly screen a large number of potential kinase inhibitors against a large number of protein kinases. There are roughly 500 protein kinases, including about 100 tyrosine kinases, encoded in the human genome. Many of them are critical components in the signal transduction pathways that become misregulated in cancer. Chemicals that inhibit individual protein kinases could serve as important lead compounds for development of drugs that are useful in the fight against cancer (and other diseases). So you want to understand how this technique works.

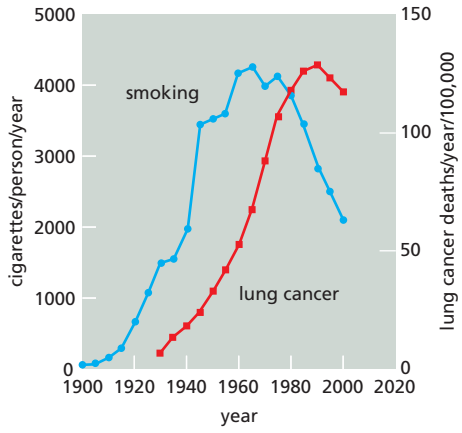


Figure 20–13 Lung cancer deaths and per capita cigarette consumption in the United States from 1930 to 2000 (Problem 20–60).

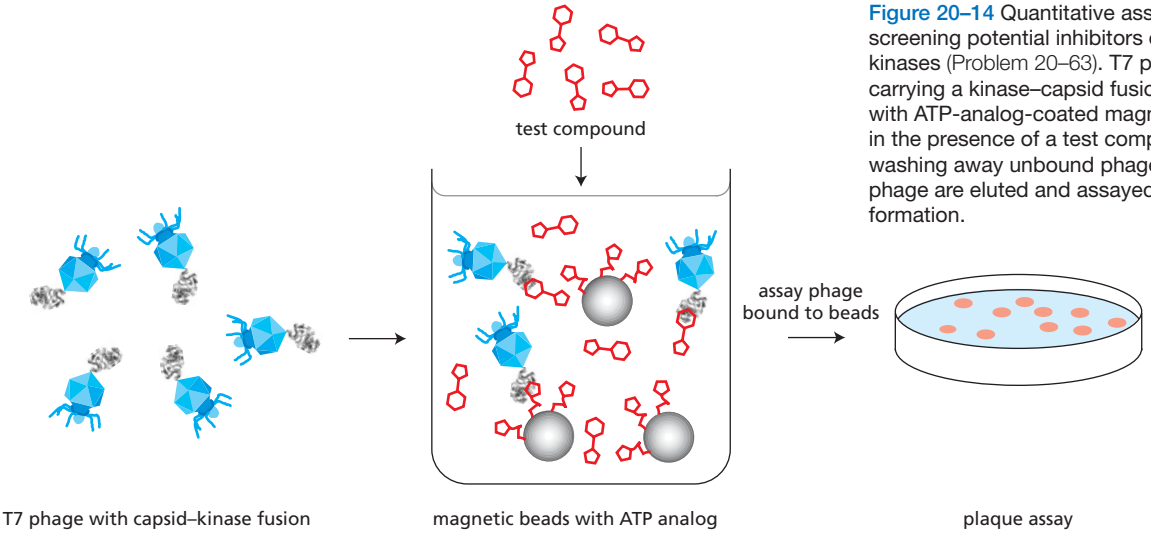


Figure 20–14 Quantitative assay for screening potential inhibitors of protein kinases (Problem 20–63). T7 phage carrying a kinase–capsid fusion are mixed with ATP-analog-coated magnetic beads in the presence of a test compound. After washing away unbound phage, attached phage are eluted and assayed for plaque formation.

There are several elements (**Figure 20–14**). First, individual protein kinase genes are fused to the major capsid (head) protein of T7 bacteriophage. When expressed in bacteria, the fusion proteins are assembled into the phage capsid, with the kinases displayed on the outer surface. Second, an analog of ATP, which can bind to the ATP-binding pocket of the kinases, is attached to magnetic beads. Third, a bank of test compounds is prepared.

To measure the ability of a test compound to inhibit the kinase, phage displaying a specific kinase are mixed with the magnetic beads in several wells of a 96-well plate. Then the test compound is added to individual wells over a range of different concentrations. The mixtures are incubated with gentle shaking for 1 hour at 25°C, the beads are pulled to the bottom with a strong magnet, and all the free (unbound) components are washed away. Finally, the remaining, attached phage are dissociated from the beads using an excess of the same ATP analog that is attached to the beads, and counted by measuring the number of plaques they form on a bacterial lawn on a Petri dish (Figure 20–14).

Although the assay is well described and the figure is clear, there are several things you just don't get. For example:

A. What is the point of the one-hour incubation?
B. How does the plaque count relate to the binding efficiency of the test compound? Will a test compound that binds a protein kinase strongly give more plaques or fewer plaques than one that binds the kinase weakly?
C. Do the test compounds compete for binding by the ATP analog? Or will a test compound that binds the kinase tightly someplace else also register in this assay?
D. Assuming that the test compounds bind to the ATP-binding site, how is it possible for them to bind one protein kinase, but not another? After all, every protein kinase has an ATP-binding site; that's how they bind to the ATP analog on the magnetic beads.

CALCULATIONS

20–64 In the high-throughput kinase-inhibitor assay described in Problem 20–63, the dissociation constant, K_d, for a test compound can be calculated readily from the curve of plaque count versus concentration of the

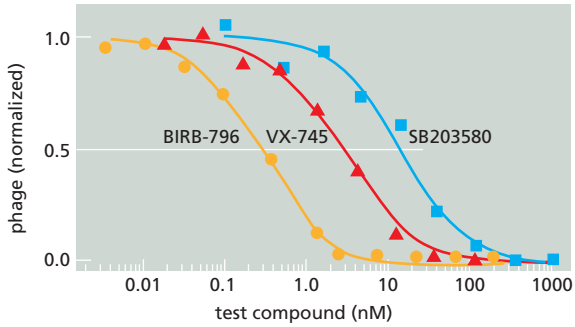


Figure 20–15 Plot of phage bound versus concentration of test compound (Problem 20–64). All plots have been normalized so that the maximum number of phage bound (highest plaque count) is 1.0 and the minimum is 0.0. For the normalized curve, the half-maximal response occurs at 0.5, which is shown by the *white* line. BIRB-796, VX-745, and SB203580 are the names of the test compounds.

test compound (**Figure 20–15**). The expression for K_d for the test compound is

$$K_{d(test)} = \frac{K_{d(analog)}}{K_{d(analog)} + [analog]} \times [test]_{½}$$

where $K_{d(analog)}$ is the dissociation constant for binding of the ATP analog to the protein kinase ATP-binding site, [analog] is the concentration of the analog bound to the magnetic beads, and $[test]_{½}$ is the concentration of free test compound that produces a half-maximal response.

If the analog concentration is kept well below $K_{d(analog)}$, then the expression becomes

$$K_{d(test)} \approx [test]_{½}$$

A. Using this approximation, determine the K_d values for the test compounds from the data in Figure 20–15.

B. The above equations assume that the phage concentration is well below $K_{d(test)}$. In these experiments, the phage were incubated with the magnetic beads and test compound at 10^{10} phage/mL. Is this a low enough concentration that the values calculated in part A are valid?

DATA HANDLING

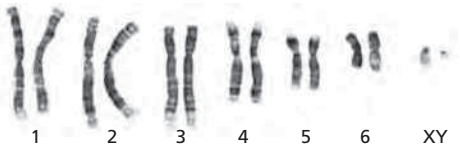
20–65 The Tasmanian devil, a carnivorous Australian marsupial, is threatened with extinction by the spread of a fatal disease in which a malignant oral–facial tumor interferes with the animal's ability to feed. You have been called in to analyze the source of this unusual cancer. It seems clear to you that the cancer somehow spreads from devil to devil, very likely by their frequent fighting, which is accompanied by biting around the face and mouth. To uncover the source of the cancer, you isolate tumors from 11 devils captured in widely separated regions and examine them. As might be expected, the karyotypes of the tumor cells are highly rearranged relative to that of the wild-type devil (**Figure 20–16**). Surprisingly,

Figure 20–16 Karyotypes of cells from Tasmanian devils (Problem 20–65). (A) A Tasmanian devil. (B) Normal karyotype for a male Tasmanian devil. The karyotype has 14 chromosomes, including XY. (C) Karyotype of cancer cells found in each of the 11 facial tumors studied. The karyotype has 13 chromosomes, no sex chromosomes, no chromosome 2 pair, one chromosome 6, two chromosomes 1 with deleted long arms, and four highly rearranged chromosomes (M1–M4).

(A)

Tasmanian devil *(Sarcophilus harrisii)*

(B)



(C)

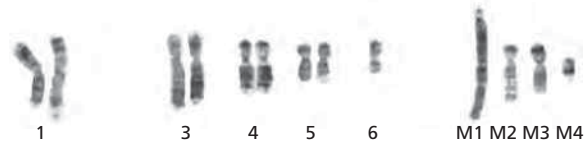


TABLE 20–2 Crosses between high-incidence and low-incidence tumor strains of mice (Problem 20–66).

Experiment	Female parent	Male parent	Tumors in F1 females
1.	D (high)	C57 (low)	36.1%
2.	C57 (low)	D (high)	5.5%
3.	A (high)	CBA (low)	86.3%
4.	CBA (low)	A (high)	0.0%
5.	Z (high)	I (low)	90.0%
6.	I (low)	Z (high)	0.0%

you find that the karyotypes from all 11 tumor samples are very similar. Moreover, one of the Tasmanian devils has an inversion on chromosome 5 that is not present in its facial tumor. How do you suppose this cancer is transmitted from devil to devil? Is it likely to arise as a consequence of an infection by a virus? Explain your reasoning.

20–66 Certain inbred strains of mice suffer tumors of the breast at a relatively high frequency, whereas other inbred strains form breast tumors rarely, or not at all. To investigate the basis for this hereditary difference, you set up a series of genetic crosses between the "high" and "low" tumor-forming strains of mice, as shown in **Table 20–2**. You are amazed to find that high frequencies of tumors appear in F1 female mice only when their mothers were from the "high-frequency" strains. When you cross the F1 progeny generated in an experiment to produce F2 mice, you find the same result: high frequencies of tumors appear in F2 female mice only when their grandmothers were from the "high-frequency" strain.

A. Can you explain these results on the basis of inheritance of a chromosomal mutation: recessive, dominant, or X-linked?

B. In Experiment 4, one of your CBA (low) mothers died and you put her pups with an A (high) mother for foster care. Much to your surprise, the fostered female pups developed breast tumors. Moreover, pups from these fostered females passed on the tendency to form breast tumors to their daughters. What do you suppose might be the basis for these results?

20–67 A major challenge in drug development is to predict clinical responses from research in the laboratory or in animals. Drug development for cancer therapy, as for other diseases, depends on two intertwined objectives. First, a drug must bind its target protein with a low K_d (in the nM range) so that the amount of drug that must be administered to the patient is kept in a reasonable range. Second, the concentration at which a drug affects its intended target protein should be 10–100-fold lower than the concentration at which it affects other (off-target) proteins.

Because protein kinases are key components in the signaling pathways that control cell behavior, they have been intense targets for anticancer drug development. The high-throughput screen described in Problem 20–64 has the potential for measuring K_d values and determining off-target effects in the same assay. The results of a screen for four kinase inhibitors are shown in **Figure 20–17**. The results are presented schematically on an evolutionary tree of the human kinases (the so-called human kinome). Only 113 of the 500 or so kinases represented on the kinome were tested in the high-throughput screen. Circles overlaid

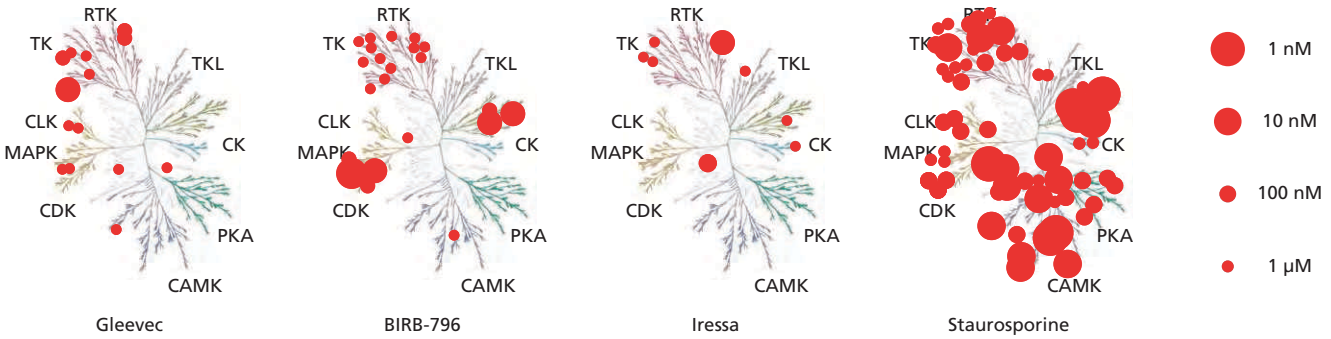


Figure 20–17 Specificity profiles for protein kinase inhibitors (Problem 20–67). The highly branched structure is the evolutionary tree for the human kinome. Circles of different sizes represent approximate binding constants, as indicated on the right. TK, nonreceptor tyrosine kinases; RTK, receptor tyrosine kinases; TKL, tyrosine kinase-like kinases; CK, casein kinase family; PKA, protein kinase A family; CAMK, calcium/calmodulin-dependent kinases; CDK, cyclin-dependent kinases; MAPK, mitogen-activated protein kinases; CLK, CDK-like kinases.

on the position of the target kinase on the kinome represent binding affinities, with larger circles indicating tighter binding (lower K_d values).

A. Assuming that the largest circle represents the main target of an inhibitor, rank in order the inhibitors from the most specific to the least specific.
B. As is true for many of the inhibitors tested in this assay, binding by BIRB-796 appears to be clustered in a few regions of the kinome. Why do you suppose that is?
C. Gleevec®, which inhibits the Abl protein kinase (its main target in the kinome), is being used with great success in the treatment of chronic myelogenous leukemia. Many patients, however, ultimately develop cancers that express a mutant form of Abl that is resistant to Gleevec. One of the most common mutant forms of Abl carries an isoleucine in place of threonine at position 315. Abl(T315I) is a poor target for Gleevec but is inhibited by BIRB-796. Do you suppose it would be possible to predict that BIRB-796 would inhibit Abl(T315I) from the data in Figure 20–17?
D. How might you adapt this high-throughput screen to finding inhibitors of clinically important resistant versions of protein kinases?

MEDICAL LINKS

20–68 Progress in cancer therapy is often measured in terms of the fraction of patients that are alive 5 years after their initial diagnosis. For example, in the United States in 1970, 7% of lung cancer patients were alive after 5 years, whereas in 2000, 14% survived for 5 years. Although this modest improvement might suggest a corresponding improvement in lung cancer therapy, many oncologists don't think therapy for this form of cancer has improved at all. In the absence of a significant change in treatment, how can it be that a higher percentage of lung cancer patients now live 5 years after their initial diagnosis?

20–69 PolyADP-ribose polymerase (PARP) plays a key role in the repair of DNA single-strand breaks. In the presence of the PARP inhibitor olaparib, single-strand breaks accumulate. When a replication fork encounters a single-strand break, it converts it to a double-strand break, which in normal cells is then repaired by homologous recombination. In cells defective for homologous recombination, however, inhibition of PARP triggers cell death.

Patients who have only one functional copy of the *Brca1* gene, which is required for homologous recombination, are at much higher risk for cancer of the breast and ovary. Cancers that arise in these tissues in these patients can be treated successfully with olaparib. Explain how it is that treatment with olaparib kills the cancer cells in these patients, but does not harm their normal cells.

MCAT STYLE

Passage 1 (Questions 20–70 to 20–72)

In rare cases, identical twins develop nearly identical leukemias, a form of cancer in which immature white blood cells proliferate out of control. In each case, analysis of cancer cells from pairs of twins demonstrated that they share an identical chromosomal rearrangement, a rearrangement not found in the twins' normal cells. In most cases, the twins developed leukemia at different times in their lives.

20–70 How might a chromosomal rearrangement contribute to development of leukemia?

A. By creating a point mutation in Ras, increasing its GTPase activity.
B. By deleting exons encoding a domain that inhibits kinase activity.
C. By deleting the promoter that controls expression of an oncogene.
D. By fusing a highly active promoter to a tumor suppressor gene.

20–71 What is the best explanation for the origin of the chromosomal rearrangement?

A. Each of the parents contributed one copy of the rearrangement to the twins.
B. The rearrangement arose from a rare somatic event that occurred *in utero*.
C. The rearrangement was caused by the genomic instability of cancer cells.
D. The rearrangement was inherited from one or the other of the two parents.

20–72 What does the different timing of the appearance of cancer in the twins say about how cancer develops?

A. Cancer cells are genetically unstable.
B. Cancer is caused by somatic mutations.
C. Cancer requires loss of tumor suppressors.
D. Cancer requires multiple mutation events.

Passage 2 (Questions 20–73 to 20–75)

Rationally designed anticancer drugs show promise and peril. This duality is evident in clinical trials of drugs designed to target the *B-Raf* oncogene. In normal cells, B-Raf functions in the MAP kinase cascade. Activation of a receptor tyrosine kinase leads to activation of Ras, which in turn activates B-Raf by promoting its dimerization.

Mutant forms of B-Raf are found in a high percentage of cancers, but are particularly prevalent in melanoma. Of the B-Raf mutations associated with cancer, 90% carry glutamic acid at position 600, instead of valine. This V600E mutation constitutively activates B-Raf in a way that is independent of Ras and the mutant form does not require dimerization.

The drug vemurafenib inhibits B-Raf by binding to its ATP-binding cleft. Vemurafenib causes spectacular shrinkage of tumors in clinical trials; however, in most cases, the tumors eventually recur (**Figure 20–18**). Paradoxically, vemurafenib causes other forms of cancer such as squamous cell carcinomas in about

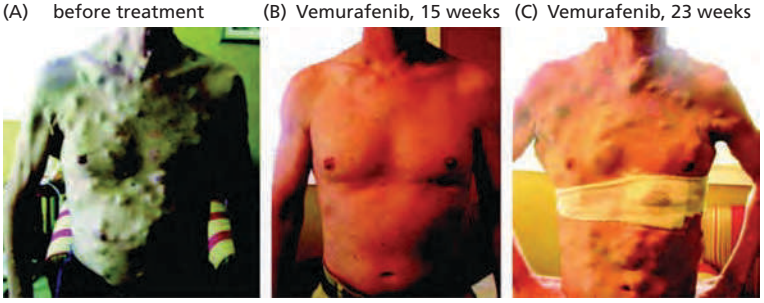


Figure 20–18 A patient with multiple myeloma treated with vemurafenib (Problems 20–73 to 20–75). (A) Tumors evident before treatment with vemurafenib. (B) Almost complete absence of tumors after 15 weeks of treatment. (C) Reappearance of tumors by 23 weeks of treatment.

25% of treated individuals. Also, vemurafenib is effective only against tumors that express the B-Raf V600E mutant; it does not shrink tumors that express hyperactive Ras instead of hyperactive B-Raf. This is odd because one might expect that inhibition of B-Raf, which is immediately downstream of Ras, would block constitutive Ras signaling in cancer cells.

20–73 Which of the following is a likely mechanism by which B-Raf (V600E) cancer cells die when exposed to vemurafenib?

A. Antibody-induced death
B. Division with DNA damage
C. Stimulation of apoptosis
D. The Warburg effect

20–74 Which of the following could be a reason why tumors come back after the majority of tumor cells have been killed by vemurafenib?

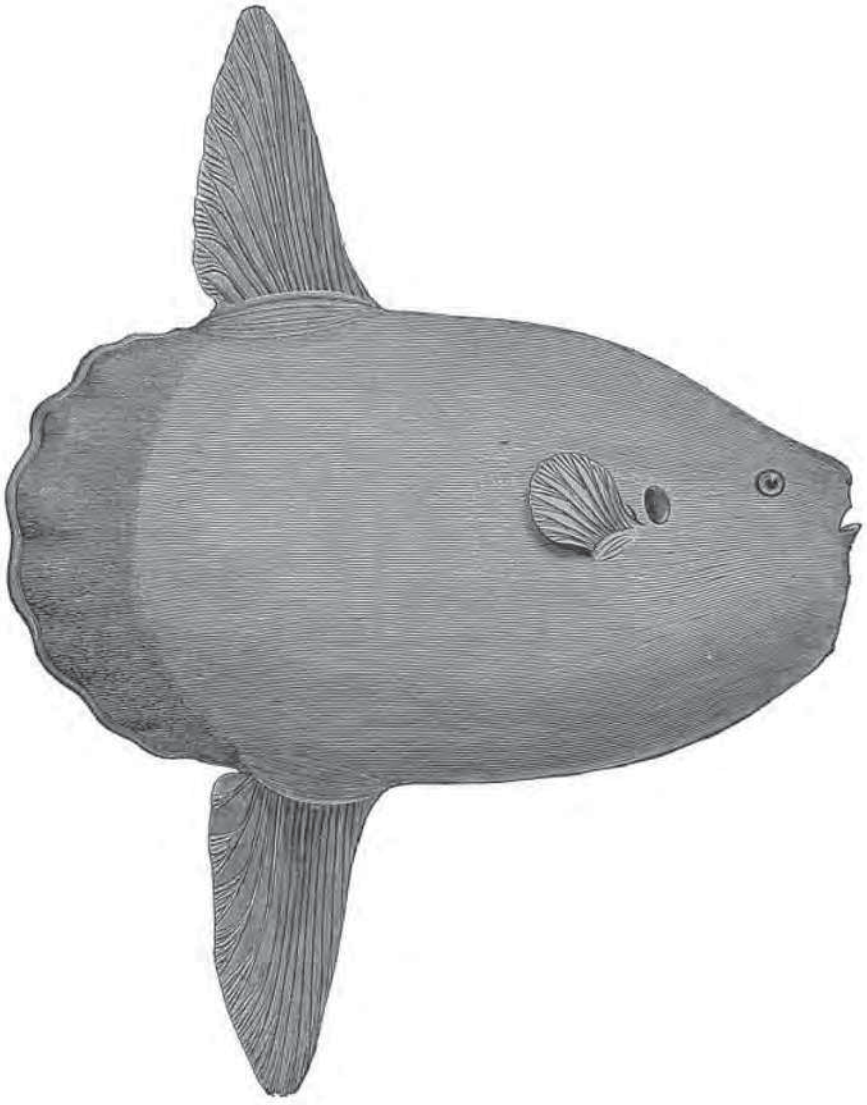
I. Some tumor cells acquire additional mutations that inactivate Mek or Erk, which are downstream components of the MAP kinase cascade.
II. Some tumor cells acquire additional mutations in other genes that promote cell survival.
III. Vemurafenib kills the majority of tumor cells, but spares a small population of cancer stem cells.

A. I
B. I and II
C. II and III
D. I, II, and III

20–75 Analysis of the effects of vemurafenib in normal cells solved the mystery of why it causes cancer in some individuals. Which of the following could explain how vemurafenib causes cancer?

A. Binding of vemurafenib to one B-Raf activates the partner B-Raf in the dimer.
B. In normal cells, binding of vemurafenib to B-Raf inhibits the associated Ras.
C. Vemurafenib mutates one copy of B-Raf, converting it into a tumor suppressor.
D. Vemurafenib prevents normal B-Raf from activating an inhibitor of apoptosis.

Answers



A Nineteenth Century Engraving of the Sunfish, *Mola mola.*
This enormous fish (up to 1000 kg in weight) is suspected to be what the artist of the sea creature sighted between Antibes and Nice actually saw. Image from Wikipedia, Originally uploaded by Citron.

CHAPTER 1

Cells and Genomes

IN THIS CHAPTER

THE UNIVERSAL FEATURES OF CELLS ON EARTH

DEFINITIONS

1–1 Plasma membrane

1–2 Enzyme

1–3 Transcription

1–4 Translation

1–5 Gene

1–6 Messenger RNA (mRNA)

1–7 Amino acid

1–8 Genome

TRUE/FALSE

1–9 True. Even in eukaryotes, where the coding regions of a gene are often interrupted by noncoding segments, the *order* of codons in the DNA is still the same as the order of amino acids in the protein.

1–10 False. The nucleotide subunits of RNA and DNA differ in two key ways. First, the backbone in RNA uses the sugar ribose instead of deoxyribose, which is used in DNA. Second, RNA uses the base uracil in place of the base thymine, which is used in DNA. Three of the four bases—A, C, and G—are the same in RNA and DNA.

THOUGHT PROBLEMS

1–11 Trying to define life in terms of properties is an elusive business, as suggested by this scoring exercise (**Table 1–2**). Cars are highly organized objects, take energy from the environment, and transform gasoline into motion, responding to stimuli from the driver as they do so. However, they cannot reproduce themselves, or grow and develop—but then neither can old animals. Cacti are not particularly responsive to stimuli, but they display other "life" attributes. It is curious that standard definitions of life usually do not mention that living organisms on Earth are largely made of organic molecules, that life is carbon-based. The first few pages of *MBoC* emphasize this point and discuss the properties of living cells mainly in terms of their "informational macromolecules"—DNA, RNA, and protein.

Reference: Pace NR (2001) The universal nature of biochemistry. *Proc. Natl Acad. Sci. USA* 98, 805–808.

TABLE 1–2 Plausible "life" scores for car, cactus, and humans (Answer 1–11).

Characteristic	Car	Cactus	Human
1. Organization	Yes	Yes	Yes
2. Homeostasis	Yes	Yes	Yes
3. Reproduction	No	Yes	Yes
4. Development	No	Yes	Yes
5. Energy	Yes	Yes	Yes
6. Responsiveness	Yes	No	Yes
7. Adaptation	No	Yes	Yes

1–12 Such modules are generally designed to look for organic molecules characteristic of life. The first Mars probe analyzed soil samples for organics, but found none. Subsequent probes have discovered a highly oxidative surface layer that would have destroyed organics. Hopeful signs of possible Martian life are an ancient abundance of water and current traces of methane in the atmosphere. The question of life on Mars remains open, as does the proper design of a module to detect it.

1–13 It is extremely unlikely that you created a new organism in this experiment. Far more probably, a spore from the air landed in your broth, germinated, and gave rise to the cells you observed. In the middle of the nineteenth century, Louis Pasteur invented a clever apparatus to disprove the then widely accepted belief that life could arise spontaneously. He showed that sealed flasks never grew anything if properly heat-sterilized first. He overcame the objections of those who pointed out the lack of oxygen or who suggested that his heat sterilization killed the life-generating principle, by using a special flask with a slender "swan's neck," which was designed to allow in oxygen but to prevent spores carried in the air from contaminating the culture (**Figure 1–4**). The cultures in these flasks never showed any signs of life; however, they were capable of supporting life, as could be demonstrated by washing some of the "dust" from the neck into the culture.

1–14 On the surface, the extraordinary mutation resistance of the genetic code argues that it was subjected to the forces of natural selection. An underlying assumption, which seems reasonable, is that resistance to mutation is a valuable feature of a genetic code, one that would allow organisms to maintain sufficient information to specify complex phenotypes. This reasoning suggests that it would have been a lucky accident indeed—roughly a one-in-a-million chance—to stumble on a code as error-proof as our own.

But all is not so simple. If resistance to mutation is an essential feature of any code that can support the complexity of organisms such as humans, then the only codes we *could* observe are ones that are error resistant. A less favorable frozen accident, giving rise to a more error-prone code, might limit the complexity of life to organisms that would never be able to contemplate their genetic code. This is akin to the anthropic principle of cosmology: many universes may be possible, but few are compatible with life that can ponder the nature of the universe.

Beyond these considerations, there is ample evidence that the code is not static, and thus could respond to the forces of natural selection. Deviant versions of the standard genetic code have been identified in the mitochondrial and nuclear genomes of several organisms. In each case, one or a few codons have taken on a new meaning.

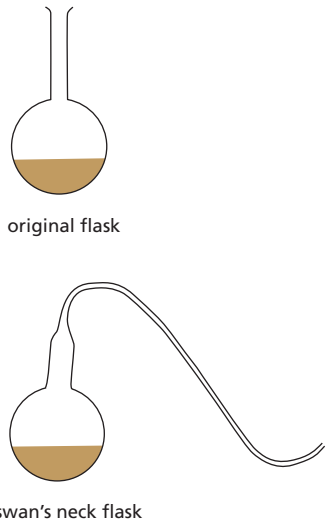


Figure 1–4 Flasks used in Pasteur's tests of spontaneous generation (Answer 1–13).

Reference: Freeland SJ & Hurst LD (1998) The genetic code is one in a million. *J. Mol. Evol.* 47, 238–248.

1–15 There are several approaches you might try.

1. Analysis of the amino acids in the proteins would indicate whether the set of amino acids used in your organism differs from the set used in Earth organisms. But even Earthly organisms contain more amino acids than the standard set of 20; for example, hydroxyproline, phosphoserine, and phosphotyrosine all result from modifications after a protein has been synthesized. Absence of one or more of the common set might be a more significant result.
2. Sequencing DNA from the "Europan" organism would allow a direct comparison with the database of sequences that are already known for Earth organisms. Matches to the database would argue for contamination. Absences of matches would constitute a less strong argument for a novel organism; it is a typical observation that about 15% to 20% of the genes identified in complete genome sequences of microorganisms do not appear to be homologous to genes in the database. Sufficiently extensive sequence comparison should resolve the issue.
3. Another approach might be to analyze the organism's genetic code. We have no reason to expect that a novel organism based on DNA, RNA, and protein would have a genetic code identical to Earth's universal genetic code.

1–16 In double-stranded DNA, which forms the genomes in all cellular life, G pairs with C, and A pairs with T. It is this requirement for base-pairing that necessitates that the number of Gs will equal the number of Cs, and that the numbers of As and Ts will be the same. In bulk samples of DNA, this translates into equivalent mole percents of G and C and of A and T.

The virus ΦX174 does not obey the "rules" because its genome is single-stranded DNA. In the absence of a requirement for systematic base-pairing, there is no constraint on the relative amounts of G and C or of A and T.

1–17 Schrödinger answered his rhetorical question as follows: "The obvious inability of present-day physics and chemistry to account for such events is no reason at all for doubting that they can be accounted for by those sciences." It is remarkable how much progress has been made since 1944, when the structure of DNA was completely unknown (its role was just beginning to come into focus), no protein had yet been sequenced, and the secret of the catalytic power of enzymes was very mysterious. Simple tests had already shown that plants and animals obeyed the laws of thermodynamics; neither cells nor organisms can create energy from nothing. All organisms require an input of energy from the environment to grow and reproduce—even to stay alive. Physicists have improved x-ray crystallography to the point where the structures of large proteins can be determined in weeks, and chemists can sequence whole bacterial genomes even more quickly. Organic chemists now understand enzyme-catalyzed reactions as well as any they study. The details of the metabolism of obscure bacteria living at extreme ocean depths on a diet of sulfur and carbon monoxide are well understood. The genes that control the intricate body plans of insects are mapped and sequenced. Yet many mysteries remain, of which one of the deepest is reflected by the enduring truth of Rudolph Virchow's famous (1859) aphorism, "*Omnis cellula e cellula*" (All cells come from cells). One cannot yet mix a defined brew of DNA, RNA, and proteins together with some lipids and expect to generate a cell from its constituents. Will the next 50 years see this overturned?

1–18

A. During replication, parental DNA serves as a template for synthesis of new DNA.

B. During transcription, DNA serves as a template for synthesis of RNA.
F. During translation, RNA (mRNA) serves as the template for synthesis of protein.

Two other processes, D. RNA → DNA, called reverse transcription, and E. RNA → RNA, called RNA replication, occur in the life cycles of RNA viruses such as HIV and poliovirus.

CALCULATIONS

1–19

A. The number (n) of generations of cell divisions required to produce 10^{13} cells is

$$2^n = 10^{13}$$

It is useful to remember that $2^{10} \approx 10^3$ (2^n produces the series: 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024; thus, $2^{10} = 1024 \approx 10^3$). If 10^3 cells result from ten generations of dividing, 10^{12} cells will result from $4 \times 10 = 40$ generations. Thus, you can estimate quickly that it will take a little over 40 generations to reach 10^{13} cells. You can get a more accurate answer, 43.2, by plugging different values of n into your calculator. Alternatively, you can solve the equation for n, which tests your familiarity with logarithms. Remember that

$$2 = 10^{\log 2} \text{ and } 2^n = 10^{n\log 2}$$

Substituting,

$$10^{n\log 2} = 10^{13}$$

Taking the log of both sides,

$$n\log 2 = 13$$

$$n = 13/\log 2 = 13/0.301$$

$$n = 43.2$$

B. If cells divided once per day and all cells continued to divide, it would take 43.2 days to generate the number of cells in an adult human.
C. Obviously we don't become adults in 43 days. The simple answer is that all cells don't continue to divide once per day and some cells are programmed to die. As cells differentiate, they generally slow their rate of division, ultimately in the adult dividing just often enough to replace cells that are lost or die. Of course, the real answer is much more complex, involving time for cell movements, for local environments to be established, for extracellular matrices to be laid down, for cells to differentiate, for global patterns to develop, and so on.

1–20 For calculations such as these, it is useful for purposes of estimation to remember that $4^5 \approx 10^3$ (4^n produces the series: 4, 16, 64, 256, 1024; thus, $4^5 = 1024 \approx 10^3$) and that $(1/4)^5 \approx (1/10)^3$. Hence, 4 different nucleotides can generate 1024 different DNA sequences, each 5 nucleotides long. Similarly, an 8-nucleotide DNA sequence can provide enough diversity to tag 21,000 protein-coding genes, there being 4^8 or 65,536 possible 8-nucleotide sequences. However, one would expect that most of these sequences would be present more than once in the 3.2×10^9 nucleotides of the human genome. Indeed, for a sequence tag to be rare enough to be expected to be present only once, it would have to be at least 16 nucleotides long. A 16-nucleotide sequence would be expected to be present about 0.7 times in the haploid human genome $[(1/4)^{16} \times (3.2 \times 10^9) = 0.75]$.

A probability calculation should properly be used to assess the likelihood that a tag is sufficiently long to be unique in the genome. For a

sequence that is present in one gene, what is the probability that it is also present elsewhere in the genome? The probability of a match (P_M) in any one comparison is the chance of a match at every nucleotide, $(1/4)^n$. Thus, for one comparison

$$P_M = (1/4)^n$$

Since the probability of all events is 1, the probability of not matching (P_N) in one comparison is

$$P_N = 1 - P_M = 1 - (1/4)^n$$

And the probability of not matching in any number of comparisons (c) is

$$P_N = \{1 - (1/4)^n\}^c$$

For a 16-nucleotide sequence and 3.2×10^9 comparisons (imagine sliding the 16-nucleotide segment one nucleotide at a time along the sequence of the human genome), the probability of not matching elsewhere is

$$P_N = 0.53$$

Or, since $P_N + P_M = 1$,

$$P_M = 0.47$$

Thus, for a 16-nucleotide sequence there is about a 1 in 2 chance that it will be present elsewhere in the human genome. As you can calculate, a 19-nucleotide sequence, for example, reduces the probability of a match to 1 in 100.

Because the sequence of the human genome is known, it is not necessary to rely on such calculations, even though they give good average estimates of length versus uniqueness. It is important to realize, however, that it is possible to find shorter sequences that are present just one time in the genome, as well as larger sequences that are present multiple times.

1–21 The surface-to-volume ratio for a sphere is $4\pi r^2/[(4/3)\pi r^3] = 3/r$; thus, the ratio is inversely proportional to radius. Consequently, relative to a human cell, a bacterium has 10 times more surface per volume of cytoplasm to allow the passage of nutrients in and waste products out. The bacteria, however, grow 72 times faster than human cells, suggesting that something besides the available surface limits the rate of growth.

THE DIVERSITY OF GENOMES AND THE TREE OF LIFE

DEFINITIONS

1–22 Virus

1–23 Model organism

1–24 Archaea

1–25 Homolog

1–26 Eukaryote

1–27 Prokaryote

TRUE/FALSE

1–28 True. Phototrophs provide the major pathway by which carbon in CO_2 is incorporated into the biosphere; however, it is not the sole mechanism. Most lithotrophs can also fix carbon, but the amounts are tiny in comparison to the carbon fixed by phototrophs.

1–29 False. The clusters of human hemoglobin genes arose during evolution by duplication from an ancient ancestral globin gene; thus, they are examples of paralogous genes. The human hemoglobin α gene is orthologous to the chimpanzee hemoglobin α gene, as are the human and chimpanzee hemoglobin β genes, and so on. All the globin genes, including the more distantly related gene for myoglobin, are homologous to one another.

THOUGHT PROBLEMS

1–30 Whether it's sunlight or inorganic chemicals, "to feed" means "to obtain free energy and building materials from." In the case of photosynthesis, photons in sunlight are used to raise electrons of certain molecules to a high-energy, unstable state. When they return to their normal, ground state, the released energy is captured by mechanisms that use it to drive the synthesis of ATP. Similarly, lithotrophs at a hydrothermal vent obtain free energy by oxidizing one or more of the reduced components from the vent (for example, $H_2S \rightarrow S + 2\ H^+$), using some common molecule in the environment to accept the electrons (for example, $2\ H^+ + \frac{1}{2}\ O_2 \rightarrow H_2O$). Lithotrophs harvest the energy released in such oxidation–reduction (electron-transfer) reactions to drive the synthesis of ATP. For both lithotrophs and phototrophs, the key to success is the evolution of a molecular mechanism to capture the available energy and couple it to ATP synthesis.

For all organisms, be they phototrophs, organotrophs, or lithotrophs, their ability to obtain the free energy needed to support life depends on the exploitation of some nonequilibrium condition. Phototrophs depend on the continual flux of radiation from the sun; organotrophs depend on a supply of organic molecules, provided ultimately by phototrophs, that can be oxidized for energy; and lithotrophs depend on a supply of reduced inorganic molecules, provided, for example, by hydrothermal vents, that can be oxidized to produce free energy.

1–31 The hemoglobin of the giant tube worms binds O_2 and H_2S and transports them to the symbiotic bacteria, which use the H_2S as an electron donor and the O_2 as an electron acceptor to generate ATP and reducing power to meet their energy needs. The resulting growth of the bacteria benefits the worms by providing increased waste products and dead bodies to live on. Moreover, in the process, the toxic H_2S is rendered harmless by oxidation to elemental sulfur, thereby preventing it from poisoning the worms.

1–32 The balanced equation for oxygenic photosynthesis, derived from experiments using water with isotopically labeled oxygen, is

$$6\ CO_2 + 12\ H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6\ H_2O + 6\ O_2$$

In this form of the equation, and by analogy to equation 2, it is apparent that the O_2 derives from H_2O, and that all the oxygen in glucose derives from CO_2.

1–33 Four (**Figure 1–5**). All could have split from the common ancestor at the same time. Bacteria–archaea could have split from eukaryotes, followed by the separation of bacteria from archaea. Bacteria–eukaryotes could have split from archaea, followed by the separation of bacteria from

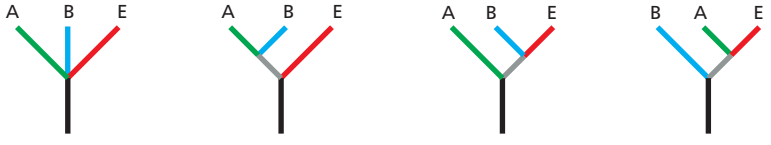


Figure 1–5 The four possible relationships for the evolution of archaea (A), bacteria (B), and eukaryotes (E) (Answer 1–33).

eukaryotes. Archaea–eukaryotes could have split from bacteria, followed by the separation of archaea from eukaryotes. Although horizontal transfers across these divisions make interpretations problematic, it is thought that archaea–eukaryotes first split from bacteria, and then archaea and eukaryotes split.

1–34 It is unlikely that any gene came into existence perfectly optimized for its function. It is thought that highly conserved genes such as ribosomal RNA genes were optimized by more rapid evolutionary change during the evolution of the common ancestor to archaea, bacteria, and eukaryotes. Since ribosomal RNAs (and the products of most highly conserved genes) participate in fundamental processes that were optimized early, there has been no evolutionary pressure (and little leeway) for change. By contrast, less conserved—more rapidly evolving—genes have been continually presented with opportunities to fill new functional niches. Consider, for example, the evolution of distinct globin genes, whose products are optimized for oxygen delivery to embryos, fetuses, and adult tissues in placental mammals.

1–35 It would be impossible to identify protein-coding genes in a vast stretch of Ts, As, Cs, and Gs if they did not have some identifying characteristics. In the absence of any knowledge of gene structure in prokaryotes, you might imagine that the sites where gene transcription begins and ends might be special and thus recognizable. Similarly, you might imagine that sequences where protein synthesis begins and ends might be distinctive and thus recognizable. In reality, it is the signals for protein synthesis that have proven most valuable for identifying prokaryotic genes.

Genes that encode proteins start with ATG (corresponding to the start codon AUG in the mRNA) and end with TAA, TAG, or TGA (corresponding to the three stop codons UAA, UAG, and UGA in mRNA). One searches for an ATG and then proceeds three nucleotides at a time (codon-by-codon) until a stop codon is reached. This procedure defines an open reading frame, or ORF. Nearly all ORFs greater than 100 codons correspond to genes. Some smaller ORFs also encode proteins and are therefore genes; however, many small ORFs occur by chance and do not correspond to genes. In some cases, real genes can be identified among the smaller ORFs by virtue of other typical signal sequences that characterize genes in prokaryotes. Nevertheless, in gene counts derived from genomic sequences, an arbitrary cutoff is used so that the smallest ORFs are not included in the count.

Gene identification in eukaryotic genome sequences is much more problematical. The protein-coding regions of eukaryotic genes are often split into segments that are not finally united until the initial RNA transcript is processed to remove the noncoding RNA. Thus, the procedure used to count genes in prokaryotes is not useful for eukaryotes. Computer algorithms to identify eukaryotic genes are not yet completely reliable.

1–36

B. It is not thought that formation of genes *de novo* from the vast amount of unused, noncoding DNA typical of eukaryotic genomes is a significant process in evolution. Mutation to generate a coding sequence complete with regulatory elements is too slow a process to account for the observed rates of evolutionary change.

1–37

A. Since it appears that genes involved in informational processes are less subject to horizontal transfer, evolutionary trees derived from such genes should provide a more reliable estimate of evolutionary relationships. Thus, archaea most likely separated from eukaryotes after the archaea–eukaryote lineage separated from bacteria.

B. Complexity is a logical explanation for the difference in rates of horizontal gene transfer (and it may even be right, although there are other possibilities). Successful transfer of an "informational" gene would require that the new gene product fit into a preexisting, functional complex, perhaps supplanting the original related protein. For a new protein to fit into a complex with other proteins, it would need to have binding surfaces that would allow it to interact with the right proteins in the appropriate geometry. If a new protein had one good binding surface, but not others, it would most likely disrupt the complex and put the recipient at a selective disadvantage. By contrast, a gene product that carries out a metabolic reaction on its own would be able to function in any organism. So long as the metabolic reaction conferred some advantage on the recipient (or at least no disadvantage), the gene transfer could be accommodated.

Reference: Jain R, Rivera MC & Lake JA (1999) Horizontal gene transfer among genomes: The complexity hypothesis. *Proc. Natl Acad. Sci. USA* 96, 3801–3806.

1–38 In single-celled organisms, the genome is the germ line and any modification is passed on to the next generation. By contrast, in multicellular organisms, most of the cells are somatic cells and make no contribution to the next generation; thus, modification of those cells by horizontal gene transfer would have no consequence for the next generation. The germ-line cells are usually sequestered into the interior of multicellular organisms, minimizing their contact with foreign cells, viruses, and DNA, thereby insulating the species from the effects of horizontal gene transfer.

1–39 It is not a simple matter to determine the function of a gene from scratch, nor is there a universal recipe for how to do it. Nevertheless, there are a variety of standard questions that help narrow down the possibilities. Below we list some of these questions.

In what tissues is the gene expressed? If the gene is expressed in all tissues, it is likely to have a general function. If it is expressed in one or a few tissues, its function is likely to be more specialized, perhaps related to the specialized functions of the tissues. If the gene is expressed in the embryo, but not the adult, it may function in development.

In what compartment of the cell is the gene expressed? Knowing the subcellular localization of the protein—nucleus, plasma membrane, mitochondria, etc.—can also help to suggest categories of potential function. For example, a protein that is localized to the plasma membrane is likely to be a transporter, a receptor or other component of a signaling pathway, a cell adhesion molecule, etc.

What are the effects of mutations in the gene? Mutations that eliminate or modify the function of the gene product can also provide clues to function. For example, if the gene product is critical at a certain time during development, the embryo will often die at that stage or develop obvious abnormalities. Unless the abnormality is very specific, it is usually difficult to deduce the function or category of function. And often the links are very indirect, becoming apparent only after the gene's function is known.

With what other proteins does the encoded protein interact? In carrying out their function, proteins often interact with other proteins involved in the same or closely related processes. If an interacting protein can be identified, and if its function is already known (through previous research or the searching of databases), the range of possible functions can be narrowed dramatically.

Mutations in what other genes can suppress effects of mutation in the unknown gene? Looking for suppressor genes can be a very powerful approach to investigating gene function in organisms such as bacteria and yeast, which have well-developed genetic systems, but this approach is not readily applicable to mouse or most higher eukaryotes at present.

The rationale for this approach is analogous to that of looking for interacting proteins: genes that interact genetically are often involved in the same or a closely related process. Identification of such an interacting gene (and knowledge of its function) would provide an important clue to the function of the unknown gene.

Addressing each of these questions requires specialized experimental expertise and a substantial time commitment from the investigator. It is no wonder that progress is made so much more rapidly when a clue to a gene's function can be found simply by identifying a similar gene of known function in the database.

CALCULATIONS

1–40 It takes only 20 hours—less than a day—before the mutant cells become more abundant in the culture. From the equation provided in the question, the number of the original ("wild-type") bacterial cells at time t minutes after the mutation occurred is $10^6 \times 2^{t/20}$. The number of mutant cells at time t is $1 \times 2^{t/15}$. At the time when the mutant cells "overtake" the wild-type cells, these two numbers are equal.

$$10^6 \times 2^{t/20} = 2^{t/15}$$

Converting to base 10 (see Answer 1–19),

$$10^6 \times 10^{(t/20)\log 2} = 10^{(t/15)\log 2}$$

Taking the log of both sides and substituting for log2 (0.301),

$$6 + (t/20)(0.301) = (t/15)(0.301)$$

Solving for t,

$$6 + 0.015t = 0.020t$$

$$0.005t = 6$$

$$t = 1200 \text{ minutes, or } 20 \text{ hours}$$

Note that it is also possible to solve this problem quickly, using the useful relationship $2^{10} \approx 10^3$, by realizing that after 1 hour the mutant cells have doubled one more time than the wild-type cells. Thus, the mutant cells double relative to the wild-type cells once per hour. After 10 hours (2^{10}) the mutant cells would have gained a factor of a thousand (10^3), and after 20 hours (2^{20}), a factor of a million (10^6), at which time they would be equal in number to the wild-type cells.

Incidentally, when the two populations of cells are equal, the culture contains 2×10^{24} cells [$(10^6 \times 2^{60}) + (1 \times 2^{80}) = (10^6 \times 10^{18}) + 10^{24} = 2 \times 10^{24}$], which at 10^{-12} g per cell, would weigh 2×10^{12} g, or two million tons! This can only have been a thought experiment.

GENETIC INFORMATION IN EUKARYOTES

TRUE/FALSE

1–41 False. Plant cells contain both mitochondria and chloroplasts.

1–42 True. Bacterial genomes seem to be pared down to the essentials: most of the DNA sequences encode proteins, a few encode functional RNAs, a small amount of DNA is devoted to regulating gene expression, and there are very few extraneous, nonfunctional sequences. By contrast, only about 1.5% of the DNA sequences in the human genome is thought to code for proteins. Even allowing for large amounts of regulatory DNA, much of the human genome is composed of DNA with no apparent function.

1–43 False. In addition to transfers from the mitochondrial genome, there are many examples of transfers of viral genomes; for example, some 1% of the mouse genome arose from copies of a sequence that originated as the genome of the mouse mammary tumor virus. What is rare is the transfer of genes from other species.

THOUGHT PROBLEMS

1–44 Like most questions about evolutionary relationships, this one was decided by comparing sequences of genes such as those for ribosomal RNA. These comparisons showed that fungi are more similar in gene sequence to animals than to plants, and probably split from the animal–plant lineage after plants separated from animals. Thus, fungi are thought never to have had chloroplasts, and fungi and plants are thought to have invented cell walls independently, as is suggested by the use of cellulose in plant cell walls and chitin in fungal cell walls.

1–45 Nucleotide sequence comparisons with other species would allow you to decide whether *Giardia* represented an ancient lineage or a more recent one. Such sequence comparisons have been done; they show that *Giardia* represents an ancient lineage (or one that has evolved very rapidly) that is almost as closely related to bacteria as it is to other eukaryotes. If *Giardia* were a stripped-down eukaryote, sequence comparisons would have revealed a closer kinship with the eukaryotic species from which it diverged. Standard sorts of sequence comparisons, of ribosomal RNA genes, for example, cannot decide the more fundamental—and more interesting—question of whether the *Giardia* lineage traces back to a time before mitochondria and internal membranes became permanent fixtures in eukaryotic cell organization.

Additional sequence comparisons can be used to address this fundamental question. The hypothesis that *Giardia* lost its mitochondria as an adaptation to its current anaerobic lifestyle in the intestinal tract implies that its ancestors once lived in aerobic environments and depended on mitochondria for energy. If that were so, then mitochondrial genes might have been transferred to the nuclear genome, and the sequence of the *Giardia* genome might reveal genes that originated from mitochondria. Sequencing targeted to genes that are likely mitochondrial markers suggests that *Giardia* at one time did indeed possess mitochondria or some related endosymbiont.

Reference: Roger AJ, Svärd SG, Tovar J, Clark CG, Smith MW, Gillin FD & Sogin ML (1998) A mitochondrial-like chaperonin 60 gene in *Giardia lamblia*: Evidence that diplomonads once harbored an endosymbiont related to the progenitor of mitochondria. *Proc. Natl Acad. Sci. USA* 95, 229–234.

1–46 Three general hypotheses have been proposed to account for the differences in rate of evolutionary change in different lineages. The individual hypotheses discussed below are not mutually exclusive and may all contribute to some extent.

The *generation-time* hypothesis proposes that rate differences are a consequence of different generation times. Species such as rat with short generation times will go through more generations and more rounds of germ-cell division, and hence more rounds of DNA replication. This hypothesis assumes that errors during DNA replication are the major source of mutations. Tests of this hypothesis in rat versus human tend to support its validity.

The *metabolic-rate* hypothesis postulates a higher rate of evolution for species with a higher metabolic rate. Species with high metabolic rates use more oxygen; hence, they generate more oxygen free radicals,

a major source of damage to DNA. This is especially relevant for mitochondrial genomes, because mitochondria are the major cellular site for oxygen utilization and free-radical production.

The *efficiency-of-repair* hypothesis proposes that the efficiency of repair of DNA damage differs in different lineages. Species with highly efficient repair of DNA damage would have a reduced fraction of damage events that lead to mutation. There is evidence in cultured human and rat cells that such differences in repair exist, in the expected direction, but it is unclear whether such differences exist in the germ lines of these organisms.

Reference: Li WH (1997) Molecular Evolution, pp. 228–230. Sunderland, MA: Sinauer Associates, Inc.

DATA HANDLING

1–47

A. The simplest hypothesis is that gene transfer occurred at the point indicated in **Figure 1–6**. Genera in many of the lineages beyond this point have a nuclear *Cox2* gene, whereas lineages that branched off prior to this point do not.

B. Five genera (*Lespedeza*, *Dumasia*, *Pseudeminia*, *Neonotonia*, and *Amphicarpa*) apparently have functional copies of both the mitochondrial and the nuclear genes, as indicated by yellow boxes in Figure 1–6.

C. Ten genera (*Eriosema*, *Atylosia*, *Erythrina*, *Ramirezella*, *Vigna*, *Phaseolus*, *Ortholobium*, *Psoralea*, *Cullen*, and *Glycine*) no longer have a functional mitochondrial gene. The minimum number of inactivation events that could account for the observed data is four, as shown by the green squares on the tree in Figure 1–6.

D. Six genera no longer have a functional nuclear gene. The minimum number of inactivation events that could account for this is five, as shown by the red circles on the tree in Figure 1–6.

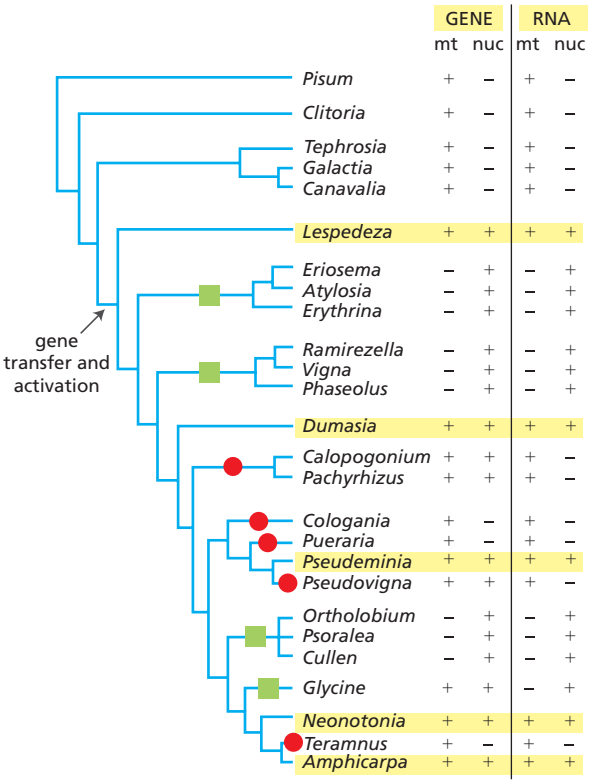


Figure 1–6 Summary of *Cox2* gene distribution and transcript data in a phylogenetic context, showing the most likely point of gene transfer and the minimum number of points for mitochondrial *(green squares)* and nuclear *(red circles)* gene inactivation (Answer 1–47). *Yellow boxes* indicate genera with apparently functional copies of both the mitochondrial and nuclear genes.

E. These data argue strongly that transfer of genes from mitochondria to the nucleus is not a one-step process; that is, simultaneous loss of the gene from mitochondria and its appearance in the nucleus. This is an unlikely scenario *a priori* since nuclear versions of mitochondrial genes must acquire a special targeting sequence that allows the encoded proteins to be delivered to mitochondria (see *MBoC* Chapter 12). The data in Figure 1–6 argue that the transfer process begins with the appearance of the gene in the nucleus (presumably followed at some point by its activation via acquisition of a targeting sequence). This first step is not accompanied by loss of the gene from the mitochondria. Once the nuclear gene is activated, there appears to be an intermediate stage in which both genes function. Subsequently, one or the other gene is inactivated. If the nuclear gene is inactivated, the transfer process is effectively aborted. If the mitochondrial gene is inactivated (often initially by point mutations), then the transfer can proceed. The final stage of transfer is deletion of the defective mitochondrial gene, a process favored by the economics of genome replication.

Reference: Adams KL, Song K, Roessler PG, Nugent JM, Doyle JL, Doyle JJ & Palmer JD (1999) Intracellular gene transfer in action: Dual transcription and multiple silencings of nuclear and mitochondrial *cox2* genes in legumes. *Proc. Natl Acad. Sci. USA* 96, 13863–13868.

1–48 If the intermediary in transfer were DNA, you would expect that the nuclear copy of the gene would have Cs at the sites of RNA editing. If the intermediary were RNA, you would expect Ts at the sites of RNA editing.

When sequences of nuclear *Cox2* genes were examined, they were found to resemble the edited RNA transcript more closely. This observation suggests that RNA was an intermediary in the transfer process. At some point, the RNA was presumably copied back into DNA by reverse transcription. Whether this is a general feature of transfer is unclear, but it fits with the multi-stage transfer described in the previous problem.

Reference: Nugent JM & Palmer JD (1991) RNA-mediated transfer of the gene *coxII* from the mitochondrion to the nucleus during flowering plant evolution. *Cell* 66, 473–481.

1–49

A. Because synonymous changes do not alter the amino acid sequence of the protein, they are not subject to selection pressures, which operate at the level of the function of the protein (and how it affects the overall fitness of the organism). By contrast, nonsynonymous changes, which substitute a new amino acid in place of the original one, have the potential to alter the function of the encoded protein (and change the fitness of the organism). Since most amino acid substitutions are deleterious to the function of the protein, they are selected against.

B. The histone H3 gene must be so exquisitely tuned to its function that virtually all amino acid substitutions are deleterious and, therefore, are selected against. The extreme conservation of histone H3 argues that its function is very tightly constrained, probably because of extensive interactions with other proteins and with its unchanging substrate, DNA.

C. Histone H3 is clearly not in a "privileged" site in the genome because it undergoes synonymous nucleotide changes at about the same rate as other genes.

Reference: Li WH (1997) Molecular Evolution. Sunderland, MA: Sinauer Associates, Inc.

1–50

A. The data in the phylogenetic tree (see Figure 1–3) refute the hypothesis that plant hemoglobin genes arose by horizontal transfer. Looking at

the more familiar parts of the tree, we see that the vertebrates (fish to human) cluster together as a closely related set of species. Moreover, the relationships in the unrooted tree shown in Figure 1–3 are compatible with the order of branching we know from the evolutionary relationships among these species: fish split off before amphibians, reptiles before birds, and mammals last of all in a tightly knit group. Plants also form a distinct group that displays accepted evolutionary relationships, with barley, a monocot, diverging before bean, alfalfa, and lotus, which are all dicots (and legumes). The sequences of the plant hemoglobins appear to have diverged long ago in evolution, at or before the time that mollusks, insects, and nematodes arose. The relationships in the tree indicate that the hemoglobin genes arose by descent from some common ancestor.

B. Had the plant hemoglobin genes arisen by horizontal transfer from a parasitic nematode, then the plant sequences would have clustered with the nematode sequences in the phylogenetic tree in Figure 1–3.

MCAT STYLE

1–51

A. All organisms have genes that encode ribosomal RNA. In addition, the sequences of ribosomal RNA genes have undergone divergent evolution between and within each of the three major divisions of life. Thus, by determining the sequence of ribosomal RNA genes, one can rapidly and comprehensively determine how many different organisms are in the sample and whether they are most closely related to bacteria, archaea, or eukaryotes. Choice B is not correct because it would not distinguish the many bacteria that are susceptible to the same antibiotic. Choice C is incorrect because there is extraordinary diversity in biochemistry and nutritional requirements. Thus, closely related species cannot be distinguished, and bacteria and archaea can appear similar. Finally, many microorganisms cannot be cultured. Choice D is not correct because bacteria and archaea can look identical when viewed by microscopy.

1–52

B. Horizontal transfer of genes between bacteria is common. In many cases, genes that encode toxins or antibiotic resistance are found on easily transferred circular plasmids. Thus, the compound of interest could be a toxin that allows the bacteria and/or sponge to survive in their environment. Choice A is unlikely because horizontal transfer of genes between eukaryotes and bacteria or archaea is extremely rare. Choice C is unlikely because all the genes in diverging organisms are subject to the changes introduced by evolution. Even genes that are highly constrained in their function—hence strongly selected for—would be expected to incorporate changes in the third positions of codons, which usually do not alter the encoded amino acid. Choice D is not correct because convergent evolution would produce similar genes, but not genes that are nearly identical.

1–53

A. Comparing new genes to known genes is usually the fastest way to gain insights into their function. If the bacterial gene is homologous to a family of genes with a known biochemical function, it is likely that the protein encoded by the new gene carries out an identical or similar function. Choices B and D would be labor intensive, and with no clues to start with, the experiments would involve much guesswork. Choice C would not provide any clues to the specific biochemical activity of the protein encoded by the gene.

Cell Chemistry and Bioenergetics

CHAPTER 2

IN THIS CHAPTER

THE CHEMICAL COMPONENTS OF A CELL

DEFINITIONS

2–1 Hydrophobic force

2–2 Hydrogen bond

2–3 Acid

2–4 van der Waals attraction

TRUE/FALSE

2–5 False. The pH of the solution will be very nearly neutral, essentially pH 7, because the few H^+ ions contributed by HCl will be outnumbered by the H^+ ions from dissociation of water. No matter how much a strong acid is diluted, it can never give rise to a basic solution. In fact, calculations that take into account both sources of H^+ ions and also the effects on the dissociation of water give a pH of 6.98 for a 10^{-8} M solution of HCl.

2–6 False. Strong acids bind protons weakly and give them up readily in a water environment.

2–7 False. Many of the functions that macromolecules perform rely on their ability to associate and dissociate readily, which would not be possible if they were linked by covalent bonds. By linking their macromolecules noncovalently, cells can, for example, quickly remodel their interior when they move or divide, and easily transport components from one organelle to another. It should be noted that some macromolecules are linked by covalent bonds. This occurs primarily in situations where extreme structural stability is required, such as in the cell walls of many bacteria, fungi, and plants, and in the extracellular matrix that provides the structural support for most animal cells.

THOUGHT PROBLEMS

2–8 The atomic weights of elements represent the average for the element as isolated from nature. Elements in nature include a mixture of isotopes. For most elements one isotope represents the vast majority; those elements have atomic weights that are nearly integers. Chlorine, however, has two abundant isotopes (75% ^{35}Cl and 25% ^{37}Cl), which average to an atomic weight of 35.5.

2–9 No. It is a coincidence that the ratio of C, H, and O in living organisms is the same as that for sugars. Much of the H and O (70%) is due to water, and the rest is from a mixture of sugars, amino acids, nucleotides, and

lipids—the whole variety of small and large molecules that make up living organisms.

2–10 B (less than 4 kJ/mole), E (4–12 kJ/mole), A (50 kJ/mole), C (350 kJ/mole), and D (2800 kJ/mole).

2–11 Because of its larger size, the outermost electrons in a sulfur atom are not as strongly attracted to the nucleus as they are in an oxygen atom. Consequently, the hydrogen–sulfur bond is much less polar than the hydrogen–oxygen bond. Because of the reduced polarity, the sulfur in H_2S is not strongly attracted to hydrogen atoms in adjacent H_2S molecules, and hydrogen bonds do not form. It is the lack of hydrogen bonds in H_2S that allows it to be a gas, and the presence of strong hydrogen bonds in water that makes it a liquid.

2–12 Although the symbol "p" in common usage denotes the "negative logarithm of," what it stands for is unclear. In the original 1909 paper in which the concept of pH was developed, the author—Danish chemist Soren P.L. Sorensen—was not explicit. In textbooks where it is commented on at all, it is most commonly reputed to stand for the French or German words for power or potential. Close examination of the original paper reveals that the "p" in pH is likely a consequence of the author's arbitrary choice to call two solutions by the letters "p" and "q." The q solution had the known H^+ concentration of 1, the p solution had the unknown H^+ concentration. If the solutions had been switched, do you think qH would ever have caught on?

Reference: Nørby JG (2000) The origin and the meaning of the little p in pH. *Trends Biochem. Sci.* 25, 36–37.

2–13 A solution of sodium chloride will be neutral. Neither the sodium ion nor the chloride ion binds H^+ or OH^- and thus neither influences the dissociation of water.

A solution of potassium acetate (the salt of a weak acid) will be basic because the acetate ion will steal sufficient numbers of protons from water to satisfy the equilibrium

$$CH_3COO^- + H_2O \rightleftharpoons CH_3COOH + OH^-$$

The increase in hydroxyl ions will cause the number of protons to decrease, satisfying the equilibrium for water ionization ($[OH^-][H^+] = 10^{-14}$) and making the solution basic.

A solution of ammonium chloride (the salt of a weak base) will be acidic because the ammonium ion will dissociate sufficiently to satisfy the equilibrium

$$NH_4^+ + H_2O \rightleftharpoons NH_3 + H_3O^+$$

The increase in hydronium ions lowers the pH and makes the solution more acidic.

2–14

A. The dissociation expression for a carboxylate group is $-COOH \rightleftharpoons H^+ + -COO^-$. The dissociation expression for an amine group is $-NH_3^+ \rightleftharpoons H^+ + -NH_2$.

B. The acidic carboxyl group gives up its proton much more readily than the protonated amine group (that's why the amine group is basic—it tends to pick up a proton from water). Thus, as shown in **Figure 2–23**, the p*K* for the carboxyl group corresponds to the point at which 0.5 equivalents of OH^- have been added, which is pH 2.3. The p*K* for the amine group corresponds to the point at which 1.5 equivalents of OH^- have been added, which is pH 9.6.

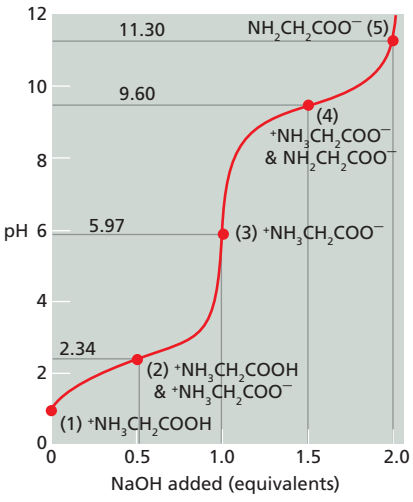


Figure 2–23 Titration of a solution of glycine (Answer 2–14).

C. The predominant ionic species of glycine are shown in Figure 2–23. At point (2), the p*K* for the carboxyl group, two species $^{+}H_3NCH_2COOH$ and $^{+}H_3NCH_2COO^{-}$ are present in equal concentrations. Similarly, at point (4), the p*K* for the amine group, two species are present at equal concentrations.

D. The isoelectric point occurs when 1.0 equivalents of OH^{-} have been added (Figure 2–23). At that point—point (3) on the curve—the predominant ionic species is $^{+}H_3NCH_2COO^{-}$, which carries no net charge. The isoelectric point for glycine occurs at pH 5.97, which is exactly halfway between the p*K* values for the carboxyl group (2.34) and the amine group (9.60). At this pH all the other minor ionic species of glycine are present in exactly balancing amounts so that there is no net charge on the solute.

2–15 The structures of these three forms of glycine are shown in **Figure 2–24**.

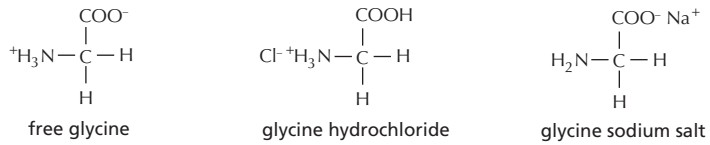


Figure 2–24 Three forms of glycine (Answer 2–15).

2–16 The titration of histidine is shown in Figure 2–4A and that for glutamate is shown in Figure 2–4B. The requirement for three equivalents of OH^{-} in both cases indicates that three ionizable groups are involved. Estimating the p*K* values from the points on the curves at which 0.5, 1.5, and 2.5 equivalents of OH^{-} were added allows a match to be made with the amino acids listed in Table 2–1.

2–17 You should advise the runner to breathe rapidly just before the race. Since a sprint will cause a lowering of blood and cell pH, the object of the pre-race routine would be to raise the pH with the idea that the runner could then sprint longer before feeling fatigue. Holding your breath or breathing rapidly both temporarily affect the amount of dissolved CO_2 in the bloodstream. Holding your breath will increase the amount of CO_2 and push the equilibrium to the right, leading to an increase in $[H^{+}]$ and a lower pH. By contrast, breathing rapidly will reduce the concentration of CO_2 and pull the equilibrium to the left, leading to a decrease in $[H^{+}]$ and a higher pH.

2–18 The majority of aspirin is absorbed into the bloodstream through the lining of the stomach. At the low pH in the stomach, which is below the p*K* of aspirin, most of the aspirin will be uncharged and will therefore diffuse through the plasma membranes of the cells that line the stomach.

2–19 The statement is correct. The hydrogen–oxygen bond in water molecules is polar; thus, the oxygen atom carries a partial negative charge and the hydrogen atoms carry partial positive charges. The partial negative charges on the oxygen atoms are attracted to the positive charges on the sodium ions but are repelled by the negative charges on the chloride ions.

2–20 Although individually weak, several such noncovalent interactions can, in aggregate, provide sufficient stability to hold a pair of molecules together. The situation is analogous to objects held together with Velcro™: a small bit holds them together weakly, whereas a large bit holds them together tightly. Such fastenings are easy to peel apart because the links can be broken a few at a time rather than all at once.

2–21 The functional groups on the three molecules are indicated and named in **Figure 2–25**.

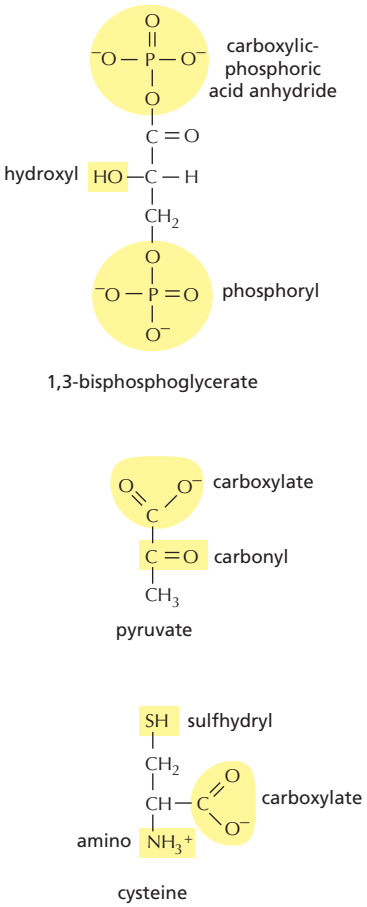


Figure 2–25 The functional groups in 1,3-bisphosphoglycerate, pyruvate, and cysteine (Answer 2–21).

2–22 A major advantage of condensation reactions is that they are readily reversible by hydrolysis (and water is readily available in the cell). This allows cells to break down their macromolecules (or macromolecules of other organisms ingested as food) and to recover the subunits intact so that they can be "recycled" to build new macromolecules.

CALCULATIONS

2–23

A. Although we do not know the molecular weight of cellulose (because the molecules contain variable numbers of $C_6H_{12}O_6$ subunits), we know it is 40% carbon {$(6 \times 12)/[(6 \times 12) + (12 \times 1) + (6 \times 16)]$}. Thus 2 g of carbon atoms, which corresponds to 10^{23} atoms of carbon, are contained in the cellulose that makes up a page.

$$\text{C atoms} = 2\text{ g} \times \frac{6 \times 10^{23}\text{ d}}{\text{g}} \times \frac{\text{C atom}}{12\text{ d}}$$
$$= 10^{23}\text{ C atoms}$$

B. The product of the number of carbon atoms in each dimension equals 10^{23} ($X \times Y \times Z = 10^{23}$). The number of carbon atoms in each dimension will be in the same ratio as the lengths. Thus, if Z is the number of carbon atoms in the thickness of the page and X is the number in the width, the ratio of X/Z is $[(21 \times 10^{-2}\text{ m})/(0.07 \times 10^{-3}\text{ m})]$, and $X = [(21 \times 10^{-2})/(0.07 \times 10^{-3})]\,Z$. Similarly, the ratio of Y/Z is $[(27.5 \times 10^{-2}\text{ m})/(0.07 \times 10^{-3}\text{ m})]$, and $Y = [(27.5 \times 10^{-2})/(0.07 \times 10^{-3})]\,Z$. Substituting for X and Y,

$$\frac{21 \times 10^{-2}\,Z}{0.07 \times 10^{-3}} \times \frac{27.5 \times 10^{-2}\,Z}{0.07 \times 10^{-3}} \times Z = 10^{23}$$
$$Z^3 = \frac{10^{23} \times (0.07 \times 10^{-3})^2}{(21 \times 10^{-2}) \times (27.5 \times 10^{-2})}$$
$$Z^3 = 8.48 \times 10^{15}$$
$$Z = 2.04 \times 10^5$$

The suggested shortcut makes the calculation a little more straightforward. The volume of the page is $4 \times 10^{-6}\text{ m}^3$ $[(21 \times 10^{-2}\text{ m}) \times (27.5 \times 10^{-2}\text{ m}) \times (0.07 \times 10^{-3}\text{ m})]$, which equals a cube with a side of 1.6×10^{-2} m. The presence of 10^{23} carbon atoms in this volume corresponds to 4.6×10^7 carbon atoms per side $(10^{23})^{0.33}$. This corresponds to about 200,000 carbon atoms to span the thickness of the page $[(4.6 \times 10^7\text{ atoms}/1.6 \times 10^{-2}\text{ m}) \times (0.07 \times 10^{-3}\text{ m})]$.

C. With a diameter of 0.4 nm each it would take 175,000 carbons atoms to span the thickness of the page $[(0.07 \times 10^6\text{ nm})/0.4\text{ nm}]$, if they were laid end to end at their van der Waals contact distance.

D. At first glance, it might seem strange that it takes more carbon atoms in cellulose, where they account for only 40% of the mass, than it takes as free, pure carbon atoms to span the thickness of the page. The key is that the atoms in cellulose are covalently bound to one another and therefore are much closer together than their van der Waals radii. The nuclei of covalently linked carbon atoms are separated by 0.15 nm, whereas those in van der Waals contact are separated by 0.4 nm.

2–24 Glucose ($C_6H_{12}O_6$) has a molecular weight of 180 $[(6 \times 12) + (12 \times 1) + (6 \times 16)]$ and therefore a mass of 180 g/mole. A concentration of 90 mg/dL corresponds to 5×10^{-3} M or 5 mM.

$$[\text{glucose}] = \frac{90\text{ mg}}{\text{dL}} \times \frac{\text{mole}}{180\text{ g}} \times \frac{10\text{ dL}}{\text{L}} \times \frac{\text{g}}{10^3\text{ mg}}$$
$$= 5 \times 10^{-3}\text{ moles/L, which is } 5 \times 10^{-3}\text{ M, or 5 mM}$$

2–25

A. Hydronium (H_3O^+) ions result from water dissociating into protons and

hydroxyl ions, each proton binding to a water molecule to form a hydronium ion ($2\ H_2O \rightarrow H_2O + H^+ + OH^- \rightarrow H_3O^+ + OH^-$). At neutral pH the concentrations of H_3O^+ ions and OH^- ions are equal. We know that at neutrality the pH is 7.0, and therefore, the H^+ (H_3O^+) concentration is 10^{-7} M.

B. The molecular weight of water is 18 and thus has a mass of 18 g/mole. The mass of 1 liter of water is 1000 g. Thus, pure water is 55.6 M [(1000 g/L) × (mole/18 g)]. (Because the mass of water is actually 18.015 g/mole, pure water is 55.5 M.)

C. The ratio of H_3O^+ ions (10^{-7} M) to H_2O molecules (55.5 M) is 1.8×10^{-9}. Thus, at neutral pH only about 2 water molecules in a billion are dissociated.

2–26

A. A solution is said to be neutral when the concentrations of H^+ and OH^- are exactly equal. This occurs when the concentration of each ion is 10^{-7} M, so that their product is $10^{-14}\ M^2$.

B. In a 1 mM solution of NaOH, the concentration of OH^- is 10^{-3} M. Thus, the concentration of H^+ is 10^{-11} M, which is pH 11.

$$[H^+] = \frac{K_w}{[OH^-]}$$
$$= \frac{10^{-14}\ M^2}{10^{-3}\ M} = 10^{-11}\ M$$

C. A pH of 5.0 corresponds to an H^+ concentration of 10^{-5} M. Thus, the OH^- concentration is 10^{-9} M ($10^{-14}\ M^2/10^{-5}$ M).

2–27

A. The values for log $[A^-]/[HA]$, $[A^-]/[HA]$, and the percentage of the acid that has dissociated are shown in **Table 2–6**. Included in the table are a set of "rule-of-thumb" values that may be easier to remember, and are handy to have mentally available for estimating answers.

B. A plot of pH versus percentage dissociation of the weak acid, HA, is shown in **Figure 2–26**. All weak acids, regardless of p*K*, yield titration curves that are identical to this one. The curves for different weak acids are shifted along the pH scale depending on their p*K* values.

This titration curve is fundamentally similar to protein–ligand binding curves and to enzyme activity curves. As pointed out in

TABLE 2–6 Dissociation of a weak acid at pH values above and below the p*K* (Answer 2–27).

pH	$\log \frac{[A^-]}{[HA]}$	$\frac{[A^-]}{[HA]}$	% Dissociation	"Rule-of-thumb" % dissociation
p*K* +4	4	10^4	99.99	99.99
p*K* +3	3	10^3	99.9	99.9
p*K* +2	2	10^2	99	99
p*K* +1	1	10^1	91	90
p*K*	0	10^0	50	50
p*K* –1	–1	10^{-1}	9.1	10
p*K* –2	–2	10^{-2}	0.99	1
p*K*–3	–3	10^{-3}	0.099	0.1
p*K* –4	–4	10^{-4}	0.0099	0.01

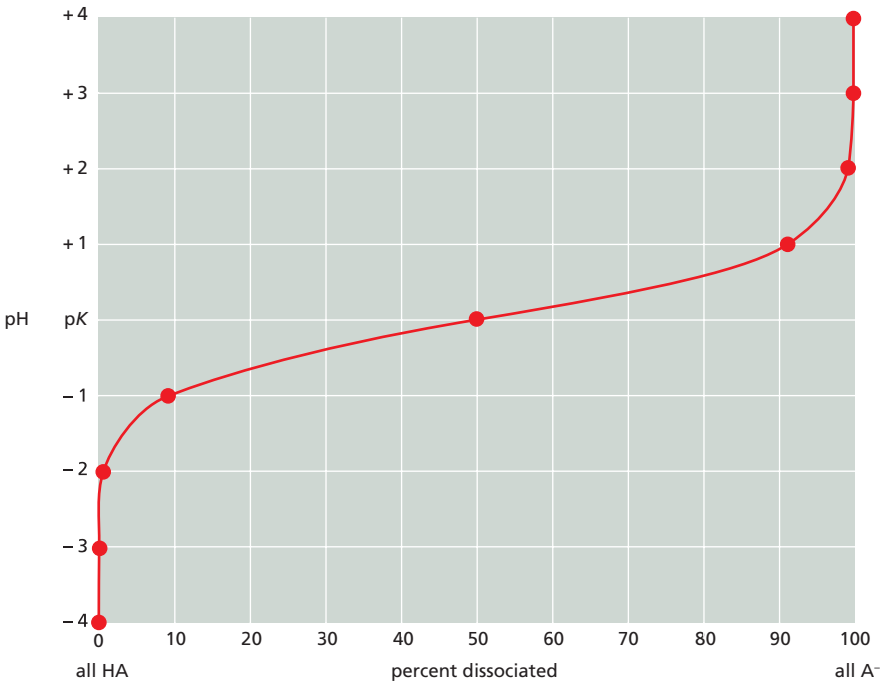


Figure 2–26 Percentage dissociation of a weak acid as a function of pH (Answer 2–27).

Problem 3–86, all three phenomena—titration of weak acids, protein–ligand binding, and enzyme activity—generate identical curves. Most importantly, the "rule-of-thumb" values pertain to each, allowing rapid estimates in all three situations.

2–28

A. Estimating the percentages of the four forms of phosphate at pH 7 is straightforward from the rule-of-thumb values derived in Problem 2–27 (see Table 2–6). Since the cytosol is about 5 pH units above the p*K* for dissociation of H_3PO_4, it will be about 99.999% ionized; thus, H_3PO_4 will account for only about 0.001% of the total. Since the cytosol is just slightly above the p*K* for dissociation of $H_2PO_4^-$, $H_2PO_4^-$ would be slightly less than 50% and HPO_4^{2-} would be slightly greater than 50% of the total. Since the cytosol is more than 5 pH units below the p*K* for dissociation of HPO_4^{2-}, it will be less than 0.001% ionized; thus, PO_4^{3-} will account for less than 0.001% of the total.

B. The ratio of $[HPO_4^{2-}]$ to $[H_2PO_4^-]$ ($[A^-]/[HA]$) in the cytosol at pH 7 can be calculated using the Henderson–Hasselbalch equation

$$pH = pK + \log \frac{[A^-]}{[HA]}$$

Substituting,

$$7.0 = 6.9 + \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]}$$

$$0.1 = \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]}$$

$$\frac{[HPO_4^{2-}]}{[H_2PO_4^-]} = 1.26$$

Because these two forms of phosphate sum to 100% (the other two forms are negligible), the ratio can be used to calculate the percentage of each form that is present (44% for $H_2PO_4^-$ and 56% for HPO_4^{2-}). Thus, if the cytosolic concentration of phosphate is 1 mM, then the concentration of $H_2PO_4^-$ is 0.44 mM and that of HPO_4^{2-} is 0.56 mM.

2–29 Since the pK values of the two buffering systems are not very different, the key consideration is overall concentration of the buffers. The concentration of globin chains in red blood cells is 6.7 mM.

$$[\text{globin}] = \frac{100\text{ mg}}{\text{mL}} \times \frac{\text{mole}}{15{,}000\text{ g}} \times \frac{\text{g}}{1000\text{ mg}} \times \frac{1000\text{ mL}}{\text{L}}$$

$$= 6.7 \times 10^{-3}\text{ mole/L} = 6.7\text{ mM}$$

If all ten histidines can interact with the cytosol (that is, are not tied up in ionic bonds, for example), then the total concentration of histidines in globin is 67 mM (10 × 6.7 mM). Thus, the potential buffering capacity of the histidines in globin, 67 mM, is much greater than that of phosphate at 1 mM.

2–30

A. The ratio of $[HCO_3^-]$ to $[CO_2(\text{dis})]$ at pH 7.4 is 20.

$$\text{pH} = \text{p}K' + \log\frac{[HCO_3^-]}{[CO_2(\text{dis})]}$$

$$7.4 = 6.1 + \log\frac{[HCO_3^-]}{[CO_2(\text{dis})]}$$

$$\log\frac{[HCO_3^-]}{[CO_2(\text{dis})]} = 1.3 \text{ and } \frac{[HCO_3^-]}{[CO_2(\text{dis})]} = 20$$

Since the total carbonate is 25 mM, HCO_3^- is 23.8 mM [(20/21) × 25 mM] and CO_2(dis) is 1.2 mM [(1/21) × 25 mM].

B. Addition of 5 mM of H^+ would drive 5 mM of HCO_3^- to CO_2(dis), thereby maintaining the equilibrium for hydration and dissociation of CO_2. Thus, addition of 5 mM H^+ would reduce $[HCO_3^-]$ to 18.8 mM, and increase $[CO_2(\text{dis})]$ to 6.2 mM. At these concentrations the pH would be 6.6.

$$\text{pH} = \text{p}K' + \log\frac{[HCO_3^-]}{[CO_2(\text{dis})]}$$

$$= 6.1 + \log\frac{18.8}{6.2}$$

$$\text{pH} = 6.1 + 0.48 = 6.6$$

In a closed system, bicarbonate/CO_2 would provide a very weak buffering system with a very small buffering capacity.

C. In an open system, addition of 5 mM H^+ would cause the same changes as above except that the excess CO_2 would be removed by exhalation, maintaining its concentration at 1.2 mM. Under these conditions the pH would be 7.3.

$$\text{pH} = \text{p}K' + \log\frac{[HCO_3^-]}{[CO_2(\text{dis})]}$$

$$= 6.1 + \log\frac{18.8}{1.2}$$

$$\text{pH} = 6.1 + 1.19 = 7.3$$

Thus, in an open system, the pH decreases by only about 0.1 pH unit. The beauty of this buffering system is that HCO_3^- is constantly being added back to the system through metabolism, which generates CO_2 that is then hydrated to HCO_3^-. Moreover, the two components of the system are independently regulated: CO_2 exhalation by the lungs can be controlled by the rate of breathing, and HCO_3^- can be excreted or retained by the kidneys.

2–31 The concentration of protein is about 200 mg/mL (0.18 × 1.1 g/mL = 198 mg/mL). Note that if you are given the density of the cell, you don't need to know its volume to calculate the concentration of protein.

DATA HANDLING

2–32 The effects on p*K* values are due to electrostatic interactions between the carboxyl and amino groups. In alanine, a large electrostatic attraction between the $-NH_3^+$ and the $-COO^-$ is present at pH 7. This favorable interaction makes it more difficult to remove a proton from $-NH_3^+$, raising its p*K*, and more difficult to add a proton to $-COO^-$, lowering its p*K*. The electrostatic attraction decreases as the amino and carboxyl groups are moved farther and farther away from one another in the oligomers of alanine, virtually disappearing by Ala_4, as reflected in the changes in p*K* values.

Reference: Cantor CR & Schimmel PR (1980) Biophysical Chemistry, Part 1: The Conformation of Biological Macromolecules, pp. 42–46. New York: WH Freeman and Company.

2–33 Assuming that the change in enzyme activity is due to the change in protonation state of histidine, the enzyme must require histidine in the protonated, charged state. The enzyme is active only below the p*K* of histidine (which is typically around 6.5 to 7.0 in proteins), where the histidine is expected to be protonated.

MEDICAL LINKS

2–34 The structures of the sedative (*R*)-thalidomide and the teratogenic (*S*)-thalidomide differ at a single chiral center (**Figure 2–27**). After the teratogen had been identified, it was assumed that if thalidomide had been synthesized as the pure, correct optical isomer, it would have caused no problems. Recent experiments, however, have shown that thalidomide is rapidly racemized (converted to a mixture of optical isomers) in animals. Thus, a protocol designed to synthesize the correct isomer would not have made a difference in the end.

2–35

A. Ethanol in 5% beer is 0.86 M. Pure ethanol is 17.2 M [(789 g/L) × (mole/46 g)], and thus 5% beer would be 0.86 M ethanol (17.2 M × 0.05).
B. At a legal limit of 80 mg/100 mL, ethanol will be 17.4 mM in the blood [(80 mg/0.1 L) × (mmol/46 mg)].

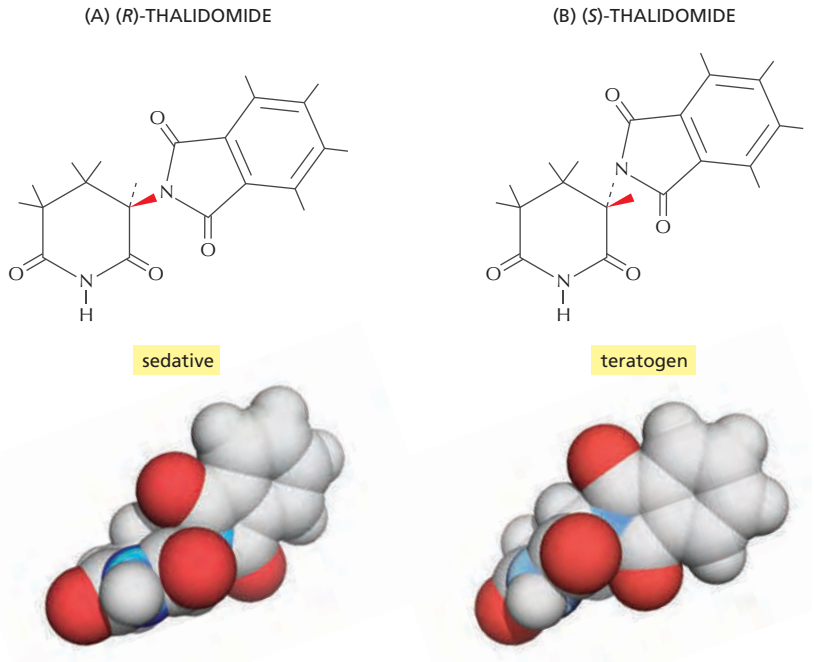


Figure 2–27 The structures of the sedative (*R*) and teratogenic (*S*) forms of thalidomide (Answer 2–34).

C. At the legal limit (17.4 mM), ethanol in 5% beer (0.86 M) has been diluted 49.4-fold (860 mM/17.4 mM). This dilution represents 809 mL in 40 L of body water (40 L/49.4). At 355 mL per beer, this equals 2.3 beers (809 mL/355 mL).
D. It would take nearly 4 hours. At twice the legal limit, the person would contain 64 g of ethanol [(0.16 g/0.1 L) × (40 L)]. The person would metabolize 8.4 g/hr [(0.12 g/hr kg) × (70 kg)]. Thus, to metabolize 32 g of ethanol (the amount in excess of the legal limit) would require 3.8 hours [(32 g) × (hr/8.4 g)].

CATALYSIS AND THE USE OF ENERGY BY CELLS

DEFINITIONS

2–36 Activation energy

2–37 Standard free-energy change ($\Delta G°$)

2–38 Oxidation

2–39 Substrate

2–40 Diffusion

2–41 Enzyme

2–42 Coupled reaction

2–43 Equilibrium

2–44 Free energy (G)

TRUE/FALSE

2–45 True. The difference between plants and animals is in how they obtain their food molecules. Plants make their own using the energy of sunlight, plus CO_2 and H_2O, whereas animals must forage for their food.

2–46 True. Oxidation–reduction reactions refer to those in which electrons are removed from one atom and transferred to another. Since the number of electrons is conserved (no loss or gain) in a chemical reaction, oxidation—removal of electrons—must be accompanied by reduction—addition of electrons.

2–47 False. The equilibrium constant for the reaction A $\rightleftharpoons$ B remains unchanged; it's a constant. Linking reactions together can convert an unfavorable reaction into a favorable one, but it does so not by altering the equilibrium constant, but rather by changing the concentration ratio of products to reactants.

THOUGHT PROBLEMS

2–48 Organic chemistry in laboratories—even the very best—is rarely carried out in a water environment because of low solubility of some components and because water is reactive and usually competes with the intended reaction. The most dramatic difference, however, is the complexity. It is critical in laboratory organic chemistry to use pure components to ensure a high yield of the intended product. By contrast, living cells carry out thousands of different reactions simultaneously with good yield and virtually no interference between reactions. The key, of course, is that cells use enzyme catalysts, which bind substrate molecules in an active site, where they are isolated from the rest of the environment. There the reactivity of individual atoms can be manipulated to encourage the

correct reaction. It is the ability of enzymes to provide such special environments—miniature reaction chambers—that allows the cell to carry out an enormous number of reactions simultaneously without cross-talk between them.

2–49 Catabolic pathways break down larger molecules (often derived from food) into smaller molecules, and abstract energy in a useful form in the process. Anabolic pathways (or biosynthetic pathways) construct larger molecules from smaller ones. The small molecules generated by catabolic pathways are used as starting points and intermediates in anabolic pathways, and the energy from catabolic pathways, harnessed in the form of activated carriers, is used to drive the energetically unfavorable process of biosynthesis.

2–50 The second law of thermodynamics applies to closed systems, which could be a chamber in a scientist's laboratory, for example, or the entire universe. Closed systems do not exchange matter or energy with their surroundings. Living organisms such as cells and human beings are not closed systems; they continually exchange matter and energy with their surroundings. It is perfectly permissible for a portion of a closed system—a human being in the universe—to increase its order, provided that the rest of the system (the rest of the universe) becomes disordered to a greater extent. This is what living organisms do: they take in food and use the energy to increase their order. But to do so they release waste products that are less complex (less ordered) than the food they took in, and much of the energy in the food is released in its most disordered form—as heat. Whatever order is created within a cell or an organism is more than paid for by the disorder introduced into its environment.

2–51 If the reaction is rewritten as its two half reactions, it is then clear that Na is oxidized and Cl is reduced.

$$2\,Na \rightarrow 2\,Na^+ + 2\,e^-$$
$$2\,e^- + Cl_2 \rightarrow 2\,Cl^-$$
$$\text{NET:}\quad 2\,Na + Cl_2 \rightarrow 2\,Na^+ + 2\,Cl^-$$

Electrons are removed from sodium; therefore it is oxidized. Electrons are added to chlorine; therefore it is reduced.

2–52

A. For polymerization to be favored at high temperature and depolymerization to be favored at low temperature, ΔH and ΔS must both be positive. At high temperature, where polymerization is favored, the $-T\Delta S$ term becomes large enough to overcome the positive ΔH term, yielding a negative, favorable ΔG for polymerization. At low temperature, where depolymerization is favored, the $-T\Delta S$ becomes small enough that it is outweighed by the positive ΔH term, giving rise to a positive, unfavorable ΔG for polymerization.

B. It seems counterintuitive that polymerization of free tubulin subunits into highly ordered microtubules should occur with an overall increase in entropy (decrease in order). But it is counterintuitive only if one considers the subunits in isolation. Remember that thermodynamics refers to the whole system, which includes the water molecules. The increase in entropy is due largely to the effects of polymerization on water molecules. The surfaces of the tubulin subunits that bind together to form microtubules are fairly hydrophobic, and constrain (order) the water molecules in their immediate vicinity. Upon polymerization, these constrained water molecules are freed up to interact with other water molecules. Their new-found disorder much exceeds the increased order of the protein subunits, and thus the net increase in entropy (disorder) favors polymerization.

2–53 The statement is correct. A reaction with a negative $\Delta G°$, for example, would not proceed spontaneously under conditions where there is already an excess of products over those that would be present at equilibrium. Conversely, a reaction with a positive $\Delta G°$ would proceed spontaneously under conditions where there is an excess of substrates compared to those present at equilibrium.

2–54 At the same concentrations, the ΔG values for the forward and reverse reactions will be the same magnitude but differ in sign. Thus, the ΔG for C + D ⟶ A + B is 20 kJ/mole.

2–55 Absolutely none. These values provide information about how energetically favorable a reaction is under standard conditions ($\Delta G°$) and under actual conditions (ΔG). They provide no information about the rate at which a favorable reaction will occur. Rates depend on other factors: in the cell, most commonly on the existence of enzymes and their properties.

2–56 The cell links these two reactions by providing an enzyme that catalyzes the net reaction directly. Thus, in the cell, phosphorylation of glucose does not occur as the sum of two reactions but as a single reaction. The enzyme hexokinase, for example, binds glucose and ATP and catalyzes the transfer of a phosphate directly from ATP to glucose.

2–57 The free energy ΔG (–45 to –55 kJ/mole) derived from ATP hydrolysis depends on both $\Delta G°$ (–30.5 kJ/mole) and the concentrations of the substrates and products.

$$\Delta G = \Delta G° + 5.9 \text{ kJ/mole} \log \frac{[\text{ADP}][\text{P}_\text{i}]}{[\text{ATP}]}$$

Given that $\Delta G°$ is –30.5 kJ/mole, the ratio of $[\text{ADP}][\text{P}_\text{i}]/[\text{ATP}]$ in cells must range from a little more than 10^{-3} (ΔG = –48 kJ/mole) to a little less than 10^{-4} (ΔG = –54 kJ/mole), as they do under different cellular conditions.

2–58 Enzyme A is beneficial. It allows the interconversion of two energy carrier molecules, both of which are required in the triphosphate form for many metabolic reactions. Any ADP that is formed is quickly converted to ATP by oxidative phosphorylation, and thus the cell maintains a high [ATP]/[ADP] ratio. Because of enzyme A, called nucleotide phosphokinase, some of the ATP is used to keep the [GTP]/[GDP] ratio similarly high.

Enzyme B would be highly detrimental to the cell. Cells use NAD^+ as an electron acceptor in catabolic reactions and must maintain a high $[NAD^+]/[NADH]$ ratio to support the breakdown of glucose and fats to make ATP. By contrast, NADPH is used as an electron donor in biosynthetic reactions; cells thus maintain a high $[NADPH]/[NADP^+]$ ratio to drive the synthesis of various biomolecules. Since enzyme B would bring both ratios to 1, it would reduce the rates of both catabolic *and* anabolic reactions.

2–59 The two lists match up as follows: A with 1; B with 5; C with 6; D with 2; E with 3; and F with 4.

2–60 Reactions B, D, and E all require coupling to other, energetically favorable reactions. In each case, molecules are made that have higher-energy bonds. By contrast, in reactions A and C, simpler molecules (A) or lower-energy bonds (C) are made.

CALCULATIONS

2–61 The enzyme catalyzes events at the rate of 1 event/3.2×10^{-5} sec, or 1 event/32 μsec [(10^{14} events/100 yr) × (yr/365 days) × (days/24 hr) × (hr/60 min) × (min/60 sec)].

2–62 The instantaneous velocities are $H_2O = 3.8 \times 10^4$ cm/sec, glucose = 1.2×10^4 cm/sec, and myoglobin = 1.3×10^3 cm/sec. The calculation for a water molecule, which has a mass of 3×10^{-23} g [(18 g/mole) × (mole/6×10^{23} molecules)], is shown below.

$$v = (kT/m)^{1/2}$$

$$v = \left(\frac{1.38 \times 10^{-16}\ \text{g cm}^2}{\text{K sec}^2} \times 310\ \text{K} \times \frac{1}{3 \times 10^{-23}\ \text{g}}\right)^{1/2}$$

$$v = 3.78 \times 10^4\ \text{cm/sec}$$

When these numbers are converted to km/hr the results are fairly astounding. Water moves at 1360 km/hr, glucose at 428 km/hr, and myoglobin at 47 km/hr. Thus, even the largest (slowest) of these molecules is moving faster than the swiftest human sprinter! And water molecules are traveling at Mach 1.1! Unlike a human sprinter, or a jet airplane, these molecules make forward progress only slowly because they are constantly colliding with other molecules in solution.

Reference: Berg HC (1993) Random Walks in Biology, Expanded Edition, pp. 5–6. Princeton, NJ: Princeton University Press.

2–63 It would take glucose an average of 0.13 second and myoglobin an average of 1.3 seconds to diffuse 20 μm. The calculation for glucose is

$$t = x^2/6D$$

$$t = (20\ \mu\text{m})^2 \times \frac{(\text{cm})^2}{(10^4\ \mu\text{m})^2} \times \frac{\text{sec}}{6 \times (5 \times 10^{-6}\ \text{cm}^2)}$$

$$t = 0.13\ \text{sec}$$

Reference: Berg HC (1993) Random Walks in Biology, Expanded Edition, pp. 5–6. Princeton, NJ: Princeton University Press.

2–64

A. At equilibrium, ΔG is zero (for any reaction); there is no tendency for the reaction to proceed in one direction over the other direction. Substituting K for [F6P]/[G6P] gives

$$0 = \Delta G^\circ + 2.3\ RT \log K$$

$$\Delta G^\circ = -2.3\ RT \log K, \text{ or } -5.9\ \text{kJ/mole} \log K$$

B. At equilibrium, ΔG is zero and ΔG° is 1.78 kJ/mole.

$$\Delta G^\circ = -2.3\ RT \log \frac{[\text{F6P}]}{[\text{G6P}]}$$

Substituting 5.9 kJ/mole for 2.3 RT,

$$= \frac{-5.9\ \text{kJ}}{\text{mole}} \log (0.5)$$

$$= 1.78\ \text{kJ/mole}$$

C. Since ΔG° relates to the equilibrium, it is unchanged; that is, $\Delta G^\circ = 1.78$ kJ/mole. At $\Delta G = -2.5$ kJ/mole, the ratio of [F6P] to [G6P] is 0.19.

$$\Delta G = \Delta G^\circ + 2.3\ RT \log \frac{[\text{F6P}]}{[\text{G6P}]}$$

$$\frac{-2.5\ \text{kJ}}{\text{mole}} = \frac{1.78\ \text{kJ}}{\text{mole}} + \frac{5.9\ \text{kJ}}{\text{mole}} \log \frac{[\text{F6P}]}{[\text{G6P}]}$$

$$\log \frac{[\text{F6P}]}{[\text{G6P}]} = \frac{-4.28\ \text{kJ/mole}}{5.9\ \text{kJ/mole}} = -0.73$$

$$\frac{[\text{F6P}]}{[\text{G6P}]} = 0.19$$

2–65 The whole population of ATP molecules in the body would turn over

(cycle) 1800 times per day, or a little more than once a minute. Conversion of 3 moles of glucose to CO_2 would generate 90 moles of ATP [(3 moles glucose) × (30 moles ATP/mole glucose)]. The whole body contains 5×10^{-2} mole ATP [$(2 \times 10^{-3}$ mole/L) × 25 L]. Since the concentration of ATP doesn't change, each ATP must cycle 1800 times per day [(90 moles ATP/day)/$(5 \times 10^{-2}$ mole ATP)].

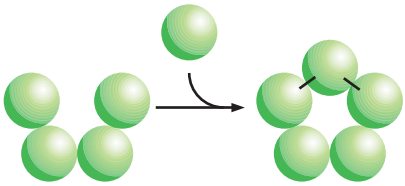


Figure 2–28 Closure of the pentameric ring with two bonds (Answer 2–66).

DATA HANDLING

2–66 Addition of the final subunit into the ring involves two sets of bonds, one to each of its neighbors (**Figure 2–28**). If the bonds were equally strong, then you might expect a squaring of the equilibrium constant (a doubling of ΔG). In reality, the situation is somewhat more complex and can give rise to equilibrium constants several orders of magnitude higher than the square. This rather simple treatment, however, serves to illustrate the stability that can be gained by closure. Icosahedral viruses, for example, are very stable as a consequence of closure in three dimensions.

Reference: Howard J (2001) Mechanics of Motor Proteins and the Cytoskeleton, pp. 151–163. Sunderland, MA: Sinauer Associates, Inc.

2–67 The theoretical underpinnings of the assertion that all ΔG values must be negative are so strong that the error must lie with the experiment. One potential source of error is that the concentrations of the intermediates have not been measured precisely enough. That is the most likely explanation given the formidable experimental challenges to such precise, instantaneous measurements of concentration. The other possibility is that the ΔG° values (equilibrium values) are slightly off relative to their true values under physiological conditions.

References: Berg JM, Tymoczko JL & Stryer L (2002) Biochemistry, Fifth Edition, pp. 436–437. New York: WH Freeman and Co.

Minakami S, Suzuki C, Saito T & Yoshikawa H (1965) Studies on erythrocyte glycolysis. I. Determination of the glycolytic intermediates in human erythrocytes. *J. Biochem.* (*Tokyo*) 58, 543–550.

HOW CELLS OBTAIN ENERGY FROM FOOD

DEFINITIONS

2–68 Citric acid cycle

2–69 Fat

2–70 Glycogen

2–71 Oxidative phosphorylation

2–72 Electron-transport chain

2–73 Glycolysis

TRUE/FALSE

2–74 False. Glycolysis is the *only* metabolic pathway that can generate ATP in the absence of oxygen. There are many circumstances in which cells are temporarily exposed to anoxic conditions, during which time they survive by glycolysis. For example, in an all-out sprint, the circulation cannot deliver adequate oxygen to leg muscles, which continue to power muscle contraction by passing large amounts of glucose (from glycogen) down the glycolytic pathway. Similarly, there are several human cell types that

do not carry out oxidative metabolism; for example, red blood cells, which have no mitochondria, make ATP via glycolysis. Thus, glycolysis is critically important, but it's sort of like insurance: it's not so important until you need it, and then it's hard to do without.

2–75 True. Oxygen is not a substrate (or a product) for any reaction in the citric acid cycle. Thus, the reactions can occur in the absence of oxygen. In cells, however, the reactions cannot proceed for very long in the absence of oxygen because NADH and $FADH_2$ cannot be converted back to NAD^+ and FAD by oxidative phosphorylation (which does depend on oxygen). In the absence of NAD^+ and FAD, four separate reactions of the cycle (see Figure 2–21) will cease to operate.

THOUGHT PROBLEMS

2–76 The two lists match up as follows: A with 2 and 3, B with 4, and C with 1.

2–77

A. One way to balance the equation for a pathway is to write down each reaction and sum them all up, as done below for the first two reactions of glycolysis.

$$\begin{array}{ll} (1) & \text{Glucose} + \text{ATP} \longrightarrow \text{G6P} + \text{ADP} + \text{H}^+ \\ (2) & \text{G6P} \longrightarrow \text{F6P} \\ \text{SUM:} & \text{Glucose} + \text{ATP} + \text{G6P} \longrightarrow \text{G6P} + \text{F6P} + \text{ADP} + \text{H}^+ \end{array}$$

Note that in the sum, the intermediate G6P appears on both sides and thus cancels out. Because the pathway intermediates always drop out of such a balanced equation, there is a less tedious way to balance the equation: all molecules at the blunt ends of the arrows are reactants and all molecules at the pointed ends are products. Ignore the intermediates, but pay careful attention to stoichiometry, since it is usually just the flow that is indicated. Using this method the equation for the first stage of glycolysis is

$$\text{Glucose} + 2\ \text{ATP} \longrightarrow 2\ \text{G3P} + 2\ \text{ADP} + 2\ \text{H}^+$$

B. The equation for the second stage of glycolysis is

$$\text{G3P} + \text{P}_\text{i} + \text{NAD}^+ + 2\ \text{ADP} \longrightarrow \text{pyruvate} + \text{NADH} + 2\ \text{ATP} + \text{H}_2\text{O}$$

C. The overall equation is just the sum of these two equations, after doubling the numbers in the second equation to get the stoichiometry right.

$$\text{Glucose} + 2\ \text{ATP} \longrightarrow 2\ \text{G3P} + 2\ \text{ADP} + 2\ \text{H}^+$$

$$2\ \text{G3P} + 2\ \text{P}_\text{i} + 2\ \text{NAD}^+ + 4\ \text{ADP} \longrightarrow 2\ \text{pyruvate} + 2\ \text{NADH} + 4\ \text{ATP} + 2\ \text{H}_2\text{O}$$

$$\text{SUM: Glucose} + 2\ \text{ADP} + 2\ \text{P}_\text{i} + 2\ \text{NAD}^+ \longrightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}_2\text{O} + 2\ \text{H}^+$$

2–78 Under anaerobic conditions, cells are unable to make use of pyruvate—the end product of the glycolytic pathway—and NADH. The electrons carried in NADH are normally delivered to the electron-transport chain for oxidative phosphorylation, but in the absence of oxygen the carried electrons are a waste product, just like pyruvate. Thus, in the absence of oxygen, pyruvate and NADH accumulate. Fermentation combines these waste products into a single molecule, either lactate or ethanol, which is shipped out of the cell.

The flow of material through the glycolytic pathway could not continue in the absence of oxygen in cells that cannot carry out fermentation. Because NAD^+ + NADH is present in cells in limited quantities, anaerobic glycolysis in the absence of fermentation would quickly convert the pool largely to NADH. The change in the ratio NAD^+/NADH would stop glycolysis at the step in which glyceraldehyde 3-phosphate (G3P) is converted

to 1,3-bisphosphoglycerate (1,3BPG), a step with only a small negative ΔG normally (see Table 2–3). The purpose of fermentation is to regenerate NAD^+ by transferring the pair of carried electrons in NADH to pyruvate and excreting the product. Thus, fermentation allows glycolysis to continue.

2–79 In the absence of oxygen, the energy needs of the cell must be met by fermentation to lactate, which requires a high rate of flow through glycolysis to generate sufficient ATP. When oxygen is added, the cell can generate ATP by oxidative phosphorylation, which generates ATP much more efficiently than glycolysis. Thus, less glucose is needed to supply ATP at the same rate.

2–80 In the presence of arsenate, 1-arseno-3-phosphoglycerate is formed instead of 1,3-bisphosphoglycerate (**Figure 2–29**). Because it is sensitive to hydrolysis in water, the arsenate high-energy bond is destroyed before the molecule that contains it can diffuse to the next enzyme. The product of the hydrolysis, 3-phosphoglycerate, is the same product normally formed, but because it is formed nonenzymatically, the reaction is not coupled to ATP formation. Arsenate wastes metabolic energy by uncoupling many phosphotransfer reactions by the same mechanism, and that is why it is so poisonous.

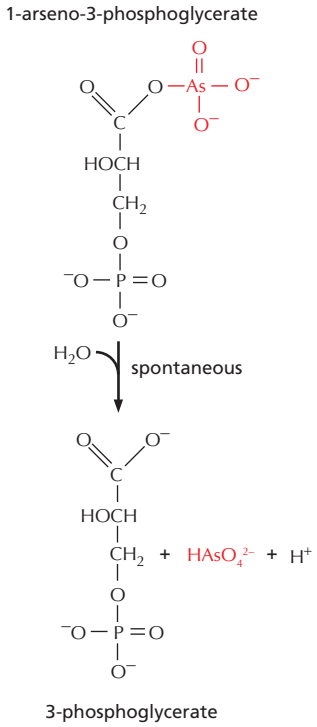


Figure 2–29 Hydrolysis of 1-arseno-3-phosphoglycerate (Answer 2–80).

2–81 The reverse of the forward reaction is simply not a possibility under physiological conditions. Recall from Problem 2–67 that flow of material through a pathway requires that the ΔG values for *every* step must be negative. Thus, for a flow from liver glycogen to serum glucose, the step from glucose 6-phosphate to glucose must have a negative ΔG. To simply reverse the forward reaction (that is, G6P + ADP $\rightarrow$ GLC + ATP, ΔG° = 16.7 kJ/mole) would require that the concentration ratio [GLC][ATP]/ [G6P][ADP] be less than $10^{-2.84}$ (0.0015) in order to bring the reaction to equilibrium ($\Delta G = 0$).

$$\Delta G = \Delta G^\circ + 5.9 \text{ kJ/mole} \log \frac{[\text{GLC}][\text{ATP}]}{[\text{G6P}][\text{ADP}]}$$

$$0 = 16.7 \text{ kJ/mole} + 5.9 \text{ kJ/mole} \log \frac{[\text{GLC}][\text{ATP}]}{[\text{G6P}][\text{ADP}]}$$

$$\log \frac{[\text{GLC}][\text{ATP}]}{[\text{G6P}][\text{ADP}]} = \frac{-16.7 \text{ kJ/mole}}{5.9 \text{ kJ/mole}} = -2.83$$

$$\frac{[\text{GLC}][\text{ATP}]}{[\text{G6P}][\text{ADP}]} = 0.0015$$

Inside a functioning cell, such as a liver cell exporting glucose, the concentration of ATP always exceeds that of ADP, but just for illustrative purposes let's assume the ratio is 1. Under these conditions, the ratio [GLC]/ [G6P] must be 0.00145; that is, the concentration of G6P must be nearly 700 times higher than that of GLC. Since the circulating concentration of glucose is maintained at between 4 and 5 mM, this corresponds to about 3 M G6P, an impossible concentration given that the total concentration of cellular phosphate is less than about 25 mM.

2–82 The statement is incorrect. The oxygen atoms that are part of CO_2 do not come from the oxygen atoms that are consumed as part of the oxidation of glucose (or of any other food molecule). The electrons that are abstracted from glucose at various stages in its oxidation are finally transferred to oxygen to produce water during oxidative phosphorylation. Thus, the oxygen used during oxidation of food in animals ends up as oxygen atoms in H_2O.

One can show this directly by incubating living cells in an atmosphere that contains molecular oxygen enriched for the isotope ^{18}O, instead of

the naturally occurring isotope, ^{16}O. In such an experiment, one finds that all the CO_2 released from cells contains only ^{16}O. Therefore, the oxygen atoms in the released CO_2 molecules do not come directly from the atmosphere, but rather from the organic molecules themselves and from H_2O.

CALCULATIONS

2–83 At this rate of ATP regeneration, the cell will consume oxygen at 6.7×10^{-15} L/min [$(0.9 \times 10^9$ ATP/min) × (1 O_2/5 ATP) × (22.4 L/6×10^{23} O_2) = 6.72×10^{-15} L/min]. The volume of the cell is 10^{-12} L [(1000 μm^3) × (cm/10^4 μm)^3 × (mL/cm^3) × (L/1000 mL)]. Dividing the cell volume by the rate of consumption of O_2 [(10^{-12} L)/(6.7×10^{-15} L/min)] indicates that the cell will consume its own volume of oxygen in 149 minutes, or about 2.5 hours. (Since air contains only 20% oxygen, a cell would consume its own volume of air in about 30 minutes.)

2–84 The human body operates at about 70 watts—about the same as a light bulb.

$$\frac{\text{watts}}{\text{body}} = \frac{10^9 \text{ ATP}}{60 \text{ sec cell}} \times \frac{5 \times 10^{13} \text{ cells}}{\text{body}} \times \frac{\text{mole}}{6 \times 10^{23} \text{ ATP}} \times \frac{50 \text{ kJ}}{\text{mole}} \times \frac{10^3 \text{ J}}{\text{kJ}}$$

$$= \frac{69.4 \text{ J/sec}}{\text{body}} = \frac{69.4 \text{ watts}}{\text{body}}$$

2–85 You would need to expend 2070 kJ in climbing from Zermatt to the top of the Matterhorn, a vertical distance of 2818 m. Substituting into the equation for work

$$\text{work} = 75 \text{ kg} \times \frac{9.8 \text{ m}}{\text{sec}^2} \times 2818 \text{ m} \times \frac{\text{J}}{\text{kg m}^2/\text{sec}^2} \times \frac{\text{kJ}}{10^3 \text{ J}}$$

$$= 2070 \text{ kJ}$$

This is equal to about 1.5 Snickers™ (2070 kJ/1360 kJ), so you would be well advised to plan a stop at Hörnli Hut to eat another one.

In reality, the human body does not convert chemical energy into external work at 100% efficiency, as assumed in this answer, but rather at an efficiency of around 25%. Moreover, you will be walking laterally as well as uphill. Thus, you would need more than 6 Snickers to make it all the way.

Reference: Frayn KN (1996) Metabolic Regulation: A Human Perspective, p. 179. London: Portland Press.

2–86

A. The ΔG in resting muscle is –0.8 kJ/mole.

$$\Delta G = \Delta G° + 5.9 \text{ kJ/mole} \log \frac{[C][ATP]}{[CP][ADP]}$$

$$= -13.8 \text{ kJ/mole} + 5.9 \text{ kJ/mole} \log \frac{(13 \times 10^{-3})(4 \times 10^{-3})}{(25 \times 10^{-3})(0.013 \times 10^{-3})}$$

$$= -13.8 \text{ kJ/mole} + 5.9 \text{ kJ/mole} \log 160$$

$$= -13.8 \text{ kJ/mole} + 13.0 \text{ kJ/mole}$$

$$\Delta G = -0.8 \text{ kJ/mole}$$

This very small negative value should not surprise you; it says that this energy-buffering system is nearly at equilibrium, which you should expect in the absence of heavy ATP usage.

B. If the concentration of ATP decreases to 3 mM and that of ADP increases to 1 mM, then the ΔG for the reaction will be –12.7 kJ/mole.

$$\Delta G = \Delta G° + 5.9 \text{ kJ/mole} \log \frac{[C][ATP]}{[CP][ADP]}$$
$$= -13.8 \text{ kJ/mole} + 5.9 \text{ kJ/mole} \log \frac{(13 \times 10^{-3})(3 \times 10^{-3})}{(25 \times 10^{-3})(1 \times 10^{-3})}$$
$$= -13.8 \text{ kJ/mole} + 5.9 \text{ kJ/mole} \log 1.56$$
$$= -13.8 \text{ kJ/mole} + 1.14 \text{ kJ/mole}$$
$$\Delta G = -12.7 \text{ kJ/mole}$$

Thus, as soon as exercise begins, the reaction will become highly favored and creatine phosphate will drive the conversion of ADP to ATP. In reality, the enzyme that catalyzes this reaction is efficient enough to keep the reaction nearly at equilibrium ($\Delta G = 0$) so that ATP levels remain high as creatine phosphate levels fall, fulfilling its role as an energy buffer.

C. If ATP (4 mM) could sustain a sprint for 1 second, then creatine phosphate (25 mM) could sustain a sprint for an additional 6 seconds by regenerating an equal amount of ATP. This is not long enough to allow a sprinter to finish 200 meters; thus, there must be another source of energy. The additional energy comes from the breakdown of muscle glycogen, which is processed through anaerobic glycolysis, producing lactate and ATP. Typical stores of muscle glycogen are sufficient for about 80 seconds of sprinting.

DATA HANDLING

2–87 Knoop's result was surprising at the time. One might have imagined that the obvious way to metabolize fatty acids to CO_2 would be to remove the carboxylate group from the end as CO_2 and then oxidize the newly exposed carbon atom until it too could be removed as CO_2. However, removal of single-carbon units is not consistent with Knoop's results, since it predicts that odd- and even-number fatty acids would generate the same final product. Similar inconsistencies crop up with removal of fragments containing more than two carbon atoms. For example, removal of three-carbon fragments would work for the eight-carbon and seven-carbon fatty acids shown in Figure 2–20, but would not work for six-carbon and five-carbon fatty acids.

Removal of two-carbon fragments from the carboxylic acid end is the only scheme that accounts for the consistent difference in the metabolism of odd- and even-number fatty acids. One might ask why the last two-carbon fragment is not removed from phenylacetate. It turns out that the benzene ring interferes with the fragmentation process, which involves modification of the third carbon from the carboxylic acid end. Since that carbon is part of the benzene ring in phenylacetate, it is protected from modification and further metabolism of phenylacetate is blocked.

Knoop's results also specify the direction of degradation. If the nonacidic end of the chain were attacked first, either the benzene ring would make the fatty acids resistant to metabolism, or the same benzene compound would always be excreted, independent of the length of the fatty acid fed to the dogs.

Reference: Knoop F (1905) Der Abbau aromatischer Fettsäuren im Tierkörper. *Beitr. Chem. Physiol.* 6, 150–162.

2–88

A. If citrate were an intermediate in a linear pathway of oxidation, its addition would be expected to lead to an increase in oxygen consumption that would match the requirements for oxidation of the carbon atoms in citrate. The balanced chemical equation shows that each mmol of citrate would require 4.5 mmol of oxygen.

B. Krebs's insight was to recognize that if citrate was an intermediate in a cyclic pathway, then a small amount could lead to substantial increases in the oxidation of other molecules (acetyl CoA) that feed into the pathway. In essence, citrate would behave catalytically.

Krebs's rationale is clearly laid out in the paper: "Since citric acid reacts catalytically in the tissue, it is probable that it is removed by a primary reaction but regenerated by a subsequent reaction. In the balance sheet no citrate disappears and no intermediate products accumulate."

C. *E. coli* and yeast do indeed use the citric acid cycle. Krebs got this point wrong because he did not realize (nor did anyone for a long time) that citrate cannot get into these cells. Therefore, when he added citrate to intact *E. coli* and yeast, he found no stimulation of oxygen consumption. Passage of citrate across a membrane requires a transport system, which is present in mitochondria but is absent from yeast and *E. coli* plasma membranes.

References: Krebs HA & Johnson WA (1973) The role of citric acid in intermediate metabolism in animal tissues. *Enzymologia* 4, 148–156.

See also Albert Szent-Györgyi's Nobel Lecture (1937) at www.nobel.se/medicine/laureates/1937/szent-gyorgyi-lecture.pdf

2–89

A. The cross-feeding experiments indicate that the three steps controlled by the products of the *TrpB*, *TrpD*, and *TrpE* genes are arranged in the order

$$X \xrightarrow{\text{TrpE}} Y \xrightarrow{\text{TrpD}} Z \xrightarrow{\text{TrpB}} \text{tryptophan}$$

where X, Y, and Z are undefined intermediates in the pathway.

The ability of the *TrpE*$^-$ strain to be cross-fed by the other two strains indicates that the *TrpD*$^-$ and *TrpB*$^-$ strains accumulate intermediates that are farther along the pathway than the step controlled by the *TrpE* gene. The ability of the *TrpD*$^-$ strain to be cross-fed by the *TrpB*$^-$ strain but not the *TrpE*$^-$ strain places it in the middle. The inability of the *TrpB*$^-$ strain to be cross-fed by either of the other strains is consistent with its controlling the step closest to tryptophan.

B. The patterns of growth on minimal medium supplemented with known intermediates in the tryptophan biosynthetic pathway are consistent with the order deduced from cross-feeding:

$$\text{chorismate} \xrightarrow{\text{TrpE}} \text{anthranilate} \xrightarrow{\text{TrpD}} \text{indole} \xrightarrow{\text{TrpB}} \text{tryptophan}$$

In reality, of course, the intermediates for the pathway were unknown (or not fully known) at the time the cross-feeding experiments were done. The intermediates were worked out by a combination of educated guesses at the likely intermediates, which could then be tested on mutant strains, and of analysis of the compounds that accumulated in the mutants.

Reference: Yanofsky C (2001) Advancing our knowledge in biochemistry, genetics, and microbiology through studies on tryptophan metabolism. *Annu. Rev. Biochem.* 70, 1–37.

MCAT STYLE

2–90

C. In cells that rely on glycolysis for ATP production, pyruvate, the end product of glycolysis, is converted to lactate, which is then released from the cell as a by-product. Production of CO_2 and oxidation of pyruvate both depend on aerobic respiration and thus would not have provided

evidence for the Warburg effect. CO_2 is released as the end product of oxidation of pyruvate, and pyruvate is oxidized in the citric acid cycle as a prelude to oxidative phosphorylation in the mitochondrion.

2–91

C. Pyruvate kinase catalyzes the final ATP-generating step in glycolysis and would be a good enzyme to target if one wanted to inhibit glycolysis. Currently, several compounds that target glycolysis are in development as anticancer drugs. The other choices are incorrect because they are all involved in aerobic respiration: acetyl CoA and isocitrate dehydrogenase function in the citric acid cycle and the electron-transport chain is central to oxidative phosphorylation.

2–92

B. Rapidly proliferating cells have increased requirements for synthesis of the macromolecules needed for cell growth, and the intermediates in glycolysis can be readily shunted to pathways for macromolecular synthesis. Aerobic respiration, in contrast, converts the carbon atoms in glucose to CO_2, which cannot be used for macromolecular synthesis. Choice A is not correct because the NADH generated in glycolysis is used for energy production, not as a source of electrons for the anabolic reactions used to produce macromolecules; the reducing power needed for macromolecular synthesis is provided by NADPH. Choice C is not correct because glycolysis produces much less NADH than aerobic respiration. Choice D is not correct because glycolysis does not produce fatty acids. Fatty acids are also not an important source of energy for cancer cells, since fatty acid metabolism requires aerobic respiration.

2–93

B. Cells undergoing increased rates of glycolysis must import large amounts of glucose. Typically, patients are given fluorodeoxyglucose, a glucose derivative that cancer cells take up just like glucose. The other choices are incorrect because each of those molecules is a product of glycolysis and would not be taken up by cells undergoing increased glycolysis.

2–94

A. At a minimum, a useful reaction for producing electrons for CO_2 reduction and energy production must occur with a decrease in free energy, which is the definition of an energetically favorable reaction. In addition, it must liberate electrons, which oxidation does, not consume them, as reduction does.

2–95

B. Oxidation of H_2S to S and 2 H^+ occurs with a decrease in free energy and liberates electrons that can be used for energy production or reduction of CO_2. Choice A is not correct because the cells can survive on CO_2 as their sole source of carbon, so they must be capable of generating energy independently of glucose. Choices C and D are incorrect because reduction reactions consume electrons.

CHAPTER 3

Proteins

IN THIS CHAPTER

THE SHAPE AND STRUCTURE OF PROTEINS

DEFINITIONS

3–1 Quaternary structure

3–2 α Helix

3–3 Primary structure

3–4 Binding site

3–5 Amyloid fibril

3–6 Polypeptide backbone

3–7 β Sheet

3–8 Protein domain

3–9 Secondary structure

TRUE/FALSE

3–10 True. In a β sheet, the amino acid side chains in each strand are alternately positioned above and below the sheet. This relationship can be seen in Figure 3–33 (see Answer 3–19), which shows that the carbonyl oxygens alternate from one side of the strand to the other. Thus, each strand in a β sheet can be viewed as a helix in which each successive amino acid is rotated 180°.

3–11 False. Intrinsically disordered regions of proteins typically have amino acid sequences with low hydrophobicity and high net charge. Low hydrophobicity reduces the effect of the hydrophobic force, which normally tends to drive the protein into a more condensed and ordered structure. A high net charge (either positive or negative) pushes similarly charged regions of the protein away from one another. By contrast, an amino acid sequence with high hydrophobicity and low net charge would tend to collapse into a defined structure.

3–12 True. Chemical groups on such protruding loops can often surround a molecule, allowing the protein to bind to it with many weak bonds.

3–13 False. In order for a prion disease to spread from one organism to another, the second organism must eat a tissue that contains a self-propagating amyloid fibril. The gene encoding the protein involved in amyloid fibril formation is not involved in the transmission from one organism to another.

THOUGHT PROBLEMS

3–14 The synthesis of a macromolecule with a unique structure requires that a single stereoisomer is used at each position. Changing one amino acid from its L to its D form would result in a different protein. Thus, if a random mixture of the D and L forms were used to build a protein, its amino acid sequence would not specify a single structure, but rather many different structures (2^n different structures would be formed, where n is the number of amino acids in the protein).

Why L-amino acids were selected in evolution as the exclusive building blocks of proteins is a mystery; we could easily imagine a cell in which certain (or even all) amino acids were used in the D forms to build proteins, as long as these particular stereoisomers were used exclusively.

3–15

A. Heating egg-white proteins denatures them, allowing them to interact with one another in ways that were not possible at the lower temperature of the hen's oviduct. This process forms a tangled meshwork of polypeptide chains. In addition to these interactions, interchain disulfide bonds also form, so that hard-boiled egg white becomes one giant macromolecule.

B. Dissolving hard-boiled egg white requires a strong detergent to overcome the noncovalent interchain bonds and mercaptoethanol to break the covalent disulfide bonds. Together, but not separately, the two reagents eliminate the bonds that hold the tangled protein chains in place. Try it for yourself!

3–16 In an α helix, the carbonyl oxygen of the first amino acid hydrogen-bonds to the amide nitrogen of amino acid 5 (**Figure 3–30**). Thus, there can be no α helix shorter than five amino acids. The single hydrogen bond that would be formed with five amino acids gives too little stability to the structure for any helicity to be detected. Only with six amino acids—two hydrogen bonds—do you begin to detect some helicity. Helicity becomes increasingly apparent as more amino acids, hence more hydrogen bonds, are added.

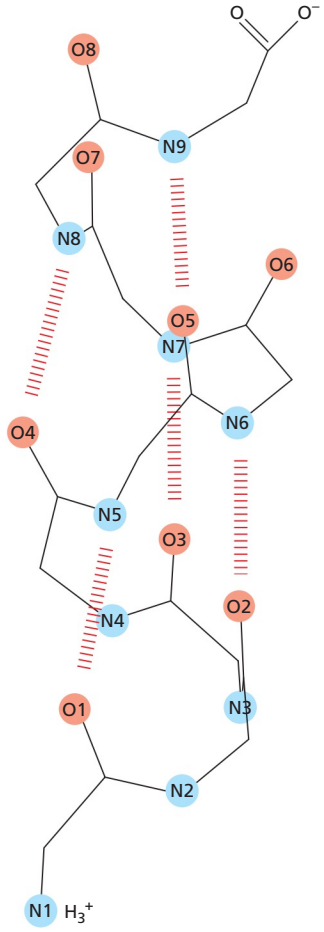


Figure 3–30 Schematic of an α helix, showing the pattern of hydrogen bonds between carbonyl oxygens and amide nitrogens within the helix (Answer 3–16).

3–17 The ends of α helices, like polar amino acids, are almost always found at the surface of a protein where they can interact with polar water molecules. In addition to their partial charge, the backbones of the four amino acids at either end of the helix carry hydrogen-bonding groups that are unsatisfied by hydrogen-bonding within the helix (**Figure 3–31**). These groups also add to the polarity of the termini of α helices.

3–18 The first two peptide sequences, but not the third, would give amphiphilic helices, as shown in **Figure 3–32**.

3–19 As illustrated in **Figure 3–33**, the first three strands of the sheet are antiparallel to their neighbors, whereas the fourth strand is parallel to the third.

3–20

D. Because the side chains of the amino acids alternately project above and below the sheet, a sequence that could form a strand in an amphiphilic β sheet should have alternating hydrophobic and hydrophilic amino acids. Only choice D satisfies this condition.

3–21 None of these folds would give a knot when stretched out. This is a general principle: proteins fold without forming knots. One might imagine that it would be difficult to thread the end of a protein through an interior loop to form a knot. A folding pathway to such a knotted form might not be achievable through random motions in a reasonable time.

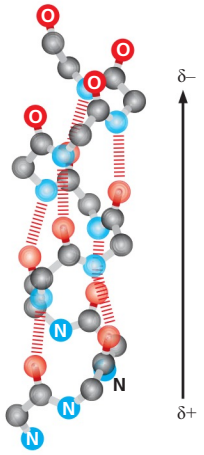


Figure 3–31 Representation of an α helix showing dipole and unsatisfied hydrogen-bonding groups at its ends (Answer 3–17). Non-hydrogen-bonded Os and Ns are labeled.

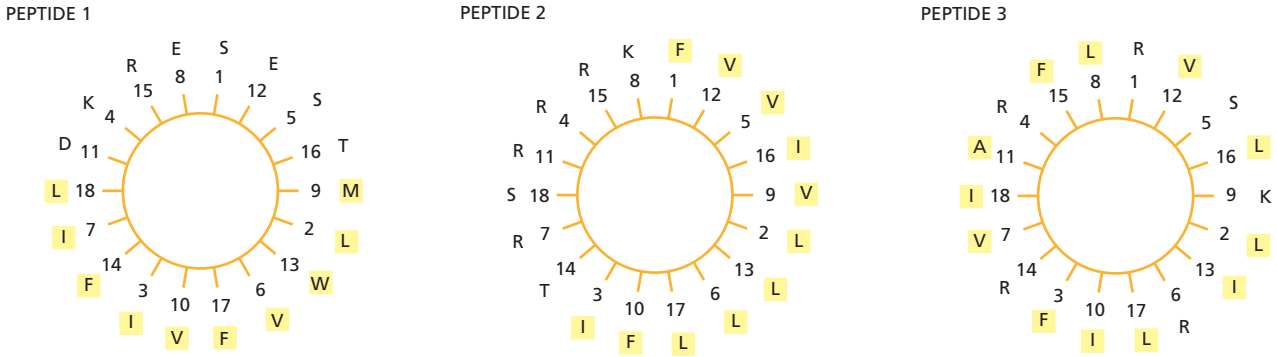


Figure 3–32 Arrangement of amino acids of the three peptide sequences around helix wheels (Answer 3–18).

3–22 Antiparallel strands are commonly formed by a polypeptide chain that folds back on itself. Thus, only a few amino acids are required to allow the polypeptide chain to make the turn. By contrast, parallel strands must be connected by a polypeptide chain that is at least as long as the strands in the sheet. For a long peptide, a common solution for satisfying backbone hydrogen-bonding requirements is an α helix (**Figure 3–34**).

3–23 Proteins obviously can't search all possible conformations on their way to finding the correct one. Thus, there must be defined pathways to simplify the search. It is now thought that weak interactions rapidly cause the protein to collapse into a molten globule, in which bonding interactions are transient and chains maintain fluidity. Within the molten globule, very weak secondary structures form and disappear, as do tertiary interactions. The formation of small elements of correct secondary structure, stabilized by appropriate tertiary interactions, then appears to nucleate formation of the final structure. This general folding pathway represents a fight between the maximization of entropy, which tends to keep the protein as random as possible, and the minimization of enthalpy through formation of weak bonds. Increasing numbers of weak interactions pull the structure through a succession of increasingly well-defined states to the final conformation. This conceptualization of folding has been likened to a funnel, and is commonly referred to as the folding funnel, with multiple routes of progress down the funnel accompanied by an increase in native-like structures.

Reference: Fersht A (1999) Structure and Mechanism in Protein Science, pp 575–600. New York: WH Freeman.

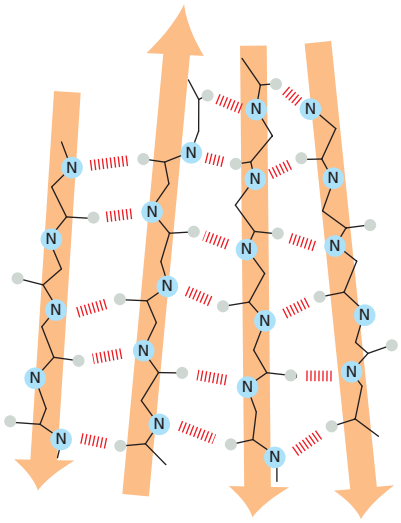


Figure 3–33 A segment of β sheet showing the polarity (N to C) of the individual strands (Answer 3–19).

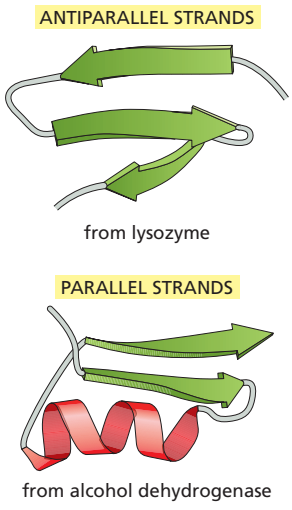


Figure 3–34 Connections between antiparallel and parallel strands of a β sheet (Answer 3–22).

3–24 Many different strings of amino acids can give rise to identical protein folds. The many amino acid differences between the homeodomain proteins from yeast and *Drosophila* are among the many possible ones that do not alter folding and function. This question could have been framed in another way; namely, how many amino acid changes are required to convert, say, an α helix into a β sheet? The answer is: surprisingly few. These two answers underscore the difficulty in predicting protein structures from amino acid sequences.

3–25

A. The protein in Figure 3–5 is composed of two domains. The protein can be cleaved in the exposed peptide segment that links the two domains (**Figure 3–35**). Fragments that correspond to individual domains are likely to fold properly. It is common experience that isolated domains are easier to crystallize than the entire protein.

B. The ability to form a crystal depends on the surface characteristics of the protein because it must be able to interact with itself in a repeating pattern to form a crystal. Homologous proteins from different species, which fold the same way (like the homeodomain proteins in Problem 3–24), differ subtly in their surface characteristics. As a result, the protein from one species may crystallize readily, while that from another species may not crystallize at all. A single amino acid change sometimes makes all the difference.

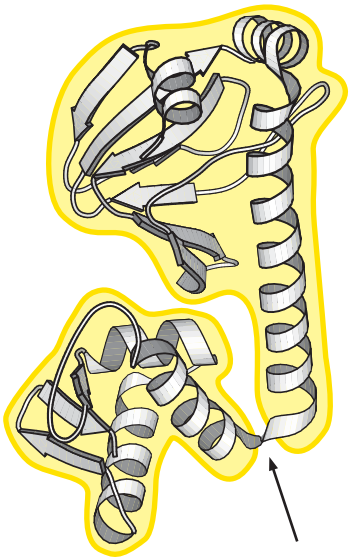


Figure 3–35 Catabolite activator protein (Answer 3–25). The arrow shows the site of cleavage in the exposed peptide segment linking the two domains.

3–26 Generally speaking, an identity of at least 30% is needed to be certain that a match has been found. Matches of 20% to 30% are problematical and difficult to distinguish from background "noise." Searching for distant relatives with the whole sequence usually drops the overall identity below 30% because the less conserved portions of the sequence dominate the comparison. Thus, searching with shorter, conserved portions of the sequence gives the best chance for finding distant relatives.

3–27 The close juxtaposition of the N- and C-termini of this kelch domain identifies it as a "plug-in" type domain. "In-line" type domains have their N- and C-termini on opposite sides of the domain.

3–28 As shown in **Figure 3–36**, the three protein monomers have distinctly different assembly properties because of the three-dimensional arrangement of their complementary binding surfaces. Monomer A would assemble into a sheet; monomer B would assemble into a long chain; monomer C would assemble into a ring composed of four subunits.

3–29 Choice B (→←) is the only arrangement of DNA binding sites that matches the arrangement of subunits in the "head-to-head" Cro dimer. The DNA that corresponds to such an arrangement is known as a palindrome:

```
ATCG·CGAT
TAGC·GCTA
```

Rotation of this sequence 180° about the central dot gives an identical sequence, just as does rotation of the arrows (→←). This demonstrates that the Cro dimer and its recognition sequence have the same symmetry, as expected.

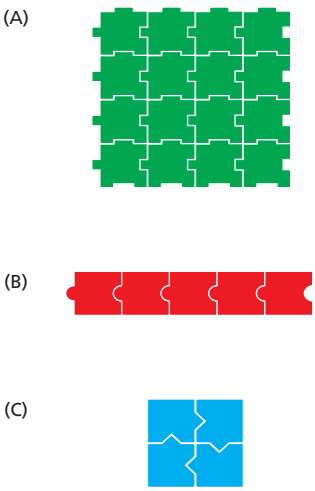


Figure 3–36 Assembly of protein monomers (Answer 3–28).

3–30 "Head-to-tail" dimers have unsatisfied binding sites at each end, which would lead to the formation of chains (see Figure 3–36B).

3–31 Proteins 1, 3, 4, and 5 can form head-to-head dimers, as illustrated for protein 1 in **Figure 3–37A**. All binding surfaces that allow proteins to interact are complementary. The binding surfaces that allow two copies

of a protein to form a "head-to-head" dimer must be self-complementary because they bind to themselves. To be self-complementary, one half of the binding site must be complementary to the other half. This means that the two halves can be folded on top of one another, with properly matched binding, across a line drawn through the center of the binding site, as illustrated for the protein 1 binding surface in **Figure 3–37B**. There is no line across which proteins 2 and 6 can be folded to make their binding partners match. Inclusion of protrusions and invaginations would not have altered this general principle: complementary binding surfaces can be folded so that a protrusion on one side inserts into an invagination on the other side.

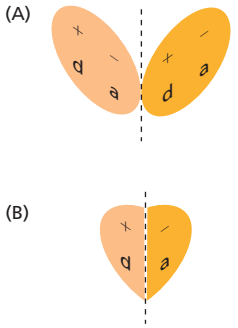


Figure 3–37 Self-complementarity in proteins (Answer 3–31). (A) Head-to-head dimer formation. (B) Self-complementary binding surface.

3–32 The coil 1A segment of nuclear lamin C matches the heptad repeat at 9 of 11 positions (**Figure 3–38**), which is very good. The match need not be perfect to allow formation of a coiled-coil. The matches to the heptad repeat in the other two marked segments (coil 1B and coil 2, see Figure 3–9) are not as good, but they are still acceptable for the formation of a coiled-coil.

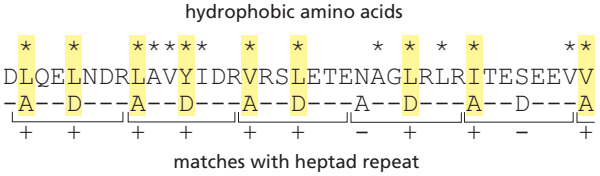


Figure 3–38 Heptad repeat motif in the coil 1A region of nuclear lamin C (Answer 3–32). Hydrophobic amino acids are marked with an asterisk (*). When a hydrophobic amino acid occurs at the A or D position in the heptad repeat, it is assigned a + and highlighted in *yellow*. The start of the heptad repeat was positioned to maximize matches.

Reference: McKeon FD, Kirschner MW & Caput D (1986) Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature* 31, 463–468.

CALCULATIONS

3–33 At equilibrium there would be 1 unfolded protein for every 10^7 folded proteins. This ratio comes from substituting values for $\Delta G°$ (41.5 kJ/mole), R, and T into the equation and solving for log$K$:

$$\log K = (41.5)/[(-2.3) \times (8.3 \times 10^{-3}) \times (310)] = -7$$

Since $K = [U]/[F]$,

$$\log K = \log([U]/[F]) = -7$$

Taking the log of both sides,

$$[U]/[F] = 10^{-7}, \text{ or } [U] = 10^{-7}\,[F]$$

3–34 Since there are 20 possible amino acids at each position in a protein 300 amino acids long, there are 20^{300} (which is 10^{390}) possible proteins. The mass of one copy of each possible protein would be

$$\text{mass} = \frac{110\text{ d}}{\text{aa}} \times \frac{300\text{ aa}}{\text{protein}} \times 10^{390}\text{ proteins} \times \frac{\text{g}}{6 \times 10^{23}\text{ d}}$$

$$\text{mass} = 5.5 \times 10^{370}\text{ g}$$

Thus, the mass of protein would exceed the mass of the observable universe (10^{80} g) by an enormous amount; more precisely by a factor of about 10^{290}!

DATA HANDLING

3–35 If unfolding of the protein simply reflected the titration of a buried histidine, it should require 2 pH units to go from 9% to 91% completion. The actual unfolding curve takes only 0.3 pH units to span this range. This sharp transition indicates a highly cooperative process; when the protein

starts to unfold, it completes the process rapidly. For example, it might be that several buried histidines can ionize when the chain starts to unfold, so that when one goes they all go together. Note also that as soon as a buried histidine (p*K* of 4 in this example) becomes accessible to solvent, its p*K* will shift toward its normal value of 6, significantly steepening its titration curve.

Reference: Creighton TE (1993) Proteins, 2nd ed, pp 288–289. New York: WH Freeman.

3–36

A. These data are consistent with the hypothesis that the springlike behavior of titin is due to the sequential unfolding of Ig domains. First, the fragment contained seven Ig domains and there are seven peaks in the force-versus-extension curve. In addition, the peaks themselves are what you might expect for sequential unfolding. Second, in the presence of a protein denaturant, conditions under which the domains will already be unfolded, the peaks disappear and the extension per unit force increases. Third, when the domains are cross-linked, and therefore unable to unfold, the peaks disappear and extension per unit force decreases.
B. The spacing between peaks, about 25 nm, is almost exactly what you would calculate for the sequential unfolding of Ig domains. The folded domain occupies 4 nm, but when unfolded, its 89 amino acids would stretch to about 30 nm (89 × 0.34 nm), a change of 26 nm.
C. The existence of separate, discrete peaks means that each domain unfolds when a characteristic force is applied, implying that each domain has a defined stability. The collection of domains unfolds in order from least stable to most stable. Thus, it takes a little more force each time to unfold the next domain.
D. The sudden collapse of the force at each unfolding event reflects an important principle of protein unfolding; namely, its cooperativity. Proteins tend to unfold in an all-or-none fashion (see Problem 3–35). A small number of hydrogen bonds are crucial for holding the folded domain together (**Figure 3–39**). The breaking of these bonds triggers cooperative unfolding.

Reference: Rief M, Gutel M, Oesterhelt F, Fernandez JM & Gaub HE (1997) Reversible folding of individual titin immunoglobulin domains by AFM. *Science* 276, 1109–1112.

3–37

A. None are detected in this experiment. Treating first with radiolabeled NEM shows that many cytosolic proteins have cysteines that are not linked by disulfide bonds. Treating first with unlabeled NEM to block these sites, followed by DTT to break disulfide bonds, should expose any –SH groups that were linked by disulfide bonds. These newly exposed –SH groups should be labeled by subsequent treatment with radiolabeled NEM. The absence of labeling indicates that no cysteines were involved in disulfide bonds.
B. BSA and insulin are labeled extensively only after their disulfide bonds have been broken by treatment with DTT. In the absence of DTT treatment, BSA is weakly labeled. Since BSA has an odd number of cysteines, at least one cannot be involved in disulfide bonds. Structural analysis confirms that one of its 37 cysteines is not involved in a disulfide bond.

Figure 3–39 Hydrogen bonds that lock the domain into its folded conformation (Answer 3–36). The indicated hydrogen bonds *(green lines)*, when broken, trigger unfolding of the domain. If you compare this topological diagram with the three-dimensional structure in Figure 3–11A, you can pick out the two short β strands that are involved in forming these hydrogen bonds.

C. Because the ER is the site where disulfide bond formation is catalyzed in preparation for export of proteins, it is expected that lysates from cells that have internal membranes would have many proteins with disulfide bonds.

PROTEIN FUNCTION

DEFINITIONS

3–38 Scaffold protein

3–39 Feedback inhibition

3–40 Antibody

3–41 Active site

3–42 Enzyme

3–43 Ubiquitin

3–44 Linkage

3–45 Protein kinase

3–46 Transition state

3–47 Allosteric protein

3–48 Proteomics

3–49 Coenzyme

TRUE/FALSE

3–50 False. The pK values of specific side-chain groups depend critically on the environment. On the surface of a protein, in the absence of surrounding charged groups, the pK of a carboxyl group is usually close to that of the free amino acid. In the neighborhood of negatively charged groups, the pK of a carboxyl group is usually higher; that is, the proton dissociates less readily, since the increase in local density of negative charge is not favored. The opposite is true in a positively charged environment. In hydrophobic surroundings, the dissociation of a proton can be substantially suppressed, since the presence of a naked charge in such an environment is highly disfavored. It is this ability to alter the reactivities of individual groups that allows proteins to fine-tune their biological functions.

3–51 False. Assuming that the three-dimensional structure of at least one family member is known, it would be possible to use evolutionary tracing—fitting the primary sequence to the structure—to determine where the conserved amino acids cluster on the surfaces of the proteins. Clusters of conserved amino acids are likely to correspond to important regions such as those involved in binding to specific ligands or other proteins. Knowing where such binding sites reside on the surface does not identify the protein's function. You would not know whether the protein was an enzyme or a structural protein, or what it bound to. Some other approach, usually biochemical, would be required to elucidate the function.

3–52 False. The turnover number is constant since it is V_{max} divided by enzyme concentration. For example, a twofold increase in enzyme concentration would give a twofold higher V_{max}, but it would give the same turnover number: $2\ V_{max}/2\ [E] = k_3$.

3–53 True. The term cooperativity embodies the idea that changes in the conformation of one subunit are communicated to the other subunits in any given multimeric assembly, so that all of these subunits are in the same conformation. Usually, these subunits are identical; however, in hemoglobin, for example, there are four subunits of two somewhat different kinds.

3–54 True. Each cycle of phosphorylation–dephosphorylation hydrolyzes one molecule of ATP; however, it is not wasteful in the sense of having no benefit. Constant cycling allows the regulated protein to switch quickly from one state to another in response to stimuli that require rapid adjustments of cellular metabolism or function. This is the essence of effective regulation.

3–55 False. Although many of the conformational changes induced by ligand binding are relatively small, in some instances these local changes are propagated through a molecule to give rise to changes of more than a nanometer. The conformational change triggered by hydrolysis of GTP by EF-Tu, for example, allows two domains of the protein to separate by 4 nm.

THOUGHT PROBLEMS

3–56 Antifreeze proteins function by binding to tiny ice crystals and arresting their growth, thereby preventing the fish from freezing. Ice crystals that form in the presence of antifreeze proteins are abnormal in that their surfaces are curved instead of straight. The various forms of the antifreeze proteins in these fishes are all composed of repeats of a simple glycotripeptide (Thr-Ala/Pro-Ala) with a disaccharide attached to each threonine. The genes for these antifreeze proteins were apparently derived by repeated duplication of a small segment of a protease gene.

References: Cheng CHC & Chen L (1999) Evolution of an antifreeze protein. *Nature* 401, 443–444.

Jia Z & Davies PL (2002) Antifreeze proteins: an unusual receptor–ligand interaction. *Trends Biochem. Sci.* 27, 101–106.

3–57 The rank order for the proportion of aspartates in the $-COO^-$ form is expected to be 4, 1, 2, 3. It is convenient to discuss the rank order starting with the aspartate side-chain carboxyl group on the surface of a protein with no other ionizable groups nearby (1). The side chain would be expected to have a p*K* around 4.5, somewhat higher than observed in the free amino acid because of the absence of the influence of the positively charged amino group, which is involved in the peptide bond in the protein. If the side chain were buried in a hydrophobic pocket on the protein (2), its p*K* would be higher, with less in the form of $-COO^-$, because the presence of a charge in a hydrophobic environment (without the easy bonding to water) would be disfavored. If there were another negative charge in the same hydrophobic environment (3), the p*K* of the aspartate side chain would be elevated even further (even more difficult to give up a proton and become charged) because of electrostatic repulsion. If there were a positively charged group in the same environment (4), then the favorable electrostatic attraction would make it very easy for the proton to come off, lowering the p*K* even below that of the side chain on the surface (1).

3–58 To bind to valine, the valyl-tRNA synthetase uses a binding pocket of the proper shape that is lined with hydrophobic residues. Such a binding site permits valine to bind well but does not fully exclude threonine, which has the same shape and a single polar hydroxyl group (**Figure 3-40**). The

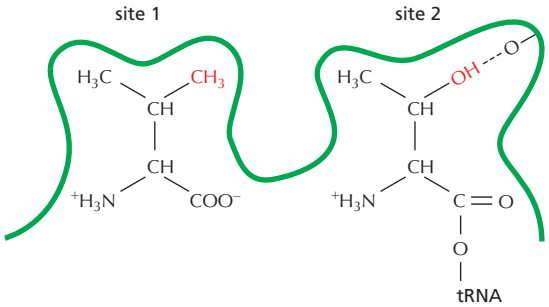


Figure 3–40 The two binding sites for valine and threonine on valyl-tRNA synthetase (Answer 3–58).

second binding site is much more specific for threonine because it contains an appropriately positioned hydrogen-bond acceptor that makes a specific hydrogen bond with threonine but not with valine. Even though valine can fit into the site, it cannot bind tightly and is thus a very poor substrate for the hydrolysis reaction.

3–59 The problem is that the off rate for the antibody–enzyme complex is too slow. In order for the peptide to displace the enzyme from the column, the enzyme must first dissociate from the antibody. The antibody binding sites would then be quickly bound by the peptide, whose high concentration would prevent the enzyme from reattaching to the antibody (any newly exposed antibody binding site would be bound by peptide). In principle, you could soak the column with peptide for several days (for several dissociation half-times, see Problem 3–77), but this usually has adverse consequences for the quality and activity of the enzyme preparation. In general, high-affinity antibodies have slow off rates and are unsuitable for affinity chromatography.

Special procedures have been devised for preparing or identifying antibodies that work in such experiments. Usually, lower-affinity antibodies are used, or chromatography is carried out under special conditions that reduce the affinity of the antibody.

Reference: Thompson NE & Burgess RR (1996) Immunoaffinity purification of RNA polymerase II and transcription factors using polyol-responsive monoclonal antibodies. *Methods Enzymol.* 274, 513–526.

3–60 The reaction rate for the altered enzyme would be substantially slower than for the normal enzyme. The reaction rate is related to the activation energy, which is the difference in energy between the trough labeled ES in Figure 3–15 and the transition state: the larger the activation energy, the slower the rate. If the altered enzyme bound the substrate with higher affinity (a lower ES trough), then the activation energy would increase and the reaction would slow down.

3–61

D. Because an enzyme has a fixed number of active sites, the rate of the reaction cannot be further increased once the substrate concentration is sufficient to bind to all the sites. It is the saturation of binding sites that leads to an enzyme's saturation behavior. The other statements are all true, but none is relevant to the question of saturation.

3–62

A. Since k_1 corresponds to the on rate and k_{-1} corresponds to the off rate,

$$K_d = [E][S]/[ES] = k_{off}/k_{on} = k_{-1}/k_1$$

B. K_m is approximately equal to K_d when k_{cat} is much less than k_{-1}; that is to say, when the ES complex dissociates much more rapidly than substrate is converted to product. This is true for many enzymes, but not all.

C. Because k_{cat} is in the numerator of the expression for K_m, K_m will always be somewhat larger than K_d. Since lower values of K_d indicate higher

binding affinity, K_m will always underestimate the binding affinity. When k_{cat} is much less than k_{-1}, the underestimate will be slight and K_m will essentially equal K_d.

3–63 All explanations have at their heart the idea that the quantity of active enzyme per total protein (the specific activity of the enzyme) is 10-fold less in bacteria. Such a situation could arise for a number of reasons: 90% of the enzyme may fold incorrectly in bacteria; an essential cofactor of the enzyme, which is normally tightly bound, may be limiting in bacteria so that only 10% of the enzyme molecules acquire it. These explanations, which propose that there are 10% normally active enzymes among otherwise dead molecules, account naturally for the observation that the K_m is identical (K_m is independent of the concentration of active enzyme) while V_{max} is lower (V_{max} is dependent on the concentration of active enzyme).

(One common suggestion is that the enzyme in bacteria folds so that each molecule has 10% of the normal activity. This possibility can be ruled out because the lower activity of each molecule would show up as a change in K_m as well as V_{max}.)

3–64

A. An enzyme composed entirely of mirror-image amino acids would be expected to fold stably into a mirror-image conformation; that is, it would look like the normal enzyme when viewed in a mirror.
B. A mirror-image enzyme would be expected to recognize the mirror image of its normal substrate. Thus, "D" hexokinase would be expected to add a phosphate to L-glucose and to ignore D-glucose.

This experiment has actually been done for HIV protease. The mirror-image protease recognizes and cleaves a mirror-image substrate.

Reference: Milton RC, Milton SC & Kent SB (1992) Total chemical synthesis of a D-enzyme: the enantiomers of HIV-1 protease show reciprocal chiral substrate specificity. *Science* 256, 1445–1448.

3–65 Phosphoglycolate is a transition-state analog for the triosephosphate isomerase reaction. It has the two characteristics that define a transition-state analog: it resembles the reaction intermediate and it binds more tightly (here, about 15 times more tightly) than the substrates.

References: Kyte J (1995) Mechanism in Protein Chemistry, pp 207–208. New York: Garland Publishing.

Pauling L (1948) Chemical achievement and hope for the future. *Am. Sci.* 36, 50–58.

3–66

A. Amino acid side chains in proteins often have quite different pK values from those in solution. Glu35 is uncharged because its local environment is nonpolar, which makes ionization less favorable (raises its pK). The local environment of Asp52 is more polar, permitting ionization near its solution pK.
B. As the pH drops below 5, Asp52 picks up a proton and becomes nonionized, interfering with the mechanism. As the pH rises above 5, Glu35 begins to release its proton, also interfering with the mechanism.

3–67 This simple question required decades of research to provide a complete and satisfying answer. At the simplest level, hemoglobin binds oxygen efficiently in the lungs because the concentration (partial pressure) of oxygen is highest there. In the tissues, the concentration of oxygen is lower because it is constantly being consumed in metabolism. Thus, hemoglobin will tend to release (bind less) oxygen in the tissues. This

natural tendency—an effect on the binding equilibrium—is enhanced by allosteric interactions among the four subunits of the hemoglobin molecule. As a consequence, much more oxygen is released in the tissues than would be predicted by a simple binding equilibrium.

3–68 When [S] >> K_m, the enzyme will be virtually saturated with substrate at all times and capable of operating at maximum rate, independent of small fluctuations in substrate concentration. For many enzymes that use ATP and a second substrate, as protein kinases do, the K_m for ATP is usually very low (a few μM for most protein kinases) relative to the concentration of ATP in the cell (1–2 mM). This situation allows the kinases to operate effectively regardless of the typical fluctuations in ATP concentration. Under these conditions, the rate of phosphorylation depends solely on the concentration of the other substrate.

When [S] ≈ K_m, the rate of the enzyme-catalyzed reaction will vary in proportion to the changes in substrate concentration. This is the typical situation for most enzymes involved in metabolic pathways, which allows them to keep up with the changing flow through the pathway.

When [S] << K_m, the enzyme will be mostly unoccupied by substrate and will be operating much below its maximum rate. This is a strategy that might be used, for example, if multiple different enzymes draw on a common pool of substrate. An enzyme with a high K_m would use only a small fraction of the pool unless the substrate concentration increased dramatically. Just such a strategy is employed in animals for routing glucose for metabolism. Most cells in the body use hexokinase, which has a low K_m, to add phosphate to glucose to initiate its metabolism. By contrast, liver cells use glucokinase, which has a high K_m, to carry out this reaction. Between meals, the circulating glucose is routed mainly to nonliver cells, which use the low K_m enzyme hexokinase. After meals, when the circulating concentration of glucose is much higher, the liver captures a much larger fraction (because glucokinase activity increases much more with higher substrate concentration than does hexokinase activity). The liver uses much of that glucose to make glycogen, which serves as a glucose reserve for use between meals.

3–69

C. Cells cannot influence rates of diffusion, which are limited by physical parameters beyond a cell's control. Cells can decrease the time it takes for substrates to reach an enzyme, however, by increasing the concentration of enzyme or by linking related enzymes in multienzyme complexes.

3–70 One reasonable proposal would be for excess AMP to feedback inhibit the enzyme for converting *E* to *F*, and excess GMP to feedback inhibit the step from *E* to *H*. Intermediate *E*, which would then accumulate, would feedback inhibit the step from R5P to *A*. Some branched pathways are regulated in just this way. Purine nucleotide synthesis is regulated somewhat differently, however (**Figure 3–41**). AMP and GMP regulate the steps from *E* to *F* and from *E* to *H*, as above, but they also regulate the step from R5P to *A*. Regulation by AMP and GMP at this step might seem problematical since it suggests that a rise in AMP, for example, could shut off the entire pathway even in the absence of GMP. The cell uses a very

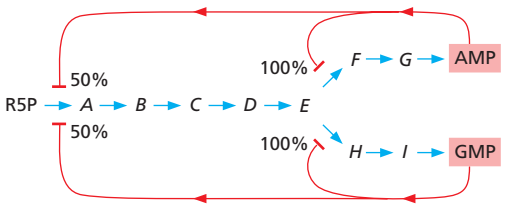


Figure 3–41 Pattern of inhibition in the metabolic pathway for purine nucleotide synthesis (Answer 3–70).

clever trick to avoid this problem. Individually, excess AMP or GMP can inhibit the enzyme to about 50% of its normal activity; together they can completely inhibit it.

3–71 In resting muscle, ATP usage is at a minimum; hence, the group of ATP-like signal metabolites accumulates. Specific members of this group inhibit glycogen phosphorylase and stimulate glycogen synthase, ensuring that glycogen reserves are maintained or increased.

In exercising muscle, ATP usage is high and AMP-like signal metabolites increase. Specific AMP-like signal metabolites stimulate glycogen phosphorylase and inhibit glycogen synthase, ensuring a breakdown of glycogen to provide glucose units for ATP production.

3–72 The substrate, phosphate, and the activator, AMP, both bind to the rarer conformation of glycogen phosphorylase, thereby shifting the conformational equilibrium in favor of the more active species. This makes good biological sense because phosphate and AMP concentrations both rise when the cell increases its rate of ATP hydrolysis, and activation of glycogen phosphorylase is one way to provide metabolic substrates for the synthesis of additional ATP. In both cases, the overall activity of the enzyme increases because the fraction of enzymes in the high-activity conformation is increased.

3–73 The first MWC postulate, which states that the subunits in the allosteric enzyme have identical conformations and are arranged symmetrically, rules out all arrangements except those shown in the leftmost and rightmost columns of the diagram. If ligand binds much more tightly to circles, then the allowed arrangements are those shown in **Figure 3–42**. If the ligand bound equally to both subunit conformations, then all the arrangements in the leftmost and rightmost columns would be allowed, consistent with MWC postulate 1.

Detailed studies on a few cooperative enzymes have found no evidence for intermediate, nonsymmetrical conformations.

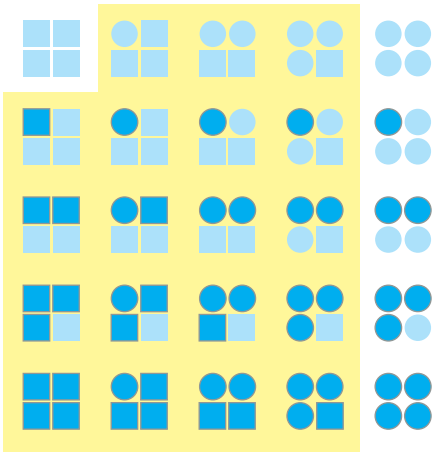


Figure 3–42 Arrangements of subunit conformations that are consistent with the MWC postulates (Answer 3–73). The area highlighted in *yellow* indicates those arrangements that are excluded by the MWC postulates for a ligand with affinity for one conformation.

3–74 The rate of the metabolic reaction depends on the population of enzyme molecules, not on an individual enzyme molecule. While an individual molecule is either on or off—depending on whether it is phosphorylated—the activity of the population of enzyme molecules depends on the *proportion* of these molecules that are phosphorylated. As the proportion of phosphorylated molecules increases from 0% to 100%, the activity of the population of enzymes (the rate of the metabolic reaction) will decrease smoothly from 100% to 0%. The phosphorylation state of a population of enzyme molecules is controlled by the balance between the opposing activities of protein kinases, which attach phosphates, and protein phosphatases, which remove them.

3–75

A. In the absence of ATP, a motor protein would stop moving. The conformational shifts that are required for movement are triggered by ATP binding and hydrolysis. In the absence of ATP, the motor protein would be stuck in its lowest-energy conformation.

B. If the free-energy change for the hydrolysis of ATP by the motor protein were zero—conditions under which ATP is as easily made as hydrolyzed—the motor protein would wander back and forth. With zero free-energy change there would be no barrier between conformations.

CALCULATIONS

3–76 The antibody binds to the second protein with an equilibrium constant, K, of $5 \times 10^7\ M^{-1}$.

A useful shortcut to problems of this sort recognizes that ΔG° is related to log K by the factor –2.3 RT, which equals –5.9 kJ/mole at 37°C.

Thus, a factor of ten increase in the equilibrium constant (an increase in log K of 1) corresponds to a decrease in $\Delta G°$ of –5.9 kJ/mole. A 100-fold increase in K corresponds to a decrease in $\Delta G°$ of –11.9 kJ/mole, and so on. For each factor of ten increase in K, $\Delta G°$ decreases by –5.9 kJ/mole; for each factor of ten decrease in K, $\Delta G°$ increases by 5.9 kJ/mole. This relationship allows a quick estimate of changes in equilibrium constant from free-energy changes and vice versa. In this problem, you are told that $\Delta G°$ increased by 11.9 kJ/mole (a weaker binding gives a less negative $\Delta G°$). According to the relationship developed above, this increase in $\Delta G°$ requires that K decrease by a factor of 100 (a decrease by 2 in log K); thus, the equilibrium constant for binding to the second protein is $5 \times 10^7\ M^{-1}$.

The solution to the problem can be calculated by first determining the free-energy change represented by the binding to the first protein:

$$\Delta G° = -2.3\ RT \log K$$

Substituting for K,

$$\Delta G° = -2.3\ (8.3 \times 10^{-3}\ \text{kJ/(mole K)})\ (310\ \text{K})\ \log\ (5 \times 10^9)$$

$$\Delta G° = -5.92\ \text{kJ/mole} \times 9.7$$

$$\Delta G° = -57.4\ \text{kJ/mole}$$

The free-energy change associated with binding to the second protein is obtained by adding 11.9 kJ/mole to the free-energy change for binding to the first protein, giving a value of –45.5 kJ/mole. Thus, the equilibrium constant for binding to the second protein is

$$\log K = (-45.5\ \text{kJ/mole})/(-5.92\ \text{kJ/mole})$$

$$= 7.7$$

$$K = 5 \times 10^7\ M^{-1}$$

3–77

A. The equilibrium constants for the two reactions are the same, $10^8\ M^{-1}$.

$$K = [\text{Ab–Pr}]/([\text{Ab}][\text{Pr}]) = k_{on}/k_{off}$$

For the first antibody–protein reaction,

$$K = k_{on}/k_{off} = 10^5\ M^{-1}\ \text{sec}^{-1}/10^{-3}\ \text{sec}^{-1}$$

$$K = 10^8\ M^{-1}$$

For the second reaction,

$$K = k_{on}/k_{off} = 10^3\ M^{-1}\ \text{sec}^{-1}/10^{-5}\ \text{sec}^{-1}$$

$$K = 10^8\ M^{-1}$$

B. Since the first reaction has both a faster association rate and a faster dissociation rate, it will come to equilibrium more quickly than the second reaction.

C. The time it takes for half the complex to dissociate can be calculated from the relationship given in the problem:

$$2.3 \log [\text{Ab–Pr}]_t/[\text{Ab–Pr}]_0 = -k_{off}t$$

Substituting 0.5 for $[\text{Ab–Pr}]_t/[\text{Ab–Pr}]_0$ and rearranging the equation:

$$t = \frac{2.3 \log 0.5}{-k_{off}}$$

For the first complex, with $k_{off} = 10^{-3}\ \text{sec}^{-1}$,

$$t = \frac{2.3 \log 0.5}{-10^{-3}\ \text{sec}^{-1}}$$

t = 692 seconds, or 11.5 minutes

For the second complex, with $k_{off} = 10^{-5}$ sec^{-1}, the calculation gives

$t = 6.9 \times 10^4$ seconds, or about 19 hours

Thus, the first complex, which falls apart relatively quickly, would be much more difficult to work with than the second complex, which falls apart more slowly. Inappropriate reliance on the equilibrium constant, instead of the off rate constant, can lead an investigator astray in this sort of experiment.

3–78

A. At equilibrium, the rates of the forward and reverse reactions are equal. This is the definition of equilibrium. The overall reaction rate at equilibrium will be 0.

B. The equilibrium constant equals 10^3. At equilibrium, the forward and reverse reactions are equal. Thus,

$$k_f\,[A] = k_r\,[B]$$

and

$$k_f/k_r = [B]/[A] = K$$

Thus,

$$K = 10^{-4}\ \text{sec}^{-1}/10^{-7}\ \text{sec}^{-1} = 10^3$$

C. Enzyme catalysis does not alter the equilibrium for a reaction; it only speeds the attainment of equilibrium. Thus, the equilibrium constant is 10^3. If the equilibrium is unchanged and k_f is increased by a factor of 10^9, then k_r must also be increased by a factor of 10^9.

3–79 At [S] = zero, the rate equals $0/K_m$ and the rate is therefore zero. At [S] = K_m, the ratio of [S]/([S] + K_m) equals 1/2 and the rate is 1/2 V_{max}. At infinite [S], the ratio of [S]/([S] + K_m) equals 1 and the rate is equal to V_{max}.

3–80 If K_m increases, then the concentration of substrate necessary to give half-maximal rate also increases. At a concentration of substrate equal to the K_m of the unphosphorylated enzyme, the phosphorylated enzyme would have a slower rate; thus, phosphorylation inhibits this enzyme.

3–81

D. The substrate concentration must be increased by a factor of 16 to increase the rate from 20% to 80% V_{max}. Substituting a rate of 20% V_{max} into the Michaelis–Menten equation gives

$$0.2\ V_{max} = (V_{max})\ [S]/([S] + K_m)$$

Canceling V_{max} and multiplying both sides by ([S] + K_m) gives

$$0.2\ [S] + 0.2\ K_m = [S]$$

$$0.8\ [S] = 0.2\ K_m$$

$$[S] = 0.25\ K_m \text{ at } 20\%\ V_{max}$$

An analogous calculation shows that

$$[S] = 4\ K_m \text{ at } 80\%\ V_{max}$$

Thus, [S] must increase by a factor of 16 (4 K_m/0.25 K_m) for the rate to go from 20% to 80% V_{max}.

3–82 The turnover number for carbonic anhydrase is 6.1×10^7/min (or 1.0×10^6/sec). For this calculation, it is necessary to express the amount of CO_2 hydrated and the amount of the enzyme on the same molecular basis,

either as molecules or moles. For CO_2,

$$\frac{0.90\ \text{g}}{\text{min mL}} \times \frac{6 \times 10^{23}\ \text{d}}{\text{g}} \times \frac{\text{molecule}}{44\ \text{d}} = 1.23 \times 10^{22}\ \text{molecules/min mL}$$

For carbonic anhydrase,

$$\frac{10\ \mu\text{g}}{\text{mL}} \times \frac{\mu\text{mole}}{30{,}000\ \mu\text{g}} \times \frac{6 \times 10^{17}\ \text{molecules}}{\mu\text{mole}} = 2.0 \times 10^{14}\ \text{molecules/min mL}$$

The turnover number

$$k_3 = V_{max}/[\text{E}] = \frac{1.22 \times 10^{22}\ \text{molecules/min mL}}{2.0 \times 10^{14}\ \text{molecules/mL}} = 6.1 \times 10^7/\text{min}$$

3–83

A. The relative concentrations of the normal and mutant Src proteins are inversely proportional to the volumes in which they are distributed. The mutant Src is distributed throughout the volume of the cell, which is

$$V_{cell} = (4/3)\pi r^3 = (4/3)\pi\ (10 \times 10^{-6}\ \text{m})^3 = 4.1888 \times 10^{-15}\ \text{m}^3$$

Normal Src is confined to the 4-nm-thick layer beneath the membrane, which has a volume equal to the volume of the cell minus the volume of a sphere with a radius 4 nm less than that of the cell:

$$V_{layer} = V_{cell} - (4/3)\pi\ (r - 4\ \text{nm})^3$$
$$= V_{cell} - (4/3)\pi\ [(10 \times 10^{-6}\ \text{m}) - (4 \times 10^{-9}\ \text{m})]^3$$
$$= (4.1888 \times 10^{-15}\ \text{m}^3) - (4.1838 \times 10^{-15}\ \text{m}^3)$$
$$V_{layer} = 0.0050 \times 10^{-15}\ \text{m}^3$$

Thus, the volume of the cell is 838 times greater than the volume of a 4-nm-thick layer beneath the membrane ($4.1888 \times 10^{-15}\ \text{m}^3/0.0050 \times 10^{-15}\ \text{m}^3$).

Even allowing for the interior regions of the cell from which it would be excluded (nucleus and organelles), the mutant Src would still be a couple of orders of magnitude less concentrated in the neighborhood of the membrane than the normal Src.

B. Its lower concentration in the region of its target X at the membrane is the reason why mutant Src does not cause cell proliferation. This notion can be quantified by a consideration of the binding equilibrium for Src and X:

$$\text{Src} + \text{X} \rightarrow \text{Src} - \text{X}$$

$$K = \frac{[\text{Src} - \text{X}]}{[\text{Src}][\text{X}]}$$

The lower concentration of the mutant Src in the region of the membrane will shift the equilibrium toward the free components, reducing the amount of complex. If the concentration is on the order of 100-fold lower, the amount of complex will be reduced up to 100-fold. Such a large decrease in complex formation could readily account for the lack of effect of the mutant Src on cell proliferation.

DATA HANDLING

3–84

A. Your results support the idea that the PI 3-kinase interacts with the activated PDGF receptor through its SH2 domains. The interaction is blocked specifically by the phosphorylated pentapeptides 708 and 719. In their nonphosphorylated forms these same pentapeptides do not block the association.

B. The common features of the seven peptides that can bind to PI 3-kinase are a phosphotyrosine and a methionine (M) located three positions away in the C-terminal direction. (Although not shown explicitly here, there seems to be no requirement for specific amino acids on the N-terminal side of the phosphotyrosine.)

C. Recognition of a couple of amino acids in a short sequence is characteristic of a surface–string interaction. Indeed, recognition of sequences by SH2 domains is often cited as a prime example of such an interaction.

3–85

A. When the concentrations of free and bound ligand are equal, their ratio becomes 1 and the concentration of free protein is equal to K_d:

$$K_d = \frac{[Pr][L]}{[Pr\text{–}L]}$$

When [L] equals [Pr–L],

$$K_d = [Pr]$$

B. Visual inspection of the data in Figure 3–23 shows that the concentrations of free and bound tmRNA are approximately equal when the concentration of SmpB is 18.8 nM. Thus, K_d is around 20 nM.

Not all of the added SmpB protein is free, since some is obviously bound to tmRNA. But because tmRNA was included at a concentration of 0.1 nM, the bound fraction at an SmpB concentration of 18.8 nM is only 0.05 nM. Thus, the correction for bound SmpB is minuscule (less than 1%) and can be neglected.

C. It is critical in this kind of experiment that the tmRNA concentration be kept well below K_d. If the concentration of tmRNA had been 100 nM, for example, the shift to 50% bound would have occurred at around 50 nM SmpB (and most of the protein would have been in the bound, not the free, form). If tmRNA were included at 100 μM, the shift to 50% bound would have occurred at around 50 μM SmpB. Thus, if tmRNA were included at a concentration above K_d, the point at which 50% was shifted to the bound form would bear no relationship to K_d.

Reference: Karzai AW, Susskind MM & Sauer RT (1999) SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J.* 18, 3793–3799.

3–86 The calculated values of fraction bound versus protein concentration are shown in **Table 3–5**. Also shown are rule-of-thumb values, which are easier to remember (see the answer to Question 2–27).

These relationships are useful not only for thinking about K_d, but also for enzyme kinetics, which we cover in other problems. The rate of a reaction expressed as a fraction of the maximum rate is

$$\frac{\text{rate}}{V_{max}} = \frac{[S]}{[S] + K_m}$$

which has the same form as the equation for fraction bound. Thus, when the concentration of substrate, [S], is tenfold above the Michaelis constant, K_m, the rate is 90% of the maximum, V_{max}. When [S] is 100-fold below K_m, the rate is 1% of V_{max}.

The relationship also works for the fractional dissociation of an acidic group, HA, as a function of pH. When the pH is 2 units above pK, 99% of the acidic group is ionized. When the pH is 1 unit less than pK, 10% is ionized.

TABLE 3–5 Calculated values for fraction bound versus protein concentration (Answer 3–86).

[Protein]	Fraction Bound (%)	Rule of Thumb
$10^4\ K_d$	99.99	99.99
$10^3\ K_d$	99.9	99.9
$10^2\ K_d$	99	99
$10^1\ K_d$	91	90
K_d	50	50
$10^{-1}\ K_d$	9.1	10
$10^{-2}\ K_d$	0.99	1
$10^{-3}\ K_d$	0.099	0.1
$10^{-4}\ K_d$	0.0099	0.01

3–87

A. It is important that only a small quantity of product is made, because otherwise the rate of the reaction would decrease as the substrate was

depleted and product accumulated. Thus, the measured rates would be lower than they should be, and the kinetic parameters would be incorrect.

B. The Michaelis–Menten plot, shown in **Figure 3–43**, is a rectangular hyperbola, as expected if this enzyme obeys Michaelis–Menten kinetics. To determine values for K_m and V_{max} from this plot by visual inspection, you must estimate the rate at infinite substrate concentration. From the curve of the line in the figure, you might reasonably estimate V_{max} as anywhere from 1.8 to 2.0 µmol/min. (As developed in the answer to Problem 3–86, a useful rule of thumb is that at a concentration of substrate 10-fold above K_m, the rate is about 90% of V_{max}.) If you chose 2.0 µmol/min, then 0.5 V_{max} (1.0 µmol/min) corresponds to a substrate concentration of 1.0 µM, which is the value of K_m. The visual uncertainty in this plot led early researchers to transform the equation into a straight-line form so a line could be fitted to the data and the kinetic parameters could be more accurately determined.

C. As indicated in the Lineweaver–Burk plot in Figure 3–43, the *y* intercept is 0.5 ($1/V_{max}$) and the *x* intercept is –1.0 ($-1/K_m$). Thus, V_{max} equals 2.0 µmol/min and K_m equals 1.0 µM. Although this form of a straight-line plot is commonly discussed in textbooks, it is rarely used in practice because the data points that are most reliable are tightly grouped at one end of the line. Consequently, the slope of the line is unduly influenced by the low (and usually less accurately determined) rates at low substrate concentration. Other straight-line transformations of the Michaelis–Menten equation such as the Eadie–Hofstee plot, which is analogous in form to a Scatchard plot, are generally preferred. In this era of computers, however, the data can be fitted perfectly well to the nonlinear Michaelis–Menten equation, although it is still common to present such data in a linear form.

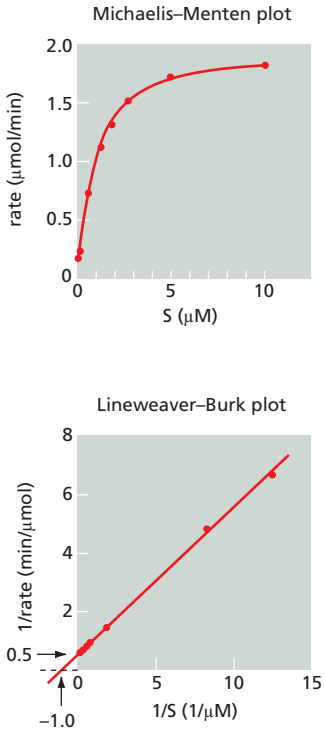


Figure 3–43 Michaelis–Menten and Lineweaver–Burk plots of the data in Table 3–2 (Answer 3–87). The *x* and *y* intercepts are indicated on the Lineweaver–Burk plot.

3–88

D. Since NAM cannot occupy site C, that site must normally be occupied by NAG; and since the cell-wall polysaccharide is an alternating polymer of NAM and NAG, the NAM monomers must occupy sites B, D, and F. Because cleavage occurs after NAM monomers, the site of cleavage must be between sites B and C or between sites D and E. Since tri-NAG occupies sites A–C but is not cleaved, whereas longer NAG polymers are, the catalytic groups for cleavage must lie between sites D and E.

3–89

A. Binding of aspartate normally shifts the conformation of ATCase from the low-activity to the high-activity state. At low aspartate concentrations, not all of the ATCase will have been shifted to the high-activity conformation. The peculiar activating effect of malate occurs because its binding helps complete the shift of ATCase to the high-activity conformation. In the presence of a low concentration of malate, the number of active sites in the high-activity conformation increases; thus, enzyme activity increases.

B. This peculiar activating effect of malate is not observed at high aspartate concentrations because ATCase is already entirely shifted to its high-activity conformation. Under these conditions, each molecule of malate that binds to an active site will reduce the total number of sites accessible to aspartate and thus reduce the overall activity of ATCase.

References: Cantor CR & Schimmel PR (1980) Biophysical Chemistry, pp 944–945. New York: WH Freeman.

Gerhart JH & Pardee AB (1963) The effect of the feedback inhibitor, CTP, on subunit interactions in aspartate transcarbamylase. *Cold Spring Harbor Symp. Quant. Biol.* 28, 491–496.

3–90

A. Both cyclin A and phosphorylation of Cdk2 are required to activate Cdk2 for efficient phosphorylation of histone H1 (see Figure 3–27, lane 5). Absence of cyclin A (lane 1) or absence of phosphorylation of Cdk2 (lane 3) results in much reduced levels of histone H1 phosphorylation.

B. Cyclin A, which binds tightly to both forms of Cdk2 (K_d = 0.05 μM), dramatically improves the binding of both forms to histone H1. In the absence of cyclin A, P-Cdk2, for example, binds histone H1 with a K_d of 100 μM, whereas in the presence of cyclin A, it binds histone H1 with a K_d of 0.7 μM, an increase in the tightness of binding of more than a factor of 100. In addition, as shown in Figure 3–27, phosphorylation of Cdk2 activates its protein kinase activity, allowing it to phosphorylate histone H1, when cyclin A is present to increase its ability to bind to histone H1.

C. Given that the intracellular concentrations of ATP and ADP are more than 10-fold higher than the measured dissociation constants, the changes in affinity for ATP and ADP are unlikely to be critical for the function of Cdk2. The binding sites for ATP will be nearly saturated regardless of the phosphorylation state of Cdk2. ADP, which binds at the same site as ATP, is unlikely to interfere significantly with ATP binding, because ADP has a higher K_d and its cellular concentration is generally lower than that of ATP.

Reference: Brown NR, Noble MEM, Lawrie AM, Morris MC, Tunnah P, Divita G, Johnson LN & Endicott JA (1999) Effects of phosphorylation of threonine 160 on cyclin-dependent kinase 2 structure and activity. *J. Biol. Chem.* 274, 8746–8756.

3–91 The slower migrating forms of β-catenin are due to ubiquitylation, not phosphorylation. Because the protein phosphatase had no effect on the slower migrating forms of β-catenin that arose in the presence of ALLN (see Figure 3–28, compare lanes 2 and 4), the difference in migration cannot be due to phosphorylation. By contrast, when His-tagged ubiquitylated proteins were first purified from cells treated with ALLN, the slower migrating forms of β-catenin were specifically detected (see lane 6).

Reference: Aberle H, Bauer A, Stappert J, Kispert A & Kemler R (1997) β-Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* 16, 3797–3804.

3–92

A. Mutant 2 is Asp181 → Ala (D181A). It is the best candidate because it has a K_m close to that of the wild-type enzyme, but a very low turnover number (k_{cat}). With these kinetic parameters it might be expected to bind normally to its target substrates but not remove phosphate from tyrosine. The next most likely candidate would be Arg221 → Lys, which has a slightly lower K_m than wild-type PTP1B and turns over slowly, although about 20 times faster than D181A. (Further studies identified the band at 180 kd as the epidermal growth factor—EGF—receptor.)

B. C215S showed no activity, as expected since the –SH group of cysteine is required for catalysis. Because C215S was not active, it was not possible to determine its K_m, which might have been similar to that of the wild-type enzyme. (Measurements of K_d were not made.) Thus, C215S was a reasonable candidate to test. Lack of success with C215S suggests that it binds phosphotyrosine-containing proteins very poorly.

Reference: Flint AJ, Tiganis T, Barford D & Tonks NK (1997) Development of substrate-trapping mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl Acad. Sci. USA* 94, 1680–1685.

MEDICAL LINKS

3–93

E. A mutation that decreases the rate of GTP hydrolysis by Ras would prolong its activated state, leading to excessive stimulation of cell proliferation. Indeed, many cancers contain just such a mutant form of Ras. The mutant Ras proteins in all other choices would lead to a decreased ability to transmit the downstream signal, thus decreasing cell proliferation. For example, a mutation that increased the affinity of Ras for GDP (choice B) would prolong the *inactive* state of Ras, thereby interfering with the growth signal and decreasing cell proliferation.

3–94 A nonfunctional GAP (choice A) or a permanently active GEF (choice D) would allow Ras to remain in the active state (with GTP bound) longer than normal, and thus might cause excessive cell proliferation.

MCAT STYLE

3–95

A. A domain is a sequence of amino acids that folds into a discrete structural unit that serves a particular function in the protein. Choice C is not correct because, although the amino acid sequences of protein domains tend to be conserved, an evolutionarily conserved sequence by itself is not sufficient to define a domain; a conserved sequence can be very short and may not fold into a discrete structural or functional unit.

3–96

B. K_m will increase. K_m is the substrate concentration at which an enzyme reaction works at half maximal velocity. Binding interactions between an enzyme and its substrate increase the effective local concentration of substrate, thereby increasing the rate of the reaction. Thus, if the binding interaction between Cdk and Rb is inactivated, a higher concentration of Rb will be required to achieve maximal velocity. Thus, K_m will increase. By contrast, a mutation that inactivates only the binding site between Cdk and Rb would not be expected to change the V_{max} of Cdk. V_{max} is the maximal rate at which an enzyme can carry out its reaction; it is achieved when substrate concentration is very high. The binding interactions between Cdk and Rb will decrease the concentration needed to achieve V_{max}, but will not change V_{max}. Thus, inactivating the binding site will not change V_{max}.

3–97

D. Protein kinases commonly transfer a phosphate group to the hydroxyl groups of the amino acids serine, threonine, and tyrosine. A much more rare class of protein kinase transfers a phosphate group to a ring nitrogen of histidine, creating a P–N bond, which is intrinsically much less stable than a P–O bond.

3–98

D. The covalent addition of a phosphate group to a protein adds a large negatively charged group that can affect protein conformation or function in each of the ways described.

3–99

B. Protein phosphorylation commonly induces conformational changes that can alter the function of the phosphorylated protein, which is why choice B is the most likely correct answer. Choice A is a less likely possibility because it is improbable that phosphorylation of Rb would cause a conformational change in an associated protein (although it cannot be ruled out absolutely). Choice C is unlikely because inactivating the Rb binding site for Cdk would not affect E2F binding, since the Rb binding sites for Cdk and E2F do not overlap (Cdk binds Rb in the Rb-E2F

complex). Choice D is incorrect because phosphate adds a negative charge, which would favor binding to a protein with a positively charged region.

3–100

C. The binding of a ligand such as a steroid hormone often causes the protein to undergo a conformational change that alters its activity. Choice A is incorrect because small molecules in general and steroid hormones in particular do not catalyze chemical reactions. Choice B is incorrect because K_m refers to the interaction of an enzyme with its substrate. Had the question been framed in terms of the dissociation constant, K_d, this choice would have been a possibility. Choice D is incorrect because simple binding interactions between two proteins do not involve positive feedback.

3–101

C. The binding of one protein to another is driven by the hydrophobic force, which tends to drive hydrophobic amino acid side chains into the interface between the two proteins, and by weak, noncovalent bonds that include hydrogen bonds, electrostatic bonds, and van der Waals interactions. Covalent bonds are generally not used to bind proteins together.

3–102

B. Binding reactions between proteins rarely require an input of energy such as that derived from ATP hydrolysis.

3–103

A. The equilibrium binding constant (K) provides a measure of the strength of the binding interaction. Recall that K is directly related to the free-energy change ($\Delta G°$) for the binding reaction. The strength of the binding interaction is not related to the rate of association or dissociation. V_{max} refers to the maximum rate of catalysis in enzyme reactions and is not relevant to the binding interactions between two proteins.

DNA, Chromosomes, and Genomes

CHAPTER 4

IN THIS CHAPTER

THE STRUCTURE AND FUNCTION OF DNA

DEFINITIONS

4–1 Genome

4–2 Double helix

4–3 Gene

4–4 Antiparallel

4–5 Base pair

TRUE/FALSE

4–6 False. The human genome consists only of linear molecules, but human cells also contain thousands of mitochondrial DNA molecules, which are circular. They constitute about 1% of the DNA in a human cell.

THOUGHT PROBLEMS

4–7 The complementary strand for this DNA is 5′-GTGCACCAT-3′. By convention, all DNA strands are written 5′ to 3′, so that the complement of 5′-ATGGTGCAC is 5′-GTGCACCAT. Keeping polarities in mind is a key to following DNA structure, DNA replication, DNA transcription, DNA repair, and recombination—virtually all aspects of DNA metabolism.

4–8 The phosphate groups in the backbone of DNA are strong acids (p*K* 1.0) and completely ionized, giving DNA its customary negative charge. These charges are normally neutralized in cells by positively charged inorganic and organic cations. The customs agent probably should be cautious, not because of the acidic nature of DNA but because of its information content.

4–9

A. The positions of the major and minor grooves are shown for all four base pairs in **Figure 4–34**. If a circle is drawn through the points of attachment to the sugar-phosphate backbones, the larger arc of the circle corresponds to the major groove and the smaller arc corresponds to the minor groove.

B. The structures of an A-T base pair and a T-A base pair are shown in Figure 4–34A and B. The two structures are identical except they have been flipped 180° about a north–south axis. The same relationship holds for the G-C and C-G base pairs (Figure 4–34 C and D).

C. As shown in Figure 4–34, the same edges of the bases, hence the same chemical moieties, always project into the same groove. Thus, for example, the methyl group of thymine is always found in the major groove, as is the amino group of cytosine.

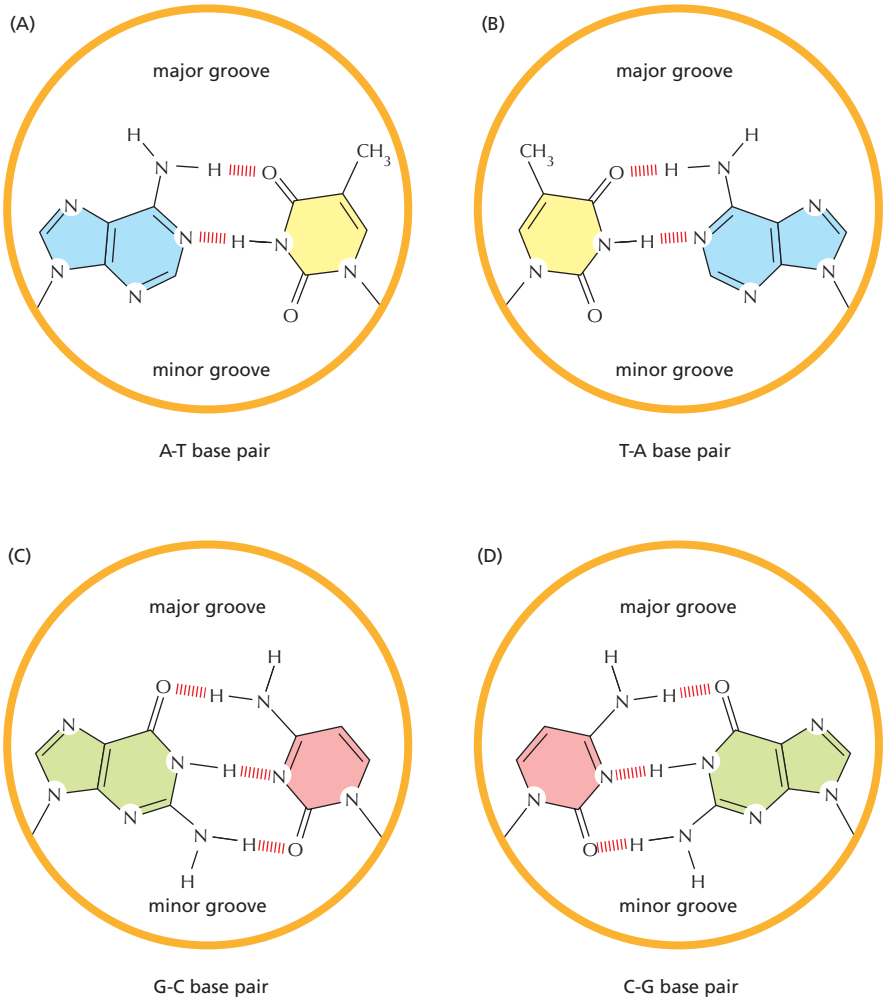


Figure 4–34 All four base pairs with the major and minor grooves indicated (Answer 4–9).

4–10 The base pairs are T-A (see Figure 4–2A) and C-G (see Figure 4–2B). The components of the base pairs, along with their stick representations, are shown in **Figure 4–35**. Thymine and cytosine are pyrimidines; adenine and guanine are purines.

4–11 In *all* samples of double-stranded DNA, the numbers of As and Ts (hence their percentages) are equal since they always pair with each other. The same is true for G and C. Results such as this one stood out as odd in the days before the structure of DNA was known. Now it is clear that, while all cellular DNA is double stranded, certain viruses contain single-stranded DNA. The genomic DNA of the M13 virus, for example, is single stranded.

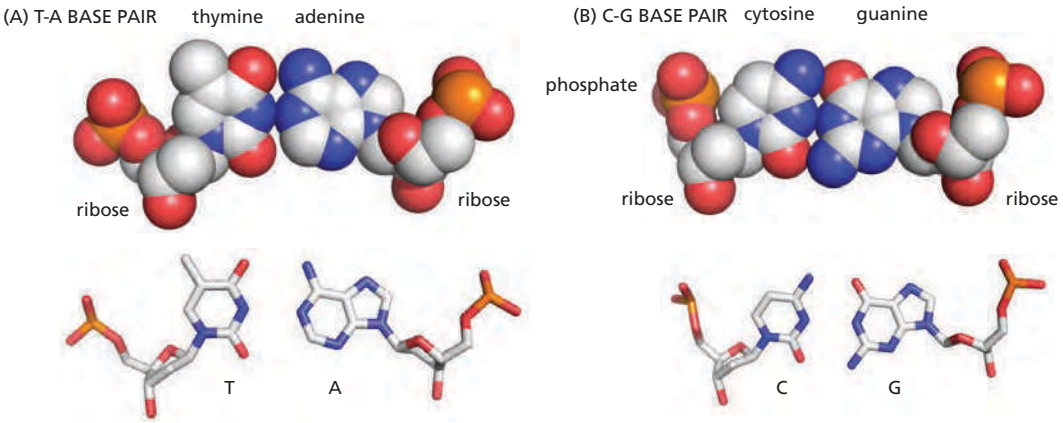


Figure 4–35 Space-filling and stick representations of two base pairs (Answer 4–10). (A) T-A base pair. (B) C-G base pair. Individual bases, ribose, and phosphates are labeled. The stick representations *below* each base pair are shown in the same orientations as the base pairs (carbon and phosphorus atoms are *gray* and *orange*, respectively, nitrogen atoms are *blue*, and oxygen atoms are *red*).

In single-stranded DNA, A is not paired with T, nor G with C, and so the A = T and C = G rules do not apply.

4–12 The segment of DNA in Figure 4–3 reads, from top to bottom, 5′-ACT-3′. The carbons in the ribose sugar are numbered clockwise around the ring, starting with C1′, the carbon to which the base is attached, and ending with C5′, the carbon that lies outside the ribose ring.

4–13 Helix A is right-handed. Helix C is left-handed. Helix B has one right-handed strand and one left-handed strand. There are several ways to tell the handedness of a helix. For a vertically oriented helix, like the ones in Figure 4–4, if the strands in front point up to the right, the helix is right-handed; if they point up to the left, the helix is left-handed. Once you are comfortable identifying the handedness of a helix, you will be amused to note that nearly 50% of the "DNA" helices in advertisements are left-handed, as are a surprisingly high number of the ones in books. Amazingly, a version of helix B was used in advertisements for a prominent international conference, celebrating the 30-year anniversary of the discovery of the DNA helix.

CALCULATIONS

4–14 Because C always pairs with G in duplex DNA, their mole percents must be equal. Thus, the mole percent of G, like C, is 20%. The mole percents of A and T account for the remaining 60%. Since A and T always pair, each of their mole percents is equal to half this value: 30%.

4–15

A. The DNA in a human cell is about 2.2 meters in length.

$$\text{length} = 6.4 \times 10^9 \text{ bp} \times \frac{0.34 \text{ nm}}{\text{bp}} \times \frac{\text{m}}{10^9 \text{ nm}}$$

$$\text{length} = 2.18 \text{ m}$$

B. The DNA occupies about 9% of the volume of the nucleus. The volume of the nucleus is

$$V = (4/3) \times 3.14 \times (3 \times 10^3 \text{ nm})^3$$

$$V = 1.13 \times 10^{11} \text{ nm}^3$$

The volume of DNA is

$$V = 3.14 \times (1.2 \text{ nm})^2 (2.2 \times 10^9 \text{ nm})$$

$$V = 9.95 \times 10^9 \text{ nm}^3$$

The ratio of DNA volume to nuclear volume is about 0.09 [$(9.95 \times 10^9 \text{ nm}^3)/(1.13 \times 10^{11} \text{ nm}^3)$]; thus, the DNA occupies about 9% of the nuclear volume.

4–16 The 1.0 g sample contains about 7.0 mg of DNA (about 0.7%):

$$\text{mass of DNA} = 10^9 \text{ cells} \times \frac{6.4 \times 10^9 \text{ bp}}{\text{cell}} \times \frac{660 \text{ d}}{\text{bp}} \times \frac{\text{g}}{6 \times 10^{23} \text{ d}}$$

$$= 7.0 \times 10^{-3} \text{ g, or } 7.0 \text{ mg}$$

Since there are 2.2 m of DNA in a human cell (see Problem 4–15) and 10^9 cells in the sample, there are 2.2×10^9 m (2.2×10^6 km) of DNA in the sample: enough to reach from the Earth to the Moon more than five times!

DATA HANDLING

4–17 This experiment implicates DNA as the genetic material. Clearly, by giving rise to progeny, bacteriophage T4 must possess some form of genetic

material. Since T4 contains only protein and DNA, there are only two choices for the genetic material. By separating the bacteria from the bacteriophage after infection and showing that the bacteria contained only the ^{32}P label (DNA), Hershey and Chase were able to demonstrate a clean separation of DNA from protein. The ability of these infected cells (in the absence of T4 proteins) to generate progeny virus shows that DNA must be the bacteriophage's genetic material.

There are caveats associated with these experiments, as the authors clearly recognized. For example, an absolute separation of bacteriophage from bacteria could not be accomplished: there was 1% ^{35}S associated with the infected cells. Also, not all proteins can be labeled with ^{35}S-methionine (some don't have methionine in their amino acid sequence). Thus, these experiments don't absolutely rule out protein as the genetic material. Nevertheless, the weight of the argument falls on the side of DNA. These experiments, coupled with other experiments such as the demonstration by Avery, MacLeod, and McCarty that DNA was the transforming principle of *Streptococcus pneumoniae*, convinced scientists of the role of DNA as the genetic material.

Reference: Hershey AD & Chase M (1952) Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J. Gen. Physiol.* 36, 39–56.

CHROMOSOMAL DNA AND ITS PACKAGING IN THE CHROMATIN FIBER

DEFINITIONS

4–18 Karyotype

4–19 Centromere

4–20 Histone

4–21 Chromosome

4–22 Cell cycle

4–23 Chromatin

4–24 Homologous chromosome (homolog)

4–25 Nucleosome

TRUE/FALSE

4–26 True. The human karyotype comprises 22 autosomes and the two sex chromosomes, X and Y. Females have 22 autosomes and two X chromosomes for a total of 23 different chromosomes. Males also have 22 autosomes, but have an X and a Y chromosome for a total of 24 different chromosomes.

4–27 False. In living cells, nucleosomes are packed upon one another to generate regular arrays in which the DNA is more highly condensed, usually in the form of a 30-nm fiber. The beads-on-a-string form of chromatin is usually observed only after the 30-nm fiber has been experimentally treated to unpack it.

4–28 True. All the core histones are rich in lysine and arginine, which have basic—positively charged—side chains that can neutralize the negatively charged DNA backbone.

4–29 False. By using the energy of ATP hydrolysis, chromatin remodeling complexes can catalyze the movement of nucleosomes along DNA, or even dissociate a nucleosome completely from the DNA.

THOUGHT PROBLEMS

4–30 The DNA molecules in chromosomes are long and exceedingly thin, and therefore very fragile. The techniques in use in the 1950s, which were gentle enough for the isolation of proteins, were much too harsh for DNA. For example, the shearing force exerted by pipetting DNA—sucking it through a small aperture—was sufficient to break it into the observed small pieces. It was a major technical achievement to demonstrate that chromosomes contain a single long DNA molecule.

4–31 The number of molecules of DNA in a human cell depends on the type of cell and its stage in the cell cycle. For the vast majority of somatic cells, there are 46 molecules of DNA (chromosomes) per cell prior to replication. After replication, but before completion of cell division, there are 92 molecules of DNA per cell. For a few somatic cells these numbers are very different. For example, red blood cells have no DNA molecules, having lost their nuclei (and mitochondria) during differentiation from reticulocytes into red cells. By contrast, skeletal muscle cells, which arise by the fusion of multiple precursor cells, have many nuclei and, thus, a very large number of DNA molecules per cell. Sex gametes are also different; they are haploid and thus have only 23 molecules of DNA per cell.

4–32 The intermediate chromosome and the sites of the inversions are indicated in **Figure 4–36**.

4–33 A gene is any DNA sequence that produces a functional RNA—structural, catalytic, or regulatory—or encodes a protein (or a set of closely related protein isoforms).

4–34 *Replication origins* are the specialized sequences that control the beginning of DNA replication, the process that allows chromosomes to be duplicated. *Centromeres* are the specialized sequences that permit one copy each of the duplicated chromosomes to be pulled into each daughter cell at cell division. *Telomeres* are the specialized sequences at the ends of chromosomes that allow ends to be efficiently replicated and also prevent chromosome ends from being recognized as breaks in need of repair.

4–35 In contrast to most proteins, which accumulate amino acid changes over evolutionary time, the functions of histone proteins must involve nearly all of their amino acids, so that a change in any position is deleterious to the cell. Histone proteins are exquisitely refined for their function.

4–36 Coiling a DNA duplex in a tight spiral around a histone octamer requires that the duplex be distorted from its preferred, gently meandering path in solution. Thus, a fair fraction of the binding energy available in the interaction of a histone octamer with a DNA molecule is used to distort

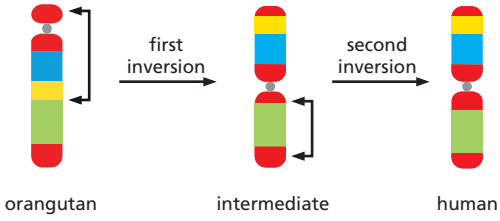


Figure 4–36 Inversions and intermediate chromosome in the evolution of chromosome 3 in orangutans and humans (Answer 4–32).

the duplex. For a more flexible CTG/CAG segment of duplex, less of the binding energy is invested in distorting the DNA; hence, more energy is available for binding the DNA to the histone octamer. This energetic consideration may be especially important during the formation of a nucleosome, in which case an especially flexible site may be the preferred site of nucleosome assembly.

Reference: Wang YH & Griffith J (1995) Expanded CTG triplet blocks from the myotonic dystrophy gene create the strongest known natural nucleosome positioning elements. *Genomics* 25, 570–573.

CALCULATIONS

4–37

A. The total length of DNA in chromosome 1 is 9.5×10^7 nm [$(2.8 \times 10^8$ bp) × (0.34 nm/bp)], which is 9.5×10^4 μm. In mitosis the chromosome measures 10 μm. Therefore the DNA molecule in chromosome 1 is compacted 9500-fold (9.5×10^4 μm/10 μm) at mitosis.

B. The packing ratio within a nucleosome core particle is 4.5 [(147 bp × 0.34 nm/bp)/(11 nm) = 4.5]. This first level of packing represents only 0.047% (4.5/9500) of the total condensation that occurs at mitosis.

C. With the stated assumptions, the DNA is compacted 27-fold in 30-nm fibers relative to the extended DNA. The total length of duplex DNA in 50 nm of the fiber is 1360 nm [(20 nucleosomes) × (200 bp/nucleosome) × (0.34 nm/bp) = 1360 nm]; 1360 nm of duplex DNA reduced to 50 nm of chromatin fiber represents a 27-fold condensation [(1360 nm/50 nm) = 27.2]. This level of packing represents 0.28% (27/9500) of the total condensation that occurs at mitosis, still a long way from what is needed.

4–38 Extrapolating from the number of genes on chromosome 22 to the whole genome gives an estimate of about 47,000 genes (700/0.015 = 46,667), which is well in excess of the likely value of about 21,000 protein-coding genes. The error in the calculation is the assumption that the *density* of genes on chromosome 22 is the same as that for the whole genome. The average genome-wide density of genes is about 6.6 genes per Mb (21,000 genes/3200 Mb). The gene density on chromosome 22 is much higher than the genome average (700 genes/48 Mb = 14.6 genes/Mb).

References: Dunham I, Shimizu N, Roe B et al. (1999) The DNA sequence of human chromosome 22. *Nature* 402, 489–495.

Lander ES, Linton LM, Birren B et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

4–39 About 7.6% of each gene is converted to mRNA [(5.4 exons/gene × 266 bp/exon)/(19,000 bp/gene) = 7.6%]. Genes occupy about 28% of chromosome 22 [(700 genes × 19,000 bp/gene)/(48×10^6 bp) = 27.7%].

Reference: Dunham I, Shimizu N, Roe B et al. (1999) The DNA sequence of human chromosome 22. *Nature* 402, 489–495.

4–40 Histone octamers occupy about 9% of the volume of the nucleus. The volume of the nucleus is

$$V = (4/3) \times 3.14 \times (3 \times 10^3 \text{ nm})^3$$

$$V = 1.13 \times 10^{11} \text{ nm}^3$$

The volume of the histone octamers is

$$V = 3.14 \times (4.5 \text{ nm})^2 \times (5 \text{ nm}) \times (32 \times 10^6)$$

$$V = 1.02 \times 10^{10} \text{ nm}^3$$

The ratio of the volume of histone octamers to the nuclear volume is 0.09; thus, histone octamers occupy about 9% of the nuclear volume. Since the DNA also occupies about 9% of the nuclear volume (see Problem 4–15), together they occupy about 18% of the volume of the nucleus.

DATA HANDLING

4–41 There are 15 bands on the gel in Figure 4–6, suggesting that *S. cerevisiae* has 15 chromosomes. From other studies, it is known that two chromosomes of very nearly the same length are present in the third band from the top. Thus, *S. cerevisiae* has 16 chromosomes.

4–42

A. The restriction analysis of the plasmid indicates that it is a linear molecule. If the plasmid were a circle, digestion with single-cut restriction nucleases would have generated only one band at 12 kb in each case—which was not observed. On the other hand, a linear molecule generates two bands when cut once, and they should sum to 12 kb. Since two bands that summed to 12 kb were generated with each of the three single-cut restriction nucleases, the plasmid must be a linear molecule.

B. Digestions with BamHI or BglII yield DNA fragments with identical 5′ extensions (5′-GATC). Thus, a mixture of these fragments can join in all possible combinations. Neither enzyme, however, can cut the hybrid sites created by joining a telomere fragment to a plasmid end (a BamHI end to a BglII end, 5′-AGATCC and 5′-GGATCT). By contrast, the appropriate enzyme can cut the sites created by joining two BamHI ends or two BglII ends. Therefore, in the presence of DNA ligase, BamHI, and BglII, joints that create a BamHI site or a BglII site are quickly recut, whereas the hybrid sites, which cannot be cut, accumulate. This strategy neatly selects for formation of hybrid joints—in this case, the telomere fragment joined to the plasmid.

Reference: Szostak JW & Blackburn EH (1982) Cloning yeast telomeres on linear plasmid vectors. *Cell* 29, 245–255.

4–43 If nucleosomes were randomly positioned on the 225-bp segment of DNA, then the 147-bp fragments would be a collection of all possible 147-bp segments of the original DNA. Such a random collection would give a highly diverse set of fragments upon digestion with a restriction nuclease that cuts at a unique location. The generation of only two fragments after restriction digestion means that there is a strongly preferred location for a nucleosome on this piece of DNA, which gives rise to a unique 147-bp fragment. When this fragment is cut, it gives a 37-bp and a 110-bp fragment, which sum to 147. If the position of the restriction cut were given in the problem, you could have deduced where the nucleosome is situated on the 225-bp segment.

4–44 The presence of a nuclease-resistant fraction in chromatin—but not in naked DNA—suggests that the Martian DNA is associated with a nucleosome-like structure that protects it from micrococcal nuclease. Since extensive digestion produced a limit product of about 300 nucleotides, the nucleosome-like structure must protect about this length of DNA. The smear of digestion products indicates that the nucleosome-like structures are not regularly spaced along the DNA as Earthly nucleosomes are. If they were regularly spaced, they would have given a ladder of bands analogous to those seen in rat liver.

4–45 These results argue strongly that the SWI/SNF complex slides nucleosomes along the DNA in an ATP-dependent manner. Two key observations support this model. First, incubation with SWI/SNF causes the nucleosome to disappear from the small fragment released by NheI (see

Figure 4–10B, lane 6), but the nucleosome remains associated with the large fragment released by EcoRI. Second, cleavage with NheI before incubation prevents loss of the nucleosome from the small fragment (see lane 4), suggesting that the nucleosome can be moved only if there is contiguous DNA.

If the mechanism of action of the SWI/SNF complex were to release the nucleosome from the DNA, then the nucleosome should have been released regardless of the restriction enzyme used or the order of incubation. Retention of the nucleosome on the large EcoRI fragment and on the precleaved NheI fragment argues against this mechanism.

If the SWI/SNF complex transferred the nucleosome from one duplex to another, then the nucleosome should have been released from the precleaved NheI fragment and transferred to the other duplex (the rest of the DNA in the substrate). This mechanism cannot strictly be ruled out by these experiments because of concentration effects. When the NheI fragment is attached to the rest of the DNA, the local concentration of the receptor duplex (the rest of the DNA) is very high. By contrast, when it is detached, the concentration of receptor duplex is low. The authors of this study ruled out this possibility by placing a barrier to sliding (but presumably not to transfer) adjacent to the NheI site. Under these conditions the nucleosome remained associated with the NheI fragment when the substrate was incubated with the SWI/SNF complex before cleavage with NheI (in contrast to the results in the absence of a barrier, as shown in Figure 4–10B, lane 6).

Reference: Whitehouse I, Flaus A, Cairns BR, White MF, Workman JL & Owen-Hughes T (1999) Nucleosome mobilization catalysed by the yeast SWI/SNF complex. *Nature* 400, 784–787.

MEDICAL LINKS

4–46 By comparing the normal chromosomes 9 and 22 with their abnormal counterparts, it would appear that the bottom portion of chromosome 22 was translocated to the bottom of chromosome 9. The presence of two X chromosomes indicates that this patient is female.

CHROMATIN STRUCTURE AND FUNCTION

DEFINITIONS

4–47 Euchromatin

4–48 Epigenetic inheritance

4–49 Position effect

TRUE/FALSE

4–50 True. Deacetylation increases the positive charge on the histone tails by unmasking the positive charges on lysines. The increased charge tends to stabilize chromatin structure, perhaps by allowing the tails to interact more strongly with the DNA.

4–51 True. The variant histones are inserted into nucleosomes via a histone-exchange process catalyzed by ATP-dependent chromatin remodeling complexes.

THOUGHT PROBLEMS

4–52 The structures of serine and lysine residues in histones and their modifications are shown in **Figure 4–37**. Phosphorylation of serine converts

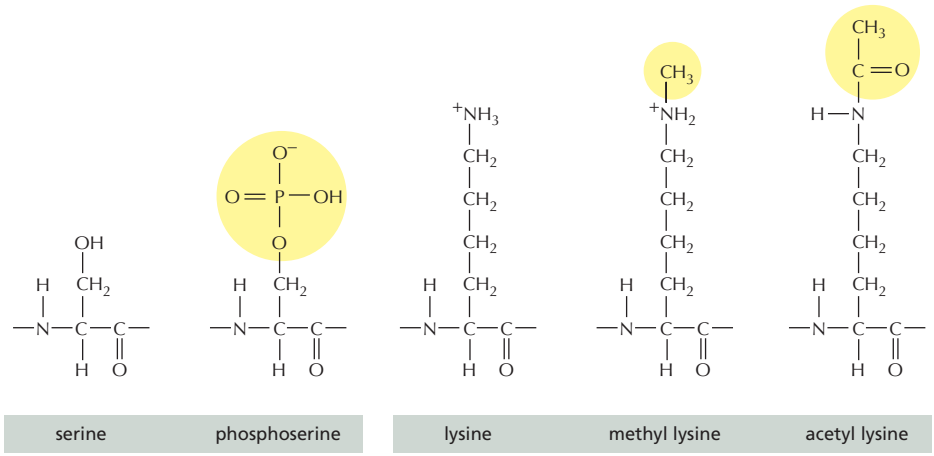


Figure 4–37 Structures of serine, lysine, and their modifications in histone tails (Answer 4–52). Each of the amino acid residues is shown as it would exist in the peptide structure of a histone.

an uncharged amino acid to a negatively charged one. Methylation of lysine does not alter the charge. Acetylation of lysine removes the positive charge, leaving the modified lysine neutral. The introduction of a negative charge by phosphorylation of serine and removal of a positive charge by acetylation of lysine would both be expected to decrease the interaction of the histone tails with DNA, which is a negatively charged polymer.

4–53 The biological outcome associated with histone methylation depends on the site that is modified. Each site of methylation has different surrounding amino acid context, which allows the binding of distinct reader complexes. It is the binding of different downstream effector proteins that gives rise to different biological outcomes.

4–54 A dicentric chromosome is unstable because the two kinetochores have the potential to interfere with one another. Normally, microtubules from the two poles of the spindle apparatus attach to opposite faces of a single kinetochore in order to separate the individual chromatids at mitosis. If a chromosome contains two centromeres, half of the time the microtubules from one of the poles will attach to the two kinetochores associated with one chromatid, while the microtubules from the other pole will attach to the two kinetochores associated with the other chromatid. Division can then occur satisfactorily. The other half of the time, the microtubules from each pole will attach to kinetochores that are associated with different chromatids. When that happens, each chromatid will be pulled to opposite spindle poles with enough force to snap it in two. Thus, two centromeres are bad for a chromosome, causing chromosome breaks—rendering it unstable.

DATA HANDLING

4–55

A. DNase I preferentially digests active chromatin, but micrococcal nuclease shows no such preference. Red cells express globin, and treatment of red-cell nuclei with DNase I reduced the ability of the DNA to protect globin cDNA. Thus, DNase I preferentially degraded the chromatin from which globin RNA was transcribed. By contrast, fibroblasts do not express globin, and treatment of fibroblast nuclei with DNase I did not reduce the ability of the DNA to protect globin cDNA. Thus, in fibroblasts the globin genes are no more sensitive than the bulk of the chromatin.

B. Trypsin treatment of nucleosome monomers affects a specific population of monomers—namely, those that were present in active chromatin. This conclusion comes from a comparison of trypsin-treated monomers

with DNase I-treated red-cell DNA. Digestion of trypsin-treated nucleosome monomers with micrococcal nuclease yielded DNA that protected globin cDNA to the same extent as DNase I-treated red-cell DNA (see Table 4–1).

If a random population of nucleosomes had been affected, all the DNA sequences present in the untreated nucleosomes would still be present in the trypsin-treated nucleosomes. Since hybridization was carried out in a vast excess of DNA, both the untreated and treated monomers would have behaved identically in their capacity to protect globin cDNA and total red-cell DNA.

C. Since the DNA in individual nucleosome monomers showed the same sensitivity to DNase I as chromatin in nuclei, the property of active chromatin that distinguishes it from bulk chromatin must be present in individual nucleosomes. This viewpoint is supported further by the observation that trypsin treatment of nucleosome monomers renders those from active chromatin sensitive to micrococcal nuclease. Individual monomers from regions of active chromatin must be physically distinct from other nucleosome monomers.

Reference: Weintraub H & Groudine M (1976) Chromosomal subunits in active genes have an altered conformation. *Science* 193, 848–856.

4–56 The control proteins either do not bind to any of the histone N-terminal peptides (Pax5) or bind to all of them (Pc1 and Suv39h1). By contrast, the HP1 proteins all bind specifically to the Lys9-dimethylated form of the H3 N-terminal peptide. The strong association of HP1 proteins with heterochromatin suggests that the Lys9-dimethylated form of H3 will be found in heterochromatin.

Reference: Lachner M, O'Carroll D, Rea S, Mechtler K & Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410, 116–120.

4–57 Colonies are clumps of cells that originate from a single founder cell and grow outward as the cells divide repeatedly. In the red colony of Figure 4–13, the *Ade2* gene has been inactivated by its position next to the telomere. The inactivation is inherited, but at a low frequency the gene is reactivated. This gives rise to white cells whose descendants are also white (producing the white sectors), even though the gene has not moved away from the telomere. This pattern shows that the inactivation of a telomere-proximal gene is passed on to daughter cells in a way that is not completely stable, and that both the off and the on stare are heritable. An epigenetic mechanism is thought to be involved, based on the tendency of a condensed chromatin state to be inherited following DNA replication.

4–58

A. It is apparent by visual inspection of the chromosomes in Figure 4–14A that there is a higher density of genes with increased expression (black bars) near telomeres than elsewhere after depletion of histone H4. This impression is confirmed by a more rigorous analysis of the data, as shown in **Figure 4–38**. This analysis indicates that the fraction of telomere-proximal genes that have increased expression is more than threefold higher than the genome-wide average of 15%.

B. It is more difficult to be certain by visual inspection alone that deletion of the *Sir3* gene preferentially increases the expression of genes near telomeres. Statistical analysis of the data, as described above, makes it clearer (Figure 4–38). The fraction of telomere-proximal genes that have increased expression is nearly tenfold higher than the genome average of 1.5%.

C. The loss of Sir3 would inactivate the Sir protein complex, which would dramatically inhibit deacetylation of histones in the region of the

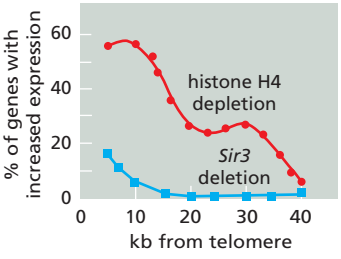


Figure 4–38 Extent of telomeric gene activation after depletion of histone H4 or deletion of the *Sir3* gene (Answer 4–58). For this analysis all the chromosomes were aligned by their telomeres and the results were summed. Windows 50-genes wide were moved a gene at a time across the chromosomes starting at the telomere. At each position the fraction of genes in the window with increased expression was determined and plotted as kb from the midpoint of the window to the telomere.

telomere. The normally deacetylated histones near telomeres allow the nucleosomes to pack together into tighter arrays, which are associated with lower levels of expression. In the absence of deacetylation, nucleosomes would be expected to pack less tightly near telomeres and gene expression should be increased, as it is. From the analysis shown in Figure 4–38, the effect of Sir3 is most apparent within 10 kb of the chromosome ends.

Depletion of histone H4 is thought to cause a general, genome-wide reduction in the number of nucleosomes. A specific effect on gene expression of telomere-proximal genes was not expected. The effect extends out to 15 kb or so away from the telomere, which is farther than the effect of loss of Sir3. This result suggests that some genes near telomeres are normally repressed by a nucleosome-specific mechanism that may be independent of the effects of the Sir protein complex. The mechanism of this effect is not yet defined. These observations suggest that a special form of chromatin may extend beyond the region to which the Sir protein complex is bound.

Reference: Wyrick JJ, Holstege FC, Jennings EG, Causton HC, Shore D, Grunstein M, Lander ES & Young RA (1999) Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. *Nature* 402, 418–421.

4–59

A. Micrococcal nuclease generates fragments whose lengths vary depending on the spacing of the internucleosomal cleavages that define the ends of the fragments (**Figure 4–39**). Since micrococcal nuclease does not cleave at precise sites within the linker DNA, there is some variability in the lengths of fragments produced by cleavage even between the same two pairs of nucleosomes. Furthermore, similar-sized fragments can be produced by cleavage between several different pairs of internucleosomal sites. These sorts of variability obscure the fine-structure details of the ordering of adjacent nucleosomes.

Digestion with BamHI sharpens the pattern of bands because it precisely defines one end of each DNA fragment. As shown in Figure 4–39, only the fragments to the right of the BamHI cleavage site hybridize to the radioactive probe. The resulting pattern is easy to interpret because the length of each fragment gives the distance from the nuclease cleavage site to the BamHI site directly. In the absence of BamHI cleavage, the bands are defined by micrococcal-nuclease cleavage at both ends. Such a pattern does not allow one to deduce the exact sites of nuclease cleavage relative to the probe. The method for mapping nuclease cut sites illustrated in this problem is called indirect end labeling because a defined end (the BamHI cleavage site) is labeled indirectly through hybridization to a radiolabeled probe.

B. The sizes of the bands indicate the distances between the nuclease cut sites and the BamHI cleavage site (Figure 4–39). Since micrococcal nuclease cleaves between nucleosomes, the cut sites define the positions

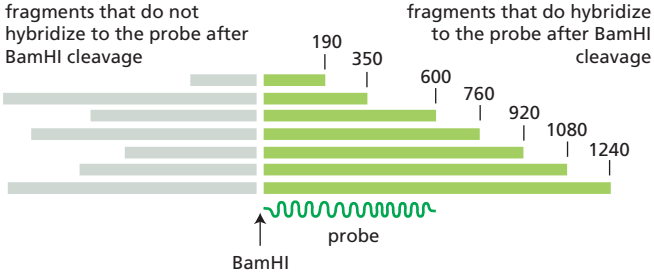


Figure 4–39 Diagram relating indirect end labeling to the fragment lengths observed in Figure 4–16 (Answer 4–59). *Vertical lines* indicate sites of micrococcal-nuclease cleavage. The small *gap* in the micrococcal-nuclease fragments shows the position of BamHI cleavage. Numbers refer to the lengths of the fragments that hybridize to the probe; they correspond to the lengths shown in Figure 4–16.

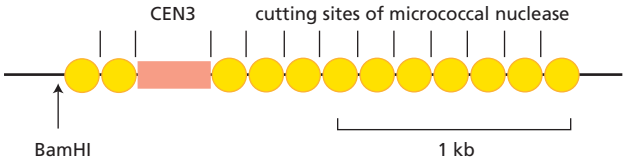


Figure 4–40 Positions of micrococcal-nuclease cleavage sites and arrangement of nucleosomes around the centromere (Answer 4–59). Nucleosomes are shown as *yellow circles.*

of the nucleosomes relative to the BamHI site (**Figure 4–40**). With the exception of the region around the centromere, the cut sites are spaced at 160-nucleotide intervals, suggesting that the nucleosomes occupy about 160 nucleotides of DNA. The cut sites on either side of the centromere are 250 nucleotides apart, suggesting that some special structure covers the centromere. It is thought that centromere-specific proteins bind to a centromere-specific nucleosome and protect the centromere from nuclease digestion. The cleavage sites on either side of the centromere indicate that there is unprotected DNA between the centromere-specific structure and the adjacent nucleosomes on either side.

C. The naked DNA control is important because all DNA sequences are not equally susceptible to micrococcal-nuclease cleavage. It is essential to know the susceptibility of the specific DNA sequence under investigation. Otherwise, one can be fooled into thinking that a specific band results from the binding of a protein adjacent to the cleavage site, when it actually derives from the cleavage specificity of the nuclease. Indeed, the centromere itself is a preferred site of cleavage (although that was left out of the naked DNA digestion shown in Figure 4–16); the absence of cutting at this sensitive site in chromatin is all the more evidence that the centromere is specifically protected.

D. The results in Figure 4–16 answer this question very elegantly. The band patterns from the three plasmids are the key results. If the nucleosomes were ordered simply because they were lined up next to the special structure at the centromere, then it should not make any difference what DNA sequence was present beyond the centromere. Results with plasmids 2 and 3 show clearly that the ordered arrangement disappears at the point where the bacterial sequences (plasmid 2) or the noncentromeric yeast sequences (plasmid 3) begin. This result argues strongly that the regular ordering of nucleosomes around the centromere is due to some feature of the sequence of the neighboring DNA itself.

Reference: Bloom KS & Carbon J (1982) Yeast centromere DNA is in a unique and highly ordered structure in chromosomes and small circular minichromosomes. *Cell* 29, 305–317.

THE GLOBAL STRUCTURE OF CHROMOSOMES

DEFINITIONS

4–60 Polytene chromosome

4–61 Lampbrush chromosome

4–62 Mitotic chromosome

TRUE/FALSE

4–63 False. The loops are actively transcribed, but contain only a minority of the DNA. Most of the DNA in lampbrush chromosomes is highly condensed on the chromosome axis and transcriptionally inactive.

4–64 True. It is thought the interphase chromosomes of all eukaryotes are arranged in loops similar to those observed for lampbrush chromosomes.

THOUGHT PROBLEMS

4–65 The results of these heterologous injection experiments show very clearly that loop structure is not an intrinsic property of the chromosome: the same chromosomes adopt different loop structures depending on the type of oocyte into which they are injected. Thus, loop structure seems to be determined by the proteins that are in the oocyte.

Reference: Gall JG & Murphy C (1998) Assembly of lampbrush chromosomes from sperm chromatin. *Mol. Biol. Cell* 9, 733–747.

DATA HANDLING

4–66 Throughout the 315-kb chromosomal segment that was analyzed, the relative amounts of DNA in polytene and diploid chromosomes varied by no more than ±50%. This variation is rather small considering that polytene chromosomes are amplified about 1000-fold. Furthermore, there is no indication that the low values are clustered at interbands. Thus, these results argue strongly that bands are darker than interbands because they are differentially stained rather than differentially replicated. The darker staining presumably reflects a more condensed chromatin structure, a higher density of proteins, or both.

The identical restriction pattern in diploid and polytene DNA also supports this conclusion in a more subtle way. If the differential replication model were correct, there would be occasional restriction fragments that spanned a replication fork. These branched fragments would migrate very differently from their unbranched counterparts (see Problem 5–61); no such anomalously migrating fragments were observed.

Reference: Spierer A & Spierer P (1984) Similar levels of polyteny in bands and interbands of *Drosophila* giant chromosomes. *Nature* 307, 176–178.

4–67

A. A few of the untreated plasmid molecules are relaxed because they contain one or more single-strand breaks in the DNA. DNA that is carefully handled during the isolation procedure is mostly supercoiled, as shown in Figure 4–20, lane 1. Harsh treatment during isolation will yield a DNA preparation that is almost entirely relaxed.

B. The bands that run at intermediate positions are a collection of topoisomers that differ only in their linking number; that is, the number of times one strand is wrapped around the other. Adjacent bands on the gel contain topoisomers that differ by a linking number of one. The rate at which DNA molecules migrate through a gel depends on how compact they are, with more compact molecules moving faster. Relaxed circular molecules are the least compact and therefore move the slowest, whereas highly supercoiled molecules are the most compact and run the fastest. Treatment with topoisomerase removes supercoils one at a time, making the molecule progressively less compact and slower in migrating.

C. The number of supercoils in the original plasmid can be estimated by counting the number of bands between the highly supercoiled and relaxed positions of the gel. About eight intermediate bands can be counted in the treated samples; thus, there must be at least nine supercoils in the original plasmid. This number is likely to be an underestimate because of the limited resolving power of such gels. Once a molecule reaches a certain degree of compactness, a gel cannot resolve molecules with further increases in supercoiling.

D. Since the supercoils are removed by *E. coli* topoisomerase I, the original supercoils must have been negative. (*E. coli* topoisomerase I will not relax positive supercoils.)

4–68 In order for the circular DNA molecules to retain a net supercoiling of zero, plectonemic supercoils and solenoidal supercoils must have the opposite sign. Thus, a negative solenoidal supercoil can be compensated for by a positive plectonemic supercoil, and vice versa. Arrangements B and C are the only structures with zero net supercoiling, as shown in **Figure 4–41**.

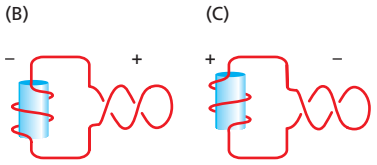


Figure 4–41 The two compensating arrangements of solenoidal and plectonemic supercoils (Answer 4–68). The signs of the solenoidal and plectonemic supercoils are indicated by "+" and "–."

4–69 Arrangements B and C (see Figure 4–21) are the two that have compensating solenoidal and plectonemic supercoils. These two alternative arrangements are nicely distinguished by incubation with *E. coli* and calf thymus topoisomerases, as shown in **Figure 4–42**. For arrangement B, incubation with *E. coli* topoisomerase I will not affect the positive plectonemic supercoils. Thus, when the histones are removed, the molecules will have the same supercoiling that they started with: zero. By contrast, calf thymus topoisomerase I will remove the positive plectonemic supercoils, so that when the histones are removed, the molecules will have two negative plectonemic supercoils—derived from the two negative solenoidal supercoils around the nucleosome.

Arrangement C gives different predictions for both incubations. Arrangement C has negative plectonemic supercoils that can be removed by both the *E. coli* and calf thymus enzymes. Thus, when the histones are removed, the molecules will have two positive plectonemic supercoils—derived from the two positive solenoidal supercoils around the nucleosome.

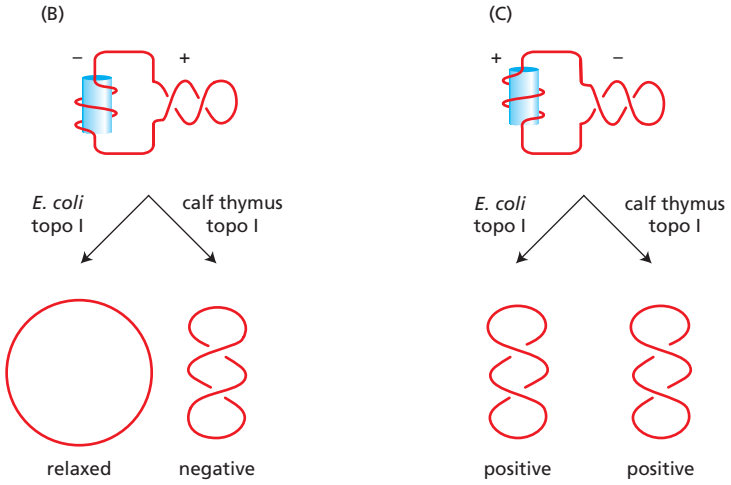


Figure 4–42 Predicted outcomes of incubating alternative arrangements B and C with *E. coli* and calf thymus topoisomerase (topo) I (Answer 4–69). The signs of the solenoidal and plectonemic supercoils are indicated by "+" and "–." Following the indicated incubations with the topoisomerases, histones were removed.

HOW GENOMES EVOLVE

DEFINITIONS

4–70 Pseudogene

4–71 Copy number variation (CNV)

4–72 Purifying selection

4–73 Single-nucleotide polymorphism (SNP)

TRUE/FALSE

4–74 True. Humans and mice diverged from a common ancestor long enough ago for roughly two out of three nucleotides to have been changed by random mutation. The regions that have been conserved are those with important functions, where mutations with deleterious effects were eliminated by natural selection. Other regions have not been conserved

because natural selection cannot operate to eliminate changes in nonfunctional DNA.

4–75 True. Although this statement is not true for all human genes, it is true for many. Even more remarkably, in some cases the human gene can indeed substitute for the corresponding gene in yeast.

4–76 False. About 5% of the human genome is subjected to purifying selection, but only about 1.5% encodes proteins. This comparison implies that 3.5% of the human genome—more than the amount that encodes proteins—has important functions that we do not understand.

4–77 True. Duplication of chromosomal segments, which may include one or more genes, allows one of the two genes to diverge over time to acquire different, but related functions. The process of gene duplication and divergence is thought to have played a major role in the evolution of biological complexity.

THOUGHT PROBLEMS

4–78 The *Hox* gene clusters are packed with complex and extensive regulatory sequences that ensure the proper expression of individual *Hox* genes at the correct time and place during development. Insertions of transposable elements into the *Hox* clusters are eliminated by purifying selection, presumably because they disrupt proper regulation of the *Hox* genes. Comparison of the *Hox* cluster sequences in mouse, rat, and baboon reveals a high density of conserved noncoding segments, supporting the presence of a high density of regulatory elements.

Reference: Lander ES, Linton LM, Birren B et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

CALCULATIONS

4–79

A. The missing values in Table 4–2 can be obtained by counting the differences between the pairwise combinations of the hemoglobin α chains in Figure 4–23. The differences are 18 for human versus frog, 17 for frog versus chicken, 12 for chicken versus whale, and 17 for whale versus fish.

B. The difference matrix shows that human and whale are the two most closely related species. The underlying assumption is that the fewer the differences, the less the evolutionary distance between the species.

C. In the cluster-analysis method the two closest species, human and whale, are combined and the average differences are determined for the other species.

	Frog	Chicken	Fish
Human/whale	17.5	11.5	17

This analysis shows that the chicken is the next closest species. It is then combined with human and whale and used to determine the average differences relative to the remaining species.

	Frog	Fish
Human/whale/chicken	17.3	18

This establishes the order of the final two species and gives the overall branching structure shown in **Figure 4–43**.

D. If you simply used the number of differences relative to human to place the other species on the tree, the order of frogs and fish would have been reversed. The cluster-analysis method is superior because it makes use of all of the information in the difference-matrix table and reduces error due to random variation in the mutational history of individual species.

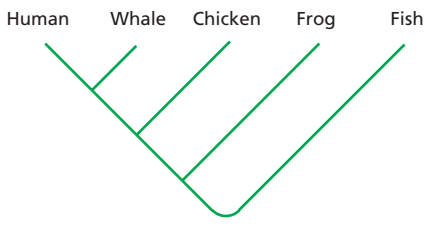


Figure 4–43 Branching order for the five species, based on the differences in the first 30 amino acids of their hemoglobin α chains (Answer 4–79).

4–80

A. For five species there are 10 equations, which is more than enough to solve for the 7 line segments that make up the tree.

1.	Human/whale	$= a + b$	$= 8$
2.	Human/chicken	$= a + c + d$	$= 11$
3.	Human/frog	$= a + c + e + f$	$= 18$
4.	Human/fish	$= a + c + e + g$	$= 17$
5.	Whale/chicken	$= b + c + d$	$= 12$
6.	Whale/frog	$= b + c + e + f$	$= 17$
7.	Whale/fish	$= b + c + e + g$	$= 17$
8.	Chicken/frog	$= d + e + f$	$= 17$
9.	Chicken/fish	$= d + e + g$	$= 20$
10.	Frog/fish	$= f + g$	$= 20$

B. Solutions to the two three-at-a-time equations are shown below. In each case, the three equations are summed in such a way as to eliminate all but one variable. For human/whale/chicken this involves subtracting equation 5 from the sum of equations 1 and 2.

Human/whale/chicken

1.	Human/whale	$= a + b$	$= 8$
2.	Human/chicken	$= a + c + d$	$= 11$
5.	Whale/chicken	$= b + c + d$	$= 12$
	(1. + 2. – 5.)	$= 2a$	$= 7$
			$a = 3.5, b = 4.5$

Human/whale/frog

1.	Human/whale	$= a + b$	$= 8$
3.	Human/frog	$= a + c + e + f$	$= 18$
6.	Whale/frog	$= b + c + e + f$	$= 17$
	(1. + 3. – 6.)	$= 2a$	$= 9$
			$a = 4.5, b = 3.5$

Note that the values for a and b are not the same. Because there are more equations than unknowns, multiple values for the unknowns are obtained. These are averaged to get the distances represented in the phylogenetic trees. Three common representations of the phylogenetic tree obtained from the data in this problem are shown in **Figure 4–44**.

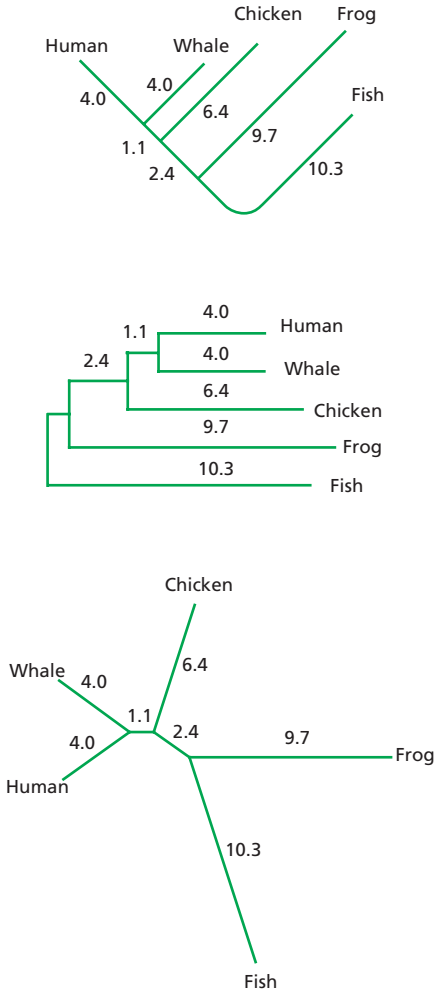


Figure 4–44 Three representations of the phylogenetic tree derived from the data in this problem (Answer 4–80). These trees are termed unrooted trees because the node where this cluster of species diverged from more primitive species is not shown, nor can it be derived from the data in the problem.

DATA HANDLING

4–81

A. The exons in the human β-globin gene correspond to the positions of homology with the cDNA, which is a direct copy of the mRNA and thus contains no introns. The introns correspond to the regions between the exons. The positions of the introns and exons in the human β-globin gene are indicated in **Figure 4–45A**.

B. From the positions of the exons, as defined in Figure 4–45A, it is clear that the first two exons of the human β-globin gene have homologous counterparts in the mouse β-globin gene (**Figure 4–45B**). Only the first half of the third exon of the human β-globin gene is homologous to the mouse β-globin gene. The homologous portion of the third exon contains sequences that encode protein, whereas the nonhomologous portion represents the 3′ untranslated region of the gene. Since this portion of the gene does not encode protein (nor does it contain extensive regulatory sequences), it evolves at a rate similar to that of introns.

C. The human and mouse β-globin genes are also homologous at their 5′ ends, as indicated by the cluster of points along the same diagonal as the first exon (Figure 4–45B). These sequences correspond to the regulatory regions in front of the start sites for transcription. The regulatory function of this region has limited its evolutionary divergence, much as the coding

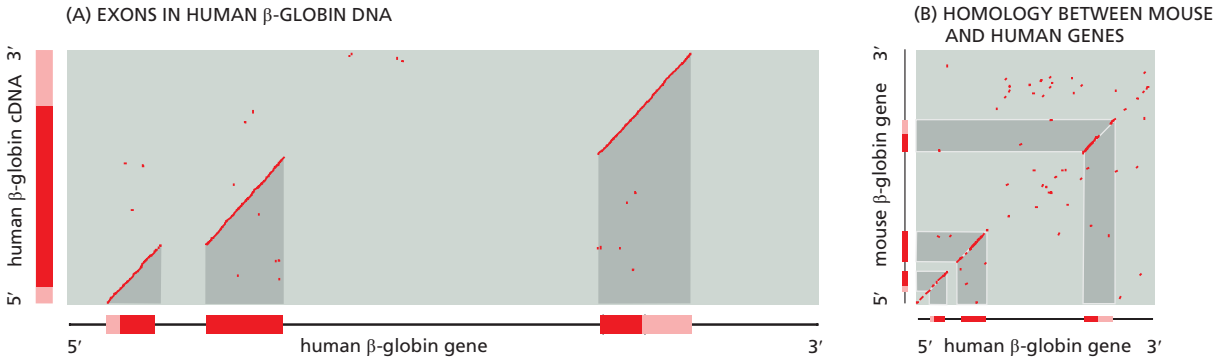


Figure 4–45 Interpretation of diagon plots (Answer 4–81). (A) Positions of the exons in the human β-globin gene. (B) The relationship of the homologous mouse sequences to the exons of the human gene. Exons are shown as *boxes*; the *pink* areas indicate the 5′ and 3′ noncoding sequences.

function of exons has limited their divergence. Functional sequences, which are under selective pressure, diverge much more slowly than sequences without function.

D. The diagon plot shows that the first intron is nearly the same length in the human and mouse genes, but the length of the second intron is noticeably different (Figure 4–45B). If the introns were the same length, the line segments that represent homology would fall on the same diagonal. The easiest way to test for the co-linearity of the line segments is to tilt the page and sight along the diagonal. It is impossible to tell from this comparison if the change in length is due to a shortening of the mouse intron or to a lengthening of the human intron, or to some combination of those possibilities.

Reference: Konkel DA, Maizel JV & Leder P (1979) The evolution and sequence comparison of two recently diverged mouse chromosomal β-globin genes. *Cell* 18, 865–873.

4–82

A. The majority of sequences isolated from the Neanderthal bone differ by seven substitutions and a single base insertion compared with the human reference sequence. Although several clones show additional individual differences, these differences, because they are represented infrequently, are most likely to be due to misincorporation of nucleotides during PCR. They are probably caused by damage to the original template and so can be ignored. The variability among contemporary human lineages, as shown in Figure 4–28, is too little to encompass the sequence obtained from Neanderthal bone. Thus, it is very likely that you have indeed determined the sequence of Neanderthal DNA.

B. Eight of the 44 sequences show an identical match, or differ only by a single base from the human sequence. These sequences almost certainly arose by amplification of contaminating human DNA. In the paper on which this problem is based, the authors estimated that they were amplifying from a starting population of about 50 DNA molecules in 5 μL of bone extract. A single contaminating human cell would have added 500–1000 additional contaminating mitochondrial sequences, so their precautions worked very well.

C. The main reason for using the mitochondrial DNA for these studies is its abundance compared with nuclear DNA. Cells typically contain 500–1000 copies of mitochondrial DNA molecules, compared with 2 copies of nuclear DNA molecules. With the accumulation of DNA damage over the 30,000 years or so since this individual died, abundance is critical for success. A second reason is that mitochondrial DNA is much more variable than nuclear DNA. The ability to detect multiple differences is also critical for showing that this one Neanderthal sample is different from human DNA. Since this problem was written, advances in the handling of archaeological DNA and in the technologies for DNA sequencing have

permitted scientists to sequence the nuclear genome of Neanderthals and define the genetic differences from modern humans.

D. The most important way to verify these results is to isolate DNA from a second Neanderthal sample. Three years after this initial report, the same segment of mitochondrial DNA from a second sufficiently preserved specimen was sequenced. Although these two individuals differed at several positions in the sequence, as expected, they shared 19 substitutions relative to the reference human sample. At present, sequences of mitochondrial DNA from more than a dozen Neanderthal individuals have been published. It is clear that Neanderthals form a group that is distinct from modern humans. It is estimated that the modern human and Neanderthal lineages diverged between 360,000 and 850,000 years ago.

References: Krings M, Stone A, Schmitz RW, Krainitzki H, Stoneking M & Pääbo S (1997) Neandertal DNA sequences and the origin of modern humans. *Cell* 90, 19–30.

Ovchinnikov IV, Götherström A, Romanova GP, Kharitonov VM, Lidén K & Goodwin W (2000) Molecular analysis of Neanderthal DNA from the northern Caucasus. *Nature* 404, 490–493.

Green, R et al. (2010) A draft sequence of the Neandertal genome. *Science* 328, 710–722.

4–83

A. The left and right boundaries of the inserted *Alu* sequences and the mutational changes in the flanking chromosomal sequences are indicated in **Figure 4–46**. The boundaries can be located unambiguously using two complementary approaches. By comparing the sequences vertically, one can make a tentative assignment of the boundaries based on the point at which the sequences diverge. The second approach makes use of a common feature of the *Alu* insertion process, namely, the duplication of chromosomal sequences at the target of insertion. A comparison of the sequences to the left and right of each *Alu* sequence shows that they are tandemly duplicated—one end of each duplication is precisely at the tentative boundary assigned on the basis of the vertical comparison.

The mutations in the flanking sequences can be located easily by comparing the repeated segments that were generated when each *Alu* inserted into the chromosome.

B. As indicated in Figure 4–46, there are five nucleotide changes in the 120 nucleotides of duplicated DNA that flank the *Alu* sequences. Using the estimate of 3×10^{-3} substitutions per site per million years, the *Alu* sequences inserted into the human albumin-family genes about 14 million years ago.

$$\text{years after insertion} = \frac{10^6 \text{ years} \times 1 \text{ site}}{3 \times 10^{-3} \text{ mutations}} \times \frac{5 \text{ mutations}}{120 \text{ sites}}$$

$$= 14 \times 10^6 \text{ years}$$

C. These particular flanking sequences are critical for the calculation because they were generated by *Alu* insertion into the human

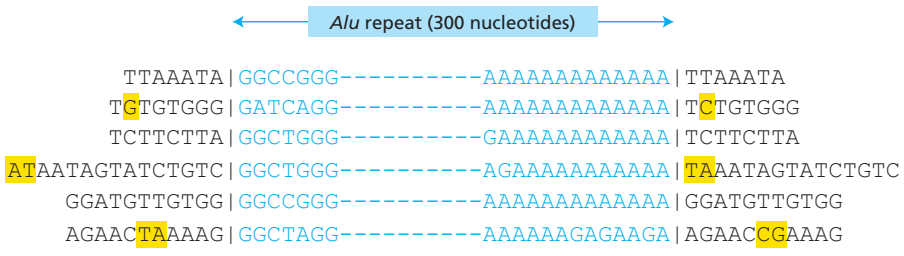


Figure 4–46 Boundaries of *Alu* inserts and mutational alterations in the flanking target-site duplications (Answer 4–83). Boundaries are indicated by *vertical lines*; mutational differences are indicated by *yellow highlights*.

albumin-family genes. Thus, the sequences that constitute the target-site duplications mark the time of *Alu* insertion.

Additional intron sequences would not help in the calculation because mutations outside the target-site duplication are unrelated to the time of *Alu* insertion. The mutations in the *Alu* sequences themselves are also not useful for estimating the time of insertion. Some of the observed mutations in the *Alu* sequences very likely were generated while they sat in the genome at another location—prior to the time at which a copy inserted into the human albumin-gene family.

D. The calculation in part B indicates that these *Alu* sequences invaded the human albumin-gene family about 14 million years ago; that is, well after the mammalian radiation and the separation of the lineages leading to rats and humans (85 million years ago).

Reference: Ruffner DE, Sprung CN, Minghetti PP, Gibbs PEM & Dugaiczyk A (1987) Invasion of the human albumin-α-fetoprotein gene family by *Alu, Kpn*, and two novel repetitive DNA elements. *Mol. Biol. Evol.* 4, 1–9.

MEDICAL LINKS

4–84

A. The *RsaI-A* gene is missing in males that are green-blind, and the *RsaI-B* gene is missing in males that are red-blind (see Figure 4–30). Therefore, the *RsaI-A* gene encodes the green visual pigment, and the *RsaI-B* gene encodes the red visual pigment.

B. The presence of very similar (98% identical) genes in close proximity raises the very likely possibility that the variability among males with normal color vision arises by homologous recombination (unequal crossing-over) between the duplicated segments. This idea is supported by the uniform increments in sizes of the NotI fragments from individuals with increasing numbers of genes (see Figure 4–31).

If there is frequent unequal crossing-over between these genes, as is suggested by the variability among normal males, then color-blind males could arise by the same process. Unequal crossing-over could eliminate a gene by deletion or alter the function of a pigment by creating a hybrid gene (**Figure 4–47**). A powerful argument in favor of this possibility is that the color-blind males (see Figure 4–30, individuals 8, 9, and 10) have the same sort of variation in intensity of their RsaI-A fragment as normal

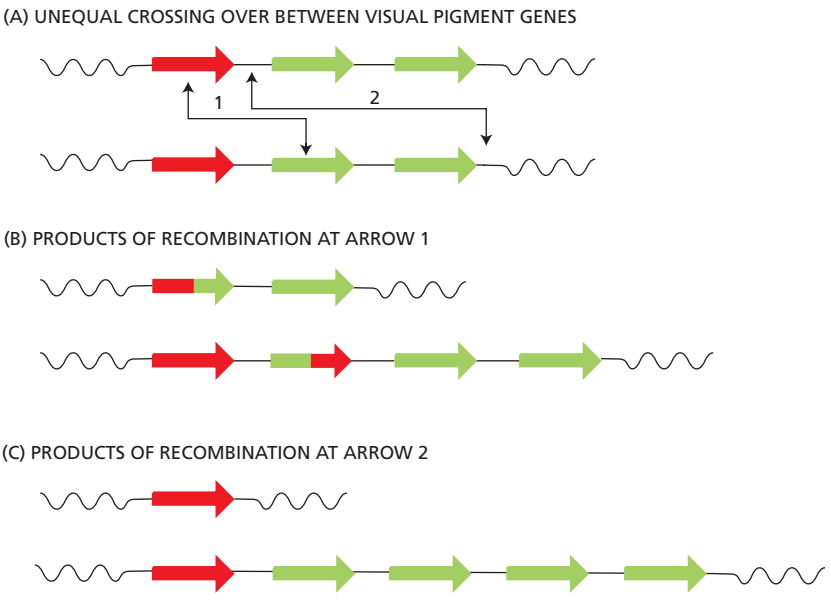


Figure 4–47 Unequal crossing-over between repeated segments (Answer 4–84). (A) Two potential sites of homologous crossover. Homologous sites are indicated by the *linked arrowheads.* (B) Recombination at sites labeled with *arrow 1*. Both products of recombination include a hybrid gene. One product is one repeat shorter and the other is one repeat longer than the parent molecules. (C) Recombination at sites labeled with *arrow 2.* The products do not include hybrid genes, but have an altered number of repeats.

males. The simplest way to account for this variation is by unequal crossing-over within a family of tandemly repeated genes.

C. The length of the duplicated segment is equal to the incremental change in NotI-fragment length with the addition of each gene. Each fragment in Figure 4–31 differs from the next higher fragment by 39 kb. For example, the NotI fragment from a male with two genes (105 kb for individual 1) differs from the NotI fragment from a male with three genes (144 kb for individual 3) by 39 kb. Thus, the duplicated segment at this locus on the X chromosome is 39 kb.

References: Lander ES, Linton LM, Birren B et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

Nathans J, Piantanida TP, Eddy RL, Shows TB & Hogness DS (1986) Molecular genetics of inherited variation in human color vision. *Science* 232, 203–210.

Nathans J, Thomas D & Hogness DS (1986) Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. *Science* 232, 193–202.

Vollrath D, Nathans J & Davis RW (1988) Tandem array of human visual pigment genes at Xq28. *Science* 240, 1669–1672.

4–85

A. Infants 2 and 8 have identical patterns and therefore must be brothers. Infants 3 and 6 also have identical patterns and must be brothers. These two sets of brothers are identical twins. The other two sets of twins must be fraternal twins because no other pairs of patterns are identical. Fraternal twins, like any pair of siblings born to the same parents, will have roughly half their genome in common. Thus, roughly half the simple-sequence polymorphisms in fraternal twins will be identical. Using this criterion, you can identify infants 1 and 7 as brothers and infants 4 and 5 as brothers.

B. You can match infants to their parents using the same sort of analysis of simple-sequence polymorphisms. Every band present in the analysis of an infant should have a matching band in one or the other of the parents, and, on average, each infant will share half of its simple-sequence polymorphisms with each parent. Thus, the degree of match between each child and each parent will be the same as that between fraternal twins.

Reference: Gelehrter TD & Collins FS (1990) Principles of Medical Genetics, p 80. Baltimore: Williams & Wilkins.

MCAT STYLE

4–86

B. Formation of heterochromatin structures is one way that gene expression is repressed and is the way sequences E and I operate. Choice A is incorrect because chromatin remodeling complexes, although they can inhibit transcription, bind to histones; they do not bind to the underlying DNA sequences. Choice C is not correct because gene repression is associated with *increased* nucleosome density, and histone chaperones only work in concert with chromatin remodeling complexes. Choice D is not correct because telomeric sequences are found only at the ends of chromosomes.

4–87

D. Each of these suggested modes of action would tend to decrease gene expression. Analysis of the SIR proteins has shown that SIR4 binds to the E and I sites. This binding allows SIR2 to deacetylate neighboring histones. SIR3 binds to the deacetylated histones, which propagates further

histone deacetylation. Deacetylation of histones allows heterochromatin to form at the HML locus, which represses gene expression. Thus, the SIR proteins comprise a reader–writer complex for chromatin modification.

4–88

D. Repressed genes are often located near the nuclear envelope, which is thought to contribute to repression of gene expression. The binding of barrier proteins near HML would block the spread of heterochromatin away from the HML gene, but would not be required for establishment of heterochromatin that represses gene expression. The formation of chromatin loops is associated with active gene expression rather than its repression.

4–89

C. Deletion of the tRNA gene allows the spread of repressive chromatin from the HML locus, indicating that the tRNA gene in some way serves as a barrier to the spread of heterochromatin. Since heterochromatin is characterized by a high density of nucleosomes, mechanisms that maintain a low nucleosome density would likely serve as a barrier. Choice A is incorrect because deletion of the site that localizes the *HML* gene to the nuclear envelope would be expected to promote gene expression rather than inhibit it. Choice B is not correct because histone chaperones act to facilitate the exchange of histones, not to create barriers. Choice D is incorrect because the highly transcribed tRNA gene is unlikely to be covered by heterochromatin, and in any case, the presence of heterochromatin would be more likely to promote the spread of repressive heterochromatin from the HML locus than to block it.

4–90

C. One of the reasons that cancer cells proliferate out of control is that they have undergone epigenetic changes that drive abnormal gene expression. The cytoplasm of the oocyte erases many of these epigenetic modifications, allowing the hybrid cells carrying the cancer cell nuclei to proliferate normally through the early stages of development. Erasing epigenetic marks is part of the normal sequence of events during development, so that a new set of marks can be established in a germ line and tissue-specific manner. These normal mechanisms permit the "reprogramming" of nuclei taken from differentiated cells, as well as cancer cells. Choices A and B are incorrect because modifications that block centromere function would block all cell division and highly condensed chromosomes would block all gene expression. Both mechanisms are incompatible with the normal cell proliferation that occurs in the nuclear transfer experiments. Choice D is incorrect because there is no information in the oocyte that could be used to repair the cancer cell DNA back to the wild-type sequence.

4–91

D. II and III are correct because formation of heterochromatin can lead to repression of gene expression, and heterochromatin is an epigenetic modification that can be initiated by modification of histone tails. Statement I is not correct because euchromatin is found at expressed genes.

DNA Replication, Repair, and Recombination

CHAPTER 5

IN THIS CHAPTER

THE MAINTENANCE OF DNA SEQUENCES

DEFINITIONS

5–1 Mutation

5–2 Germ cell

5–3 Somatic cell

TRUE/FALSE

5–4 True. If the DNA in somatic cells were not sufficiently stable (that is, if it accumulated mutations too rapidly), the organism would die (of cancer, for example), and if organisms died before they could reproduce, the species would be at risk. If the DNA in reproductive cells were not sufficiently stable, many mutations would accumulate and be passed on to future generations, increasing the risk that the species would die out.

THOUGHT PROBLEMS

5–5 For either hypothesis you might expect to see about 10 surviving colonies per plate. If the bacteriophages induced resistance, the surviving colonies would appear in random positions on each of the replica plates. If the mutations preexisted, the resistant colonies would appear at the same locations on each of the three replica plates. In actual experiments of this kind, the surviving colonies appear at the same locations, indicating that the mutations preexist in the population.

Reference: Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM & Veres RC (2000) Genetics: From Genes to Genomes, pp. 217–218. New York: McGraw Hill.

5–6 Each time the genome is copied in preparation for cell division, there is a chance that mistakes (mutations) will be introduced. The rate of mutation for humans is estimated to be 1 nucleotide change per 10^{10} nucleotides each time the DNA is replicated. Since there are 6.4×10^9 nucleotides in each diploid cell, an average of 0.64 random mutations will be introduced into the genome each time it is copied. Thus, the two daughter cells from a cell division will often differ from one another and from the parent cell that gave rise to them. Even genomes that are copied perfectly, giving rise to identical daughter cells, will often be altered in subsequent replication cycles. The proportion of identical cells depends on the exact mutation rate.

5–7 Natural selection alone is not sufficient to eliminate recessive lethal genes from the population. Consider the following line of reasoning. Homozygous defective individuals can arise only as the offspring of a

mating between two heterozygous individuals. By the rules of Mendelian genetics, offspring of such a mating will be in the ratio of 1 homozygous, normal: 2 heterozygous: 1 homozygous, defective. Thus, statistically, heterozygous individuals should always be more numerous than the homozygous defective individuals. And although natural selection effectively eliminates the defective genes in homozygous individuals through death, it can't touch them in heterozygous individuals, if the defective genes do not affect the phenotype. Natural selection will keep the frequency of the defective gene low in the population, and indeed act to reduce it, but in the absence of any other effect, there will always be a reservoir of defective genes in the heterozygous individuals.

At low frequencies of the defective gene, another important factor—chance—comes into play. Chance variation can increase or decrease the frequency of heterozygous individuals (and thereby the frequency of the defective gene). By chance, the offspring of a mating between heterozygotes could all be homozygous normal, which would eliminate the defective gene from that lineage. Increases in the frequency of a deleterious gene are opposed by natural selection; however, decreases are unopposed and can, by chance, lead to elimination of the defective gene from the population.

CALCULATIONS

5–8 In the initial population, there are 10^6 copies of your 1000-bp gene, or a total of 10^9 bp to be replicated when the population doubles. At a mutation rate of 1 mutation per 10^9 bp per generation, you might expect one copy of your 1000-bp gene to carry a mutation (one mutant cell) in the population of 2×10^6 cells, which is a frequency of 5×10^{-7} [1 mutant cell/(2×10^6 total cells)].

After the first doubling, there will be 2×10^6 copies of your 1000-bp gene, for a total of 2×10^9 bp to be replicated at the next population doubling. At the same rate of mutation, you would now expect two mutant copies of the gene to be generated, which is a frequency of new mutants of 5×10^{-7} [2 mutant cells/(4×10^6 total cells)]. The frequency of total mutations in the population is greater because the mutant cell that was generated in the first doubling will divide to produce two mutant cells in the second generation, for an overall frequency of mutants equal to 10^{-6} [$4/(4 \times 10^6)$].

After the second doubling, there will be 4×10^6 copies of your 1000-bp gene for a total of 4×10^9 bp to be replicated. You would expect four mutant copies of the gene to be generated, which is a frequency of new mutants of 5×10^{-7} [$4/(8 \times 10^6)$] in the third generation. The four mutant cells present after the second doubling would also double to generate eight mutant cells; thus, the overall frequency of mutants would be 1.5×10^{-6} [$12/(8 \times 10^6)$].

This exercise illustrates a key difference between *rates* of mutation and *frequencies* of mutation. Rates are constant under constant conditions, whereas frequencies increase with increasing growth of the cell population.

DATA HANDLING

5–9 The variation in frequency of mutants in different cultures exists because of variations in the time at which the mutations arose. For example, cultures with only one mutant bacterium must have acquired the mutation in the last generation; cultures with two mutants likely acquired a mutation in the next-to-last generation and produced two mutant daughter cells; cultures with four mutants likely acquired the mutation in the third-to-last generation and the mutant cell divided twice. Cultures with

large numbers of mutant cells acquired a mutation early in growth and those cells divided many times. To understand this variability, it is best to think of the mutation rate (1 mutation per 10^9 bp per generation) as a probability: a 10^{-9} chance of making a mutation each time a nucleotide pair is copied. Thus, sometimes a mutation will occur before 10^9 nucleotides have been copied and sometimes after.

Analysis of the variation in frequencies among cultures grown in this way (which is known as fluctuation analysis) is a common method for determining rates of mutation. Luria and Delbrück originally devised the method to show that mutations preexist in populations of bacteria; that is, they do not arise as a result of the selective methods used to reveal their presence.

Reference: Luria SE & Delbrück M (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28, 491–511.

DNA REPLICATION MECHANISMS

DEFINITIONS

5–10 RNA primer

5–11 DNA ligase

5–12 Strand-directed mismatch repair

5–13 DNA helicase

5–14 Sliding clamp

5–15 DNA topoisomerase

5–16 Replication fork

5–17 Lagging strand

TRUE/FALSE

5–18 False. The sequence of nucleotides in a newly synthesized strand is very different from that of the parental strand used as the template for its synthesis; the new strand is complementary to the 3′-to-5′ sequence of the parental template strand.

5–19 True. At each replication fork, the leading strand is synthesized continuously and the lagging strand is synthesized as Okazaki fragments. Since half the DNA at each replication fork is stitched together from Okazaki fragments, half the genome must be made this way.

5–20 True. If the replication fork moves forward at 500 nucleotide pairs per second, the DNA ahead of it must rotate at 48 revolutions per second (500 nucleotides per second/10.5 nucleotides per helical turn) or 2880 revolutions per minute. The havoc this would wreak on the chromosome is prevented by a DNA topoisomerase that introduces transient nicks just in front of the replication fork. The action confines the rotation to a short, single-stranded segment of DNA.

5–21 True. When topoisomerase I cleaves DNA, it stores the energy of the backbone phosphodiester bond in a phosphotyrosine bond to the enzyme, which it then uses to remake the phosphodiester bond in DNA.

THOUGHT PROBLEMS

5–22 The complementary strand is 5′-TGATTGTGGACAAAAATCC-3′. Recall that the two strands of a DNA double helix are antiparallel; that is, they

run in opposite directions. By convention, sequences of single strands are written in the 5′-to-3′ direction.

5–23 Because the two strands of the DNA double helix are antiparallel, the indicated phosphate is at the 5′ end of the fragment to which it is attached.

5–24

A. Dideoxycytidine triphosphate (ddCTP) is identical to dCTP except that it lacks the 3′-hydroxyl group on the sugar ring. ddCTP is recognized by DNA polymerase as dCTP and becomes incorporated into DNA. Because it lacks the crucial 3′-hydroxyl group, its addition to a growing DNA strand creates a dead end to which no further nucleotides can be added. Thus, when ddCTP is added in large excess, each new strand will be synthesized until the first G is encountered in its template strand. ddCTP will then be incorporated in place of C, and the extension of this strand will be terminated.

B. If ddCTP is added at 10% of the concentration of dCTP, there is a 1 in 10 chance of its being incorporated whenever a G is encountered in the template strand. Thus, a population of DNA fragments will be synthesized, and from their lengths the location of the G nucleotides in the template strand can be deduced. The use of such terminator nucleotides forms the basis of several methods for DNA sequencing.

C. Dideoxycytidine monophosphate (ddCMP) lacks the 5′-triphosphate group as well as the 3′-hydroxyl group of the sugar ring. The absence of the triphosphate means that ddCMP cannot provide the free energy that drives the polymerization of nucleotides into DNA. It is not a substrate for DNA polymerase and will not be incorporated into the replicating DNA. The molecule, at either concentration, is therefore not expected to affect DNA replication.

5–25 If the proofreading exonuclease activity of DNA polymerase were lost, you would expect the fidelity of DNA synthesis to be compromised. The proofreading exonuclease accounts for about a factor of 100 in the overall fidelity of DNA synthesis in *E. coli*, and its loss might be expected to lower overall fidelity by this amount.

Loss of proofreading activity would also be expected to affect the rate of DNA synthesis. When a nucleotide is misincorporated, a normal DNA polymerase can quickly remove it with its proofreading activity and then continue on. By contrast, a misincorporated nucleotide might affect a DNA polymerase lacking a proofreading exonuclease more dramatically, since DNA polymerase requires a base-paired primer. Thus, a proofreading-deficient DNA polymerase might be expected to pause or stall at each misincorporated nucleotide. Because the normal frequency of misincorporation is so low, about 1 in 10^5, it might be difficult to demonstrate such a rate change in practice.

5–26 While the process may seem wasteful, it provides an elegant solution to the difficulty of proofreading during primer formation. To start a new primer on a piece of single-strand DNA, one nucleotide must be put in place and then linked to a second and then to a third and so on. Even if these first nucleotides were perfectly matched to the template strand, such short oligonucleotides bind with very low affinity and it would consequently be difficult to distinguish the correct from incorrect bases by proofreading. The task of the primase is to "just get anything down that binds reasonably well and don't worry about accuracy." Later, these sequences are removed and replaced by DNA polymerase, which uses the accurately synthesized DNA of the adjacent Okazaki fragment as its primer. DNA polymerase has the advantage—which primase lacks—of putting the new nucleotides onto the end of an already existing strand. The newly added nucleotide is held firmly in place, and the accuracy of its

base-pairing to the next nucleotide on the template strand can be accurately assessed. Therefore, as DNA polymerase fills the gap, it can proofread from the start of the new DNA strand that it makes. What appears at first glance as energetically wasteful is really just a necessary price to be paid for accuracy.

5–27 Sequences in single-strand DNA that can form hairpin helices are self-complementary, which means that they can base-pair and that the resulting duplex will have strands running in opposite directions. An example of such a sequence is shown in **Figure 5–36**.

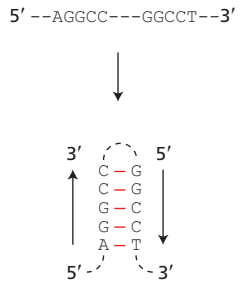


Figure 5–36 An example of a sequence of single-strand DNA that could form a hairpin helix (Answer 5–27).

5–28

A. In general, proteins that are required for movement of the replication fork will display a quick-stop phenotype because the fork will be unable to progress in the absence of their function. Thus, temperature-sensitive mutants of DNA topoisomerase I (inability to relieve winding tension ahead of the replication fork), SSB protein (inability to stabilize the single-strand DNA at the fork), DNA helicase (inability to melt the DNA ahead of the replication fork), and DNA primase will display the quick-stop phenotype. Of these, only the phenotype of DNA primase is difficult to predict. DNA primase directly affects synthesis of the lagging strand, but it is not required for synthesis of the leading strand. Its quick-stop phenotype may result from either of two indirect effects: (1) exposure of sufficient single-strand DNA to use up all the SSB protein or (2) interference with the DNA helicase that is linked to DNA primase as part of the complex of proteins required to carry out the priming reaction.

   Proteins that are not involved in the movement of the replication fork will display a slow-stop phenotype. Thus, a temperature-sensitive initiator protein would show the slow-stop pattern of replication, since DNA molecules that had passed the initiation step before the temperature was increased would continue to replicate the chromosome until an initiation step was required in the next cycle. Similarly, a temperature-sensitive DNA ligase would show a slow-stop phenotype, since the progress of the replication fork would not be stopped. Replication would cease only during the next cycle when the nicks were "uncovered" on the template strand.

B. The mixed extracts should be fully competent for DNA replication at 42°C; that is, the mixture should exhibit a nonmutant phenotype. The defective DNA helicase extract would provide normal DNA ligase, and the defective DNA ligase extract would provide normal DNA helicase. Thus, the entire complement of normal proteins would be present in the mixed extract. This mutual correction by extracts with different deficiencies is called complementation. (Because of the extreme complexity of DNA replication and the large number of proteins involved, cell-free extracts are not capable of maintaining DNA replication indefinitely. In practice, the behaviors of extracts from slow-stop mutants and from nonmutant cells are often difficult to distinguish.)

5–29 Mismatch repair normally corrects a mistake in the new strand, using information in the old, parental strand. If the old strand were "repaired" using the new strand that contains a replication error as the template, then the error would become a permanent in the genome, with the "correct" information being erased in the process. Therefore, if repair enzymes did not distinguish between the two strands, there would be only a 50% chance that any given replication error would be corrected.

   Overall, such indiscriminate repair would introduce the same number of mutations as would be introduced if mismatch repair did not exist. In the absence of repair, a mismatch would persist until the next replication. When the replication fork passed the mismatch, and the strands were separated, properly paired nucleotides would be inserted opposite

each of the nucleotides involved in the mismatch. A normal, nonmutant duplex would be made from the strand containing the original information; a mutant duplex would be made from the strand that carried the misincorporated nucleotide. Thus, the original misincorporation event would lead to 50% mutants and 50% nonmutants in the progeny. This outcome is equivalent to that of indiscriminate repair: averaged over all misincorporation events, indiscriminate repair would also yield 50% mutants and 50% nonmutants among the progeny.

5–30 Clearly, DNA polymerases must be able to extend a mismatched primer occasionally; otherwise no mismatches would be present in the newly synthesized DNA. Most mismatches are removed by the 3′-to-5′ proofreading exonuclease associated with the DNA polymerase. When the exonuclease does not remove the mismatch, the polymerase can extend the growing chain. In reality, DNA polymerase and the proofreading exonuclease are in competition with each other. In the case of bacteriophage T7 DNA polymerase, numbers are available that illustrate this competition. Normally, T7 DNA polymerase synthesizes DNA at 300 nucleotides per second, while the exonuclease removes terminal nucleotides at 0.2 nucleotides per second, suggesting that 1 in 1500 (0.2/300) correctly added nucleotides are removed by the exonuclease. When an incorrect nucleotide has been incorporated, the rate of removal increases 10-fold to 2.3 nucleotides per second and the rate of polymerization decreases 3×10^4-fold to 0.01 nucleotide per second. Comparison of these rates for a mismatched primer suggests that about 1 in 200 (0.01/2.3) mismatched primers will be extended by T7 DNA polymerase.

Reference: Johnson KA (1993) Conformational coupling in DNA polymerase fidelity. *Annu. Rev. Biochem.* 62, 685–713.

5–31 When DNA polymerase encounters a nick in either template for the leading or lagging strands, the replication fork collapses, generating a double-strand break, as shown in **Figure 5–37A**. When DNA polymerase encounters a thymine dimer on the template for the leading or lagging strand, the DNA polymerase stops. The polymerase on the opposite, nonblocked strand, however, continues for a while before it stops (**Figure 5–37B**).

5–32 The enzyme topoisomerase II is responsible for unlinking SV40 daughter duplexes. Topoisomerase II introduces a transient double-strand break into one circle, and then guides the second duplex through the first before it reseals the break.

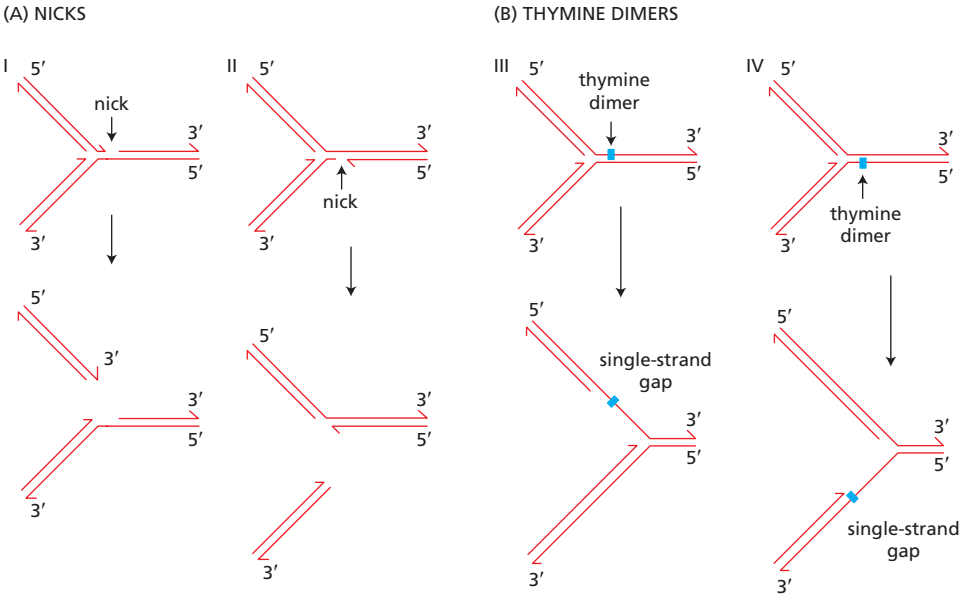


Figure 5–37 Consequences of replication across a damaged site on the templates for the leading and lagging strands (Answer 5–31). (A) Nicks. (B) Thymine dimers.

CALCULATIONS

5–33

A. A 1:12 weight ratio of nucleotides to SSB protein corresponds to an 8.8:1 ratio of nucleotides to SSB molecules.

$$\frac{\text{nucleotides}}{\text{SSB molecule}} = \frac{1}{12} \times \frac{35{,}000\ \text{d}}{\text{SSB molecule}} \times \frac{1\ \text{nucleotide}}{330\ \text{d}}$$

$$= 8.8\ \text{nucleotides/SSB molecule}$$

B. Since there are 10.4 nucleotides per 3.4 nm of single-strand DNA, the 8.8 nucleotides would stretch about 3 nm. (If the single-strand DNA were fully extended, it would stretch about twice as far.) The 12-nm length of an SSB molecule suggests that at saturation SSB proteins are in contact with one another and probably overlap considerably.

C. The absence of significant binding at a low SSB concentration, but nearly quantitative binding at 14-fold higher concentration, suggests that SSB protein binds cooperatively to DNA. In essence, cooperative binding means that the binding of one monomer makes it easier for additional monomers to bind. If the monomers overlap with one another when bound, as suggested by the calculation in part B, cooperativity likely arises because each monomer has two binding sites—one for DNA and one for other monomers. Binding of the first monomer to DNA will be weak because it can bind only through its DNA-binding site. By binding adjacent to bound monomers, subsequent monomers can make use of both their binding sites. Mathematically, this type of interaction leads to a steep dependence of binding on concentration.

Reference: Alberts BM & Frey L (1970) T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA. *Nature* 227, 1313–1318.

5–34 Since both strands of the *E. coli* genome must be copied, a total of 9.2×10^6 nucleotides must be polymerized. Polymerization of nucleoside triphosphates into DNA consumes two high-energy, phosphoanhydride bonds for each nucleotide added: the nucleoside triphosphate is hydrolyzed to add the nucleoside monophosphate to the growing strand, and the released pyrophosphate is hydrolyzed to phosphate. Therefore, 1.8×10^7 high-energy phosphate bonds are hydrolyzed during each round of replication.

Since each glucose can provide 30 high-energy phosphate bonds, 6×10^5 ($1.8 \times 10^7/30$) molecules are required to provide sufficient energy for one round of replication.

At 180 d/molecule, 6×10^5 glucoses would have a mass of 1.8×10^{-16} g [$(6 \times 10^5$ molecules) × (180 d/molecule) × ($1\ \text{g}/6 \times 10^{23}$ d)]. This amount of glucose is roughly 0.02% (1.8×10^{-16} g glucose/1×10^{-12} g *E. coli*) of the mass of one *E. coli* cell.

DATA HANDLING

5–35 DNA isolated from your starting cells has a heavy density, as you would expect. After one generation in normal, light medium, the DNA has uniformly shifted to medium density: synthesis of the new DNA from light nucleotides results in hybrid DNA molecules that contain one heavy maternal strand and one light newly synthesized strand (**Figure 5–38**). After another round of replication in light medium, two forms of DNA appear in about equal proportions: one form is again a hybrid of a light and a heavy strand and has medium density, while the other form is composed of two light strands and has a low density (Figure 5–38). During the subsequent rounds of replication, more light DNA is formed, and the

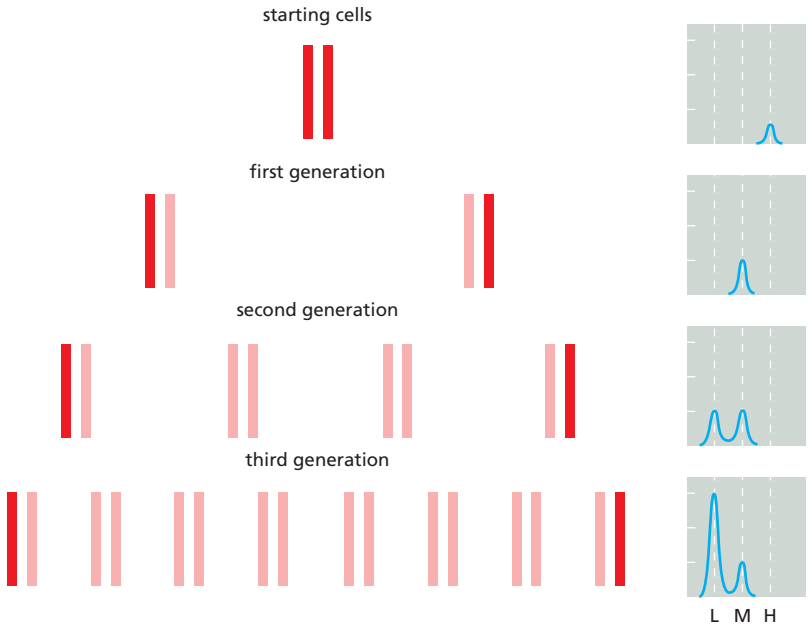


Figure 5–38 Change in density of DNA from cells grown initially in heavy medium and then for various numbers of generations in light medium (Answer 5–35). L, M, and H refer to light, medium, and heavy density, respectively.

proportion of medium-density DNA diminishes. Your results are therefore in complete agreement with the hypothesis that you set out to test.

5–36

A. ATP hydrolysis is required for unwinding because energy is needed to melt DNA. Strand separation is energetically unfavorable because stacking interactions between the planar base pairs are largely lost upon strand separation. In addition, the hydrogen bonds that link the bases present a kinetic barrier to strand separation.

B. Since dnaB melts off only the 3′ fragment of substrate 3 (lanes 9 and 10, Figure 5–7), it must bind to the long single strand and move along it in the 5′-to-3′ direction. When it reaches the double-strand region formed by the 3′ fragment, it unwinds the fragment. The 5′-to-3′ movement of dnaB suggests that it unwinds the parental duplex at the replication fork by moving along the template for the lagging strand.

   If dnaB moves in the 5′-to-3′ direction, why does it not melt the 5′ fragment off substrate 3 by binding to the short 5′ tail? Pat yourself on the back if you wondered about this. In real experiments, a small amount of the 5′ fragment is melted off. This is a rarer event because of the difference in target size: dnaB is much more likely to bind to the long single strand.

C. If SSB is added first, it inhibits dnaB-mediated unwinding because it coats the single-strand DNA, preventing dnaB from binding. By contrast, if SSB is added after dnaB has bound, it stimulates unwinding by preventing the unwound DNA from reannealing.

Reference: LeBowitz JH & McMacken R (1986) The *Escherichia coli* dnaB replication protein is a DNA helicase. *J. Biol. Chem.* 261, 4738–4748.

5–37

A. There is a similar increase in reversions of the *LacZ* allele in the R orientation in mismatch-repair-deficient (1.9-fold) and proofreading-deficient (2.5-fold) strains of *E. coli*. For the *Rif* gene, however, there was no difference in mismatch-repair-deficient (1.1-fold) and proofreading-deficient (1.0-fold) strains, as expected. Since reversion results from misincorporation of G opposite T, which occurs on the leading strand in orientation R (see Figure 5–8B), leading-strand DNA synthesis appears to be less accurate than lagging-strand synthesis.

B. The reason for the apparent difference in fidelity of DNA synthesis on the leading and lagging strands is not clear. (Four different alleles of *LacZ* were tested in this study; all showed the same 2- to 5-fold lower fidelity of synthesis on the leading strand.) The difference is unlikely to be due to the intrinsic properties of the DNA polymerase since the same polymerase is used to make both strands. If you thought about transcription of the *LacZ* gene, good for you. But transcription occurs at a very low level under the conditions used here and the direction of transcription did not correlate with mutation frequency for the four alleles that were studied.

The authors suggested the following explanation. Because the polymerase on the lagging strand must dissociate and rebind each time it comes to the end of an Okazaki fragment, it might dissociate with greater ease than the polymerase on the leading strand. If the polymerase on the lagging strand dissociated from mismatches more readily, as well, the mismatched primer would be exposed more often on the lagging strand than on the leading strand. They argue that such an exposed mismatch might be subject to repair by other 3′-to-5′ exonucleases in the cell. In effect this would mean that the lagging strand has two ways to repair a mismatched primer, while the leading strand has only one. Such an explanation might account for the difference in fidelity, but it remains to be proven.

Reference: Fijalkowska IJ, Jonczyk P, Tkaczyk MM, Bialoskorska M & Schaaper RM (1998) Unequal fidelity of leading strand and lagging strand DNA replication on the *Escherichia coli* chromosome. *Proc. Natl Acad. Sci. USA* 95, 10020–10025.

THE INITIATION AND COMPLETION OF DNA REPLICATION IN CHROMOSOMES

DEFINITIONS

5–38 S phase

5–39 Origin recognition complex (ORC)

5–40 Replication origin

5–41 Telomerase

TRUE/FALSE

5–42 True. See **Figure 5–39**.

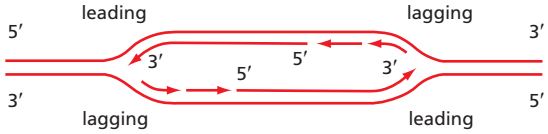


Figure 5–39 A labeled diagram of a replication bubble (Answer 5–42).

5–43 True. Consider a single template strand, with its 5′ end on the left and its 3′ end on the right. No matter where the origin is, synthesis to the left on this strand will be continuous (leading strand), and synthesis to the right will be discontinuous (lagging strand). Thus, when replication forks from adjacent origins collide, a rightward-moving (lagging) strand will always meet a leftward-moving (leading) strand.

5–44 False. If one origin is deleted, the adjacent DNA, which would normally be replicated from that origin, will be replicated instead from a neighboring origin. Thus, replication of the DNA adjacent to the deleted origin may occur somewhat later than normal, but it will occur.

THOUGHT PROBLEMS

5–45 As always, you come through with flying colors. Although you were initially bewildered by the variety of structures, you quickly realized that H forms were just like the bubbles except that cleavage occurred within the bubble instead of outside it. Next you realized that by reordering the molecules according to the increasing size of the bubble (and flipping some structures end-for-end), you could present a convincing visual case for bidirectional replication away from a unique origin of replication (**Figure 5–40**). The case for bidirectional replication is clear since unidirectional replication would give a set of bubbles with one end in common. Replication from a unique origin is likely, but not certain, because you cannot rule out the possibility that there are two origins on either side of and equidistant from the restriction site used to linearize the DNA. Repeating the experiment using a different restriction nuclease will resolve this issue and define the exact position of the origin(s) on the viral DNA. Your advisor is pleased.

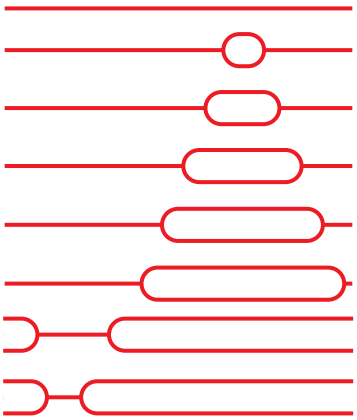


Figure 5–40 Bidirectional replication from a unique origin (Answer 5–45).

5–46

F. Each newly synthesized strand in a daughter duplex was synthesized by a mixture of continuous and discontinuous DNA synthesis from multiple origins. Consider a single replication origin. The fork moving in one direction synthesizes a daughter strand continuously as part of leading-strand synthesis. The fork moving in the opposite direction synthesizes a portion of the same daughter strand discontinuously as part of lagging-strand synthesis.

5–47

E. The two daughter chromosomes will be shorter at opposite ends. This outcome is illustrated in **Figure 5–41**, which shows replication from a single origin to the ends of the chromosome. (Multiple origins make no difference to the outcome.) The leading strand can continue all the way to the end of the chromosome, but the lagging strand cannot. The very last RNA primer cannot be replaced by DNA because there is no upstream primer for DNA polymerase to extend.

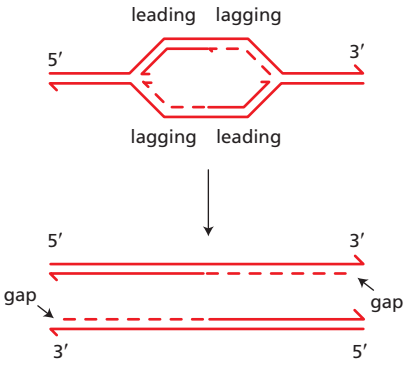


Figure 5–41 Consequences of the end-replication problem for daughter duplexes (Answer 5–47).

CALCULATIONS

5–48

A. The approximate positions of the origins of replication and their associated replication forks are labeled in **Figure 5–42** on a schematic diagram of the electron micrograph.

B. The distance between replication forks 4 and 5 is about 0.3 μm (300 nm), which corresponds to about 880 nucleotides [(300 nm)/(0.34 nm/nucleotide)]. If replication forks 4 and 5 were each traveling at 50 nucleotides/second, they would collide in about 9 seconds [880/(50 × 2)].

Replication forks 7 and 8 are moving in opposite directions and would therefore never collide.

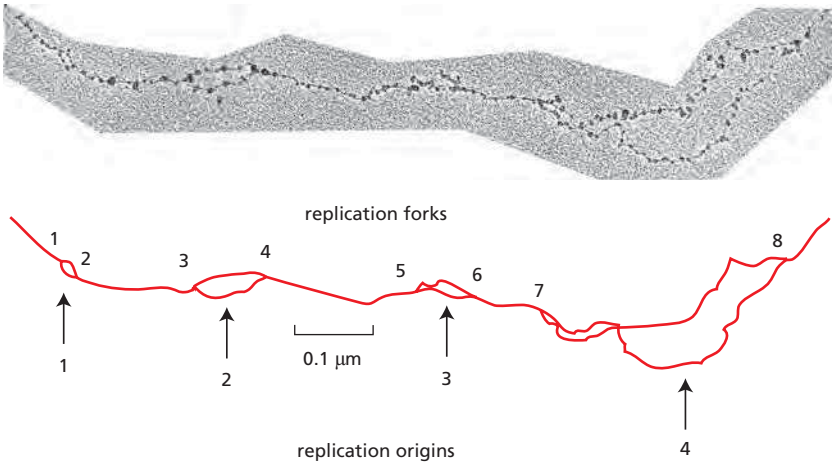


Figure 5–42 Replication bubbles in a *Drosophila* chromosome (Answer 5–48). The schematic diagram of the electron micrograph shows the origins of replication and the replication forks.

5–49 If there were no time constraints on replication, one origin would be required for each chromosome; thus, a minimum of 46 origins, equal to the number of chromosomes in a human cell, would be needed.

In 8 hours (28,800 seconds), the two replication forks from one origin would synthesize 2.88×10^6 nucleotides [(2 forks) × (50 nucleotides/second) × (2.88×10^4 seconds)]. To replicate the entire genome would require about 2200 equally spaced origins [(6.4×10^9 nucleotides)/(2.88×10^6 nucleotides/origin) = 2222 origins]. It is estimated that the human genome has in excess of 50,000 origins of replication, more than enough to finish replication within the time allotted in the cell cycle.

DATA HANDLING

5–50

A. The regions of the tracks that are dense with silver grains correspond to those segments of DNA that were replicated when the concentration of ^{3}H-thymidine was high. The less-dense regions mark segments of DNA that were replicated when the concentration of ^{3}H-thymidine was low.

B. The difference in the arrangements of the dark and light sections of the tracks derives from the difference in the labeling schemes in the two experiments. In the first experiment (see Figure 5–11A), ^{3}H-thymidine was added immediately after release of the synchronizing block. Thus, replication was initiated at origins in the presence of ^{3}H-thymidine, giving a continuous dark section on both sides of the origin. When the concentration of label was lowered, replication proceeded in both directions away from the origin, leaving light sections at both ends of the dark sections. In the second experiment (see Figure 5–11B), replication began at origins in the absence of ^{3}H-thymidine so that the origin was unlabeled. Addition of a high concentration of label followed by a low concentration gave rise to a dark section with a light section at one end. Adjacent dark sections are part of the same replicating DNA molecule; they are linked by the unlabeled (therefore invisible) segment that contains the replication origin.

C. The approximate rate of fork movement can be estimated from the labeling times and the lengths of the labeled sections. In the first experiment, segments roughly 100 µm in length were labeled during the 45-minute labeling period. Because two replication forks were involved in synthesizing each labeled segment, each replication fork synthesized about 50 µm of DNA in 45 minutes. Therefore, the rate of fork movement is about 1.1 µm/min (50 µm/45 min). In the second experiment, segments roughly 50 µm in length were labeled; however, each was synthesized by only one replication fork. Thus, the rate of fork movement was also about 1.1 µm/min.

This information is not sufficient to estimate the time required to replicate the entire genome. The missing information is the number of active origins of replication and their distribution. Assuming that all origins are activated at the same time and all forks move at the same rate, the minimum time required to replicate the genome (regardless of its size) is fixed by the distance between the two origins that are farthest apart.

Reference: Huberman JA & Riggs AD (1968) On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol.* 32, 327–341.

5–51

A. Hybridization at the 4.5-kb position is due to plasmid molecules that were not replicating at the time DNA was isolated. The intensity of this spot indicates that the majority of plasmid molecules were not replicating. The low frequency of replicating molecules, even during S phase, was one of the contributing factors in the difficulty of proving that an ARS was an origin of replication.

B. The results in Figure 5–13B indicate that *Ars1* behaves as an origin of replication. The gel pattern with BglII-digested DNA looks like the pattern due to replicating molecules with two branches (see Figure 5–12C). The gel pattern with PvuI-digested DNA looks much like the pattern for replication intermediates with symmetrically located replication bubbles (see Figure 5–12B). The very short tail on the spot at 9 kb in Figure 5–13B indicates that the replication bubbles are slightly asymmetrically situated. These gel patterns are exactly what would be expected if replication began at *Ars1*. As shown in **Figure 5–43**, cleavage with BglII, which cuts at *Ars1,* would generate molecules with two branches. Cleavage with PvuI, which cuts almost half way around the circle from *Ars1,* generates molecules with nearly symmetrical replication bubbles.

C. The discontinuity in the arc of hybridization of PvuI-cut plasmids (see Figure 5–13B) results from the difference in migration of bubble forms and branched forms. Molecules that have just begun replicating will be converted to bubble forms by PvuI cleavage, whereas molecules replicated past the PvuI site will be converted to branched forms. Thus, a replicating molecule that is cleaved either has a bubble or it is branched—there is no intermediate. Since the two forms migrate differently, there is a gap in the electrophoretic pattern.

Reference: Brewer BJ & Fangman WL (1987) The localization of replication origins on ARS plasmids in *S. cerevisiae. Cell* 51, 463–471.

5–52

A. The DNA from an amplified cluster is an "onion-skin" structure as illustrated in **Figure 5–44**. When examined by electron microscopy, DNA from late-stage follicle cells shows multiple, nested replication forks, just as expected for this mechanism of amplification.

B. If every origin were activated, each round of replication would double the number of chorion genes. Therefore, it would take six rounds of replication to achieve a 60-fold amplification ($2^6 = 64$).

C. Given the overreplication of the chorion gene cluster, the 510-nucleotide amplification-control element is probably an origin of replication. It cannot, however, be a standard origin; it must also contain a sequence that allows it to escape the block to re-replication in follicle cells at specific stages. It is known that the origin recognition complex (ORC) binds throughout the nucleus until a specific stage of development at which it is cleared from all origins except those that are to be amplified. The clearing of ORC from most origins and its continued binding at amplification sites are dependent on the activities of other proteins, but the details of these processes are not yet defined.

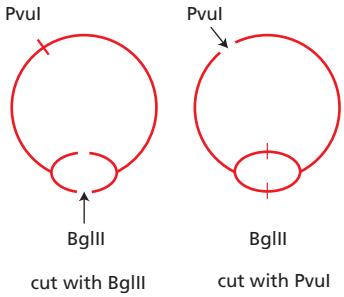


Figure 5–43 Conversion of replicating plasmid molecules into linear forms with two branches by BglII, or with replication bubbles by PvuI (Answer 5–51).

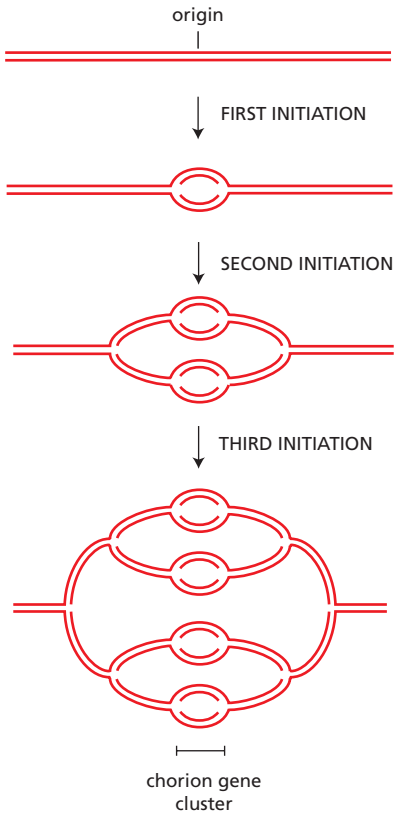


Figure 5–44 Onion-skin structure of an amplified cluster of chorion genes (Answer 5–52). Three initiation events are illustrated; six would be required to amplify the chorion gene cluster 60-fold.

References: Orr-Weaver TL & Spradling AD (1986) *Drosophila* chorion gene amplification requires an upstream region regulating s18 transcription. *Mol. Cell. Biol.* 6, 4624–4633.

Royzman I, Austin RJ, Bosco G, Bell SP & Orr-Weaver TL (1999) ORC localization in *Drosophila* follicle cells and the effects of mutations in *dE2F* and *dDP*. *Genes Dev.* 13, 827–840.

5–53

A. ORC binding protects two neighboring locations on the origin DNA, as indicated by blank regions where bands that were visible in the absence of ORC are missing in its presence (**Figure 5–45**, regions marked with P). Note that a couple of bands (marked by an *) are more intense when ORC is bound, indicating that they are *more* accessible to DNase I in the complex than in native DNA.

B. ATP is required for binding by both the wild-type and the mutant ORCs. For the wild-type ORC, about 100 nM ATP gives full binding (Figure 5–45, lane 3); for the mutant ORC, a whopping 10 mM (10^5 more than for wild-type ORC) is required (lane 14).

C. Because exactly the same results were obtained with ATP and the non-hydrolyzable analog ATPγS, ATP hydrolysis cannot be required for ORC binding. The authors of this study suggest that ATP hydrolysis is important for a subsequent step in the complex process that enables an origin for replication.

D. The Walker motif in Orc1 is important to the function of ORC, as shown by the dramatically different binding results when the motif is mutated. The binding of mutant ORC at very high ATP concentrations suggests that the mutation lowers the affinity of Orc1 for ATP, but probably does not compromise other functions of the protein.

Reference: Klemm RD, Austin RJ & Bell SP (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* 88, 493–502.

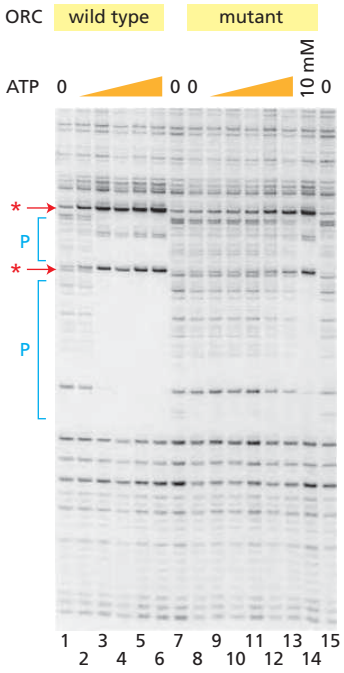


Figure 5–45 ORC binding sites on origin DNA as revealed by DNase footprinting (Answer 5–53). "P" indicates protected areas; * indicates enhanced cleavage.

5–54

A. Most of the SV40 DNA is not replicated, as is clear from comparison of the CAF1-treated samples (Figure 5–16). CAF1 treatment moves most of the replicated (labeled) DNA to the supercoiled position, but does not significantly alter the distribution of bulk DNA, as indicated on the stained gel. The small increase in stained DNA at the supercoiled position in the CAF1-treated sample shows how little of the total DNA has been replicated.

B. CAF1 assembles nucleosomes only on replicated DNA, as indicated by the dramatic change in the migration of replicated (labeled) DNA, in the absence of any significant effect on the bulk (stained) DNA (Figure 5–16).

C. Because CAF1 specifically targets replicated DNA for assembly into nucleosomes, the replicated DNA must bear some "mark" of replication. Further experiments by the authors of this study identified the mark as the sliding clamp (PCNA), which tethers DNA polymerase to the duplex. The interaction between the clamp and CAF1 is clearly useful, allowing nucleosome assembly to occur immediately in the wake of the DNA polymerase.

Reference: Shibahara K & Stillman B (1999) Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* 96, 575–585.

5–55

A. The region of intense hybridization to telomeres in the unaffected spores (1 and 3) extends from less than 200 nucleotides to just over 300 nucleotides, averaging about 250 nucleotides. Since the cleavage site is

35 nucleotides from the beginning of the telomere repeats, the average length of the telomere repeat in fission yeast is just over 200 nucleotides.

B. The descendants of spores 2 and 4 show telomere shortening with time, whereas the descendants of spores 1 and 3 remain the same size. Thus, spores 2 and 4 appear to lack telomerase, and it looks as though your identification of the fission-yeast telomerase gene was correct. It is worth noting that a number of genes in yeast cause a similar telomere-shortening phenotype, but only one of them encodes the catalytic subunit of telomerase. Such genes are known as *Est* genes, for ever shorter telomeres.

C. Although it is somewhat difficult to estimate precisely, it looks as though telomeres lose about 60 nucleotides every 3 days. At four generations per day [(24 hours/day)/(6 hours/generation)], the yeast go through about 12 generations in 3 days. Thus, they lose about five nucleotides per generation (60 nucleotides/12 generations).

D. This problem doesn't give you a firm basis for a prediction but, in fact, the majority of fission yeast that lose their telomeres stop dividing but continue to grow in size, forming abnormally large cells.

Reference: Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB & Cech TR (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science* 277, 955–959.

DNA REPAIR

DEFINITIONS

5–56 Nonhomologous end joining

5–57 DNA repair

TRUE/FALSE

5–58 False. Repair of damage to a single strand by base excision repair or nucleotide excision repair, for example, depends on just the two copies of genetic information contained in the two strands of the DNA double helix. By contrast, precise repair of damage to both strands of a duplex—a double-strand break, for example—requires information from a second duplex, either a sister chromatid or a homolog.

5–59 True. Both spontaneous depurination and removal of deaminated C by uracil DNA glycosylase leave a sugar that is missing its base, which is the substrate recognized by AP endonuclease.

5–60 True. The initial steps, including recognition of damage and DNA incision, are specific for repair, whereas the later steps tend to be catalyzed by enzymes such as helicases, DNA polymerases, and ligases whose activities are common features of DNA metabolism.

THOUGHT PROBLEMS

5–61 The statement is incorrect. DNA defects introduced by deamination and depurination reactions occur spontaneously. They do not arise from replication errors and are therefore equally likely to occur on either strand. If DNA repair enzymes recognized such defects only on newly synthesized DNA strands, half of the defects would go uncorrected. Also there is no fundamental reason to link such repair events to replication. The bases produced by deamination and depurination are distinct from the normal bases and can be recognized in any sequence context. By contrast, misincorporation during replication adds *normal* bases that are mispaired.

The only way to identify them correctly is to search the newly synthesized strand.

5–62 At many sites in vertebrate cells, the sequence 5′-CG-3′ is selectively methylated on the cytosine base. Spontaneous deamination of methyl-C produces T. A special DNA glycosylase recognizes a mismatched base pair involving T in the sequence TG, and removes the T. This DNA repair mechanism is clearly not 100% effective, as methylated C nucleotides are common sites for mutation in vertebrate DNA. Over time, the enhanced mutation rate of CG dinucleotides has led to their preferential loss, accounting for their underrepresentation in the human genome.

CALCULATIONS

5–63 The average distance between the centers of the Ku dimers is 65 nm, which is equal to the width of about eight Ku dimers. (The average distance from the *edge* of one dimer to the next is equal to the width of about seven Ku dimers.) This means that usually there will be a Ku dimer within half that distance, within four Ku diameters, of any potential double-strand break. Having Ku dimers in such close proximity suggests that double-strand breaks will be rapidly recognized.

The volume of the nucleus is 1.13×10^{11} nm^3 [$4\pi/3 \times (3000\text{ nm})^3$]. The nuclear volume per Ku dimer is 2.8×10^5 nm^3 [$(1.13 \times 10^{11}\text{ nm}^3)/(4 \times 10^5)$], which is equal to a cube 65 nm on a side [$(2.8 \times 10^5\text{ nm}^3)^{0.33}$]. If you imagine a Ku dimer in the middle of each cube, the average separation of their centers will be 65 nm, or the equivalent of eight times the width of a Ku dimer.

Reference: Lieber MR, Ma Y, Pannicke U & Schwarz K (2003) Mechanism and regulation of human non-homologous DNA end-joining. *Nat. Rev. Mol. Cell Biol.* 4, 712–720.

5–64 If the inaccurately repaired breaks were randomly distributed around the genome, then 2% of them would be expected to alter crucial coding or regulatory information. Thus, the functions of about 40 genes (0.02×2000) would be compromised in each cell, although the specific genes would vary from cell to cell. Because not all genes are expressed in every cell, gene mutations in some cells would be without consequence. In addition, because the human genome is diploid, the effect of mutations in expressed genes would be mitigated by the remaining allele. For most loci, one functional allele (50% of normal protein) is adequate for normal cell function; however, for some loci, 50% is not adequate. Thus, the mutations would be expected to compromise the functions of some cells.

Reference: Lieber MR, Ma Y, Pannicke U & Schwarz K (2003) Mechanism and regulation of human non-homologous DNA end-joining. *Nat. Rev. Mol. Cell Biol.* 4, 712–720.

DATA HANDLING

5–65

A. The extreme UV sensitivity of *UvrARecA* double mutants, relative to cells with mutations in two *Uvr* genes, suggests that there are two separate pathways for dealing with UV damage. The *Uvr* gene products are involved in one pathway, whereas RecA is involved in a different pathway. It turns out that the *Uvr* genes are involved in nucleotide excision repair, whereas *RecA* is critical for homologous recombination. These two pathways are required to deal with the variety of damage introduced by UV light. As a rule of thumb, if a combination of mutant genes produces a phenotype that is no more defective than those of the individual mutant genes, the gene products are likely to act in the same pathway.

B. A lethal hit in the *UvrARecA* strain corresponds to about one pyrimidine dimer. The number of pyrimidine dimers per lethal hit can be calculated as follows. Since *E. coli* is 50% GC, all four bases are equally represented in the genome. If they were arranged randomly (which they are not, but this assumption is a reasonable approximation), then of the 16 possible dinucleotide pairs in DNA, one-quarter would be pyrimidine pairs. Therefore, the *E. coli* genome (4.6×10^6 base pairs) contains 1.2×10^6 possible UV targets. Given that a dose of 400 J/m^2 converts 1% of the pyrimidine (pyr) pairs into pyrimidine dimers, the number of pyrimidine dimers per lethal hit in *E. coli* is

$$\frac{\text{pyr dimers}}{\text{lethal hit}} = \frac{1.2 \times 10^6 \text{ pyr pairs}}{\textit{E. coli}} \times \frac{0.04 \text{ J/m}^2}{\text{lethal hit}} \times \frac{1 \text{ pyr dimer}}{100 \text{ pyr pairs}} \times \frac{1}{400 \text{ J/m}^2}$$

$$\frac{\text{pyr dimers}}{\text{lethal hit}} = 1.2$$

5–66

A. As shown in Figure 5–19, untreated bacteria and bacteria adapted to MNNG by a brief exposure to low levels differ only in the presence or absence of O^6-methylguanine. The absence of O^6-methylguanine in adapted bacteria correlates with the low level of mutation, suggesting that it is the mutagenic lesion. O^6-methylguanine is thought to be mutagenic because it can mispair with T during replication.

B. The kinetics of removal of the methyl group from O^6-methylguanine are peculiar because the amount removed does not increase with time, as one might expect for a typical enzyme. In addition, the amount that is demethylated is directly proportional to the amount of purified protein added to the reaction. One possible explanation for such behavior is that the enzyme is very unstable; however, the identical end points at 5°C and 37°C argue against this explanation, since enzyme stability usually varies with temperature.

C. A calculation of the number of mutagenic bases that are demethylated per enzyme molecule indicates that each enzyme removes only one methyl group. This calculation shows that the protein is used stoichiometrically instead of catalytically, which explains the peculiar kinetics.

For example, 2.5 ng of protein removes half of the initial number of O^6-methylguanines, or 0.13 pmol (0.5 × 0.26 pmol) from the DNA. Thus, the number of enzyme molecules is

$$\text{enzymes} = 2.5 \text{ ng} \times \frac{\text{nmol}}{19{,}000 \text{ ng}} \times \frac{6.0 \times 10^{14} \text{ molecules}}{\text{nmol}}$$

$$= 7.9 \times 10^{10} \text{ molecules}$$

and the number of methyl groups is

$$\text{methyl groups} = 0.13 \text{ pmol} \times \frac{6.0 \times 10^{11} \text{ methyl groups}}{\text{pmol}}$$

$$= 7.8 \times 10^{10} \text{ methyl groups}$$

It turns out that methyl groups are transferred to one particular cysteine in the protein. Once methylated, the protein is dead and ultimately is degraded. Because the protein inactivates itself during the reaction, it is not an enzyme in the usual sense. (An enzyme is a catalyst, which by definition is not consumed during the reaction.)

Reference: Lindahl T, Demple B & Robins P (1982) Suicide inactivation of the *E. coli* O^6-methylguanine-DNA methyltransferase. *EMBO J.* 1, 1359–1363.

5–67 The calculated *P* value for chi-square analysis of these two distributions is less than 0.001. Thus, the observed distribution is significantly different from the distribution expected by chance. There is less than a 1/1000 possibility that the observed distribution is the same as the distribution expected by chance. This analysis says that microhomologies are relevant to the mechanism of NHEJ, but it doesn't specify how. It is thought that the microhomologies—in conjunction with NHEJ proteins—may help to align the two duplexes so that the other manipulations required to link the duplexes together can occur.

Reference: Roth DB, Porter TN & Wilson JH (1985) Mechanisms of nonhomologous recombination in mammalian cells. *Mol. Cell. Biol.* 5, 2599–2607.

MEDICAL LINKS

5–68

A. The patterns of radioactivity in Figure 5–22B, lanes 6 and 10, show that the XP-V enzyme can elongate the labeled primer; thus, it is a DNA polymerase. This enzyme is known as DNA polymerase η. If the XP-V enzyme simply helped a normal polymerase to overcome a chemical block, for example, it would not be able to elongate the primer by itself.

B. Both the XP-V enzyme (DNA polymerase η) and DNA polymerase α can elongate the primer on an undamaged template. As shown by the bands just below the full-length band (see Figure 5–22B, lanes 6 and 10), DNA polymerase η did not always make a full-length product, whereas DNA polymerase α did.

   On the template containing the cyclobutane dimer, DNA polymerase η can still synthesize a product that is essentially the same as on an undamaged template (lanes 6 and 10); however, DNA polymerase α stops synthesis when it reaches the site of the damage (lane 9).

   On the template containing the 6-4 photoproduct, DNA polymerase η adds one nucleotide at the site of the 6-4 photoproduct and stops (lane 14). Once again, DNA polymerase α stops synthesis when it reaches the site of the damage (lane 13). DNA polymerase α also cannot extend synthesis of the primer containing one nucleotide added by DNA polymerase η across from the 6-4 photoproduct (lane 15).

C. As you might imagine, if the specificity of DNA polymerase η is relaxed sufficiently so that it can insert nucleotides opposite a cyclobutane dimer, it might also be sloppy when copying normal DNA. In fact, DNA polymerase η makes mistakes on normal DNA at a frequency of about 1 in 32. Thus, it is an error-prone DNA polymerase. It lacks a proofreading exonuclease and does not require an accurately base-paired 3′ end to initiate synthesis. This is not surprising given that it has evolved to recognize a completely different kind of structure in its template. Under normal circumstances, specialized polymerases such as DNA polymerase η are allowed to operate only at sites of damage; thus, their error-proneness on normal DNA is not an issue.

D. In normal individuals, NER does not fix all the UV damage before the replication fork arrives. The residual damage blocks the replicative DNA polymerase and triggers its replacement by DNA polymerase η. This specialized polymerase adds As across from cyclobutane dimers (which are mostly T-T dimers) thereby minimizing mutation. Patients with XP-V are sensitive to sunlight and prone to cancer because they are missing DNA polymerase η. When UV damage appears at a replication fork in XP-V patients, another polymerase—less specialized for UV damage—carries out the bypass synthesis. As a result, higher frequencies of incorrect nucleotides are introduced, which ultimately leads to mutations and cancer.

Reference: Masutani C, Araki M, Yamada A, Kusumoto R, Nogimori T, Maekawa T, Iwai S & Hanaoka F (1999) Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity. *EMBO J.* 18, 3491–3501.

5–69

A. The adaptive response to low levels of MNNG must require the synthesis of new proteins, since the response is blocked by chloramphenicol (see Figure 5–24). If activation of a preexisting protein were all that was required, chloramphenicol would not be expected to block adaptation.

B. The adaptive response might be short-lived for several reasons. Presumably, once the signal for adaptation (ultimately MNNG) is removed, the induced synthesis of new proteins would halt. The resistance to MNNG mutagenesis and killing would then depend on the stability of the induced proteins. If they were relatively unstable, the resistant state would decay rapidly as the proteins became inactive. Even if the proteins were stable, the resistant state of the population of bacteria would decay fairly rapidly due to their growth and the resulting dilution of the protein.

Reference: Teo I, Sedgwick B, Kilpatrick MW, McCarthy TV & Lindahl T (1986) The intracellular signal for induction of resistance to alkylating agents in *E. coli. Cell* 45, 315–324.

HOMOLOGOUS RECOMBINATION

DEFINITIONS

5–70 Allele

5–71 Gene conversion

5–72 Hybridization

5–73 Holliday junction

TRUE/FALSE

5–74 True. The mechanisms of homologous recombination require fairly large stretches of nearly identical DNA in order to initiate and complete a recombination event. These long regions of near identity are incorporated into the mechanism of homologous recombination to ensure that duplexes recombine only at corresponding points along the chromosome, and not, for example, between closely related repeated DNA elements that litter the genomes of higher eukaryotes.

5–75 True. Conversion is a change in frequency of markers (nucleotide differences) during recombination. In the starting duplexes, each marker is present equally: once in each duplex (or twice in each duplex if the individual strands are counted). In the products of a recombination event associated with conversion, the frequency of markers is altered, so that they are no longer equal. Instead of 1:1 (or 2:2) as in the input duplexes, the frequency becomes 2:0 (or 4:0 or 3:1) in the output duplexes. The two common mechanisms for generating this change—mismatch repair and DNA synthesis—both involve some amount of DNA synthesis.

THOUGHT PROBLEMS

5–76 The recombination substrates and products are shown in **Figure 5–46**. The first rule for deducing the recombination products is to align the homologous segments; that is, to draw the arrows one above the other so that they are pointing in the same direction. Alignment requires a

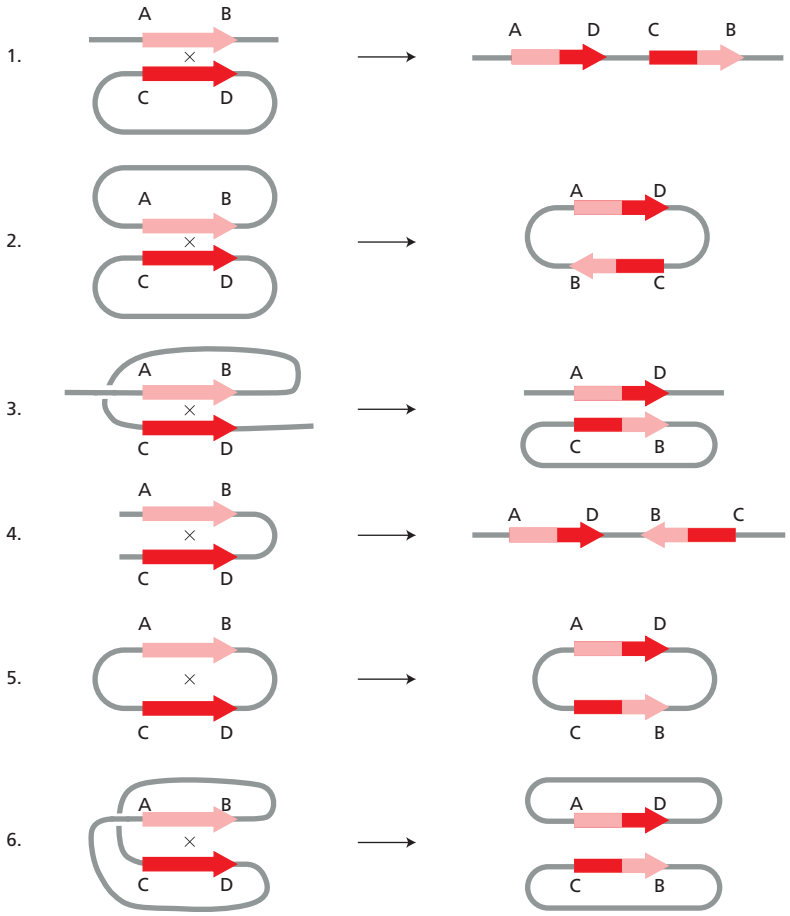


Figure 5–46 Alignments and crossovers in various recombination substrates (Answer 5–76).

twisting of substrates 3, 4, and 6. Alignment is necessary in order to form a Holliday junction, as would be more apparent if real sequences were used instead of arrows.

Substrates 3 and 4 illustrate a useful rule. Recombination between direct repeats in a chromosome (as in substrate 3) deletes one copy of the repeat and the intervening DNA. Recombination between inverted repeats in a chromosome (as in substrate 4) simply inverts the DNA between the repeats.

5–77 Repair of a double-strand break by homologous recombination requires an intact homologous chromosome as a template for repair. In a haploid cell in G_1, each chromosome is present in only one copy. Thus, when a break occurs in G_1, there is no intact homologous template to use for repair. In haploid cells in G_2, there are two copies of each chromosome (sister chromatids), so that a broken copy can be repaired from the intact sister chromatid.

5–78

A. Your friend is correct. Because the crossover point in any individual molecule is equidistant from a defined sequence (the unique site for the restriction nuclease), the sequences involved in the crossover must be homologous. Since they occur at random distances from the restriction site, there are no preferred sites for recombination.

B. If you repeated the experiments in a *RecA*-deficient strain of *E. coli*, no figure-8 forms or χ forms would be found.

C. If the figure-8 forms were intermediates in a site-specific recombination between the monomers, the χ forms would all have had exactly the same crossover point and would all look identical.

D. If the figure-8 forms were intermediates in a random, nonhomologous recombination between the monomers, the χ forms would have had four arms of different lengths.

Reference: Potter H & Dressler D (1976) On the mechanism of genetic recombination: electron microscopic observation of recombination intermediates. *Proc. Natl Acad. Sci. USA* 73, 3000–3004.

5–79 This statement is incorrect. Crossing and noncrossing pairs of strands can be interconverted by rotational movements that do not require strand breakage.

5–80 The double Holliday junction that would result from strand invasion is shown in **Figure 5–47**. Two representations are shown, both correct. The upper one looks simpler because the invading duplex has been rotated so that the marked 5′ end is on the bottom. This arrangement minimizes the number of lines that must cross, which is why most recombination diagrams are shown in this way. The lower representation is perfectly correct, but it looks more complicated. Note that both drawings represent exactly the same molecular structure. DNA synthesis uses the 3′ end of the invading duplex as a primer and fills the single-strand gap by 5′-to-3′ synthesis, as indicated.

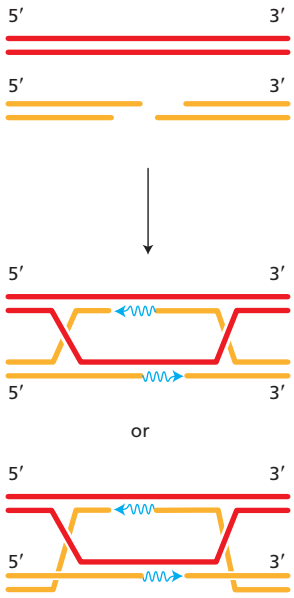


Figure 5–47 Double Holliday junction (Answer 5–80). New DNA synthesis is indicated by *wavy blue lines*.

5–81 A large percentage of the human genome is made up of repetitive elements such as *Alu* sequences, which are scattered among the chromosomes. If, for example, recombination were to occur between two such sequences that were on different chromosomes, a translocation would be generated. Unrestricted recombination between such repeated elements would quickly rearrange the genome beyond recognition. Different rearrangements in different individuals would lead to large numbers of nonviable progeny, putting the species at risk.

This calamity is avoided through the action of the mismatch repair system. Repeated sequences around the genome differ by a few percent of their sequence. When recombination intermediates form between them, many mismatches are present in the heteroduplex regions. When the mismatch repair system detects too high a frequency of mismatches, it aborts the recombination process in some way. This surveillance mechanism ensures that sequences that successfully recombine are nearly identical as expected for sequences at the same locus on homologous chromosomes.

DATA HANDLING

5–82

A. The first labeled restriction fragment to appear after starting the reaction is fragment *c*, and label appears in the other fragments progressively, with fragment *a* the last to become double-stranded. This order of appearance matches the order of the fragments in the 5′-to-3′ direction on the + single-strand circle shown in Figure 5–29B, starting from the top. Since pairing between DNA strands is antiparallel, invasion must start at the 3′ end of the minus strand of the linear DNA, and branch migration must proceed in the 3′-to-5′ direction along the minus strand (**Figure 5–48**).

B. It takes about 20 minutes for the last fragment to become well labeled. This indicates that the rate of movement of the branch point is about 350 nucleotides/minute (7000 nucleotides/20 minutes), or about 6 nucleotides/second. Compared with the rate of replication, which is about 500 nucleotides/second, the rate of branch migration catalyzed by RecA is very slow.

C. The presence of a 500-nucleotide-pair patch of nonhomologous DNA would inhibit branch migration severely. Nevertheless, RecA can catalyze branch migration through such a nonhomology at a low frequency and produce a double-strand circle with the nonhomologous DNA looped out as a single strand (Figure 5–48).

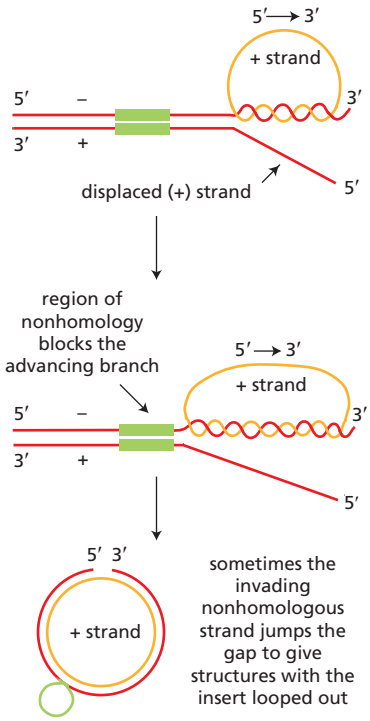


Figure 5–48 Effect of a nonhomologous patch on branch migration catalyzed by RecA (Answer 5–82).

Reference: Cox M & Lehman IR (1981) Directionality and polarity in recA protein-promoted branch migration. *Proc. Natl Acad. Sci. USA* 78, 6018–6022.

5–83

A. As evident in the micrograph in Figure 5–30A, Rad52 binds to the ends of DNA. Also, it apparently binds more effectively to ends with single-strand tails than it does to blunt-ended molecules, since DNA molecules with Rad52 bound to the ends are commonly observed only when the DNA has single-strand tails.

B. By binding to the ends of the linear DNA, Rad52 prevents access by the exonuclease, which requires a free end. Bound Rad52 does not interfere with digestion by the endonuclease, which can cleave in the interior of the DNA.

C. The preferential binding of Rad52 to ends with single-strand tails, as opposed to blunt ends, suggests that broken ends are processed first by an exonuclease to create single strands to which Rad52 can bind. The binding of Rad52 then protects against further exonuclease action. At this point it is thought that Rad52 loads Rad51, a RecA-like recombinase, onto the single strands, leading to the subsequent step of strand invasion on a homologous duplex, as shown in **Figure 5–49**.

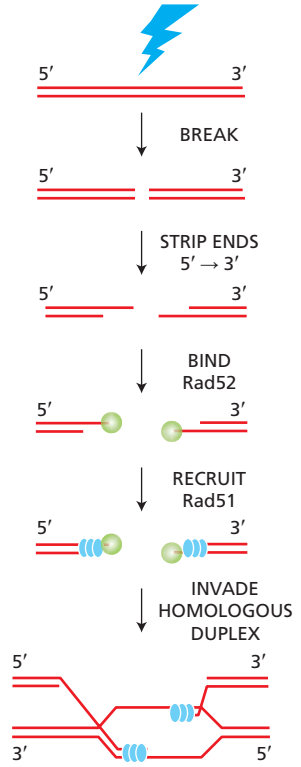


Figure 5–49 The initial steps in Rad52-promoted repair of DNA breaks by homologous recombination (Answer 5–83).

Reference: Van Dyck E, Stasiak AZ, Stasiak A & West SC (1999) Binding of double-strand breaks in DNA by human Rad52 protein. *Nature* 398, 728–731.

5–84

A. RuvC can cleave only 4 of the 256 (4^4) possible four-nucleotide sequences, which is 1/64 of all possible sequences.

B. No. One of the four four-nucleotide sequences would be expected on average every 64 nucleotides. Thus, only a small amount of branch migration would be required to juxtapose a Holliday junction with an appropriate cleavage sequence. In cells, RuvC operates in conjunction with RuvAB, which is a helicase that drives branch migration of Holliday junctions.

C. Evidently, the two subunits of RuvC coordinate their cleavages. Only when both have encountered an appropriate cleavage sequence does either site get cleaved. This conclusion is apparent in the results with the hybrid junction in Figure 5–31B. When one duplex carries a resolution sequence but the other does not, the sequence is not cleaved. This indicates that the two subunits do not operate independently of one another. Additional experiments in the reference below showed that in Holliday junctions with two cleavable but nonidentical sequences, both sequences were cleaved.

D. The duplexes generated by cleavage of the indicated strands of the Holliday junction in Figure 5–31A would have the same sequences shown in the figure, except that segment *a* would be connected to segment *d*, and segment *c* would be connected to segment *b*. Thus, a crossover would be generated. In the absence of any proteins but RuvC, the two product duplexes would each carry a nick at the site of RuvC cleavage.

Reference: Shah R, Cosstick R & West SC (1997) The RuvC protein dimer resolves Holliday junctions by a dual incision mechanism that involves base-specific contacts. *EMBO J.* 16, 1464–1472.

TRANSPOSITION AND CONSERVATIVE SITE-SPECIFIC RECOMBINATION

DEFINITIONS

5–85 DNA-only transposon

5–86 Reverse transcriptase

5–87 Retrovirus

5–88 Conservative site-specific recombination

TRUE/FALSE

5–89 False. Transposable elements integrate nearly randomly and genes often are destroyed or altered by the integration event. While it is true that some of these events are lethal to the cell and to the transposable element, most events are not. Spreading throughout the genome, even at the cost of a few cells (and transposons), ensures that the transposable element will survive with the species.

THOUGHT PROBLEMS

5–90 Cre-mediated recombination between oppositely oriented LoxP sites inverts the sequence between the sites, whereas recombination between LoxP sites in the same orientation deletes the sequence from the genome, releasing it as a circle (**Figure A5–3**). Since the circle likely lacks an origin of DNA replication, it will be lost as the cells divide. This result should remind you of the similar outcome obtained for homologous recombination between direct repeats and inverted repeats in Problem 5–76 (see Figure 5–46, substrates 3 and 4). As in that problem, the easiest way to work out the products is to align the LoxP sites and then follow the crossover between them.

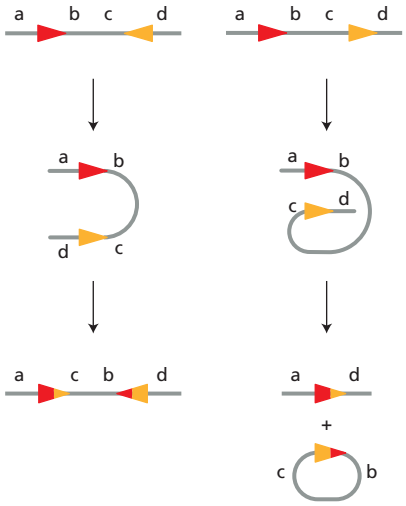


Figure 5–50 Products of Cre-mediated recombination between oppositely oriented and directly repeated LoxP sites (Answer 5–90).

DATA HANDLING

5–91

A. All colonies must have arisen by transposition of Tn10 into the bacterial genome because survival depends on the presence of the tetracycline-resistance gene carried by Tn10. The presence of mixed colonies with blue and white sectors is the key observation. Since the frequency of sectored colonies is high but transposition is rare, sectored colonies must arise commonly in individual transposition events. A replicative mechanism can transfer only one strand of the parent heteroduplex and, thus, can generate only white or blue colonies, depending on which strand is transferred. A cut-and-paste mechanism transfers both strands of the heteroduplex, which upon replication and segregation into daughter bacteria will produce a sectored colony. (Once the bacteria are spread onto a Petri dish, all the descendants of the original infected cell are confined to the immediate vicinity and, thus, grow together to form the colony. If two different daughters are produced at the first division, their descendants will grow together to produce a single colony with sectors containing the two different kinds of bacteria.) The pure blue and pure white colonies arise from transposition events that involve the homoduplexes. The proportions of blue, white, and sectored colonies are as expected from the equal mixture of heteroduplexes (which give rise to the sectored colonies) and homoduplexes (which give rise to the pure colonies).

B. Each heteroduplex contains a mismatched region of DNA corresponding to the position of the mutation in the *LacZ* gene. If these heteroduplexes were introduced into bacteria that could repair such mismatches, then the frequency of sectored colonies would be expected to decrease markedly. In essence, each repair event would convert a heteroduplex into a homoduplex. If the mismatch repair were unbiased, the frequencies of blue colonies and white colonies would each increase equally. These experiments were carried out in bacteria defective in mismatch repair precisely to avoid that distortion of the data.

Reference: Bender J & Kleckner N (1986) Genetic evidence that Tn10 transposes by a nonreplicative mechanism. *Cell* 45, 801–815.

5–92

A. Transposition of the Ty element depends on reverse transcription of an RNA intermediate. Normally, reverse transcriptase is expressed at a very low level. Your modified plasmid, however, places the gene under control of the galactose control elements. In the presence of glucose (absence of galactose), the galactose control elements turn the gene off and, as a result, the expression of reverse transcriptase is very low. In the presence of galactose, the reverse transcriptase gene is expressed at very high levels. Thus, the frequency of transposition increases substantially.

B. The frequency of Ty-induced *His*$^+$ colonies is low because a very specific kind of transposition event is required to activate the defective histidine gene: the Ty element must transpose to a site near the 5′ end of the gene. Thus, even though nearly all cells show evidence for transposition, insertion near the defective histidine gene is still relatively rare.

C. The data in Figure 5–35 indicate that nearly every cell harboring the Ty-bearing plasmid suffers one or more transposition events when grown on galactose. Each Ty transposition has the potential for altering the function or expression of genes near the site of integration. If the element integrates into the coding portion of a gene, it can eliminate the encoded function; if it integrates in the noncoding region near a gene, it may alter the gene's expression. In organisms such as yeasts, which have been finely tuned to their environmental niche by evolutionary pressure, it is unlikely that random insertion of a Ty element will improve growth characteristics. Thus, it is not unreasonable that a high rate of transposition should cause cells to grow poorly.

These data do not prove that the cells grow more slowly because of the high rate of transposition, even though that explanation is very likely to be correct. As the authors point out, the high level of expression of reverse transcriptase might interfere directly with RNA metabolism. For example, mRNA molecules could be inactivated by reverse transcription. Alternatively, the reverse transcripts of the cellular mRNAs could be mutagenic to the nuclear genes. Errors introduced during reverse transcription into DNA could be incorporated into the nuclear genes by recombination.

Reference: Boeke JD, Garfinkel DJ, Styles CA & Fink GR (1985) Ty elements transpose through an RNA intermediate. *Cell* 40, 491–500.

MCAT STYLE

5–93

B. A key feature of highly accurate DNA polymerases is that they possess a 3′-to-5′ proofreading exonuclease activity that detects and removes mismatched bases at the 3′ end of the growing DNA strand. Choice A is not correct because Okazaki fragments are not involved in PCR. Choice C is incorrect because prematurely falling off the DNA does not introduce errors, although it may produce short segments and lower the efficiency of amplification. Choice D is not correct because all DNA polymerases, including Taq polymerase, synthesize DNA in the 5′-to-3′ direction.

5–94

B. All DNA polymerases have a strong preference for a matched base at the 3′ end of the growing DNA strand. In the absence of a 3′-to-5′ proofreading exonuclease to remove a 3′ mismatched base, Taq polymerase will tend to stall, thereby limiting the average length of synthesized fragments. Choice A is not correct because Okazaki fragments are not involved in the DNA synthesis associated with PCR amplification. Choice C is not correct because, although Taq polymerase does not possess helicase activity, no helicase is required in PCR amplification, which synthesizes DNA from a single-strand DNA template. Choice D is incorrect because

the primers are present in the PCR mix from the start; they are not made during the reaction.

5–95

C. Normally, the bacterial mismatch repair machinery can detect which strand to correct because the parental strand is marked by methylation; thus, by correcting the unmethylated strand to the sequence on the methylated strand, the mismatch repair machinery will preserve the wild-type sequence. In the case of a PCR product, however, the DNA is not marked. If the mismatches are repaired independently of one another and randomly with respect to which strand is used as the template for repair, then both mismatches would be converted to mutations 25% of the time.

5–96

A. The polymerase that modifies Okazaki fragments to prepare them for joining into intact strands must be able to remove the RNA primer at their 5′ ends and to fill in the small gap that is left behind once the primer is removed. The properties of DNA polymerase I match these requirements: its 5′-to-3′ exonuclease activity can remove the RNA primer at the start of the Okazaki fragment and it can synthesize the short stretches of DNA necessary to fill the resulting gap. Choice B is incorrect because, although DNA can be damaged when single strands are exposed during transcription, DNA polymerase is not equipped with the repair activities required to remove a damaged base from an otherwise intact strand. Choice C is not correct because cellular DNA synthesis is carried out by DNA polymerase III, whose properties are much better suited for copying the large bacterial genome. Choice D is not correct because primers for Okazaki fragments are synthesized by DNA primase, which is specialized for making RNA primers.

How Cells Read the Genome: From DNA to Protein

CHAPTER 6

IN THIS CHAPTER

FROM DNA TO RNA

DEFINITIONS

6–1 General transcription factor

6–2 snRNA (small nuclear RNA)

6–3 Promoter

6–4 Exosome

6–5 RNA polymerase

6–6 mRNA (messenger RNA)

6–7 RNA splicing

6–8 Terminator

6–9 Exon

6–10 Nuclear pore complex

TRUE/FALSE

6–11 True. Errors in DNA replication have the potential to affect future generations of cells, while errors in transcription have no genetic consequence. Errors in transcription lead to mistakes in a small fraction of RNAs, whose functions are further monitored by downstream quality control mechanisms. They are not passed on to progeny cells. In contrast, errors in DNA replication change the gene and, thereby, affect all the copies of RNA (and protein) made in the original cell and all its progeny cells.

These considerations are reflected in the intrinsic error rates for RNA and DNA polymerases: RNA polymerases typically make 1 mistake in copying 10^4 nucleotides, while DNA polymerases make about 1 error per 10^7 nucleotides. Such significant differences in error rates suggest that natural selection is stronger against errors in replication than against errors in transcription.

6–12 False. The σ subunit associates with the bacterial RNA polymerase core enzyme to form the RNA polymerase holoenzyme only during the initiation phase of RNA synthesis. The σ subunit helps the core enzyme bind to the promoter and stays associated with the core enzyme until RNA synthesis has been properly initiated, and then it dissociates.

6–13 True. At its 3′ end, each eukaryotic mRNA has a string of adenine nucleotides, the last of which has a terminal ribose with a free 3′-OH group. At its 5′ end, each mRNA carries a 7-methylguanosine that is linked 5′ to 5′

with the first nucleotide in the mRNA. This linkage leaves a free 3′-OH group on the ribose of the capping nucleotide.

6–14 False. Although intron sequences are mostly dispensable, they must be removed precisely. An error of even one nucleotide during removal would shift the reading frame in the spliced mRNA molecule and produce an aberrant protein.

6–15 False. The 3′ ends of most pre-mRNA transcripts produced by RNA polymerase II are defined not by the termination point of transcription, but by cleavage of the RNA chain 10–30 nucleotides downstream of the sequence AAUAAA.

THOUGHT PROBLEMS

6–16 The answer is best given by Francis Crick himself, who in 1957 coined the terms "the sequence hypothesis," which proposed that genetic information is encoded in the sequence of the DNA bases, and "the central dogma," which stated that DNA makes RNA makes protein.

"I called this idea the central dogma, for two reasons, I suspect. I had already used the obvious word hypothesis in the sequence hypothesis, and in addition I wanted to suggest that this new assumption was more central and more powerful. I did remark that their speculative nature was emphasized by their names.

As it turned out, the use of the word dogma caused almost more trouble than it was worth. Many years later Jacques Monod pointed out to me that I did not appear to understand the correct use of the word dogma, which is a belief *that cannot be doubted*. I did apprehend this in a vague sort of way but since I thought that *all* religious beliefs were without serious foundation, I used the word in the way I myself thought about it, not as most of the rest of the world does, and simply applied it to a grand hypothesis that, however plausible, had little direct experimental support."

Reference: Crick F (1988) What Mad Pursuit: A Personal View of Scientific Discovery, p. 109. New York: Basic Books, Inc.

6–17 Actually, the RNA polymerases are not moving at all because they have been fixed and coated with metal to prepare the sample for viewing in the electron microscope. Before they were fixed, however, they were moving from left to right, as indicated by the gradual lengthening of the RNA transcripts. The RNA transcripts are shorter than the DNA that encodes them because they begin to fold up (acquire a three-dimensional structure) as soon as they are synthesized, whereas the DNA is an extended double helix.

6–18 A with 7, B with 4, C with 2, D with 5, E with 8, F with 3, G with 1, and H with 6.

6–19 If the polymerase transcribes the sequence from left to right, it will use the bottom strand as a template to make the sequence 5′-GUAACGGAUG (the RNA sequence corresponding to the top strand of the DNA). If the polymerase moves from right to left, it will use the top strand as a template to make the sequence 5′-CAUCCGUUAC (the RNA sequence corresponding to the bottom strand of the DNA, written 5′ → 3′).

6–20 General transcription factors play several roles in promoting transcription by RNA polymerase II. They help position the RNA polymerase correctly at the promoter, they aid in pulling apart the two strands of DNA to allow transcription to begin, and they release RNA polymerase from

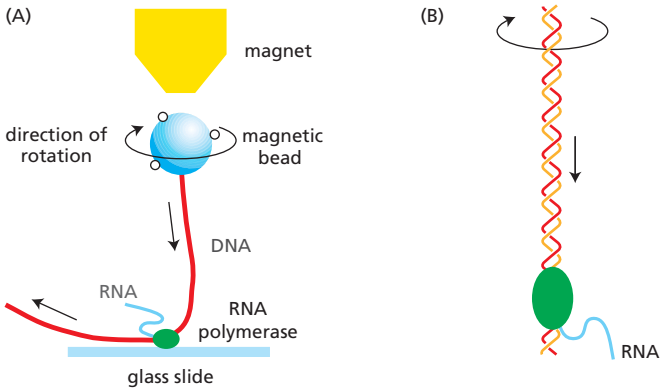


Figure 6–39 Rotation of DNA duplex due to movement relative to RNA polymerase (Answer 6–22). (A) Direction of rotation of the magnetic bead. (B) Direction of rotation of the DNA duplex.

the promoter once transcription has begun. They are called "general" because they assemble on all promoters used by RNA polymerase II; they are identified by names beginning with TFII (transcription factor for RNA polymerase II). Labeling them "general" transcription factors also serves to distinguish them from more specialized gene regulatory proteins that enhance transcription at selected promoters in certain cell types.

6–21 The RNA polymerase must be moving from right to left in Figure 6–2. If the RNA polymerase does not rotate around the template as it moves, it will overwind the DNA ahead of it, causing positive supercoils, and underwind the DNA behind it, causing negative supercoils. If the RNA polymerase were free to rotate about the template as it moved along the DNA, it would not overwind or underwind the DNA, and no supercoils would be generated.

Reference: Liu LF & Wang JC (1987) Supercoiling of the DNA template during transcription. *Proc. Natl Acad. Sci. USA* 84, 7024–7027.

6–22 The bead would rotate clockwise from the perspective of the magnet, as shown in **Figure 6–39A**. As shown in **Figure 6–39B**, the motion of the helix relative to a fixed RNA polymerase causes the helix to rotate.

Reference: Harada Y, Ohara O, Takatsuki A, Itoh H, Shimamoto N & Kinosita K (2001) Direct observation of DNA rotation during transcription by *Escherichia coli* RNA polymerase. *Nature* 409, 113–115.

6–23 From electron micrographs such as that shown in Figure 6–1 it seems clear that RNA does not become wrapped around the DNA as it is spun out behind RNA polymerase. Thus, RNA polymerase doesn't seem to revolve around DNA as it moves. Consistent with this, other evidence suggests that RNA polymerase does induce positive supercoiling ahead of it and negative supercoiling behind. But the level of supercoiling tension is not so high as you would expect if the continued movement of RNA polymerase generated ever-higher levels of coiling. These observations suggest that supercoiling tension is relieved by the action of topoisomerases in the cell, so that supercoiling around the RNA polymerase is maintained at optimal levels.

6–24 The tails arise because the ends of the mRNA are not complementary to the ends of the DNA restriction fragment. Thus, one of the single-strand tails at each end is DNA from the restriction fragment. A single-strand tail at one end corresponds to the 5′ end of the mRNA, which must come from an upstream exon that is not present in the restriction fragment. A single-strand tail at the other end corresponds to the 3′ end of the mRNA, which may come from a downstream exon not present in the restriction

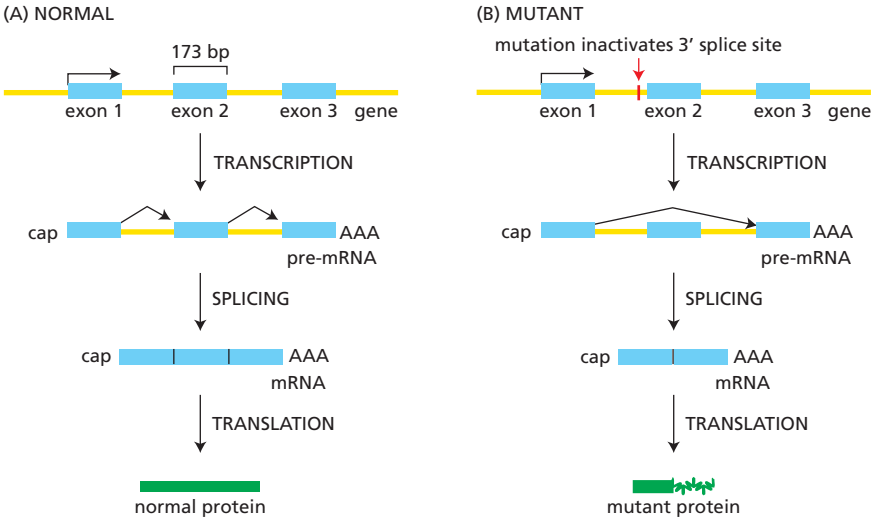


Figure 6–40 Splicing of the Smilin transcript (Answer 6–25). (A) Normal transcript. (B) Mutant transcript.

fragment or may simply be the poly-A tail itself. Without additional information you cannot identify which single strand comes from which source.

Reference: Berget SM, Berk AJ, Harrison T & Sharp PA (1978) Spliced segments at the 5′ termini of adenovirus-2 late mRNA: a role for heterogeneous nuclear RNA in mammalian cells. *Cold Spring Harb. Symp. Quant. Biol.* 42, 523–529.

6–25

A. A single nucleotide change in a gene could cause an internal deletion in the mRNA if it altered splicing so that an exon that was usually incorporated was skipped instead.

B. Removal of 173 nucleotides from the protein-coding portion of the mRNA would cause a shift in the reading frame for translation into amino acids. Because a codon is three nucleotides, a loss of 173 nucleotides does not correspond to an integral number of codons. Thus, the Smilin encoded by the mRNA lacking exon 2 would be fine up to the missing exon, but would encode an unrelated sequence of amino acids thereafter until a stop codon was reached.

C. The simplest explanation is that the Smilin gene contains a 173-nucleotide-long exon (exon 2 in **Figure 6–40A**) that is lost during the processing of the mutant precursor mRNA. This could occur, for example, if the mutation changed the 3′ splice site in the preceding intron so that it was no longer recognized by the splicing machinery (a change in the conserved AG at the intron/exon boundary could do this). Use of the next available 3′ splice site—adjacent to exon 3—would cause loss of exon 2 from the mutant mRNA (**Figure 6–40B**). During protein synthesis, the absence of exon 2 (173 nucleotides) would throw the ribosomes out of the correct reading frame as they moved from exon 1 to exon 3. At that junction, the ribosomes would begin synthesizing a protein sequence unrelated to the one normally encoded by exon 3.

6–26 Statement C is the only one that is necessarily true for exons 2 and 3. It is also the only one true for exons 7 and 8. While the conditions given in A and B could be the case, they need not be. However, because the encoded protein sequence is the same in segments of the mRNA that correspond to exons 1 and 10, neither choice of alternative exons (2 versus 3, or 7 versus 8) can be allowed to alter the reading frame. To maintain the normal reading frame—whatever that is—the alternative exons must have a number of nucleotides that when divided by 3 (the number of nucleotides in a codon) gives the same remainder.

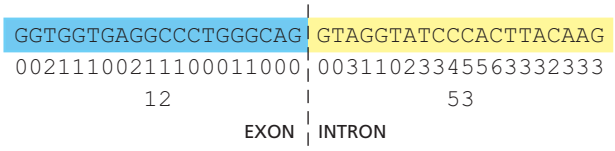


Figure 6–41 Sum of differences from the cow β-globin sequence (Answer 6–27). The β-globin sequence from the cow was compared nucleotide by nucleotide with the β-globin sequences from the other six species. The number of differences at each position is summed *below* each nucleotide of the β-globin sequence. The total number of differences on each side of the exon/intron boundary (*dashed line*) is shown at the *bottom*.

Because the sequence of the α-tropomyosin gene is known, the actual state of affairs can be checked. Exons 2 and 3 both contain the same number of nucleotides, 126, which is divisible by 3 with no remainder. Exons 7 and 8 also contain the same number of nucleotides, 76, which, when divided by 3, leaves a remainder of 1.

6–27 Since introns evolve faster than exons, the introns of the different species will be more variable than the exons. It is difficult to scan these sequences by eye and decide, with confidence, which side is the more conserved. One way to quantify the differences is to pick one sequence, for example, the cow, and count up how often the other sequences differ at each position, as shown in **Figure 6–41**. Summing the differences on each side of the junction makes it clear that sequences on the left are much more similar to one another than are the sequences on the right. (Similar differences exist no matter which sequence is chosen for comparison.) Thus, the more conserved sequences, which are on the left in Figure 6–6, correspond to exons, and the less conserved sequences, which are on the right, correspond to introns.

6–28

A. If the splicing machinery bound to one splice site and scanned across the intron to find its complementary splice site, it would use the first appropriate splice site it encounters. The products predicted from the intron-scanning model are shown in **Figure 6–42**. If the splicing machinery bound to a 5′ splice site and scanned toward a 3′ splice site, minigene 1 would generate one product (Figure 6–42A) and minigene 2 would generate two products (Figure 6–42B). By contrast, if the splicing machinery bound to a 3′ splice site and scanned toward a 5′ splice site, minigene 1 would generate two products (Figure 6–42A) and minigene 2 would generate one product (Figure 6–42B).

B. The results of this experiment do not match the expectations for either scanning model, suggesting that scanning models are incorrect. The ordering mechanism by which cells avoid exon skipping probably depends on two factors: first, that assembly of the spliceosome occurs as the pre-mRNA emerges from the RNA polymerase; and second, that exons may be defined as an independent step prior to the assembly of the spliceosome.

Reference: Kühne T, Wieringa B, Reiser J & Weissmann C (1983) Evidence against a scanning model of RNA splicing. *EMBO J.* 2, 727–733.

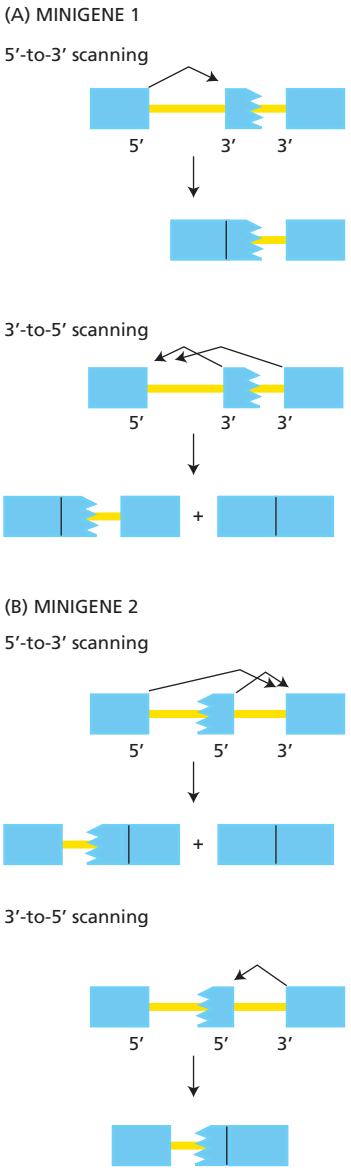


Figure 6–42 Expected products in a test of intron scanning (Answer 6–28). (A) Expected products for 5′-to-3′ scanning and 3′-to-5′ scanning in minigene 1. (B) Expected products for 5′-to-3′ scanning and 3′-to-5′ scanning in minigene 2. *Rectangles* indicate complete exons; boxes with *ragged edges* represent partial exons.

6–29 "Export ready" means that an mRNA is bound by the appropriate set of proteins. Proteins such as the cap-binding complex, the exon junction complex, and the poly-A-binding protein must be present, while proteins such as spliceosome components must be absent. RNA fragments from excised introns do not acquire the necessary set of proteins and are thus doomed to degradation.

6–30 The structure of the nucleolus depends on the rRNA genes, which are located in clusters at the tips of each copy of five different chromosomes in humans. During interphase, the transcribed rRNA genes associate to form the visible nucleolus. At mitosis, the chromosomes disperse and the

nucleolus breaks up. After mitosis, the tips of the chromosomes again coalesce and the nucleolus re-forms in a process that depends on transcription of the rRNA genes.

CALCULATIONS

6–31

A. At the plateau there are 7.2×10^9 transcripts per reaction. The reaction incorporates 2.4 pmol of CMP, and each transcript is 400 nucleotides long, of which 200 nucleotides are CMP.

$$\frac{\text{transcripts}}{\text{reaction}} = \frac{2.4 \text{ pmol CMP}}{\text{reaction}} \times \frac{6 \times 10^{23} \text{ CMP}}{\text{mole}} \times \frac{\text{mole}}{10^{12} \text{ pmol}} \times \frac{\text{transcripts}}{200 \text{ CMP}}$$
$$= 7.2 \times 10^9 \text{ transcripts/reaction}$$

B. Each reaction contains 1.0×10^{11} templates. At 16 µg/mL, a 25 µL reaction volume contains 0.4 µg of template (16 µg/mL × 0.025 mL = 0.4 µg). Each template is 3500 nucleotide pairs (np) long.

$$\frac{\text{templates}}{\text{reaction}} = \frac{0.4 \text{ µg}}{\text{reaction}} \times \frac{6 \times 10^{17} \text{ d}}{\text{µg}} \times \frac{\text{np}}{660 \text{ d}} \times \frac{\text{template}}{3500 \text{ np}}$$
$$= 1.0 \times 10^{11} \text{ templates/reaction}$$

C. There are about 0.07 transcripts/template, which is equivalent to about 1 RNA transcript per 14 template molecules.

$$\frac{\text{transcripts}}{\text{template}} = \frac{7.2 \times 10^9 \text{ transcripts}}{\text{reaction}} \times \frac{\text{reaction}}{1.0 \times 10^{11} \text{ templates}}$$
$$= 0.07 \text{ transcripts/template}$$

The poor efficiency of the reaction is typical of *in vitro* transcription. The ratio of 1 transcript per 14 templates does not necessarily mean that 1 out of 14 templates functions in RNA synthesis. It may be that a much smaller fraction of the templates makes a large number of transcripts. For example, 1 in 140 templates might synthesize 10 transcripts each.

Reference: Sawadogo M & Roeder RG (1985) Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. *Proc. Natl Acad. Sci. USA* 82, 4394–4398.

DATA HANDLING

6–32

A. Starting with a complex in which a C had been incorporated at position +34, the RNA polymerase could be walked down to position +43 in two steps: (1) incubate with a mixture of ATP and UTP, and then wash away the nucleotides; and (2) incubate with a mixture of UTP and CTP, and then wash. This protocol will allow the polymerase to incorporate all the nucleotides up to position +43, but not the A at position +44.

B. Incorporation of the correct A nucleotide occurred 130 times faster than the incorrect G nucleotide at position +44 (0.20/0.0015 = 133; see Table 6–1). Incorporation of the next nucleotide—a C—after the correct A occurred about 5 times faster than incorporation of C after the incorrect G (0.17/0.036 = 4.7). Thus, when the RNA polymerase makes a mistake, it incorporates the next nucleotide more slowly. This pause allows a window of opportunity for removal of the incorrect nucleotide before the next nucleotide is added.

Reference: Thomas MJ, Platas AA & Hawley DK (1998) Transcriptional fidelity and proofreading by RNA polymerase II. *Cell* 93, 627–637.

6–33 The consensus sequence for this set of promoters is shown in **Figure 6–43**. In these 13 promoters, there are clear examples of common nucleotides

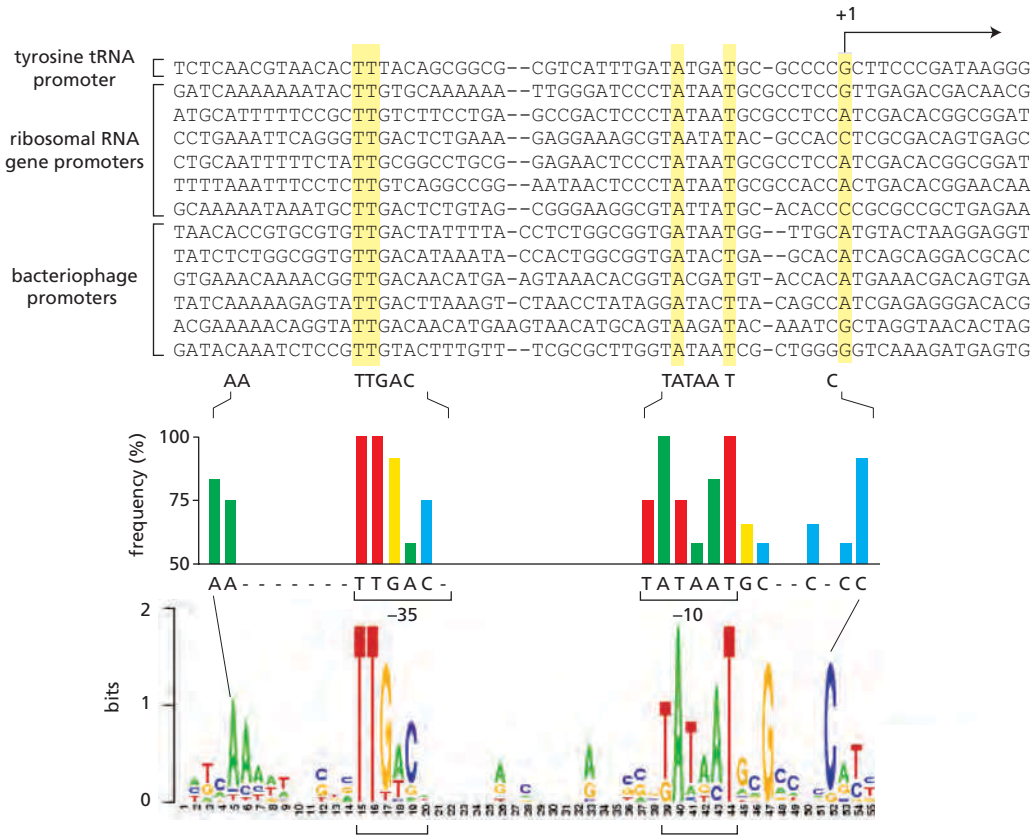


Figure 6–43 Consensus sequence for promoters recognized by σ^{70} factor (Answer 6–33). Nucleotides that are perfectly conserved and the first nucleotide of the transcripts are *highlighted* for reference. *Below* the sequences themselves are indicated the consensus nucleotides and, on an expanded scale, the frequencies of the conserved nucleotides around the –35 and –10 regions. Finally, at the *bottom* the consensus sequence is shown as a sequence logo.

outside the –35 and –10 regions. Also, one of the accepted consensus nucleotides (the terminal A in the –35 sequence) doesn't even show up as common. When 300 promoters recognized by σ^{70} are compared, the consensus sequence is TTGACA (–35) and TATAAT (–10). It's always better to compare more sequences!

6–34

A. There are three locations where the linker-scanning mutations drastically decreased the amount of transcript. These locations are from about –15 to –30, –45 to –60, and –80 to –105. Linker-scanning through the promoter element at –15 to –30 seems to have the most dramatic effect, whereas scanning through the other two sensitive sites gives lesser effects that are about equal to one another.

You may also have noticed the effect of a linker-scanning mutation that overlapped the start site of transcription. In the absence of the usual sequence at the start site, the transcript is initiated at a variety of positions.

B. The segment from –15 to –30 likely includes the TATA box, which is usually located about 25 nucleotides or so upstream of the transcription start site.

Reference: McKnight SL & Kingsbury R (1982) Transcriptional control signals of a eukaryotic protein-coding gene. *Science* 217, 316–324.

6–35

A. The 400-nucleotide transcript is absent from lane 4 (Figure 6–11B) because GTP was included in the reaction mixture. GTP allows transcription to proceed beyond the end of the C-minus sequence (the synthetic sequence lacking C nucleotides), thereby generating transcripts longer than 400 nucleotides. In the absence of GTP (see lane 2), transcription cannot proceed beyond the C-minus sequence. In the presence of GTP

and RNAse T1 (see lane 6), the longer transcripts are cleaved at the first G to yield the 400-nucleotide transcript. In the presence of GTP, RNAse T1, and 3′ *O*-methyl GTP (see lane 8), any long transcripts that escape termination by 3′ *O*-methyl GTP are cleaved by RNAse T1 to yield the 400-nucleotide transcript.

B. One of the difficulties in assaying promoter function *in vitro* is the high background of nonspecific initiation of transcription. It is this background that is so evident in Figure 6–11B, lane 3. Its source is not altogether clear, but transcription may start at sequences in the rest of the plasmid that weakly resemble true RNA polymerase II promoters.

C. A transcript of about 400 nucleotides is present in Figure 6–11B, lane 5, because cleavage with RNAse T1 liberates it from any randomly initiated transcript that has traversed the C-minus sequence. It is actually a few nucleotides longer than the specifically initiated transcript since its 5′ and 3′ ends are defined, respectively, by the G nucleotides that immediately precede and follow the C-minus sequence.

   The 400-nucleotide transcript is absent from Figure 6–11B, lane 7, because 3′ *O*-methyl GTP will terminate most transcripts that are initiated in front of the C-minus sequence. The combination of 3′ *O*-methyl GTP and RNAse T1 eliminates virtually all the background synthesis from the control plasmid.

D. As shown in Figure 6–11B, lanes 7 and 8, specific transcription can be assayed in the presence of G nucleotides if 3′ *O*-methyl GTP and RNAse T1 are included (to inhibit background transcription and to cleave any random transcripts to small pieces).

Reference: Sawadogo M & Roeder RG (1985) Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. *Proc. Natl Acad. Sci. USA* 82, 4394–4398.

6–36 These experiments provided the most convincing early demonstration that there were three different RNA polymerases in eukaryotic cells: one—peak 1—that was insensitive to α-amanitin (RNA polymerase I), one—peak 2—that was inhibited by both 1 μg/mL and 10 μg/mL α-amanitin (RNA polymerase II), and one—peak 3—that was inhibited by 10 μg/mL α-amanitin, but not by 1 μg/mL (RNA polymerase III). It would be unlikely that different forms of the same polymerase could have such different sensitivities to the same molecule. These results also indicate the ways the RNA polymerases were named: by the order in which they were eluted from the column.

Reference: Roeder RG (1974) Multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in *Xenopus laevis*. Isolation and partial characterization. *J. Biol. Chem.* 249, 241–248.

6–37

A. Since an equal amount of transcription from each template was observed when the preincubation was carried out with the individual templates or a mixture (see Figure 6–13C, lanes 1 to 3), Srb2 protein does not show a preference for either template.

B. Srb2 acts stoichiometrically. If Srb2 acted catalytically, it should have been able to modify the second template after the two were mixed. Catalytic activity would have produced transcripts from both templates regardless of which one was originally included in the preincubation with Srb2. When *excess* Srb2 was added at the beginning of the preincubation with one template, transcription was observed from both templates after mixing. This is consistent with a stoichiometric requirement for Srb2 and rules out the possibility that Srb2 was inactivated during the preincubation—and was for that reason unable to act (catalytically) on the second template after mixing.

C. The production of transcripts solely from the template that was preincubated with Srb2 indicates that Srb2 is part of the preinitiation complex. If Srb2 were able to act after transcription had begun, transcripts would have been produced from both templates regardless of which one was included in the preincubation.

D. During preincubation of the template with the extract and Srb2, a number of proteins including Srb2 bind at the promoter to form a preinitiation complex. Evidently, the preinitiation complex, once formed, is stable and does not readily exchange proteins with other templates that are added later.

E. Although the *Srb2* gene was originally identified as a suppressor of the cold-sensitive phenotype of yeast carrying an RNA polymerase II gene with a short CTD, neither those genetic results nor the transcription assays described here provide evidence that Srb2 binds to the CTD. (Nor do they argue against direct interaction; they simply do not speak to the issue.) Additional experiments have shown that Srb2 is part of a complex of proteins known as the Mediator. The Mediator binds to the dephosphorylated CTD, entering and leaving initiation complexes at every round of transcription in a process that may be coupled to CTD phosphorylation and the release of RNA polymerase at initiation of transcription (**Figure 6–44**).

References: Koleske AJ, Buratowski S, Nonet M & Young RA (1992) A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* 69, 883–894.

Svejstrup JQ, Li Yang, Fellows J, Gnatt A, Bjorklund S & Kornberg RD (1997) Evidence for a mediator cycle at the initiation of transcription. *Proc. Natl Acad. Sci. USA* 94, 6075–6078.

Thompson CM, Koleske AJ, Chao DM & Young RA (1993) A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* 73, 1361–1375.

6–38 The pattern of reaction with DNA and protein is strikingly clear. When the U analog was closer than 10 nucleotides to the 3′ end of the RNA, it reacted predominantly with its pairing partner in the template strand. By contrast, when it was 10 nucleotides or farther from the 3′ end, it no longer reacted with DNA at all, and reacted strongly with protein. These patterns of reactivity indicate that the newly synthesized RNA remains paired with the DNA template over a stretch of 8–9 nucleotides from the 3′ end, and then separates from the template strand. It also seems that the U analog must be closely associated with the RNA polymerase even when it is up to 24 nucleotides from the 3′ end.

Reference: Nudler E, Mustaev A, Lukhtanov E & Goldfarb A (1997) The RNA–DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. *Cell* 89, 33–41.

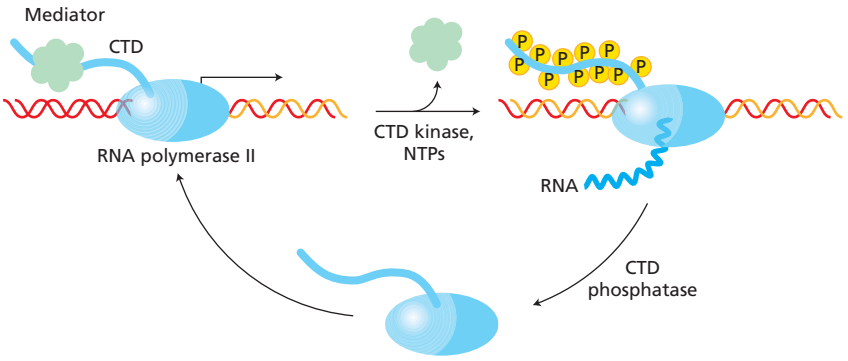


Figure 6–44 A summary of the proposed role of the Mediator complex in initiation of transcription (Answer 6–37).

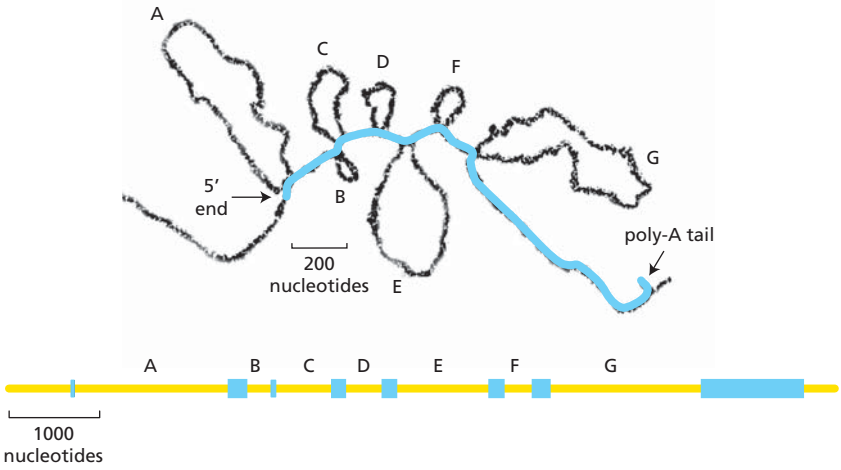


Figure 6–45 Interpretation of the electron micrograph and the intron–exon structure of the chicken ovalbumin gene (Answer 6–39). The letters A to G identify introns and the *blue boxes* identify exons.

6–39 A schematic diagram of the structure of the mRNA–DNA hybrid and the intron–exon structure of the gene are shown in **Figure 6–45**.

References: Dugaiczyk A, Woo SL, Lai EC, Mace Jr ML, McReynolds L & O'Malley BW (1978) The natural ovalbumin gene contains seven intervening sequences. *Nature* 274, 328–333.

Garapin AC, Cami B, Roskam W, Kourilsky P, Le Pennec JP, Perrin F, Gerlinger P, Cochet M & Chambon P (1978) Electron microscopy and restriction enzyme mapping reveal additional intervening sequences in the chicken ovalbumin split gene. *Cell* 14, 629–639.

6–40 The results of these experiments argue convincingly that base-pairing occurs between the pre-mRNA and the U1 RNA. The base-pairing between the U1 snRNA and the pre-mRNA is not extensive, but it is better when splicing occurs successfully than when it does not. These experiments illustrate a classic approach for testing the reality of proposed base-pairing schemes. If a scheme is important, then a base change in one component should interfere with the process. A compensating change in the second component (to restore base-pairing) should then reestablish the process. That is just what was observed in these experiments. A change of G → A in the pre-mRNA inhibited splicing, whereas a compensating C → U change in the U1 snRNA, which should restore base-pairing (GC → AU), reestablished splicing. Moreover, none of the other mutations in the U1 snRNA (which would not restore base-pairing) overcame the splicing defect.

Reference: Zhuang Y & Weiner AM (1986) A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. *Cell* 46, 827–835.

6–41

A. The 5′ ends of the RNA molecules were labeled. Only labeled fragments show up in the autoradiograph (see Figure 6–17). Thus, if the shortest fragments (those that were at the bottom of the gel) are from the 5′ end, the 5′ end must have been labeled.

B. The bands corresponding to the As in the AAUAAA signal sequence are missing from the ladder of bands in polyadenylated and cleaved RNA (see Figure 6–17, lanes 3 and 4) because modification of any one of those As interfered with cleavage and with polyadenylation. Thus, RNA molecules that carry a single modification in the signal sequence are not recognized by the components of the extract and, as a result, do not show up in the population of molecules that carry poly-A tails (see lane 3) or in the population of molecules that are cleaved (see lane 4).

C. The band at the arrow in Figure 6–17 is absent from the polyadenylated RNA but present in the cleaved RNA because modification of this A does not prevent cleavage, but it does prevent polyadenylation. Thus, RNA molecules with this A modified are present in the cleaved molecules (see Figure 6–17, lane 4) but are not present in the polyadenylated molecules (see lane 3).

D. The analysis of the missing bands in parts B and C indicates that the AAUAAA signal sequence is important for the cleavage of precursor RNAs and that the AAUAAA sequence and the single A are required for polyadenylation.

E. If the other end—the 3′ end—of the RNA molecules were labeled, it would have been possible to determine whether any of the As or Gs on the 3′ side of the cleavage site were important for polyadenylation. These experiments have been done; they show that no single modification 3′ of the polyadenylation site prevents polyadenylation. The sequence requirements on the 3′ side of the cleavage site (GU- or U-rich) are not so specific as those on the 5′ side and would not be expected to be inactivated by single changes.

Reference: Conway L & Wickens M (1987) Analysis of mRNA 3′ end formation by modification interference: the only modifications which prevent processing lie in AAUAAA and the poly(A) site. *EMBO J.* 6, 4177–4184.

6–42

A. The idea behind the oligonucleotide experiment was to try to cleave the RNA component of the snRNP that was suspected of interacting with the conserved sequence at the 3′ end of the histone precursor. If the snRNP interacted by hybridizing to the precursor RNA, then an oligonucleotide that matched the sequence in the precursor RNA should be able to hybridize to the snRNA. Formation of a DNA–RNA hybrid would render the snRNA sensitive to cleavage by added RNAse H. Cleavage of the snRNA in this critical region of interaction should render the extract incapable of processing the precursor. This result was the one observed for the mouse and consensus oligonucleotides.

B. The inability of the human oligonucleotide to block processing was not anticipated, since a human extract was being used. Examination of the hybrids that can form between the various oligonucleotides and the U7 snRNA reveals that the mouse and consensus oligonucleotides can hybridize perfectly for an 11-nucleotide and a 10-nucleotide stretch, respectively (**Figure 6–46**). By contrast, hybridization to the human oligonucleotide is split by an unmatched nucleotide into two segments of 6 and 5 nucleotides. The stability of pairing of two separate segments is not so great as for a continuous pairing segment. Hence, the human oligonucleotide does not pair with sufficient stability to render the U7 snRNA sensitive to RNAse H cleavage.

Reference: Mowry KL & Steitz JA (1987) Identification of the human U7 snRNP as one of several factors involved in the 3′ end maturation of histone premessenger RNA's. *Science* 238, 1682–1687.

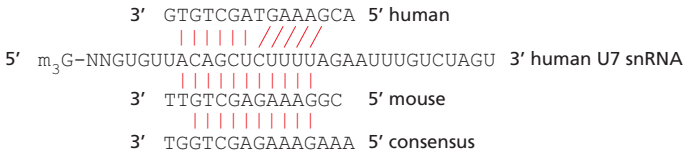


Figure 6–46 Pairing between the three oligonucleotides and the human U7 snRNA (Answer 6–42).

6–43 For nucleotides in U5 snRNA that are true targets for U85 snoRNA-dependent modification, the expectation is that the modification will be dependent on pairing between U5 snRNA and U85 snoRNA. Thus, a bona fide modification should be present in the transfection with U2–U5, which can be modified by the endogenous U85 snoRNA; however, it should be absent in the transfection with U2–U5m, which cannot pair stably with the endogenous U85 snoRNA. Most importantly, modification of U2–U5m should be restored in the presence of U85m snoRNA, which can pair with U2–U5m. Both pseudouridine ψ46 and methylated C45 behave according to these expectations (see Table 6–2). Thus, pseudouridine ψ46 and 2′-*O*-methylation at C45 are both dependent on U85 snoRNA.

Methylated U41 is present in all transfections, indicating that it is modified by some other component of the cell in a way that is not dependent on the nearby sequences that were modified in U2–U5m. Pseudouridine ψ43 is not modified under any conditions in these experiments, suggesting that it cannot be modified as part of the fragment of U5 that was inserted into U2. Thus, these experiments do not rule it out as a potential target for modification by U85 snoRNA. Nevertheless, studies with many other box H/ACA snoRNAs indicate that the guide sequences universally position the U to be converted to ψ in the location shown for ψ46 in Figure 6–20.

Reference: Jády B & Kiss T (2001) A small nucleolar guide RNA functions both in 2′-*O*-ribose methylation and pseudouridylation of the U5 spliceosomal RNA. *EMBO J.* 20, 541–551.

MEDICAL LINKS

6–44

A. Since RNA polymerase is blocked by pyrimidine dimers, the sensitivity of transcription to UV damage will depend on the distance between the promoter for a gene and the probe. It is a simple measure of the target size for UV damage. If the polymerase must travel twice as far to complete a transcript, the chances of its encountering a block to transcription are twice as great.

B. Transcription through the *Vsg* gene is seven times more sensitive to UV irradiation than transcription through the ribosomal transcription unit at the site of rRNA probe 4, which is about 7 kb from its promoter (see Figure 6–21A). Thus, the beginning of the *Vsg* gene, which is where the probe was located, is about 50 kb (7 × 7 kb) away from its promoter. This calculation assumes that the DNA between the *Vsg* promoter and the *Vsg* gene has about the same sensitivity to UV light as the DNA in the ribosomal RNA transcription unit. It also assumes that multiple UV-induced pyrimidine dimers are not common enough to skew the linear relationship between UV dose and distance.

C. If the nearby gene is 20% less sensitive to UV irradiation than the *Vsg* gene, it is inactivated at 80% the rate of the *Vsg* gene. Therefore, its promoter is 40 kb away (0.80 × 50 kb). Given that the nearby gene is 10 kb in front of the *Vsg* gene, its promoter must map very near the promoter for the *Vsg* gene. Thus, it is likely that the two genes are transcribed from the same promoter.

Reference: Johnson PJ, Kooter JM & Borst P (1987) Inactivation of transcription by UV irradiation of *T. brucei* provides evidence for a multicistronic transcription unit including a VSG gene. *Cell* 51, 273–281.

FROM RNA TO PROTEIN

DEFINITIONS

6–45 Proteasome

6–46 Genetic code

6–47 Initiator tRNA

6–48 Anticodon

6–49 Ribozyme

6–50 Nonsense-mediated mRNA decay

6–51 Reading frame

6–52 Aminoacyl-tRNA synthetase

6–53 Molecular chaperone

TRUE/FALSE

6–54 False. Wobble pairing occurs between the third position in the codon and the first position in the anticodon.

6–55 False. Because correct base-pairing is only about 10- to 100-fold more stable than incorrect matches, additional mechanisms, beyond the simple thermodynamics of base-pairing, must be used to reach the accuracy of protein synthesis routinely achieved in the cell. Two such mechanisms are induced fit, where the ribosome folds around the correct base pairs, and kinetic proofreading, which introduces delays that allow poorly matched bases to dissociate.

THOUGHT PROBLEMS

6–56 The amino acids encoded in each of the three reading frames are shown in **Figure 6–47**. If this segment of RNA encoded part of a larger protein, it would have to be translated in reading frame 1, which is the only one that does not contain a stop codon.

6–57 The only codon assignments consistent with the observed changes, and with the assumption that single-nucleotide changes were involved, are GUG for valine, GCG for alanine, AUG for methionine, and ACG for threonine. It is unlikely that you would be able to isolate a valine-to-threonine mutant in one step because that would require two nucleotide changes. Typically, two changes would be expected to occur at a frequency equal to the product of the frequencies for each of the single changes; hence, the double mutant would be very rare.

6–58

A. UUUUUUUUUUUU... codes for a polymer of phenylalanine.

B. AUAUAUAUAUAU... codes for a polymer of alternating isoleucines and tyrosines. Because the start point of the ribosome on the RNA is random, the ribosomes will generate a mixture of polymers, some of which start with isoleucine and some with tyrosine.

C. AUCAUCAUCAUC... codes for a mixture of three different polymers. Ribosomes start translation in each of the three reading frames: AUC–AUC–AUC–AUC... codes for a polymer of isoleucine; UCA–UCA–UCA–UCA...

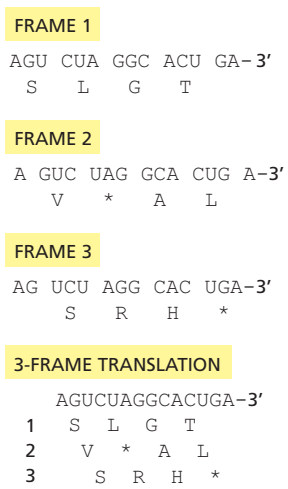


Figure 6–47 Amino acids encoded in the three reading frames of an RNA (Answer 6–56). The amino acids encoded in each reading frame are shown separately and all together, as they are usually represented. *Asterisks* identify stop codons.

codes for a polymer of serine; and CAU–CAU–CAU–CAU... codes for a polymer of histidine.

6–59

A. The genetic code can be used to convert the two amino acid sequences into a set of potential mRNA sequences. At the sites where two or more nucleotides are shown, different mRNA sequences are consistent with the same sequence of encoded amino acids.

wild type:

```
 N    M    N    G    K
AAU  AUG  AAU  GGU  AAA
  C         C    C    G
             A
             G
```

mutant:

```
 N    M    I    W    Q    I    C    V    M    K    D
AAU  AUG  AUU  UGG  CAA  AUU  UGU  GUU  AUG  AAA  GAU
  C         C         G    C    C    C         G    C
            A              A         A
                           G
```

Comparison of the potential mRNA sequences shows that the mutant carries an extra U at the eighth position, the first one that differs between the two potential mRNA sequences. If you compare only the codons for N (wild type) with I (mutant), there are three possibilities for the difference: insertion of a U, deletion of an A, or a nucleotide change of A → U. Comparison of the next codon in the two sequences rules out a nucleotide change, which would leave the adjacent codon unaffected and thus would not give rise to a frameshift mutation. It also allows one to distinguish between insertion and deletion of a nucleotide. Note that the Gs in the first and second positions of the glycine codon (GG–) in the wild type have become the Gs in the second and third positions in the tryptophan codon (–GG) in the mutant. This shift can only be explained by an insertion. Thus, the frameshift mutation arose by insertion of an A-T base pair at the eighth position in this DNA sequence.

B. Removing the inserted U from the mutant sequence gives the extended sequence of the wild type, which can be converted back into an amino acid sequence as shown below.

```
                              *
                         W    *
                         S    S
 N    M    N    G    K   F    V    L    *
AAU  AUG  AAU  GGC  AAA  UUU  GUG  UUA  UGA  AAG
  C                      C    C    C         G
                         A         A
                                   G
```

The first seven codons in the extended mRNA clearly code for amino acids, although the identities of some are ambiguous because the mRNA sequence is not fully defined. The eighth codon could either code for an amino acid or be a stop codon. The ninth codon is definitely a stop codon. Thus, since the asparagine (N) at the beginning of the peptide sequence is amino acid 263, the intact protein would be either 269 or 270 amino acids in length. Two stop codons in tandem are commonly found in the end of coding sequences in bacteria.

6–60 Mutations of the type described in 2 and 4 are often the most harmful. In both cases, the reading frame would be changed. Because these frameshifts occur early in the coding sequence or in the middle of it, the encoded protein will contain a nonsensical and usually truncated

sequence of amino acids. In contrast, a reading frameshift that occurs toward the end of the coding sequence, as described in 1, will result in a largely correct protein that may be functional.

Deletion of three consecutive nucleotides, as in scenario 3, leads to the deletion of one amino acid, if it cleanly deletes a codon, or to the deletion of one amino acid and the substitution of another, if the deletion overlaps two adjacent codons. Importantly, deletion of three nucleotides would not alter the reading frame. The deleted amino acid (or altered amino acid) may or may not be important for the folding or activity of the protein. In many cases such mutations are silent; that is, they have insignificant consequences for the organism.

Substitution of one nucleotide for another, as in scenario 5, is often completely harmless, because it does not change the encoded amino acid. In other cases, it may change an amino acid, and the consequences may be deleterious or benign, depending on the location and functional significance of that particular amino acid. Often, the most deleterious kind of single-nucleotide change creates a new stop codon, which gives rise to a truncated protein.

6–61

A. A genetic code that used pairs of nucleotides would have 16 different codons (4 possible nucleotides in the first position × 4 possible nucleotides in the second position). Thus, it could specify a maximum of 16 different amino acids.

B. A triplet code that depended only on codon composition would have 20 different codons (4 codons composed all of one base; 12 codons with two bases the same and one different; and 4 codons with three different bases). Such a code could specify a maximum of 20 different amino acids.

C. It is relatively easy to see how a doublet code could be translated by a mechanism similar to that used with the standard genetic code. It is more difficult to see how the nucleotide composition of a stretch of three nucleotides could be translated without regard to the order of nucleotides, because base-pairing could no longer be used. An AUG, for example, would not base-pair with the same anticodon as a UGA.

6–62 In present-day cells, there is wobble in the matching of codons to anticodons. In several cases, the same tRNA pairs with multiple codons that specify the same amino acid but differ in their nucleotide sequence, generally at the third position of the codon. It seems likely that in the early biological world, without highly evolved ribosomes to help in the pairing process, the converse may also have been true: several different tRNAs, with similar anticodons, may have been able to bind to the same codon. This would have played havoc with the translation of the genetic message into protein, unless the amino acids carried by these tRNAs were chemically similar. In the absence of perfect specificity, natural selection may have operated to ensure that tRNAs with related anticodons carried chemically similar amino acids.

Alternatively, in the early world, before modern aminoacyl-tRNA synthetases had evolved, there was probably some ambiguity in the matching of tRNAs with appropriate amino acids. The same tRNA might have become coupled to any of a number of amino acids that were chemically similar. One can imagine the evolution of the genetic code by refinement of a matching process that was originally imprecise and gave only a blurred relationship between sets of roughly similar codons and sets of roughly similar amino acids.

6–63 The rules for wobble base-pairing between the anticodon and the codon, expressed both ways, are shown in **Table 6–5**. It is striking that A in the wobble position of the anticodon in eukaryotes does not have a pairing

TABLE 6–5 Rules for wobble base-pairing between codon and anticodon (Problem 6–63).

	Wobble codon base	Possible anticodon base		Wobble anticodon base	Possible codon base
Bacteria	U C A G	A, G, or I G or I U or I C or U	Bacteria	U C A G I	A or G G U U or C U, C, or A
Eukaryotes	U C A G	G or I G or I U C	Eukaryotes	U C A G I	A G U or C U or C

partner in codons. It turns out that A is not used in the wobble position in eukaryotic tRNAs. In many cases an A is encoded in the wobble position, but it is changed to inosine (I) after transcription, giving rise to a mature tRNA that recognizes U or C in the wobble position of the codon.

Reference: Lander ES et al (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

6–64 In eukaryotes, a minimum of 45 tRNAs would be required to recognize all 61 codons, given the rules for wobble base-pairing. Pairs of codons that end in U or C always encode the same amino acid (for example, CAU and CAC encode histidine, and GGU and GGC encode glycine). Thus a single tRNA with an I (or a G) in the wobble position of the anticodon would be required for each such pair of codons (see Table 6–5, Answer 6–63). The 16 pairs of such codons (32 codons) would require 16 tRNAs. Each of the remaining 29 codons, which end in either an A or a G, would require a specific tRNA with a corresponding U or C in the wobble position. Thus, the minimum number of tRNAs is 16 plus 29, or 45.

6–65 The single codon for tryptophan is 5′-UGG. The anticodon of the normal tryptophan tRNA is 5′-CCA, which pairs specifically with this codon. A mutation that changes the anticodon to 5′-*U*CA would allow the tRNA to recognize the 5′-UGA stop codon, which would lead to insertion of tryptophan at UGA and prevent termination of translation. Because of wobble (see Table 6–5), the mutant anticodon would also recognize the normal 5′-UGG codon, so that, in principle, its ability to insert tryptophan at the normal UGG codons would not be compromised.

Many genes use UGA codons as the natural stop sites for their encoded proteins. These stop codons would also be affected by the mutant tRNA. In reality, there is a competition between the mutant tRNA and the termination factors. Whenever the tRNA wins the race, the affected proteins would be made with additional amino acids at their C-terminal ends. The additional lengths would depend on the number of codons before the ribosomes encounter another stop codon in the mRNA. The potential chaos that such mutations might cause is mitigated by two factors: the efficiency of translation of stop codons by such mutant tRNAs is usually low, and many bacterial genes are “protected” by double stop codons at their ends. In reality, such suppressors have been invaluable for genetic studies in bacteria.

6–66 This experiment beautifully demonstrates that the ribosome does not check the amino acid that is attached to a tRNA. Once an amino acid has been coupled to a tRNA, the ribosome will "blindly" incorporate that amino acid according to the match between the codon and anticodon. We can therefore conclude that a significant part of the correct reading of the genetic code—namely, the matching of a codon with the correct amino acid—is performed by the synthetase enzymes that attach amino acids to tRNAs.

Reference: Chapeville F, Lipmann F, von Ehrenstein G, Weisblum B, Ray WJ & Benzer S (1962) On the role of soluble ribonucleic acid in coding for amino acids. *Proc. Natl Acad. Sci. USA* 48, 1086–1092.

6–67 One effective way of driving a reaction to completion is to remove one of the products. The flow of substrates to products then increases to reestablish the equilibrium ratio—the principle of mass action. All three of the products of this reaction are removed. The concentration of AMP is constantly reduced by conversion to ADP and then to ATP by other reactions in the cell. Similarly, the aminoacyl-tRNAs are used in protein synthesis, constantly decreasing their concentrations. But by far the most dramatic influence is the removal of PP_i by hydrolysis to two phosphates. That reaction yields as much free energy as the hydrolysis of ATP to ADP, which means that essentially all of the PP_i will be converted to free phosphates. As a result, the linked reactions for charging a tRNA and hydrolyzing PP_i—the reactions as they occur in cells—have a $\Delta G°$ of –28.8 kJ/mole.

6–68

A. The ratio of N-terminal to total radioactivity will increase with increasing time of exposure. Because you isolate only complete protein for your analysis, the position that will be labeled at the shortest time point will be the C-terminus. At the shortest time point, the radioactivity at the N-terminus will be nearly nonexistent, giving a very low ratio of N-terminal to total radioactivity. With increasing time, more and more protein will carry label at the N-terminus so the ratio is expected to rise. By the time the N-terminus becomes labeled, all the rest of the leucines in the protein will also be labeled; thus, at late times, the ratio of N-terminal to total radioactivity will equal the ratio of N-terminal to total leucines, which is 1:5 or 0.2.

6–69 The proportion of a cell's total energy devoted to protein synthesis is typically determined by measuring oxygen consumption in the presence and absence of inhibitors of protein synthesis. Because oxygen is used principally for generation of ATP via oxidative phosphorylation, and nearly all the cell's energy is derived from oxidative phosphorylation, oxygen consumption is a fairly direct measure of energy usage. The fractional drop in oxygen consumption in the absence of protein synthesis (when it's inhibited) indicates the proportion of the cell's energy normally devoted to protein synthesis.

6–70

A. 5′-GUAGCCUACCCAUAGG-3′

B. This short mRNA encodes three different peptides because there are three different reading frames. In the second reading frame, the first codon is the stop codon UAG; thus, it is unlikely that the subsequent codons will be translated.

```
          5′-GUAGCCUACCCAUAGG-3′
Frame 1   V  A  Y  P  *
Frame 2    *  P  T  H  R
Frame 3     S  L  P  I
```

The other possible mRNA from this DNA would read

```
      5′-CCUAUGGGUAGGCUAC-3′
Frame 1   P  M  G  R  L
Frame 2    L  W  V  G  Y
Frame 3     Y  G  *  A
```

Thus, the sequences of the peptides encoded by the complementary DNA strand would be completely different. (Be careful to keep the polarity of the strands correct; don't fall into the trap of thinking that the complementary sequence of the first mRNA is 5′-CAUCGGAUGGGUAUCC-3′. That sequence is incorrect because it has the wrong polarity.)

C. If translation begins at the 5′ end of the mRNA, the synthesized protein would be valine-alanine-tyrosine-proline (VAYP). Only after a peptide bond has been formed between alanine and tyrosine will $tRNA^{Ala}$ leave the ribosome. Thus, the next tRNA that will bind to the ribosome after $tRNA^{Ala}$ has left is $tRNA^{Pro}$. When the amino group of alanine forms a peptide bond, the ester bond between valine and $tRNA^{Val}$ is broken, $tRNA^{Val}$ moves from the P site to the E site (exit site), and $tRNA^{Ala}$ moves from the A site to the P site.

6–71 In eukaryotic cells, protein synthesis is normally initiated by scanning from the 5′ end of the mRNA until the first AUG codon is found. (Sometimes the second or third AUG codon may be used instead—a phenomenon known as leaky scanning.) This mechanism of initiation ensures that ribosomes will all start translating near the 5′ end of the mRNA. When the ribosomes complete synthesis of the protein, they fall off the mRNA and must reinitiate by scanning from the 5′ end. By contrast, in prokaryotic cells, protein synthesis is initiated by base-pairing between mRNA sequences adjacent to an initiation AUG codon and sequences in the 16S rRNA of the small ribosomal subunit. The prokaryotic initiation strategy allows ribosomes to recognize several start sites in the same mRNA. This key difference in mechanism underlies their ability to make several proteins from a single polycistronic mRNA.

6–72 A broken mRNA when translated would produce a truncated protein that could be harmful to the cell. A protein fragment can retain some of the functions of the whole protein, allowing it, for example, to bind to a target protein but trap it in an unproductive complex. Alternatively, a protein fragment can display new, aberrant binding surfaces that allow it to bind to novel partners, interfering with their function. Finally, the deletion could remove the portion of the protein that normally controls its activity. In this case, the truncated protein could be locked into its active state, with dire consequences for the cell.

6–73

A. Edeine specifically inhibits initiation of protein synthesis by preventing the joining of the 60S ribosomal subunit to the 40S subunit/mRNA/initiator tRNA complex. Since elongation is not blocked, ribosomes that have already begun synthesis complete their individual chains and fall off the mRNA, leaving attached only the small subunit and the initiator tRNA. Edeine is an antibiotic produced by certain strains of *Bacillus brevis*.

B. A lag occurs before protein synthesis shuts off because edeine inhibits initiation but has no effect on elongation. Thus, a ribosome that has just started making a new polypeptide is free to complete it. Incorporation of label continues for just the length of time it takes to complete the protein (in this case, the globin chains of hemoglobin), which takes about a minute.

C. If cycloheximide (or any other elongation inhibitor) is added at the same time as an initiation inhibitor, the polyribosomes are "frozen." Polyribosome breakdown by initiation inhibitors requires ribosome movement, which is blocked by elongation inhibitors.

Reference: Safer B, Kemper W & Jagus R (1978) Identification of a 48S preinitiation complex in reticulocyte lysate. *J. Biol. Chem.* 253, 3384–3386.

6–74 D. The formation of one peptide bond, but no more, eliminates all choices except D. If farsomycin inhibited formation of the 80S initiation complex (choice A), inhibited binding of aminoacyl-tRNAs to the A site (choice B), or inactivated peptidyl transferase (choice C), no peptide would have been formed. If it interfered with chain termination and release (choice E), the entire peptide would have been made.

6–75 In a well-folded protein, the majority of hydrophobic amino acids will be sequestered in the interior, away from water. Exposed hydrophobic patches thus indicate that a protein is abnormal in some way. Some proteins initially fold with exposed hydrophobic patches that are used in binding to other proteins, ultimately burying those hydrophobic amino acids as well. As a result, hydrophobic amino acids are usually not exposed on the surface of a protein, and any significant patch is a good indicator that something has gone awry. The protein may have failed to fold properly after leaving the ribosome, it may have suffered an accident that partly unfolded it at a later time, or it may have failed to find its normal partner subunit in a larger protein complex.

6–76 Molecular chaperones fold like any other protein. Molecules in the act of synthesis on ribosomes are bound by hsp70 chaperones. And incorrectly folded molecules are helped by hsp60-like chaperones. That they function as chaperones when they have folded correctly makes no difference to the way they are treated before they reach their final, functional conformation. Of course, properly folded hsp60-like and hsp70 chaperones must already be present to help fold the newly made chaperones. At cell division, each daughter cell inherits a starter set of such chaperones from the parental cell.

6–77 Computer algorithms that search for exons are complex affairs, as you might imagine. They combine statistical information derived from known genes in searching for unidentified genes. The list of common features includes:

1. Exons that encode protein will have an open reading frame, and the reading frames in adjacent exons will match up.
2. Internal exons (excluding the first and the last) will have splicing signals at each end; most of the time (98.1%) these will be AG at the 5′ ends of the exons and GT at the 3′ ends.
3. The multiple codons for most individual amino acids are not used with equal frequency. This so-called coding bias can be factored in to aid in the recognition of true exons.
4. Exons and introns have characteristic length distributions. The median length of exons in human genes is about 120 bp. Introns tend to be much larger: a median length of about 2 kb in genomic regions of 30–40% GC content, and a median length of about 500 bp in regions above 50% GC.
5. So-called CpG islands are often located just upstream of the 5′ ends of genes. The dinucleotide 5′-CpG is greatly underrepresented in the human genome, occurring at about 20% its expected frequency. Cs in CpG dinucleotides are a target for methylation. Deamination of methyl C produces T, which accounts for the deficit of CpG. In CpG islands, however, CpG dinucleotides are not methylated and occur at their expected frequency. CpG islands often contain binding sites for gene regulatory proteins.
6. The initiation codon for protein synthesis (nearly always AUG) has a statistical association with adjacent nucleotides that seem to enhance its recognition by translation factors.
7. The terminal exon will have a signal (most commonly AATAAA) for cleavage and polyadenylation close to its 3′ end.

The statistical nature of these features coupled with the low frequency of coding information in the genome (2–3%) and the frequency

of alternative splicing (30–50% of genes), makes it especially impressive that current algorithms can identify about 70% of individual exons and about 20% of complete genes in the human genome.

Reference: Lander ES et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

CALCULATIONS

6–78

A. As shown in **Figure 6–48**, the rate of synthesis is linear with time. The curvature so apparent in the autoradiograph in Figure 6–24 results from the nonlinear migration of proteins in SDS polyacrylamide gels.

B. The rate of protein synthesis can be determined from the slope of the line in Figure 6–48. This system is synthesizing roughly 52,000 daltons of protein per 10 minutes, or 5200 daltons per minute, which corresponds to about 47 amino acids per minute [(5200 daltons/minute)/(110 daltons/amino acid)]. This rate is less than the rate in *E. coli,* which is about 10 times faster. The rate is also about three times less than that of globin synthesis in the same reticulocyte lysate. As discussed in part C, part of the reason for the low rates may be that the mix of tRNAs in the reticulocyte lysate is not optimal for this plant virus protein.

C. The autoradiograph contains many bands, rather than just a few, because ribosomes keep loading onto the mRNA throughout the course of the experiment. You could obtain the theoretical result in Figure 6–25B by adding an inhibitor of initiation of protein synthesis after 5 minutes or, alternatively, by adding unlabeled methionine in vast excess after 5 minutes.

The presence of discrete bands rather than a continuous background fuzz suggests that there are specific hang-up points along the mRNA, perhaps where ribosomes must wait for rare tRNAs. The tRNA population in the reticulocyte is specialized for making globin, not a protein from a plant virus!

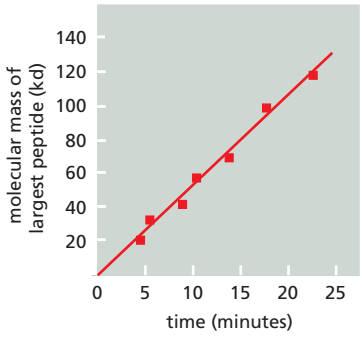


Figure 6–48 Rate of synthesis of a TMV protein (Answer 6–78).

6–79

A. Since an average protein contains about 455 amino acids [(50,000 d/protein) × (amino acid/110 d)], it will take a muscle cell about 3.8 minutes to make it [(455 amino acids) × (sec/2 amino acids) × (min/60 sec)]. Since titin is 60 times the size of an average protein, the muscle cell will require 3.8 hours to make it [(3,000,000 d/titin) × (amino acid/110 d) × (sec/2 amino acids) × (hr/3600 sec)].

B. It will take a muscle cell about 23 minutes to transcribe an average gene and 23 hours to transcribe titin. For the average protein, 455 amino acids corresponds to 1365 nucleotides of RNA [(3 nt/codon) × (455 codons)]. Given that 5% of the initial transcript is converted to mRNA, the initial transcript is 2.7×10^4 nucleotides (1365 nt × 20), which would require about 23 minutes to transcribe [$(2.73 \times 10^4$ nt) × (sec/20 nt) × (min/60 sec)]. Because titin is 60 times as big, the muscle cell will require about 23 hours to transcribe it.

6–80 The energy cost of translation and transcription will be equal when 30 protein molecules have been made from one mRNA. Protein synthesis requires four high-energy phosphate bonds per codon (per three nucleotides). Transcription consumes six high-energy phosphate bonds to make a codon, but also consumes 19 times more energy synthesizing RNA that will be discarded (95%). Thus, transcription consumes a grand total of 120 high-energy phosphate bonds per codon (6 + 114), compared to four per codon for translation. The ratio of energy costs per codon (120/4 = 30) defines the number of protein molecules that will have been made when the energy cost of translation matches that of transcription.

Because most mRNAs are used to make hundreds to thousands of proteins, translation consumes a much higher fraction of the cell's energy than does transcription.

6–81

A. Since the bacteria were labeled for one generation, which represents a doubling in mass, 4 μg of the 8 μg of flagellin isolated from the gel were synthesized in the presence of ^{35}S-cysteine. The amount of radioactivity in the sample indicates that about 1 out of every 1670 flagellin (flgn) molecules contains a cysteine.

$$\frac{\text{Cys}}{\text{flgn}} = \frac{300 \text{ cpm Cys}}{4 \text{ μg flgn}} \times \frac{\text{pmol Cys}}{5 \times 10^3 \text{ cpm}} \times \frac{4 \times 10^4 \text{ μg flgn}}{\text{μmol flgn}} \times \frac{\text{μmol}}{10^6 \text{ pmol}}$$

$$= \frac{6 \times 10^{-2} \text{ pmol Cys}}{100 \text{ pmol flgn}}$$

$$\frac{\text{Cys}}{\text{flgn}} = 6 \times 10^{-4}$$

which is equal to 1 cysteine per 1670 flagellin molecules $[1/(6 \times 10^{-4})]$.

B. The normal codons for cysteine are UGU and UGC. Thus, the error in anticodon–codon interaction is a mistake at the first position of the codon (third position of the anticodon). This experiment, as well as others, suggests that ribosomes tend to mistake U for C and C for U in the first two positions of the codon, and C and U for A in the first position.

C. Assuming that all six arginine codons are equally frequent, there should be six sensitive (CGC and CGU) arginine codons $[(2/6) \times 18]$ in a flagellin molecule. Therefore, the actual error frequency per codon-at-risk is

$$\text{error frequency} = \frac{1 \text{ cysteine}}{1670 \text{ flagellin molecules}} \times \frac{\text{flagellin molecule}}{6 \text{ sensitive codons}}$$

$$\text{error frequency} = 10^{-4}$$

D. If the probability of making a mistake at each codon is 10^{-4}, the probability of not making a mistake at each codon is $(1 - 10^{-4})$. The probability of not making a mistake at n codons is then $(1 - 10^{-4})^n$. Thus, the percentage of correctly synthesized molecules 100 amino acids in length is $(1 - 10^{-4})^{100}$, or 99%. For a protein 1000 amino acids long, 90% are correct. For a protein 10,000 amino acids long, only 37% are correct. Given these sorts of estimates, it is perhaps not surprising that proteins more than 3000 amino acids long are rare. If you are curious, you might calculate the fraction of titin molecules that you would expect to be made correctly (see Problem 6–79).

Reference: Edelmann P & Gallant J (1977) Mistranslation in *E. coli*. *Cell* 10, 131–137.

DATA HANDLING

6–82

A. A simple change of anticodon allows tRNA^{Val} to be charged by IleRS at about 60% normal efficiency. Thus, the anticodon is the most important part for charging. As the rest of the tRNA^{Val} molecule becomes more like tRNA^{Ile}, the efficiency steadily increases, suggesting that additional sequences that aid in the charging with isoleucine are dispersed throughout tRNA^{Ile}.

B. Results with the chimeric tRNAs show that a tRNA^{Val} carrying just the D loop and anticodon from tRNA^{Ile} is very nearly as effective for valine editing as normal tRNA^{Ile}. In fact, a close examination of the sequences of the D loops from the two tRNAs reveals just three nucleotide differences. These nucleotides are located at the elbow in the "L"-shaped three-dimensional structure of tRNAs (**Figure 6–49**).

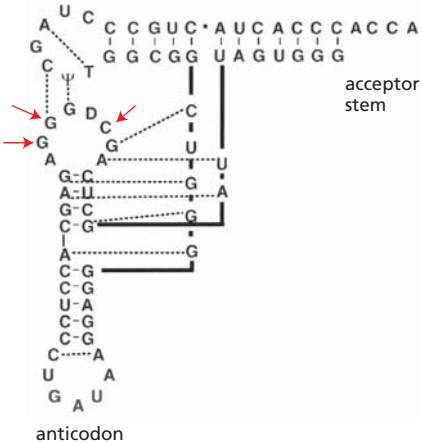


Figure 6–49 Schematic structure of tRNA^{Ile} (Answer 6–82). *Arrows* indicate the locations of the nucleotide differences between the D loops in tRNA^{Ile} and tRNA^{Val}. *Dotted lines* indicate hydrogen bonds between nucleotides that help to establish the three-dimensional structure.

C. Generally no. However, the D loop is crucial to valine editing and it also improves the efficiency of $tRNA^{Ile}$-charging by IleRS.

Reference: Hale SP, Auld DS, Schmidt E & Schimmel P (1997) Discrete determinants in transfer RNA for editing and aminoacylation. *Science* 276, 1250–1252.

6–83

A. The data in Figure 6–27 indicate that the N-terminus of the protein is synthesized first. The steadily decreasing level of radioactivity from the N-terminus to the C-terminus is exactly what you would expect if synthesis began at the N-terminus. As illustrated in **Figure 6–50A**, all the ribosomes carry a labeled lysine at position 8 in their nascent chains, but the ribosome at the 5′ end of the mRNA has not yet reached the lysine at position 16. Thus, when digested with trypsin, all of these nascent chains will yield a labeled N-terminal peptide, but a smaller fraction will yield the second peptide. Fewer still will contain the third peptide, and so on. Almost none of the ribosomes will carry a nascent chain with the labeled lysine nearest the C-terminus.

B. The lines for the α and β chains in Figure 6–27 are very similar, with nearly identical intercepts on both axes, which indicates that roughly equal *numbers* of each chain are being synthesized. However, there is not enough information to decide whether the numbers of α- and β-globin mRNA molecules are equal. You would need to know how many ribosomes there were on each mRNA—the average polyribosome size for α- and β-globin mRNAs—to deduce their relative abundance from these kinds of data. Actually, there is about twice as much α-globin mRNA as β-globin mRNA, but the α-globin mRNA is less efficiently translated; that is, fewer ribosomes initiate synthesis on α-globin mRNA per unit time than on β-globin mRNA. These factors cancel out to give a balanced production of the two chains.

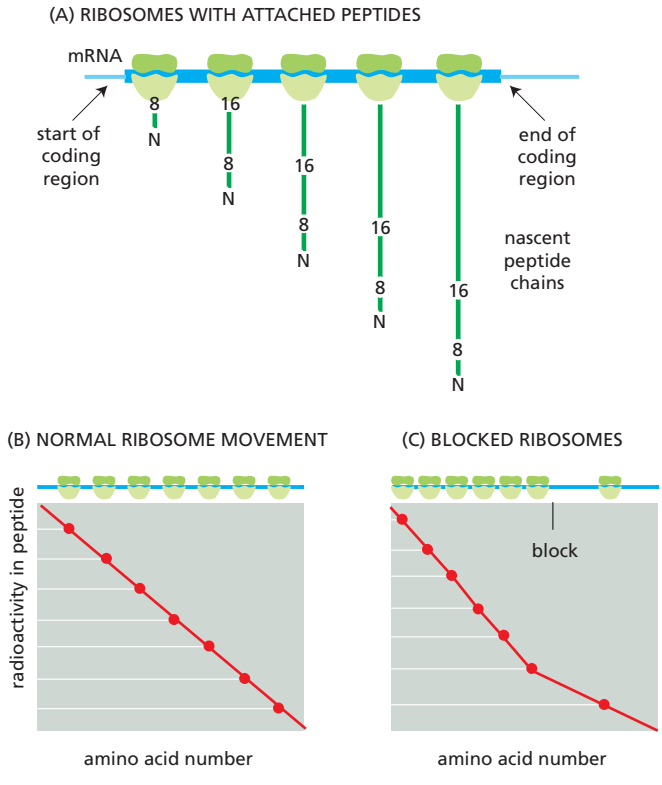


Figure 6–50 Relationship of ribosome position to peptide length and labeling pattern (Answer 6–83). (A) Lengths of peptides associated with ribosomes at various positions along β-globin mRNA. Numbers refer to positions of the first two lysines. (B) Pattern of peptide labeling for evenly spaced ribosomes. (C) Pattern of peptide labeling for ribosomes whose movement is inhibited at a point about midway down the mRNA. Peptides associated with each ribosome are shown on the graphs in (B) and (C) as *lines*. *Red circles* correspond to the C-termini of the polypeptides and are aligned immediately *below* the ribosome on which the peptides are synthesized. *Red lines* through the circles show the expected patterns of peptide labeling.

C. The graph in Figure 6–27 hits zero right at the end of the coding region, which indicates that chains are released from ribosomes as soon as they encounter the stop codon—or at least they do so without a measurable pause on this time scale.

D. If there were a significant roadblock to ribosome movement, the data would resemble that in Figure 6–28A. A roadblock would result in more densely packed ribosomes in front of the block and less densely packed ribosomes beyond the block. The consequences of normal and inhibited ribosome movement are illustrated schematically in **Figure 6–50B and C**.

6–84

A. The DNA sequence GGG TAT CTT TGA CTA CGA CGC C should not encode the protein sequence of RF2, since UGA is a termination codon. It appears that this sequence must break the usual rules of the triplet code, with a leucyl-tRNA decoding the quadruplet shown in italics below.

GGG TAT CTTT GAC TAC GAC GCC

In essence, the ribosome must shift its reading frame in the middle of the gene!

Frameshift mutations were originally isolated by Seymour Benzer in his work on the r_{II} genes of bacteriophage T4 and exploited by Francis Crick in a proof of the triplet nature of the genetic code. Later, mutant tRNA molecules that could read four bases at a time were isolated by clever genetic selection and shown to suppress certain frameshift mutations. It came as a great surprise, however, to find *natural* examples of frameshift suppression. The first example was found in the bacteriophage T7 gene 10. Since then, several retroviruses and retroposons have been found to use frameshift suppression of termination codons as a way of making minor gene products. The mechanism of suppression in these cases is not clear.

B. The occurrence of an in-frame suppressible UGA codon (which is recognized uniquely by RF2) in the sequence of RF2 immediately suggests a novel form of gene control. Although the mechanism of frameshifting is undefined, frameshifting and termination at the UGA codon probably compete with one another. When the level of RF2 in the cell is high, termination should occur more frequently at the UGA codon than when the level of RF2 is low. Thus, new RF2 would be synthesized infrequently when its levels were already adequate, but if the levels fell, the chances of ribosomal frameshifting would increase and more RF2 would be made. Thus, this situation seems to be a very cleverly appropriate autoregulation.

References: Craigen WJ, Cook RG, Tate WP & Caskey CT (1985) Bacterial peptide chain release factors: conserved primary structure and possible frameshift regulation of release factor 2. *Proc. Natl Acad. Sci. USA* 82, 3616–3620.

Jacks T & Varmus H (1985) Expression of the Rous sarcoma virus *pol* gene by ribosomal frameshifting. *Science* 230, 1237–1242.

6–85

A. The sequence data for the *Tetrahymena* protein are unusual because they indicate that UAG and UAA, which are stop codons in other organisms, specify glutamine (Q) in *Tetrahymena*.

B. The minor protein above the full-length, 116 kd protein is produced from the pure TMV mRNA by readthrough of the normal stop codon. Although it is difficult to be sure exactly how such a rare event occurs, the amount of this protein is thought to represent the frequency with which the reticulocyte translation system mistakenly inserts an amino acid at the site of the stop codon instead of terminating properly. It is a little surprising that

a second termination codon is not encountered for 506 codons (about 50 kd of additional protein).

C. Given that *Tetrahymena* uses UAG and UAA as codons for glutamine, the increase in the proportion of the readthrough TMV protein is most likely due to the presence of a $tRNA^{Gln}$ species with an anticodon complementary to the normal TMV stop codon (which is UAG). The addition of *Tetrahymena* RNA causes a small shift in the proportions because it contains some charged $tRNA^{Gln}$. The cytoplasm causes a larger shift because it also contains the appropriate aminoacyl-tRNA synthetase. (The additional shift with the cytoplasm suggests that the tRNA synthetases in the reticulocyte lysate cannot recharge the special *Tetrahymena* tRNA.) These results suggest that at least two components from *Tetrahymena*—a special tRNA and its cognate aminoacyl-tRNA synthetase—must be added to a reticulocyte lysate to allow *Tetrahymena* mRNA to be translated efficiently. These components compete effectively with the reticulocyte release factors, allowing the *Tetrahymena* mRNAs to be read.

D. Although slight variations in the genetic code were originally discovered in mitochondrial genomes, they were not as surprising as the *Tetrahymena* changes. After all, mitochondrial genomes are small and encode relatively few proteins, so it is less difficult to imagine how changes might occur. By contrast, the *Tetrahymena* genome encodes thousands of proteins. It is much more surprising that it managed to survive the presumptive transition from the standard code to its present-day code.

References: Andreasen PH, Dreisig H & Kristiansen K (1987) Unusual ciliate-specific codons in *Tetrahymena* mRNAs are translated correctly in a rabbit reticulocyte lysate supplemented with a subcellular fraction from *Tetrahymena*. *Biochem. J.* 244, 331–335.

Horowitz S & Gorovsky MA (1985) An unusual genetic code in nuclear genes of *Tetrahymena*. *Proc. Natl Acad. Sci. USA* 82, 2452–2455.

6–86

A. The set of control experiments argues convincingly that the association between dnaK and the labeled proteins is meaningful; that is, it reflects some biological function.

In the presence of SDS, which eliminates protein–protein interactions, antibodies precipitate only dnaK (see Figure 6–31A, lane 2), suggesting that protein–protein associations are required for precipitation of the labeled proteins. The absence of labeled proteins from *dnaK*-deletion cells (see lane 3) indicates that precipitation depends on dnaK and is not the result, for example, of nonspecific association with the antibodies. The lack of precipitation of labeled proteins from a mixture of labeled *dnaK*-deletion cells and unlabeled wild-type cells (see lane 4) argues that the associations of proteins with dnaK were established in cells and not during subsequent experimental procedures.

B. ATP would be expected to interfere with precipitation of labeled proteins if hsp70 used it in the normal way; that is, to power the cycling of hsp70 on and off the protein. In the absence of ATP, dnaK will have hydrolyzed a bound molecule of ATP—that it acquired in the cell—to ADP, altering its own conformation and allowing it to latch onto a hydrophobic patch in a nascent protein. If ATP is present in the extract, it will displace the ADP, reversing the conformational change and releasing dnaK from the nascent protein. In the more dilute conditions in the extract, the presence of ATP greatly favors the off reaction, and as a result labeled proteins are not precipitated.

C. The pulse-chase experiment in Figure 6–31B indicates that dnaK binds the labeled proteins only for a few minutes after ^{35}S-methionine has been incorporated. A natural interpretation of this experiment is that some of the labeled proteins are incorrectly folded initially, and bind dnaK as a

consequence. Antibodies against dnaK precipitate these bound proteins. With time and with help from dnaK, the proteins correctly fold and are no longer substrates for dnaK binding; hence, they disappear from the immunoprecipitates.

D. Nothing in these experiments shows directly that the proteins bound by dnaK are in the process of being translated on ribosomes. The very short duration of the labeling pulse (15 seconds) would be expected to label proteins in the process of being translated, but it would also label proteins that were completed during the pulse and, therefore, clear of the ribosome. The category of protein that dnaK binds is not clear from these experiments. In additional experiments, the authors show convincingly that some of the proteins that are bound by dnaK were indeed attached to ribosomes.

References: Deuerling E, Schulze-Specking A, Tomoyasu T, Mogk A & Bukau B (1999) Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature* 400, 693–696.

Teter SA, Houry WA, Ang D, Tradler T, Rockabrand D, Fischer G, Blum P, Georgopoulos C & Hartl FU (1999) Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell* 97, 755–765.

6–87

A. The results suggest that tritium exchange occurs within one cycle. Although there is time for multiple cycles—and they presumably occur in the presence of ATP—the results with AMPPNP indicate that a single cycle is sufficient. Because AMPPNP cannot be hydrolyzed, the chaperone will not be able to eject the protein and repeat the cycle.

B. The accelerated exchange of tritium in the presence of the chaperone and ATP indicates that the protein is unfolded before it is refolded. The isolation-chamber model starts from the premise that aggregation limits the folding of a protein. If the cavity of GroEL facilitated proper folding by reducing inappropriate interactions, it would not seem essential that the protein first be unfolded. By contrast, if a stable but incorrectly folded domain blocked correct folding, the protein would, by necessity, have to be unfolded first. Thus, the results with the particular protein used in these experiments, which was the plant CO_2-fixation protein, RuBisCo, support an active-unfolding model, which is powered not by ATP hydrolysis but by ATP binding. More recent experiments indicate that accurate refolding of RuBisCo occurs inside the GroEL chamber, which in some way helps the protein to avoid misfolded intermediates by encouraging it along the productive folding pathway.

References: Brinker A, Pfeifer G, Kerner MJ, Naylor DJ, Hartl FU & Hayer-Hartl M (2001) Dual function of protein confinement in chaperonin-assisted protein folding. *Cell* 107, 223–233.

Shtilerman M, Lorimer GH & Englander SW (1999) Chaperonin function: folding by forced unfolding. *Science* 284, 822–825.

6–88

A. Although the first codon of β-galactosidase could have been changed by recombinant DNA techniques, it would no longer have served as a start site for translation. All proteins, bacterial and eukaryotic, are initially translated with methionine at their N-termini. In many cases, methionine is removed (and occasionally additional amino acids as well), leaving a new N-terminus.

The procedure described here was arrived at by chance! The investigators were originally interested in whether ubiquitin at the N-terminus would cause a protein to be degraded. This question led them to generate the fusion gene. In bacteria, which do not have a ubiquitin-dependent

protease, the fusion protein was made as they anticipated; however, in yeast, the same plasmid produced only β-galactosidase, suggesting that the ubiquitin was removed. To try to prevent this cleavage, they altered the codons at the junction. The ubiquitin was still removed, but now the resulting β-galactosidases differed remarkably in stability. The focus of their study quickly changed, leading to insights into the role of the N-terminus in determining the stability of proteins.

B. The half-lives of the different β-galactosidases can be estimated from the graph in Figure 6–35B by finding the time at which half the β-galactosidase remains. The three β-galactosidases have very different half-lives: R-β-galactosidase has a half-life of about 2 minutes; I-β-galactosidase has a half-life of about 30 minutes; and M-β-galactosidase has a half-life that is too long to be measured in this experiment (it was estimated to be greater than 20 hours).

Reference: Bachmair A, Finley D & Varshavsky A (1986) *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* 234, 179–186.

THE RNA WORLD AND THE ORIGINS OF LIFE

DEFINITIONS

6–89 RNA world

TRUE/FALSE

6–90 False. Although only a few types of reactions are represented among the ribozymes in present-day cells, ribozymes that have been selected in the laboratory can catalyze a wide variety of biochemical reactions, with reaction rates similar to those of proteins. In light of these results, it is unclear why ribozymes are so underrepresented in modern cells. It seems likely that the availability of 20 amino acids versus 4 bases affords proteins a greater number of catalytic strategies than ribozymes, as well as endowing them with the ability to bind productively to a wider range of substrates (for example, hydrophobic substrates, which ribozymes have difficulty with).

THOUGHT PROBLEMS

6–91 RNA has the ability to store genetic information like DNA and the ability to catalyze chemical reactions like proteins. Having both of these essential features of "life" in a single type of molecule makes it easier to understand how life might have arisen from nonliving matter. The use of RNA molecules as catalysts in several fundamental reactions in modern-day cells supports this idea. Nevertheless, it is not yet possible to specify a plausible pathway from the "primordial" soup to an RNA world. Because RNA molecules are highly susceptible to chain breakage (see below), many have speculated that there may have been an "RNA-like" precursor molecule to RNA—one that likewise had catalytic and informational properties, but was more stable.

6–92 Although RNA is thought to have played an important role in the evolution of life on Earth, possibly as a replicating catalyst, it is unclear that it was the *first* replicating catalyst. Other, less efficient molecular systems that combined informational and catalytic properties may have preceded RNA. Regardless of its original role, it is clear that RNA now plays a larger role than that of mere messenger in information flow; RNA provides critical functions in replication, gene regulation, splicing, translation, peptide bond formation, membrane transport of proteins, and telomere maintenance, among others.

6–93 The RNA molecule will not be able to catalyze its own replication. As a single molecule with a single catalytic site, it cannot be both template and catalyst simultaneously. (To visualize the critical difficulty, try to imagine how the active site of the RNA could copy itself.) Once a second molecule—either template or catalyst—was generated, then replication could begin.

Reference: Bartel DP & Szostak JW (1993) Isolation of new ribozymes from a large pool of random sequences. *Science* 261, 1411–1418.

6–94 The complement of this hairpin RNA could also form a similar hairpin, as shown in **Figure 6–51**. The two structures would be identical in the double-stranded regions that involved standard G-C and A-U base pairs. They would differ in the sequence of the single-stranded regions. Because G-U base pairs can form in RNA, unlike C-A base pairs, one hairpin would be predicted to contain an additional base pair, as shown.

Figure 6–51 Hairpins formed by an RNA strand and by its complement (Answer 6–94). An RNA *(black)* and its complement *(blue)* are shown as double-stranded RNA in the middle. The structures formed by each strand are shown *above* and *below* the duplex. The nonstandard G-U base pair in the lower hairpin is highlighted with a *dashed box*.

6–95 The deoxyribose sugar of DNA makes the molecule much less susceptible to breakage. The hydroxyl group on carbon 2 of the ribose sugar is an agent for catalysis of the adjacent 3′-5′ phosphodiester bond that links nucleotides together in RNA. Its absence from DNA eliminates that mechanism of chain breakage. In addition, the double-helical structure of DNA provides two complementary strands, which allows damage in one strand to be repaired accurately by reference to the sequence of the second strand. Finally, the use of T in DNA instead of U, as in RNA, builds in a protection against the effects of deamination—a common form of damage. Deamination of T produces an aberrant base (methyl C), whereas deamination of U generates C, a normal base. The cell's job of recognizing damaged bases is much easier when the damage produces an abnormal base.

6–96

A. Ligation of the substrate oligonucleotide to the pool RNA is analogous to chain elongation during RNA polymerization (**Figure 6–52**). In both cases, the growing strand (primer) and the nucleoside triphosphate (NTP) or its analog base-pairs to a template. In both cases, the 3′ hydroxyl of the growing strand attacks the α-phosphate of the 5′ triphosphate and displaces pyrophosphate (PP_i) with concomitant formation of a 3′-5′ phosphodiester bond (Figure 6–52).

B. It is critical to the selection and amplification scheme that the catalytic RNA becomes attached to the tag. The tag is used to fish out specific RNA molecules from the large pool of random molecules. If the tag were not attached to the ribozyme that catalyzed the linkage, no selection and amplification of the relevant ribozyme (the point of the whole scheme) would occur.

C. The random segment in the middle is the part of the molecule that guarantees that a very large number of different sequences—hence, conformations and catalytic activities—will be present in the starting pool. It is your hope that one or a few such molecules can catalyze the intended reaction.

The constant regions at the ends of each pool RNA molecule serve different purposes. The constant region at the 5′ end of the pool RNAs serves as a binding site for the substrate oligonucleotide, so that the ends can be juxtaposed to create the substrate for ligation. This constant region also serves as one site required for regenerating a pool of RNA by T7 RNA polymerase transcription. This is an essential step if the cycle of selection and amplification is to be repeated. The constant region at the 3′ end of the pool RNAs serves as a site for attachment to the agarose bead for ease of manipulation, for specific amplification of linked substrate and

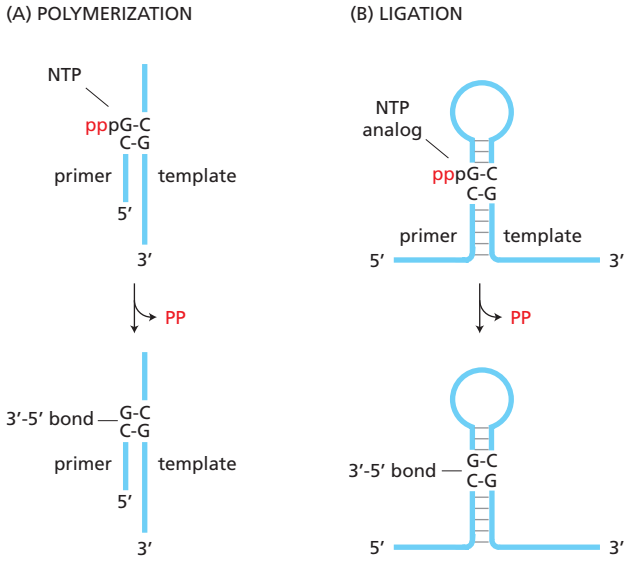


Figure 6–52 Similarity of polymerization and ligation (Answer 6–96). (A) RNA polymerization. (B) RNA ligation. Analogous parts of the reactions are labeled.

catalytic RNAs, and for amplification to link the T7 promoter so that the DNA oligonucleotides can be reconverted to RNA oligonucleotides for subsequent cycles.

D. A catalytic RNA molecule is selected by passing the pool of RNA through an affinity column that carries oligonucleotides that are complementary to the substrate oligonucleotide. Only in those molecules that have undergone a ligation reaction will the catalytic RNA be attached to the substrate. The vast majority of noncatalytic RNAs will pass through such an affinity column. When the RNA is eluted from the column, it will contain a mixture of the sought-after catalytic RNA and contaminating noncatalytic RNA. The catalytic RNA can be specifically amplified using PCR primers, one of which is specific for the substrate RNA and the other for the pool RNA. Such a pair of PCR primers will selectively amplify catalytic RNAs, because only the catalytic RNAs will be attached to the substrate RNAs and be amplified.

E. Even assuming that one cycle of selection and amplification is sufficient to remove all contaminating noncatalytic RNA molecules, which is probably not the case, there is still a critical reason for carrying out multiple cycles of selection and amplification. In the starting pool of RNA molecules, it is unlikely that any molecule will be represented more than once. Thus, at the end of the first time period for ligation, the very best catalyst in the population, many much weaker catalysts, and even some noncatalytic RNAs that are linked by an uncatalyzed mechanism, will all be attached to the substrate RNA. They will all be represented equally in the amplified pool. Purification at this stage would yield an extensive mixture of RNA molecules with a very wide range of catalytic activities.

Subsequent rounds of selection and amplification allow the best catalysts to win out over the weaker ones. Consider, for example, the second cycle. In the window for ligation, most of the good catalysts will attach themselves to the substrate, while many fewer of the weaker catalysts and essentially none of the noncatalytic RNAs will do so. Thus, the amplification step in the second cycle will enrich considerably for the better catalysts. By decreasing the time for ligation in subsequent cycles, better and better catalysts can be selectively amplified.

Reference: Bartel DP & Szostak JW (1993) Isolation of new ribozymes from a large pool of random sequences. *Science* 261, 1411–1418.

CALCULATIONS

6–97

A. There are 6×10^{15} molecules, 300 nucleotides (nt) in length, in 1 mg of RNA.

$$\text{number} = \frac{1 \text{ RNA molecule}}{300 \text{ nt}} \times \frac{\text{nt}}{330 \text{ d}} \times \frac{6 \times 10^{20} \text{ d}}{1 \text{ mg}}$$
$$= 6 \times 10^{15} \text{ RNA molecules}$$

B. If the 220-nucleotide segment were completely random, there would be 4 choices of nucleotide at each of 220 positions, which is 4^{220} or about 3×10^{132} possible different RNA molecules. Thus, in a 1 mg sample, there will be 2×10^{-117} $[(6 \times 10^{15})/(3 \times 10^{132})]$ of all possible sequences represented … a trivial fraction of the whole. (A sample large enough to have one copy of each possible RNA would outweigh the known universe by more than 30 orders of magnitude.)

C. If a single 50-nucleotide RNA were required to catalyze the ligation, your chances of success would be close to nil. There are about 10^{18} different 50-nucleotide sequences represented in a 1 mg sample of RNA. Considering just the random 220 nucleotides, there would be about 170 different 50-mers in each of 6×10^{15} molecules (imagine sliding a 50-nucleotide window across the 220 nucleotides one nucleotide at a time) for a total of $170 \times 6 \times 10^{15}$, or 10^{18} different molecules. Since there are 4^{50} or about 10^{30} ($4^5 \approx 10^3$) different 50-mers, your chances would be roughly 1 in a trillion (10^{-12}) of having the unique catalytic RNA in your sample. Are you feelin' lucky?

That so many ribozymes have been successfully isolated from such pools argues that a very large number of different sequences must be able to catalyze any given reaction, or that the catalytic RNAs must be very small. Since the identified ribozymes are not particularly small, it must be that many different sequences are capable of catalysis.

Reference: Bartel DP & Szostak JW (1993) Isolation of new ribozymes from a large pool of random sequences. *Science* 261, 1411–1418.

DATA HANDLING

6–98

A. Error-prone PCR was used to introduce mutations into the pool of RNA molecules in some rounds in order to try to generate ever more efficient catalysts of ligation. Because all possible molecules cannot be present in the starting material (see Problem 6–97), this technique gives you a way to increase the diversity of molecules that are closely related to those with demonstrated catalytic activity. It is likely that better catalysts will be found in the "sequence neighborhood" of existing catalysts. You waited until round 5 to apply error-prone PCR to give time for some moderately good catalysts to arise.

B. By making ligation more and more difficult—by lowering the concentration of Mg^{2+} and by decreasing the time available for ligation—you are selecting for better and better catalysts.

C. Your scheme for selection and amplification has improved the ligation rate about 3 million-fold from 0.000003 ligations per hour for the starting RNA pool to 8.0 per hour after round 10. Thus, your final pool of ribozymes catalyzes ligation about 3×10^6-fold faster than the uncatalyzed reaction.

D. The diversity evident in your round-10 pool of RNA molecules indicates that many sequences can carry out efficient ligation. Since 11 of 15 of the cloned and sequenced molecules are clearly similar, they form a single sequence family, presumably with a very similar overall conformation. The other molecules may represent additional catalytically active

conformations. You will tell your audience that additional structural and enzymological studies will be needed to determine the catalytic mechanism(s) represented in your pool of ribozymes.

Reference: Bartel DP & Szostak JW (1993) Isolation of new ribozymes from a large pool of random sequences. *Science* 261, 1411–1418.

MCAT STYLE

6–99

C. Mutations that lead to incorporation of the wrong amino acid often cause folding defects, and the incorrectly folded protein is recognized and targeted for destruction by ubiquitin-dependent proteolysis. Choice A is unlikely because mutations that block binding of Tbx5 to Nkx2-5 would cause defects in the transcription of genes regulated by Tbx5, but not in the transcription or translation of Tbx5 itself. Choice B is not correct because a splicing defect would cause failure to form the normal, mature mRNA. Choice D is probably not correct because a premature stop codon would most likely cause the mature mRNA to be destroyed by nonsense-mediated decay. This choice cannot be ruled out entirely, however, because some premature stop codons do not trigger nonsense-mediated decay.

6–100

D. Choice II is correct because a short deletion that disrupts the reading frame will cause the mRNA to be destroyed via nonsense-mediated decay, which would lead to decreased levels of the full-length mRNA. Choice III is correct because a mutation in the promoter region could cause defects in the initiation of transcription, which would lead to decreased mRNA levels. Choice I is not correct because a short deletion that preserves the reading frame would not be recognized as an abnormal mRNA.

6–101

D. Since normal levels of Tbx5 mRNA and protein are produced, yet Tbx5 does not function properly, there must be a mutation in Tbx5 that affects its activity. Since Tbx5 binds to Nkx2-5 to carry out its functions, a mutation that affects this interaction is likely to be responsible for the observed phenotype. Choice A is not correct because a mutation that blocks binding to the ribosome would cause decreased levels of Tbx5 protein. Choice B is not correct because mRNAs must be transported to the cytoplasm to be translated; thus, a cell with a defect in nuclear export of Tbx5 mRNA would fail to produce protein. Choice C is not correct because a splicing defect would cause decreased levels of full-length, mature Tbx5 mRNA.

6–102

B. Many transcription factors bind to DNA in promoter regions to help recruit RNA polymerase II to initiate transcription. The other choices are not correct because 5′ cap formation, recruitment of splicing factors, and transcription elongation occur after initiation of transcription.

6–103

B. The ability of snRNAs to bind to pre-mRNA splice site sequences by base-pairing guides the splicing process. Choice A is not correct because snRNPs do not bind to ribosomes; they carry out their functions in the nucleus. Choice C is not correct because introns are found in pre-mRNA, not in snRNAs. Even if they were found in snRNAs, they would not have provided a clue to snRNA function. Choice D is not correct because lariats form in the pre-mRNA during the splicing process; they are not found in snRNAs.

6–104

C. Since snRNPs carry out pre-mRNA splicing, and RNA transcription and pre-mRNA splicing are coupled processes that occur in the nucleus, one would expect to find snRNPs in the nucleus near RNA polymerase II, which transcribes the vast majority of transcripts with introns. Choices A and B are not correct because splicing occurs in the nucleus, so one would not expect to find snRNPs in the cytoplasm. Choice D is not correct because RNA polymerase III does not transcribe pre-mRNA, and only pre-mRNAs are spliced by snRNPs.

6–105

B. The complex rearrangements that occur during splicing require an energy source such as ATP, so one might expect to find a protein capable of hydrolyzing ATP—an ATPase—in a snRNP. Choices A and C are not correct because DNA helicases and enhancer proteins operate on DNA and thus would not be expected to play a role in the splicing of RNA. Choice D is not correct because specific bases in the mRNA carry out the cleavage of pre-mRNA during splicing; thus, a site-specific ribonuclease would not be expected in a snRNP.

CHAPTER 7

Control of Gene Expression

AN OVERVIEW OF GENE CONTROL

DEFINITIONS

7–1 RNA transport control

7–2 Transcriptional control

7–3 mRNA degradation control

7–4 Translational control

7–5 RNA processing control

TRUE/FALSE

7–6 False. Carrots can be grown from single carrot cells, and tadpoles can be gotten by injecting differentiated frog nuclei into frog eggs. But carrots cannot be gotten from frog eggs no matter what.

7–7 False. Comparison of the patterns of expressed mRNAs underestimates the differences in the patterns of proteins produced in different specialized cell types because gene expression can be regulated at many steps after transcription. Differences in post-transcriptional regulation introduce additional differences into the protein profiles observed in different cell types.

THOUGHT PROBLEMS

7–8 Each added phosphate alters the charge by one unit, but has relatively little effect on the molecular mass. As a consequence, proteins that differ only in the number of attached phosphates will appear to be the same size, but will have different isoelectric points, forming a set of horizontal spots, as shown for a few proteins in **Figure 7–44**. It is important to keep in mind that a horizontal array of spots does not prove that the proteins are related by phosphorylation; they could be different proteins

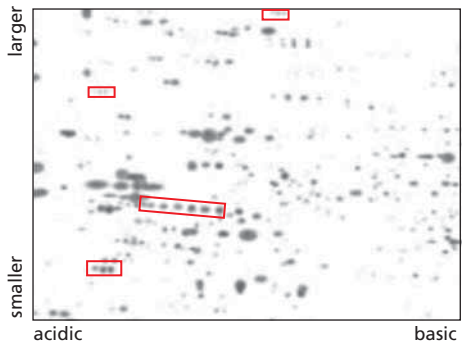


Figure 7–44 Protein spots in a two-dimensional gel that might differ by the number of attached phosphates (Answer 7–8). A few sets of horizontal spots that could be related by phosphorylation are *boxed*. Not all such sets of proteins are indicated.

IN THIS CHAPTER

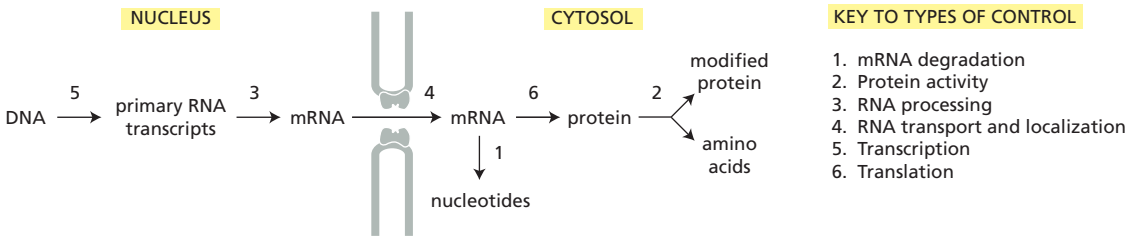


Figure 7–45 Types of control that regulate each step in the pathway for gene expression in eukaryotic cells (Answer 7–9).

with the same molecular mass and slightly different isoelectric points, or they could be the same protein with a different type of modification that affects the charge of the protein. Treatment of the proteins with a protein phosphatase before separation by gel electrophoresis could be used to resolve the issue.

7–9

A. The six types of regulation of gene expression are indicated at the appropriate points on the diagram in **Figure 7–45**.

B. Bacterial cells lack a nuclear membrane and other membrane-enclosed intracellular compartments. As a result, there is no RNA processing control or RNA transport and localization control.

DATA HANDLING

7–10

A. For each of the lambs, the microsatellite analysis at each of the four polymorphic loci matches that of the nuclear donor cell, and is distinct from that of the surrogate mother. This result indicates conclusively that the lambs were derived from the donor cell nuclei.

B. If any of the lambs had resulted from an inadvertent mating of the surrogate mother with an unknown father, then the microsatellite analysis would have given a pattern different from that of the nuclear donor cells. The pattern would also have been distinct from that of the surrogate mother. It would have consisted of one band in common with the surrogate mother and one band derived from the rogue male.

Reference: Wilmut I, Schnieke AE, McWhir J, Kind AJ & Campbell KHS (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810–813.

7–11 The V and C segments in the germ line are separated by a very long segment of DNA, which contains the sites for the restriction nuclease used in the digestion. As a result, the V and C segments are on different restriction fragments, which is why the segment-specific probes each hybridize to a band of a different size (**Figure 7–46**). After the V and C segments are brought into proximity by the deletion of the intervening DNA in B cells, the probes would be expected to hybridize to new bands. If the restriction nuclease no longer cleaves between the two segments, both probes will hybridize to the same band, as shown in Figure 7–45. In the classic paper describing these results, which are reproduced in Figure 7–45, a restriction nuclease was used that didn't cut between the rearranged segments, because that gave the clearest demonstration that a rearrangement had indeed occurred.

There are three additional possible patterns, depending on the sites at which the restriction nuclease cleaves relative to the end points of the rearrangement. If the deleted DNA removed neither of the two restriction sites that lie between the V and C segments, then the hybridization pattern in B cells would be the same as in the germ line. If the interior site near the V segment were deleted, the C segment would be on the same

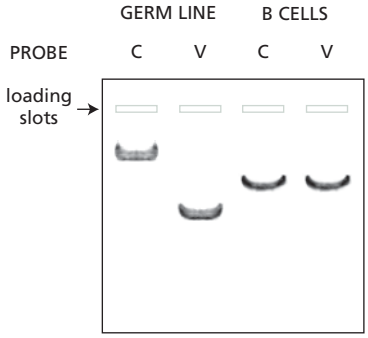


Figure 7–46 Pattern of hybridization to V- and C-segment probes in the germ line and B cells (Answer 7–11).

fragment as in the germ line, but the V segment would be on a fragment of a different size. If the interior site near the C segment were deleted, the V segment would be on the same fragment as in the germ line, but the C segment would be on a fragment of a new size.

Reference: Tonegawa S (1983) Somatic generation of antibody diversity. *Nature* 302, 575–581.

MEDICAL LINKS

7–12 Although it is true that cancer cells differ from their normal precursors, they typically differ in their expression of only relatively few genes (oncogenes and tumor suppressor genes). When the overall patterns of mRNAs in cancer cells are compared with the patterns of mRNAs in normal tissues, they match for the great majority of mRNAs. This RNA signature allows a tumor to be definitively assigned to a particular tissue type.

CONTROL OF TRANSCRIPTION BY SEQUENCE-SPECIFIC DNA-BINDING PROTEINS

DEFINITIONS

7–13 Transcription regulator

7–14 *cis*-Regulatory sequence

TRUE/FALSE

7–15 False. Although the individual contacts are weak, the 20 or so contacts that are typically formed at a protein–DNA interface add together to ensure that the interaction is both highly specific and very strong. In fact, DNA–protein interactions include some of the tightest and most specific molecular interactions in biology.

7–16 True. Both the helix–loop–helix motif and the leucine zipper motif are structural motifs that allow transcription regulators to dimerize, so that each member of the pair can position an α helix in the major groove of the DNA.

THOUGHT PROBLEMS

7–17 The major and minor grooves and the length scale are indicated in **Figure 7–46**. It is virtually impossible to determine the polarity of the strands from a picture of a space-filling model. If you could examine an actual space-filling model (not just a picture), you could determine how the deoxyribose sugars were arranged in the two strands, and thus determine the polarity. You can also do this from a more detailed ball-and-stick diagram, and we encourage you to try it. The polarities of the two strands are indicated on the ribbon model shown in Figure 7–46. One way to remember the strand polarities is as follows. Imagine that you are standing in the minor groove of a DNA duplex so that one strand is on your left and the other is on your right. The strand on your left runs 5′ to 3′ from behind you to in front of you. Note that if you turn around and face the other direction, the strand on your left still runs 5′ behind you to 3′ in front of you.

7–18 Contacts can form between the protein and the edges of the base pairs that are exposed in the grooves of the DNA, especially the major groove. The types of contact that can form in the major groove are shown in

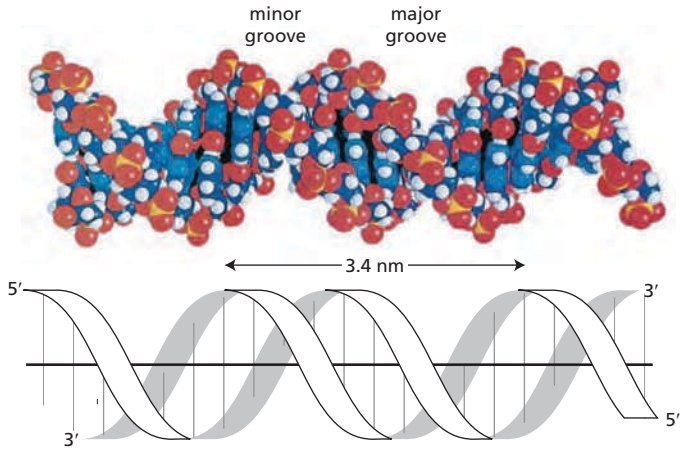


Figure 7–47 Space-filling and ribbon models of a DNA duplex (Answer 7–17). Major and minor grooves are indicated, along with a scale of 3.4 nm per 10 base pairs. The polarities of the strands are shown on the ribbon diagram.

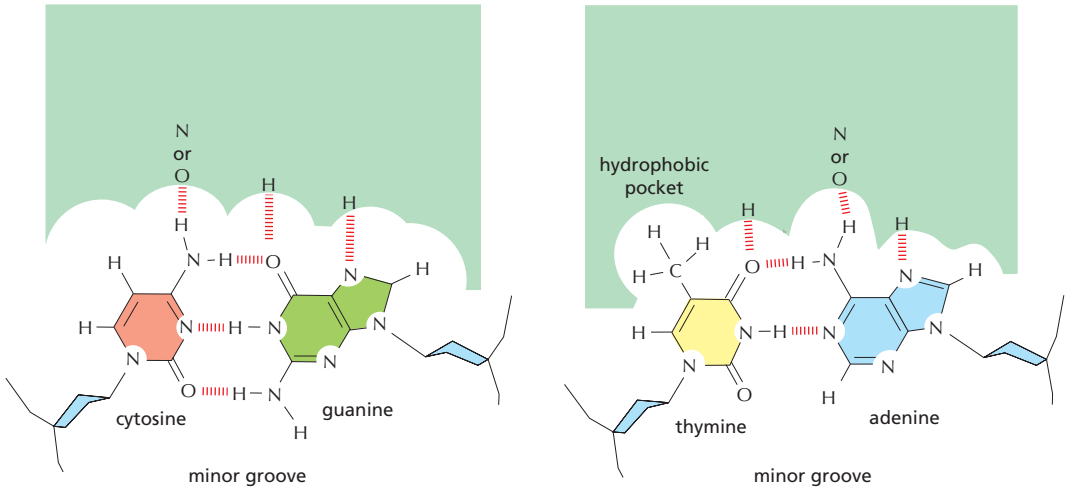
Figure 7–47. A hydrophobic interaction can sense the methyl group on the pyrimidine ring of T. Hydrogen bonds provide sequence-specific contacts. The arrangement of hydrogen-bond donors and acceptors of a T-A base pair is different from that of a C-G base pair. In addition to the specific contacts shown in the figure, electrostatic attractions between positively charged amino acid side chains and the negatively charged phosphate groups in the DNA backbone commonly stabilize DNA–protein interactions.

7–19 The two basic components of genetic switches are *cis*-regulatory DNA sequences and the transcription regulators that bind to them.

7–20 Under the specified conditions (equal concentrations of DNA and transcription regulator), the protein would occupy its recognition site equally well in the eukaryotic nucleus and in the bacterium. A nonmathematical way of thinking about this is to imagine a small volume of eukaryotic nucleus, equal in size to that of the bacterium and containing the binding site. That small volume in the eukaryotic nucleus is directly comparable to the interior of the bacterium. In those equal volumes, the ability of the transcription regulator to find its binding site is equivalent. So long as the *concentrations* of the DNA and transcription regulator are the same, the total volume will make no difference. This means, of course, that the total *number* of transcription regulator molecules is 500 times higher in a single nucleus than in a single bacterium.

Reference: Ptashne M (1986) A Genetic Switch: Gene Control and Phage λ, p. 114. Oxford, UK: Blackwell Scientific Press.

Figure 7–48 Arrangements of noncovalent bonds that allow proteins to recognize C-G and T-A base pairs selectively by binding in the major groove (Answer 7–18).



7–21 The strength and specificity of the DNA–protein interaction can be adjusted with this motif by changing the binding properties of individual zinc fingers, as well as by altering the number of zinc finger repeats. The other types of DNA-binding motifs, which function primarily as head-to-head dimers, cannot be so readily formed into repeating chains.

7–22 There are at least two advantages of dimerization. First, the binding affinity of a dimer can be higher because the number of potential contacts with DNA is double that possible with a monomer. Second, combinatorial pairing of different subunits can increase the number of DNA-binding specificities that are available to a cell.

7–23 The affinity of the dimeric lambda repressor for its binding site is the sum of all the interactions made by each DNA-binding domain. An individual DNA-binding domain will make just half the contacts and provide just half the binding energy as the dimer. Thus, although the concentration of binding domains is unchanged, their binding as monomers is sufficiently weak that they do not compete with the binding of RNA polymerase. As a result, the genes for lytic growth are turned on.

7–24 The phosphate interferes with the function of the DNA-binding domain by adding a negative charge and by creating steric problems. Typically, DNA-binding domains are positively charged, which helps them bind to the negatively charged DNA. Addition of a negative charge would increase charge repulsion between the DNA and the protein, interfering with its function. If the phosphate were added to the binding surface itself, it would likely interfere directly with the interaction of myogenin with the DNA.

A heterodimer formed between myogenin and Id, a truncated helix–loop–helix protein lacking a DNA-binding domain, would be unable to bind to DNA tightly because it would make only half of the necessary contacts.

References: Benezra R, Davis RL, Lockshon D, Turner DL & Weintraub H (1990) The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. *Cell* 61, 49–59.

Li L, Zhou J, James G, Heller-Harrison R, Czech MP & Olson EN (1992) FGF inactivates myogenic helix–loop–helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. *Cell* 71, 1181–1194.

CALCULATIONS

7–25

A. In the 0.4 ng starting sample there are 4.8×10^9 oligonucleotides that are 76 nucleotide pairs (np) long.

$$\text{oligonucleotides} = 0.4\ \text{ng} \times \frac{6 \times 10^{14}\text{d}}{\text{ng}} \times \frac{\text{np}}{660\text{d}} \times \frac{\text{oligonucleotide}}{76\ \text{np}}$$
$$= 4.8 \times 10^9$$

B. Yes. The total number of different 14-base-pair-long sequences is $4^{14} = 2.7 \times 10^8$, and there were 4.8×10^9 oligonucleotide molecules in the starting sample. Moreover, each oligonucleotide contains 13 14-base-pair-long sequences in its central 26 random nucleotides. Thus, in the starting sample, each possible 14-bp sequence is likely to be represented more than a hundred times.

C. The consensus binding sequence for the transcription regulator is 5′-ATGCCCATATATGG. As shown in **Table 7–3**, where the sequences are aligned according to the consensus binding site, the consensus is very

TABLE 7–3 Sequences of selected and amplified DNAs aligned according to the consensus sequence (Answer 7–25).

GAATTCGCCTCGAGCACATCATTGCCCATATATGGCACGACAGGATCC
GAATTCGCCTCTTCTAATGCCCATATATGGACTTGCTCGACAGGATCC
GGATCCTGTCGGTCCTTTATGCCCATATATGGTCATTGAGGCGAATTC
GAATTCGCCTCATGCCCATATATGGCAATAGGTGTTTCGACAGGATCC
GAATTCGCCTCTATGCCCATATAAGGCGCCACTACCCCGACAGGATCC
GAATTCGCCTCGTTCCCAGTATGCCCATATATGGACACGACAGGATCC
GGATCCTGTCGACACCATGCCCATATTTGGTATGCTCGAGGCGAATTC
GAATTCGCCTCATTTATGAACATGCCCTTATAAGGACCGACAGGATCC
GAATTCGCCTCTAATACTGCAATGCCCAAATAAGGAGCGACAGGATCC
GAATTCGCCTCATGCCCAAATATGGTCATCACCTACACGACAGGATCC
consensus ATGCCCATATATGG

good for these 14 nucleotides, but drops off dramatically in the flanking regions.

Reference: Pollock R & Treisman R (1990) A sensitive method for the determination of protein–DNA binding specificities. *Nucleic Acids Res.* 18, 6197–6204.

DATA HANDLING

7–26

A. The results of the matings in Figure 7–10 show that bacteriophage proliferation and cell lysis occur only when the donor carries a lambda prophage and the recipient does not. These results are consistent with the idea that a repressor keeps the lytic genes of the prophage turned off. In bacteria that harbor a prophage, the presence of the repressor ensures that the prophage remains quiescent. When the prophage is transferred into a bacterium that does not carry a prophage, it finds itself in a repressor-free environment and its lytic program is induced. If the prophage is instead transferred into a bacterium that harbors a prophage of its own, the presence of the repressor keeps the prophage from being induced.

B. Exactly the same results would have been expected if lysis were controlled by a transcription regulator that activated expression of an anti-lysis protein. If the prophage entered a prophage-negative recipient, the transcription regulator would be absent, preventing expression of the anti-lysis protein and allowing prophage induction and lysis to occur. In a prophage-positive recipient, the anti-lysis protein would already be present, thereby preventing prophage induction and cell lysis. It was only with additional genetic and biochemical experiments that the mechanism was identified as involving a transcription regulator that repressed the lytic genes of the prophage.

Reference: Echols H (2001) Operators and Promoters: The Story of Molecular Biology and Its Creators, pp. 46–47. Berkeley, CA: University of California Press.

7–27

A. Some of the individual RNA polymerase molecules deviate from the bulk flow because they bind to the DNA and slide along it for some period of time before they fall off and rejoin the flow. The binding must be nonspecific since the RNA polymerase molecules slide for long distances. Yet a

portion of the RNA polymerase molecule normally involved in promoter binding must be involved in sliding, because the nonspecific binding is eliminated when the RNA polymerase molecules have already bound a DNA fragment containing a strong promoter.

B. Sliding along the DNA would allow transcription regulators to find their targets faster than expected by three-dimensional diffusion because it reduces the search to one dimension. It is thought that most transcription regulators accelerate their search of DNA by some combination of sliding and intersegment transfer (hopping from one segment to another).

C. If the target sites were present in short DNA molecules, then the search would be expected to approximate a three-dimensional search and, hence, be slow. On the other hand, if the target sites were in long DNA molecules, then the search would be accelerated by sliding. Thus, a transcription regulator would be expected to find its target faster in a population of long DNA molecules than in a population of short ones.

References: Kabata H, Kurosawa O, Arai I, Washizu M, Margarson SA, Glass RE & Shimamoto N (1993) Visualization of single molecules of RNA polymerase sliding along DNA. *Science* 262, 1561–1563.

Shimamoto N (1999) One-dimensional diffusion of proteins along DNA. *J. Biol. Chem.* 274, 15293–15296.

7–28

A. At the point of minimum relative migration, the CAP sites are separated by 85 nucleotide pairs (see Figure 7–12C). At 10.6 nucleotides per turn, this number of nucleotides corresponds to 8 helical turns (85/10.6 = 8). At the point of maximum relative migration, the CAP sites are separated by 79 nucleotide pairs, which equals 7.5 helical turns.

B. Yes. The two CAP sites must be bent exactly the same way since they are identical. Therefore, they will both have the same groove of the helix facing the inside of the bend at the center of bending. In order for the DNA to bend into the *cis* configuration, the two centers of bending must be on the same side of the helix. The major grooves (or minor grooves) are on the same side of the helix at integral numbers of helical turns. Thus, it is expected that the point of minimum relative migration (the *cis* configuration) will occur after an integral number of helical turns. Similarly, the point of maximum relative migration (the *trans* configuration) will occur when the centers of bending are on opposite sides of the helix—that is, at half-integral numbers of helical turns.

C. At the point of minimum migration of the construct with one CAP site and one $(A_5N_5)_4$ site, the centers of bending are separated by 101 nucleotides (see Figure 7–12D). At 10.6 nucleotide pairs per helical turn, the centers of bending are separated by 9.5 helical turns (101/10.6 = 9.5).

D. Since the point of minimum relative migration (the *cis* configuration) occurs at a half-integral number of turns, the two centers of bending cannot have the same groove of the helix facing the inside of the bend. As discussed in part B, if the same groove of the helix faced the inside of the bend, the centers of bending in the *cis* configuration would be separated by an integral number of turns. Therefore, the two centers of bending must have opposite grooves facing the inside of the bend. Because the $(A_5N_5)_4$ site is known to bend with the major groove facing the inside of the bend (as was stated in the problem), the CAP-binding site must be bent so that the minor groove faces the inside of the bend at the center of bending.

References: Zinkel SS & Crothers DM (1987) DNA bend direction by phase sensitive detection. *Nature* 328, 178–181.

Gartenberg MR & Crothers DM (1988) DNA sequence determinants of CAP-induced bending and protein binding affinity. *Nature* 333, 824–829.

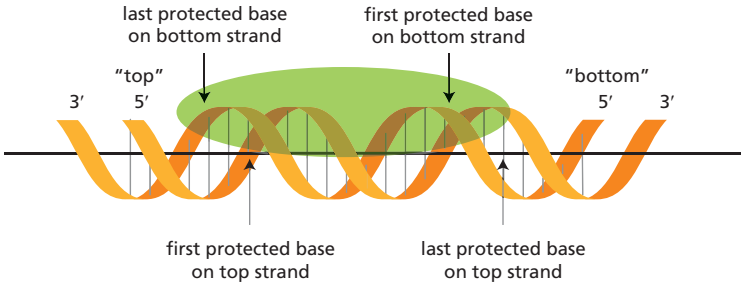


Figure 7–49 Binding of a protein to DNA (Answer 7–30).

7–29

A. Free heterodimers rapidly exchange subunits with the population of Fos monomers in solution. As shown in Figure 7–13C, rhodamine fluorescence at 603 nm is abolished within a few seconds of addition of excess unlabeled Fos. This is the expected outcome if heterodimers rapidly dissociate and re-form. In the presence of excess Fos, most of the Fos–F–Jun–R heterodimers would be replaced by Fos–Jun–R heterodimers. In the absence of a nearby fluorescein, energy cannot be efficiently transferred to rhodamine and thus there would be no fluorescence at 603 nm.

In contrast to the free heterodimer, the DNA-bound heterodimer is exceedingly stable and does not readily exchange subunits with the population of monomeric Fos, as is evident from the undiminished fluorescence at 603 nm over the time of the experiment. (The authors of the study report only a 15% loss of fluorescence after 16 hours of incubation.)

The increased stability of the Fos–Jun heterodimer in the presence of DNA arises because the subunits, when bound to DNA, are linked in two ways: via their leucine zipper motifs and via their interactions with the DNA. By contrast, the subunits in a free heterodimer are held together just by the interaction between their leucine zipper motifs.

B. If these *in vitro* results with Fos and Jun can be extrapolated to the cell, they would imply that free heterodimers can rapidly exchange partners, forming an equilibrium mixture of heterodimers in the cell. Once bound to sites in DNA, the subunits would be effectively locked together, and would no longer communicate with the unbound pool of subunits. (It is important to remember that all leucine zippers may not behave like Fos and Jun, and it is not certain that measurements made *in vitro* reflect the true situation in cells.)

Reference: Patel LR, Curran T & Kerppola TK (1994) Energy transfer analysis of Fos–Jun dimerization and DNA binding. *Proc. Natl Acad. Sci. USA* 91, 7360–7364.

7–30 It is not surprising that the DNA footprint on the two strands should be slightly different. DNA is a three-dimensional object, as is the protein. The interaction of the protein with the DNA need not protect the two strands identically, as shown in **Figure 7–49**. Especially at the margins of the footprint, one strand is likely to be more protected than the other.

TRANSCRIPTION REGULATORS AS GENE SWITCHES

DEFINITIONS

7–31 Promoter

7–32 Gene control region

7–33 Gene

TRUE/FALSE

7–34 True. The ability of eukaryotic transcription regulators to act over long distances is one of the key differences between gene regulation in prokaryotes and in eukaryotes.

7–35 True. Although it is generally the case that bacterial control elements are grouped closely around start points of transcription, a few elements are more distant. In those cases, it is thought that the transcription regulators cause DNA looping, as is common in eukaryotes. These examples of distant regulatory sites suggest that the common, close-packed organization in bacteria may have arisen in response to evolutionary pressures to maintain a compact genome.

THOUGHT PROBLEMS

7–36 The function of these *Arg* genes is to synthesize arginine. When arginine is abundant, expression of the biosynthetic genes should be turned off. If ArgR acted as a transcription repressor (which it does in reality), then binding of arginine would be expected to increase the repressor's affinity for its regulatory sites, allowing it to bind and shut off gene expression. If ArgR acted as a transcription activator instead, then the binding of arginine would be expected to reduce its affinity for its regulatory sites, preventing its binding and shutting off gene expression.

Reference: Glandsdorff N (1987) Biosynthesis of arginine and polyamines. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology (FC Neidhardt ed), pp. 321–344. Washington DC: American Society for Microbiology.

7–37

A. When sufficient tryptophan is present in the cells, the tryptophan repressor will block expression of the biosynthetic enzymes. Likewise, when cells are starved for tryptophan, the unoccupied repressor will not bind to the DNA and the biosynthetic enzymes will be induced. This simple and elegant form of feedback inhibition allows cells to adjust the rate of tryptophan synthesis to meet their needs.
B. In both scenarios, transcription of the genes encoding the tryptophan biosynthetic enzymes would no longer be regulated by the absence or presence of tryptophan. The enzymes would be permanently on in scenario (i) because the repressor could not bind to the DNA. The enzymes would be permanently off in scenario (ii) because the repressor would always be bound to the DNA.
C. In scenario (i), the normal tryptophan repressor molecules would completely restore the regulation of the tryptophan biosynthetic enzymes. Because the mutant repressor does not bind to the DNA, it would not affect the function of the normal repressor. By contrast, expression of the normal tryptophan repressor would have no effect in scenario (ii) because the binding sites on the DNA would remain permanently occupied by the mutant repressor.

7–38 As indicated in **Figure 7–50**, CAP is bound when glucose is absent, and the *Lac* repressor is bound when lactose is absent. The operon will be turned on only when CAP is bound (glucose is absent) and the *Lac* repressor is not bound (lactose is present).

7–39

E. In the fused operon, the genes in the *Lac* operon have come under control of the regulatory region of the *Trp* operon. Thus, expression of β-galactosidase, the product of the *LacZ* gene, will be regulated by the tryptophan repressor. Because the tryptophan repressor requires

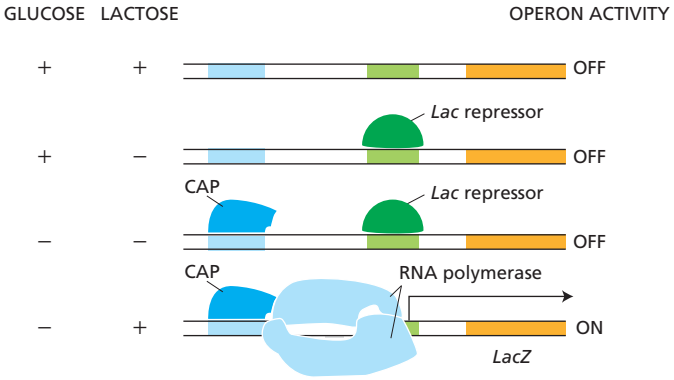


Figure 7–50 Combinations of sugars that correspond to combinations of transcription regulators and result in the indicated activity of the operon (Answer 7–38).

tryptophan in order to bind to its regulatory region and shut off the operon, expression of β-galactosidase (and the other genes in the fused operon) will occur only when tryptophan is absent from the medium (condition E).

7–40 The results of this experiment favor the DNA looping model, which would not be affected by the protein bridge (so long as it allowed the DNA to bend, which it does). By contrast, the scanning model is likely to be affected by the nature of the linkage between the regulatory sequence and the promoter. If the proteins entered at the regulatory sequence and scanned to the promoter, they would have to traverse the protein bridge. If such proteins were geared to slide on DNA, they would likely be blocked by the protein linker.

Reference: Müeller-Storm HP, Sogo JM & Schaffner W (1989) An enhancer stimulates transcription in *trans* when attached to the promoter via a protein bridge. *Cell* 58, 767–777.

7–41 Bending proteins can help to bring together distant DNA regions that normally would contact each other only rarely (**Figure 7–51**). Such proteins act to increase the local concentration of transcription regulators in the neighborhood of RNA polymerase by bringing them closer together. Bending proteins are found in both prokaryotes and eukaryotes and are involved in many examples of transcriptional regulation.

7–42 In order for Gal4 to work properly, the DNA-bound Gal4 must recruit many proteins, including RNA polymerase, to the promoter. When there is too much Gal4 in the cell, the free and DNA-bound Gal4 will compete for the limited quantities of these other components. In the presence of excess Gal4, those components are tied up in unproductive complexes with free Gal4, thereby preventing their recruitment to the promoter. As might be expected, cells that massively overexpress Gal4 grow poorly because of the reduced availability of critical components of the transcription machinery.

Reference: Ptashne M & Gann A (2002) Genes & Signals, pp. 75–76. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

7–43 Histone modification enzymes and chromatin remodeling complexes are recruited to specific regions of chromatin by transcription activators that can bind to DNA in unmodified chromatin. Once bound, histone modification enzymes can add groups to histone tails, altering their packing properties and providing binding sites for additional specific proteins. Similarly, chromatin remodeling complexes, once recruited, alter the local chromatin structure. This facilitates the binding of additional transcription activators that cannot bind to unmodified chromatin. Together,

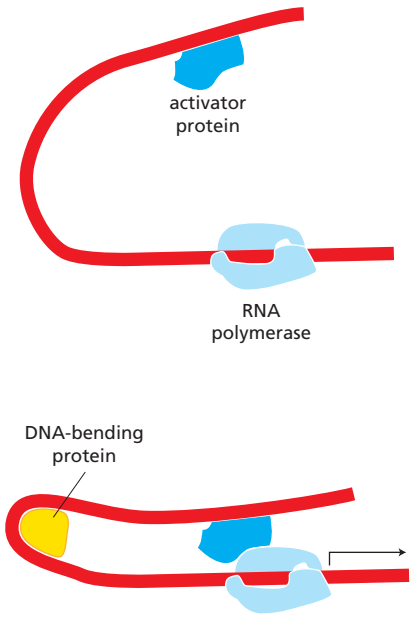


Figure 7–51 Role of a bending protein in bringing together distant transcription regulators and RNA polymerase (Answer 7–41).

these changes in histones and chromatin allow the transcription machinery to be assembled at specific promoters and to initiate transcription.

7–44 In a sense, the DNA acts as a tether, holding the proteins in close proximity so that inherently weak interactions between them can occur readily.

7–45 There is no need for an infinite number of transcription regulators. Many transcription regulators are expressed continuously, but their activities are controlled by signals from inside or outside the cell (for example, the availability of nutrients as for the tryptophan repressor, or by hormones as for the glucocorticoid receptor). In this way, the transcriptional program can be adjusted to the physiological needs of the cell in the absence of transcriptional control of the transcription regulators. Even more importantly, transcription regulators are generally used in combinations. They can affect one another's activity, thereby further increasing the possible regulatory repertoire of gene expression with a limited set of proteins. Even though a cell doesn't need an infinite number of genes for transcription regulators, the cell does devote a large fraction of its genome to the control of transcription: an estimated 10% of all genes in mammalian cells code for transcription regulators.

DATA HANDLING

7–46

A. The rapid bacterial growth at the beginning of the experiment results from the metabolism of glucose. The slower growth at the end results from metabolism of lactose. The bacteria stopped growing in the middle of the experiment because they ran out of glucose but did not yet possess the enzymes necessary for lactose metabolism. Before they could utilize the lactose in the medium, they had to induce the *Lac* operon. The delay in growth represents the time required for the induction.

B. Induction of the *Lac* operon requires that two conditions be met: lactose must be present and glucose must be absent. During the first part of the experiment, glucose and lactose are both present; therefore, the conditions for induction are not met. Only when glucose is exhausted are the requirements for induction satisfied.

CAP and the *Lac* repressor mediate induction (**Figure 7–52**). For the operon to be on, CAP must be bound and the *Lac* repressor must not be bound. The presence of lactose in the medium increases the intracellular concentration of allolactose, an isomer of lactose, which binds to the *Lac* repressor, thereby lowering its affinity for its binding site and causing its release from the DNA. Removal of the *Lac* repressor satisfies one condition for induction. The second condition is tied to the concentration of glucose. When the concentration of glucose falls, the intracellular level of cAMP rises. cAMP binds to CAP and alters its conformation so that it can bind to its binding site. When CAP is in place (and the *Lac* repressor

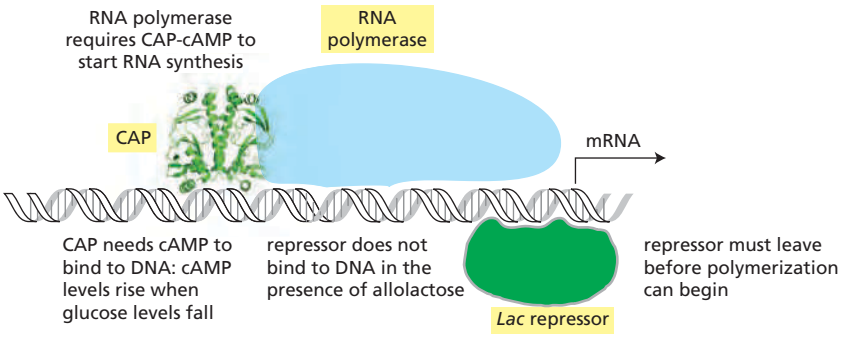


Figure 7–52 Induction of the *Lac* operon (Answer 7–46). The proteins and DNA are drawn approximately to scale.

is absent), RNA polymerase can bind to the promoter and initiate transcription.

Reference: Monod J (1947) The phenomenon of enzymatic adaptation. Growth Symposium XI:223–289. [Reprinted in Selected Papers in Molecular Biology by Jacques Monod (A Lwoff, A Ullmann eds), pp. 68–134. New York: Academic Press, 1947.]

7–47

A. RNA polymerase can bind to the promoter of the glutamine synthetase gene in the absence of NtrC, but it cannot initiate transcription because it is in an inactive complex. The interaction with phosphorylated NtrC activates the RNA polymerase, allowing it to initiate transcription.
B. Although NtrC can bind to its binding sites regardless of its state of phosphorylation, NtrC cannot activate RNA polymerase in its nonphosphorylated form. Only phosphorylated NtrC protein can bind to RNA polymerase and stimulate transcription.
C. Although transcription can be stimulated by phosphorylated NtrC in the absence of its binding sites, the activation requires high concentrations of NtrC. The NtrC-binding sites serve to increase the concentration of NtrC in the vicinity of the glutamine synthetase promoter. In the absence of NtrC-binding sites, the requisite high concentration of NtrC might inappropriately activate RNA polymerases bound to other promoters. The presence of NtrC-binding sites allows activation of the glutamine synthetase gene at a low-enough concentration of NtrC that promoters without NtrC-binding sites are not activated.

Reference: Ninfa AJ, Reitzer LJ & Magasanik B (1987) Initiation of transcription at the bacterial *glnAp2* promoter by purified *E. coli* components is facilitated by enhancers. *Cell* 50, 1039–1046.

7–48

A. If the SAGA complex served as a coactivator that bridged the Gal4 activator and the general transcription factors at the promoter, you would expect that fragment B, which contains the UAS to which Gal4 binds, would be enriched by chromatin immunoprecipitation of SAGA components. A two- to threefold enrichment of this fragment was observed in cells grown on galactose (see Figure 7–20B). You might also have expected fragment C to be enriched, since it contains the promoter region where the transcription machinery assembles. In experiments not discussed in this problem, the authors showed that SAGA does bind to a general transcription factor, but that interaction does not survive the steps of chromatin immunoprecipitation.
B. SAGA meets the criteria for a coactivator. It is demonstrably present at the UAS in cells grown on galactose, but is absent when the cells are grown on raffinose.

Reference: Larschan E & Winston F (2001) The *S. cerevisiae* SAGA complex functions *in vivo* as a coactivator for transcriptional activation by Gal4. *Genes Dev.* 15, 1946–1956.

7–49 These results show that E and I inhibit expression of any *Ura3* gene placed between them (see Figure 7–21B, sites A and B), but do not affect expression of a *Ura3* gene placed outside (sites C through F). Whereas all strains grow in complete medium, strains with *Ura3* between E and I do not grow in the absence of uracil, but do grow in the presence of FOA, indicating that expression of the *Ura3* gene in these strains is inhibited. By contrast, all the strains with *Ura3* inserted outside E and I grow in the absence of uracil, and do not grow in the presence of FOA, demonstrating that the *Ura3* genes in these strains are expressed. Thus, E and I act as insulators that can silence genes placed between them.

Reference: Bi X, Braunstein M, Shei G-J & Broach J (1999) The yeast *HML* I silencer defines a heterochromatin domain boundary by directional establishment of silencing. *Proc. Natl Acad. Sci. USA* 96, 11934–11939.

MOLECULAR GENETIC MECHANISMS THAT CREATE AND MAINTAIN SPECIALIZED CELL TYPES

DEFINITIONS

7–50 Induced pluripotent stem (iPS) cell

7–51 Cell memory

TRUE/FALSE

7–52 True. In most organisms, the extracellular information that constitutes positional cues must navigate the plasma membrane of the target cell, either by passing through the membrane, or more usually by binding to receptors on the cell surface, generating signals in the cytosol that bring appropriate transcription regulators into play.

7–53 True. This is thought to be the way that MyoD turns on the program of muscle development. All the affected genes whose regulation is understood depend on a group of transcription regulators that act collectively to determine whether a gene will be transcribed. It is thought that most, if not all, of the transcription regulators are in place and that MyoD completes the signal, turning some genes on and others off.

7–54 False. Even specialized cells must constantly respond to changes in their environment, which they do in many cases by altering the pattern of gene transcription.

THOUGHT PROBLEMS

7–55

A. UV light throws the switch from the prophage to the lytic state. When cI is destroyed, Cro is made and turns off the production of new cI. The virus starts to produce coat proteins, and new virus particles are released.
B. When the UV light is switched off, the virus remains in the lytic state. Thus, cI and Cro form a gene regulatory switch that, once thrown, is not reversible.
C. This switch makes sense for the lambda phage. UV light is likely to damage the bacterial DNA, thereby rendering the bacterium an unreliable host for the virus. A prophage will switch to the lytic state, make phage particles, and leave the irradiated cell in search of new, healthier host cells to infect.

7–56 The induction of a transcription activator that stimulates its own synthesis creates a positive feedback loop that can, depending on the stability of protein A, its affinity for its *cis*-regulatory sequence, and other parameters, lead to cell memory. The continued self-stimulated synthesis of activator A can, in principle, last for many cell generations, serving as a constant reminder of an event in the distant past. By contrast, the induction of a transcription repressor that inhibits its own synthesis creates a negative feedback loop that guarantees a transient response to the transient stimulus. Because repressor R shuts off its own synthesis, the cell will quickly return to the state that existed before the transient signal.

7–57 You could create 16 different cell types with four different transcription regulators (all the eight cell types shown in Figure 7–24, plus another

eight created by turning on an additional transcription regulator). MyoD by itself is sufficient to induce muscle differentiation when expressed in fibroblasts. The action of MyoD can be accommodated into a model like that shown in Figure 7–24. It is likely that fibroblasts have already accumulated a number of transcription regulators that can cooperate with MyoD to turn on muscle-specific genes. If muscle cells were specified, for example, by the combination of transcription regulators 1, 3, and MyoD, then the addition of MyoD would convert two of the cell types of Figure 7–24 (cells F and H) to muscle cells, but no others.

DATA HANDLING

7–58

A. Construct 1 corresponds to mutant embryo B, construct 2 corresponds to mutant embryo D, and construct 3 corresponds to mutant embryo C.

B. With the exception of one aspect of embryo D, as discussed below, the results with the various constructs validate the simple rule for *Eve* expression in stripe 2. When the Krüppel-binding sites are removed (construct 1), thereby eliminating the effects of the Krüppel repressor, the stripe 2 expression of β-galactosidase expands slightly in the posterior direction (see Figure 7–27B). This is as expected according to the rule because the activators Hunchback and Bicoid are both present slightly beyond the posterior end of stripe 2.

   When two of the Bicoid-binding sites are removed (construct 3), making the construct less sensitive to the effects of the Bicoid activator, stripe 2 expression of β-galactosidase is lessened but appears at its normal position (see Figure 7–27C).

   When the Giant-binding sites are removed (construct 2), eliminating the effects of the Giant repressor, stripe 2 expression of β-galactosidase expands substantially in the anterior direction (see Figure 7–27D). The simple rule for *Eve* stripe 2 expression, however, predicts that β-galactosidase will be expressed in the entire anterior end of the mutant embryo. The activators Bicoid and Hunchback are both present in this region, and both repressors—Giant and Krüppel—are absent. This result suggests that something is missing from the formulation of the simple rule.

C. The reason why β-galactosidase is not expressed in the anterior end of the embryo is not clear. There are several plausible mechanisms that fall into two general categories. It is possible that another protein whose expression is confined to the anterior end modifies one or both activators to render them nonfunctional for binding to the *Eve* stripe 2 control element. The modification could take the form of a phosphorylation, for example, or occur by direct binding to the activators. The second general mechanism would be the expression of another, as-yet undefined repressor that binds to the *Eve* stripe 2 control element and prevents expression.

D. The overlap of repressor-binding sites and activator-binding sites in the *Eve* stripe 2 control element is striking. It suggests that the repressors function primarily by preventing activator binding. The extensive competition for binding between activators and repressors presumably sharpens the boundary between *Eve* expression and nonexpression, giving rise to very well-defined stripes in the embryo.

Reference: Stanojevic D, Small S & Levine M (1991) Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science* 254, 1385–1387.

7–59 The two models make different predictions about the activities of the mutant receptors. If a DNA-binding domain were created by hormone binding, then mutant receptors lacking the hormone-binding domain

would fail to activate CAT expression. By contrast, if a preexisting DNA-binding domain were uncovered by hormone binding, then some mutant receptors would be expected to activate CAT expression constitutively (in the presence or absence of hormone) due to deletion of a segment of protein that masks an otherwise functional DNA-binding domain.

The experimental results match the expectations for a preexisting DNA-binding domain that is masked by the hormone-binding domain. The four mutants with the longest C-terminal segments (shortest deletions) are missing enough of the hormone-binding domain to interfere with hormone binding, but they still retain enough to mask the DNA-binding domain. The next four mutants, which constitutively activate CAT expression, have an unmasked DNA-binding domain that binds DNA whether or not the hormone is present. The two mutants with the longest deletions are nonfunctional because they lack the complete DNA-binding domain.

Reference: Godowski PJ, Rusconi S, Miesfeld R & Yamamoto KR (1987) Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* 325, 365–368.

7–60

A. In the presence of **a**1, α2 exists in two forms with different binding specificities. Your second set of experiments (see Figure 7–29, lanes 5 and 6) supports this interpretation, and rules out the possibility that α2 is present in a single form that can bind to both **a**-specific and haploid-specific regulatory sequences. Addition of excess unlabeled **a**-specific DNA eliminates binding to the radioactive **a**-specific fragments, but not to the radioactive haploid-specific fragments. Similarly, addition of excess unlabeled haploid-specific DNA eliminates binding to the radioactive haploid-specific fragments. If a single form of α2 were able to bind to both regulatory sequences, either unlabeled site in excess would have eliminated binding to both kinds of radioactive fragment.

   Your third experiment (see Figure 7–29, lanes 7 and 8) shows that the ratio of binding activities for **a**-specific and haploid-specific sequences varies depending on the amount of **a**1 protein. If the α2 repressor in a diploid cell were shifted entirely into a form that could bind both sites, by phosphorylation, for example, the ratio should be independent of the amount of **a**1 protein.

B. Your experiments are most easily explained if **a**1 acts stoichiometrically to alter the binding specificity of the α2 repressor, presumably by binding to it. A stoichiometric mechanism is supported by the fragment-binding experiments shown in lanes 7 and 8 of Figure 7–29. When **a**1 is low, the binding to haploid-specific fragments is low; when **a**1 is high, the binding to haploid-specific fragments is high. Similarly, the simplest explanation for the effects of the defective α2 repressor is that it binds to **a**1 protein, thereby reducing the availability of the **a**1 protein for binding to the normal α2 repressor and preventing the turn off of haploid-specific genes.

   Note that neither of these experiments absolutely rules out the possibility that **a**1 protein acts catalytically on the α2 repressor. However, a catalytic mechanism for **a**1 protein would require special assumptions to account for the experimental observations. Subsequent experiments showed that both α2 and **a**1 are homeodomain proteins that function as dimers. The α2/α2 homodimer binds to **a**-specific *cis*-regulatory sequences, whereas the α2/**a**1 heterodimer binds to haploid-specific *cis*-regulatory sequences. In **a**/α diploid cells, a mixture of α2/α2 and α2/**a**1 dimers is present, allowing both haploid-specific and **a**-specific genes to be turned off.

References: Goutte C & Johnson AD (1988) a1 Protein alters the DNA binding specificity of α2 repressor. *Cell* 52, 875–882.

Goutte C & Johnson AD (1993) Yeast a1 and α2 homeodomain proteins form a DNA-binding activity with properties distinct from those of either protein. *J. Mol. Biol.* 233, 359–371.

ADDITIONAL MECHANISMS THAT REINFORCE CELL MEMORY IN HUMANS AND OTHER VERTEBRATES

DEFINITIONS

7–61 X-inactivation

7–62 DNA methylation

7–63 Epigenetic inheritance

7–64 Monoallelic gene expression (X-inactivation and genomic imprinting are examples, and also valid choices)

7–65 Genomic imprinting

7–66 CG island

TRUE/FALSE

7–67 False. Because the strands of DNA are antiparallel, the 5′CG sequences in one strand are paired with 5′CG sequences in the other strand. Thus, the Cs in both strand are in the same context and can be methylated by the same methyl transferases.

7–68 True. In unmethylated regions of the genome, spontaneous deamination of C (a very common event) gives rise to the novel DNA base, uracil, which can be accurately recognized and repaired. By contrast, deamination of 5-methyl C gives rise to a T, a normal DNA base, which is more difficult for the cell's repair machinery to recognize as incorrect. As a consequence, methylated CG dinucleotides in the germ line have tended to be lost during evolution, leaving the CG islands found in modern genomes.

7–69 True. There are several epigenetic mechanisms of inheritance that allow cells to retain a memory of the gene expression patterns in their parent cells, including transcription regulators that activate their own transcription, DNA methylation, and chromatin structure, to name a few.

THOUGHT PROBLEMS

7–70 The genomic DNAs in the sperm and unfertilized egg are methylated in a characteristic way. Shortly after fertilization, both the paternal and maternal genomes are subjected to a genome-wide wave of demethylation, during which the vast majority of methyl groups—except those responsible for genomic imprinting—are lost from the DNA. Demethylation of the maternal genome occurs passively as a consequence of inhibition of the maintenance methyl transferase, resulting in the loss of methyl groups during each round of DNA replication. Demethylation of the paternal genome occurs by active demethylation. Later in development, at the time of implantation in the uterine wall, new methylation patterns are established by several *de novo* DNA methyl transferases that methylate specific CG dinucleotides. Once the new patterns of methylation are established, they are preserved by the maintenance methyl transferase, which adds methyl groups to an unmethylated CG opposite a methylated CG. In the germ line, a second wave of demethylation occurs that removes the parental genomic imprints, allowing new, sex-specific imprints to be established.

7–71 The affected individuals have one deleted gene and one inactive gene due to imprinting. Individuals who carry the deletion will produce affected offspring only if they mate to the sex in which imprinting occurs. Thus, females who carry the deletion are at risk for affected progeny only if the gene is paternally imprinted. Similarly, males who carry the deletion are at risk for affected offspring only if the gene is maternally imprinted. In the pedigree shown in Figure 7–30A, it is only females who carry the deletion that have affected children. Thus pedigree A must involve paternal imprinting. Similarly, in pedigree B, it is only males who carry the deletion that have affected children; thus this pedigree must involve maternal imprinting.

Reference: Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM & Veres RC (2000) Genetics: From Genes to Genomes, pp. 408–410. Boston: McGraw-Hill Companies.

7–72 *Igf2* is an imprinted gene. It is expressed from the paternal chromosome, but the gene on the female chromosome is imprinted and turned off. This pattern of expression is just what would be expected based on the tug-of-war hypothesis. The gene is expressed from the male, which would tend to make the embryo larger; however, it is not expressed (because of imprinting) from the female chromosome, which would tend to limit growth of the embryo. This expected pattern of parental imprinting of growth-promoting and growth-retarding genes is common, but there are exceptions that call the general hypothesis into question. Nevertheless, it provides a provocative perspective on the phenomenon of imprinting.

Reference: Moore T & Haig D (1991) Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet.* 7, 45–49.

DATA HANDLING

7–73 These results argue strongly that a single critical methylation site cannot be responsible for determining whether the γ-globin gene will be expressed. If there were such a single site, the difference between the transcriptional activities of constructs B (0%) and C (100%) would have indicated that the site was located in the region that is unmethylated in construct C but methylated in construct B; that is, in one of the five CG sequences (12 to 16) shown in the expanded region around the start site for transcription (see Figure 7–31). This expectation is contradicted by the results with constructs D and E: neither made any γ-globin transcript. Since sites 14, 15, and 16 are unmethylated in construct D, and sites 12 and 13 are unmethylated in construct E, no *one* unmethylated site can be the sole determinant for *γ-globin* gene expression. Even when all five of these sites were unmethylated (construct F), the *γ-globin* gene was expressed only at a low level.

These results indicate that there are multiple methylation sites that contribute to the decision to express the *γ-globin* gene. The authors of these experiments favored the idea that a sizable region around the promoter (larger than in construct F) must be free of methylation for maximal gene expression.

Reference: Murray EJ & Grosveld F (1987) Site specific demethylation in the promoter of human gamma-globin gene does not alleviate methylation mediated suppression. *EMBO J.* 6, 2329–2335.

7–74

A. Gene A is expressed only from active X chromosomes, as indicated by equal expression in males and females and by the pattern of expression in the hybrid cells. Gene B is expressed from both active and inactive X chromosomes, as indicated by the results with the hybrid cell lines, and

by the levels of expression in the other cells, which correlate with the total number of X chromosomes. Gene C is expressed only from inactive X chromosomes, as indicated by its expression in females but not males, and by the pattern of expression in the hybrid cell lines.

The most common pattern of expression is like that of gene A; the vast majority of genes on the inactive X chromosome are turned off. A few genes like gene B are expressed from both active and inactive X chromosomes. The pattern for gene C was very surprising. The gene that encodes this RNA is called *Xist* (for X-inactivation specific transcript); it is expressed exclusively from the X-inactivation center on the inactive X chromosome, and it plays a direct role in X-inactivation.

B. The rule for X-inactivation is that only one X chromosome remains active—all the rest are inactivated. This is apparent in the Northern analysis in Figure 7–32. Gene A, which is expressed from the active X, is expressed at uniform levels regardless of the number of X chromosomes, indicating that only one X remains active. By contrast, gene C, which is expressed only from the inactive X, is expressed in all cells that have more than one X chromosome, at levels that depend on the number of X chromosomes. (Analysis of gene B, which is expressed from both active and inactive X chromosomes, provides no information about X-inactivation.)

These rules for X-inactivation were worked out from cytological observations long before the advent of molecular biology techniques. The inactive X chromosome is highly condensed and easily visualized as a distinct entity in the nucleus—the so-called Barr body. It was noted early on that female cells have one Barr body and male cells have none. Abnormal individuals with extra X chromosomes have a number of Barr bodies equal to the number of X chromosomes minus one.

Reference: Brown CJ, Ballabio A, Rupert JL, Lafreniere RG, Grompe M, Tonlorenzi R & Willard HF (1991) A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 349, 38–44.

POST-TRANSCRIPTIONAL CONTROLS

DEFINITIONS

7–75 RNA editing

7–76 Alternative RNA splicing

7–77 Post-transcriptional control

7–78 Internal ribosome entry site (IRES)

TRUE/FALSE

7–79 False. In order for there to be a change in the amino acid sequence at the end of the protein, a new splicing event would need to accompany the change in the site for poly-A addition. Splicing would be required to eliminate one stop codon and bring a new one into play. In eukaryotic cells, the transcription of many genes yields a set of mRNAs that differ only in the position of their poly-A tails. For most such genes, ribosomes translate the mRNA until they reach the stop codon, and ignore any differences in lengths of the 3′ untranslated regions.

7–80 False. Alternative splicing of the *Drosophila Dscam* gene has the potential to produce some 38,000 proteins, but the proteins are related to one another, as are alternatively spliced versions of the products of other genes. Thus, the diversity represented by this potential collection of

38,000 *related* proteins is much less than that represented by the 21,000 protein-coding genes in the human genome. Although the *Drosophila Dscam* gene is noteworthy for the large number of alternatively spliced products, alternative splicing itself is common; it is estimated that 90% of the genes in the human genome are alternatively spliced.

THOUGHT PROBLEMS

7–81 Flavopiridol blocks the ability of Cdk9 to phosphorylate RNA polymerase; thus you would expect flavopiridol to interfere with the conversion of RNA polymerase to the form required for productive HIV transcription. Although this outcome seems likely from the brief summary of the effects of Tat on HIV transcription given in the problem, there are other protein kinases in the transcription complex that might mediate the critical phosphorylation of RNA polymerase. Direct tests, however, have shown that flavopiridol effectively blocks Tat-activated transcription and interferes with HIV replication.

References: Jones KA (1997) Taking a new TAK on Tat transactivation. *Genes Dev.* 11, 2593–2599.

Chao S-H, Fujinaga K, Marion JE, Taube R, Sausville EA, Senderowicz AM, Peterlin BM & Price DH (2000) Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. *J. Biol. Chem.* 275, 28345–28348.

7–82 The first step—common to all mechanisms—is for the mRNA to leave the nucleus through the nuclear pores and enter the cytosol. From that point, there are three basic mechanisms that might apply to different mRNAs. (1) The mRNA associates with the cytoskeleton and is transported in a given direction by molecular motors. (2) The mRNA diffuses randomly and is captured by binding to protein components that are at the target location. (3) The mRNAs are subject to rapid degradation except when bound by a protein at their target location. An additional common feature of these three localization mechanisms is that the mRNA is kept in an inactive state until it reaches its target location.

7–83 Bound IRP interferes with translation by blocking the stable association of the small ribosomal subunit with the mRNA, the first step in initiation of translation. The small ribosomal subunit—complexed with additional factors—binds at the cap structure and then scans along the mRNA until it finds a suitable AUG at which to start translation. When an IRP is bound near the cap, the small ribosomal subunit cannot bind. If IRP binding is moved far enough downstream (>60 nucleotides) by changing the location of the IRE, then the ribosome complex can bind to the 5′ end of the message. Once bound, this juggernaut unwinds any structure and releases any proteins in its way.

Reference: Hentze MW & Kühn LC (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc. Natl Acad. Sci. USA* 93, 8175–8182.

7–84

A. The opposite regulation of ferritin and the transferrin receptor makes perfect biological sense. When iron levels are high, cells prevent toxicity in two ways. They decrease the amount of iron they take in by reducing their synthesis of transferrin receptor, and they increase the amount of iron that is safely sequestered inside ferritin by increasing ferritin synthesis. When iron levels are low, cells avoid iron deficiency by reversing these two effects. They increase the amount of transferrin receptor, allowing them to bring more iron into the cells. And they decrease ferritin synthesis, thereby reducing their levels of sequestered iron.

B. At low levels of iron, when the IRPs are bound to the IREs in the mRNA, the mRNA is stable. At high levels of iron, when IRPs are bound to the iron and are released from the mRNA, the mRNA is rapidly degraded. The observation that unbound mRNA is subject to rapid degradation suggested to the original researchers that a sensitive cleavage site was being masked by IRP binding. As a result, messages were stable in the absence of iron, but rapidly degraded when the cleavage site was exposed in the presence of iron. Specific cleavage sites have now been mapped in the mRNA.

Reference: Hentze MW & Kühn LC (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc. Natl Acad. Sci. USA* 93, 8175–8182.

7–85 The observation that an IRES element overcomes translational repression suggests that cap-dependent initiation of translation is the target for inhibition. The proteins that bind to the UA-rich element could also bind to and mask the 5′ mRNA cap, so that ribosome initiation complexes cannot bind it. In this scenario, if an alternative way to initiate translation—an IRES—were provided, translation could proceed perfectly well.

Reference: Otero LJ, Devaux A & Standart N (2001) A 250-nucleotide UA-rich element in the 3′ untranslated region of *Xenopus laevis* Vg1 mRNA represses translation both *in vivo* and *in vitro*. *RNA* 7, 1753–1767.

DATA HANDLING

7–86

A. The most dramatic changes were observed in HeLa cells because the changes altered the ability of those cells to recognize the splice site at the beginning of exon 4. In F9 cells, this splice site is skipped and, for that reason, F9 cells are presumably less sensitive to changes in the repeats.

B. The results in Figure 7–33 rule out the possibility that a particular combination of repeats is required for correct splicing in HeLa cells. It seems that two repeats can be enough, as shown by construct B (see Figure 7–33); however, not just any two repeats will do, as shown by construct E. To make more general conclusions—such as more than one repeat with at least one in the intron—would require that more combinations be tested.

Reference: Hedjran F, Yeakley JM, Huh GS, Hynes RO & Rosenfeld MG (1997) Control of alternative pre-mRNA splicing by distributed pentameric repeats. *Proc. Natl Acad. Sci. USA* 94, 12343–12347.

7–87 These results indicate that the tissue-specific synthesis of ApoB100 in liver cells and ApoB48 in intestinal cells results from a difference in the way the RNA transcripts are processed. The hybridization results in Table 7–2 show that the DNA in both liver and intestine matches the oligo-Q sequence, but not the oligo-STOP sequence. Therefore, the tissue-specific differences cannot be due to separate genes that are transcriptionally regulated. Since the *ApoB* mRNA in the intestine contains a termination codon at a point where the *ApoB* mRNA in the liver contains a glutamine codon, the two mRNAs cannot encode the same protein. Thus, ApoB48 and ApoB100 cannot be related to one another by tissue-specific protein cleavage.

The identity of the DNA sequences from liver and intestine and the difference in *ApoB* mRNA sequences from the same tissues indicate that one tissue must alter at least one specific nucleotide during expression of the *ApoB* gene. The results in Table 7–2 show that sequences complementary to the oligonucleotide with the termination codon (oligo-STOP)

are present only in intestinal RNA. Thus, the intestine specifically alters a nucleotide in the transcript. This work marks one of the earliest discoveries of RNA editing.

References: Powell LM, Wallis SC, Pease RJ, Edwards YH, Knott TJ & Scott J (1987) A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. *Cell* 50, 831–840.

Chen S-H, Habib G, Yang CY, Gu ZW, Lee BR, Weng S-A, Silberman SR, Cai S-J, Deslypere JP, Rosseneu M, Gotto AM, Li W-H & Chan L (1987) Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science* 238, 363–366.

7–88 The results with the 12 mutants argue very strongly that the regulatory interaction involves the nascent β-tubulin protein, rather than the β-tubulin mRNA. The only mutants that show a wild-type response to an increase in intracellular free tubulin dimers are the ones that still encode arginine (R) and glutamic acid (E) as the second and third amino acids in the coding portion of their mRNA. It is extremely unlikely that an interaction with the mRNA would tolerate just those changes that did not alter the amino acid sequence.

Further studies using site-directed mutagenesis have determined that the N-terminal tetrapeptide MREI confers the full range of β-tubulin autoregulation. A selective mRNA degradation mechanism operates during translation, if this tetrapeptide is bound by β-tubulin as it emerges from the ribosome.

References: Gay DA, Yen TJ, Lau JTY & Cleveland DW (1987) Sequences that confer β-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a β-tubulin mRNA. *Cell* 50, 671–679.

Yen TJ, Machlin PS & Cleveland DW (1988) Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. *Nature* 334, 580–585.

7–89 One attractive hypothesis is that the length of the poly-A tail determines the efficiency with which it interacts with the cap structure at the 5′ end. There is a good correlation between length of poly-A tail and efficiency of translation. Short poly-A tails may compromise an mRNA by binding too few poly-A-binding proteins, which may limit the strength of the interactions between the two ends of the mRNA and, thereby, the efficiency of translation.

Reference: Wickens M, Goodwin EB, Kimble J, Strickland S & Hentze M (2000) Translational control of developmental decisions. In Translational Control of Gene Expression (N Sonenberg, JWB Hershey, MB Mathews eds), pp. 295–370. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

7–90

A. The mRNAs in the presence or absence of the TGEs seem to be perfectly stable. There appears to be about as much radiolabeled mRNA present at the 1024-cell stage as there was when it was injected at the one-cell stage, indicating that little, if any, of the input RNA has been lost.
B. The presence of the TGEs significantly affects the lengths of the poly-A tails. In the absence of the TGEs, the poly-A tails are slowly shortened, but retain 15–40 A nucleotides even at the 1024-cell stage. By contrast, in the presence of the TGEs, the poly-A tails are rapidly shortened, and RNAs without tails begin to accumulate as early as the four-cell stage. Thus, it appears that the binding of proteins to the TGEs promotes removal of As from the poly-A tail.

Reference: Thompson SR, Goodwin EB & Wickens M (2000) Rapid deadenylation and poly(A)-dependent translational repression mediated by the *Caenorhabditis elegans tra-2* 3′ untranslated region in *Xenopus* embryos. *Mol. Cell. Biol.* 20, 2129–2137.

7–91 These experiments provide a very convincing demonstration that IRESs allow ribosomes to initiate translation in the absence of a cap (or an end of any kind). The linear and circular mRNAs were arranged so that they would give different translation products: a 20 kd protein from the linear mRNA, and a 23 kd protein from the circular mRNA. The gels confirm that a circular mRNA with an IRES is translated into the 23 kd product, whereas a circular mRNA without an IRES gives no product at all. Arranging the linear and circular mRNAs so that they would give different-sized fragments was a key aspect of the experimental design. Had they been constructed to give the same size fragment, it would have been very difficult to rule out contamination of the circular mRNA by the linear molecule.

Reference: Chen C-Y & Sarnow P (1995) Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* 268, 415–417.

REGULATION OF GENE EXPRESSION BY NONCODING RNAs

DEFINITIONS

7–92 RNA interference (RNAi)

7–93 MicroRNA (miRNA)

7–94 piRNA (piwi-interacting RNA)

7–95 CRISPR

7–96 Long noncoding RNA (lncRNA)

TRUE/FALSE

7–97 True. siRNA-mediated defense mechanisms are crucial for plants, worms, and insects, although in mammals, a protein-based system has largely taken over the task of fighting off viruses.

7–98 False. In the limited number of examples of lncRNA functions that are known, serving as a scaffold for binding groups of proteins is one of the functions of lncRNAs that has been defined.

7–99 True. The CRISPR system of defense in bacteria keeps track of foreign invaders by incorporating samples of the invaders' genome sequences into special regions of the bacterial genome, from which they are later transcribed and processed into crRNAs that can trigger destruction of invaders the next time they appear. The piRNA system is largely analogous, incorporating sequences of transposable elements into special clusters, which are transcribed and processed to produce piRNAs that suppress expression of transposable-element genes. There are also two distinct differences: piRNAs are directed against single-stranded RNA, whereas crRNAs target double-stranded DNA; and piRNAs target invaders that are already established in the host genome, whereas crRNAs target invaders from outside the cell.

THOUGHT PROBLEMS

7–100 First, a single miRNA can regulate a whole set of different mRNAs, so long as the mRNAs carry a common sequence in their untranslated regions. Second, regulation by miRNAs can be combinatorial, allowing incremental changes in translation of mRNAs, which can fine-tune gene expression. Third, an miRNA occupies little space in the genome compared with a protein.

7–101 These characteristics of piRNAs are suggestive of an important function. Of the ones listed, the most compelling is the conservation of piRNA genes in genomes as diverse as mouse, rat, and human. In the absence of some critical function, the piRNA genes would be expected to have diverged significantly. Although it is difficult to define the function of individual piRNAs—there are so many and they likely have overlapping functions—it is much easier to show that the proteins they interact with, the Piwi proteins, are critical. Mutations in Piwi proteins in fruit flies, nematode worms, flat worms, and plants cause severe problems in germline development.

Reference: Girard A, Sachidanandam R, Hannon GJ & Carmell MA (2006) A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* 442, 199–202.

7–102 The most reasonable hypothesis is that the defective *β-galactosidase* gene is being transcribed and processed into piRNAs. Clusters of piRNAs are transcribed onto a single large RNA from which many individual piRNAs are processed by cleavage and trimming. According to this hypothesis, some of the *β-galactosidase* sequences, which would be transcribed along with the piRNAs, are processed just like normal piRNAs. The β-galactosidase piRNAs may silence expression of the normal *β-galactosidase* gene in the same way that piRNAs silence expression of genes on transposable elements.

If this hypothesis is correct, then you should be able to find *β-galactosidase* sequences in the population of piRNAs. This is indeed what is observed.

Reference: Ronsseray S, Josse T, Boivin A & Anxolabéhère D (2003) Telomeric transgenes and trans-silencing in *Drosophila*. *Genetica* 117, 327–335.

DATA HANDLING

7–103

A. There is no evidence that *Let7* miRNA causes degradation of *Daf12* mRNA. The total amount of *Daf12* mRNA, as judged by the area under the two mRNA curves in Figure 7–40B, seems to be unchanged.
B. In the absence of *Let7* miRNA, *Daf12* mRNA is present in slightly larger polysomes than when *Let7* miRNA is present (Figure 7–40B). Larger polysomes mean that there are, on average, more ribosomes on *Daf12* mRNA when *Let7* miRNA is absent.
C. Because *Let7* miRNA reduces the average number of ribosomes on the *Daf12* mRNA, it seems likely that *Let7* miRNA in some way interferes with loading of ribosomes on *Daf12* mRNA. How it might reduce loading of ribosomes is not clear, but reduced loading would decrease protein expression.

Reference: Ding XC & Grosshans H (2009) Repression of *C. elegans* microRNA targets at the initiation level of translation requires GW182 proteins. *EMBO J.* 28, 213–222.

7–104 The results of single-cell analysis support the second hypothesis; namely, that deletion of *Xist* prevents inactivation of the X chromosome from which it is deleted. Thus, in mutant ES cells that have undergone differentiation, it is always allele A—the allele on the nonmutant X chromosome—that is inactivated.

If the first hypothesis—no X-inactivation—had been correct, the differentiated mutant ES cells would have looked the same as the undifferentiated cells; that is, both alleles would have been expressed. If the third hypothesis—no effect on X-inactivation—had been correct, the differentiated mutant ES cells would have resembled the nonmutant ES cells; that is, the A and B alleles would have been expressed in different individual cells.

Reference: Penny GD, Kay GF, Sheardown SA, Rastan S & Brockdorff N (1996) Requirement for *Xist* in X chromosome inactivation. *Nature* 379, 131–137.

7–105

A. In the mutant ES cells, differentiation causes inactivation of one of the two X chromosomes. Thus, the functions responsible for chromosome counting and silencing must be intact in the *Tsix* mutant cells. The choice of which X chromosome to inactivate, however, is no longer random: the chromosome that cannot express *Tsix* is inactivated, and the normal X chromosome remains active. This result strongly suggests that *Tsix* is critical for the choice of which X chromosome to inactivate.

B. *Tsix* could regulate *Xist* in two general ways. Transcription of *Tsix* across the *Xist* promoter could interfere with *Xist* transcription, thereby keeping its levels low and preventing X-inactivation. Alternatively, *Tsix* could regulate *Xist* through an interaction between the *Tsix* and *Xist* lncRNAs, which are complementary. The pairing could prevent *Xist* function, or it could destabilize *Xist* lncRNA. For either mechanism, turning off *Tsix* transcription on one X chromosome would allow *Xist* lncRNA to accumulate on that chromosome, leading to its inactivation.

References: Lee JT & Lu N (1999) Targeted mutagenesis of *Tsix* leads to nonrandom X inactivation. *Cell* 99, 47–57.

Mlynarczyk SK & Panning B (2000) X inactivation: *Tsix* and *Xist* as yin and yang. *Curr. Biol.* 10, R899–R903.

MCAT STYLE

7–106

A. Enhancer sequences promote transcription and can be located on either end of the gene and far away from the coding sequence. Choice B is not correct because Mediator refers to a large multiprotein complex that promotes initiation of transcription; it is not a DNA element. Choice C is not correct because promoters are always found at the 5′ ends of genes. Choice D is not correct because the TATA box is always located within the promoter region at the 5′ end of a gene.

7–107

C. Many transcription regulators bind cooperatively to DNA. The closely apposed Oct4 and Sox2 binding sites allow the transcription regulators to directly interact with each other when they bind to the DNA element, which promotes formation of a complex. In this case, the direct interaction of Oct4 with Sox2 causes conformational changes in both proteins that expose transcriptional activation domains and enhance transcription. Choice A is not correct because it does not explain how a small change in spacing would affect the spread of repressive chromatin. Choices B and D are incorrect because a slight change in the spacing

between the Oct4 and Sox2 binding sites would be unlikely to affect the suggested functions.

7–108

B. Small changes in Oct4 levels can cause large changes in transcriptional output if the binding of Oct4 to DNA is cooperative. For example, if Oct4 is mostly monomeric, but forms a weakly associated dimer that is stabilized by binding to DNA, the amount of Oct4 that is bound to DNA will increase sharply with Oct4 concentration. If the Oct4-DNA complex promotes or inhibits transcription, small changes in the concentration of Oct4 can cause large changes in the transcriptional outputs that affect differentiation. Choice A is not correct because chromatin modification, although it can explain how genes are turned on or off, cannot explain how small changes in the amount of a transcription regulator can cause big effects. Choice C is incorrect because dimerization alone cannot create nonlinear effects—big changes in output from small changes in input. Choice D is not correct because negative feedback would tend to decrease the effects of small changes in transcription regulator levels, rather than amplify them.

7–109

D. Many genes in differentiated cells are kept inactive by histone modifying enzymes that create repressive chromatin. By interfering with DNA methylation, 5-azacytidine also affects the reader-writer complexes that affect histone modifications, allowing genes to be activated. Choice A is not correct because Mediator is a large multiprotein complex that helps activate RNA polymerase; it is not able to reverse the repressive chromatin found at silenced genes in differentiated cells. Choice B is incorrect because DNA methylation is usually associated with inactive genes, so inhibition of a demethylase would favor gene inactivation. Choice C is unlikely because riboswitches work primarily in prokaryotic cells.

7–110

C. If MyoD promotes its own transcription via positive feedback, an initial pulse of MyoD expression from a vector would trigger transcription of the endogenous *MyoD* gene, which could then keep itself active via positive feedback. Once MyoD is active, it will trigger differentiation and also maintain the differentiated state. During the normal process of differentiation, signals received from outside the cell trigger an initial pulse of MyoD transcription from the endogenous *MyoD* gene. The other answers are not correct because they suggest mechanisms that would not be capable of maintaining the differentiated state when MyoD expression from the vector was turned off; once MyoD was turned off, the cells would revert to their status before MyoD was turned on.

7–111

B. The circuit in B includes a positive feedback loop, which would ensure that the differentiated state persists after removal of the initial pulse of MyoD. It also includes a feed forward circuit, in which expression of late genes requires binding of MyoD, as well as a signal from the early genes. In circuits A and C, there is no positive feedback loop to maintain levels of MyoD. Circuit D does not include the binding of MyoD to the promoters of the late genes.

Analyzing Cells, Molecules, and Systems

CHAPTER 8

IN THIS CHAPTER

ISOLATING CELLS AND GROWING THEM IN CULTURE

DEFINITIONS

8–1 Monoclonal antibody

8–2 Hybridoma

TRUE/FALSE

8–3 True. Individual cells can be cut away from their neighbors with a laser beam, allowing the cells to be isolated and studied.

8–4 False. A monoclonal antibody recognizes a specific antigenic site, but this does not necessarily mean that it will bind only to one specific protein. There are two complicating factors. First, antigenic sites that are similar, but not identical, can bind to the same antibody with different affinities. If too much antibody is used in an assay, the antibody may bind productively to a protein with a high-affinity site and to other proteins with low-affinity sites. Second, it is not uncommon for different proteins to have the same antigenic site; that is, the same cluster of five or six amino acid side chains on their surfaces. This is especially true of members of protein families, which have similar amino acid sequences, and are often identical in functionally conserved regions.

THOUGHT PROBLEMS

8–5 Cells in a tissue are bound together by protein-mediated attachments to one another and to an extracellular matrix containing collagen. Treatment with trypsin, collagenase, and EDTA disrupts these attachments. Trypsin is a protease that will cleave most proteins, but generally only those portions of a native protein that are unstructured. The triple-helical structure of collagen, for example, is a poor substrate for trypsin. Collagenase, which is a protease specific for collagen, digests a principal component of the extracellular matrix. EDTA chelates Ca^{2+}, which is required for the cell-surface proteins known as cadherins to bind to one another to link cells together. Removal of Ca^{2+} prevents this binding and thereby loosens cell–cell attachments.

The treatment doesn't kill the cells because all the damage occurs to extracellular components, which the cells can replace. So long as the plasma membrane isn't breached, the cells will survive.

8–6 It is the general goal of cell biology research to discover how individual cells work, but it is very difficult to study most processes in single cells. Analysis of a population of identical cells can yield valid conclusions about the workings of the individual cells. By contrast, if the population is a mixture of different cell types, its analysis will give properties of the

mixture, which may or may not accurately describe the individual cells. Consider an analogy. We know from looking at individual human eyes that they are various shades of brown or blue or green. Yet if we could tell eye color only by looking at 1000 at a time, and if we started with a random population, we might conclude that eyes were a bluish brown—a color that doesn't apply to any single individual.

8–7 Primary cultures are prepared directly from the tissues of an organism, without cell proliferation *in vitro*. When cells in primary culture are induced to proliferate, they form what are known as secondary cultures. Secondary cultures have a limited life-span and will stop growing after a finite number of cell divisions. Cell lines are derived from rare, altered cells in a secondary culture that can proliferate without limit. Cells from a cell line will proliferate indefinitely, having overcome the blocks that limit the proliferation of the vast majority of cells in secondary cultures. When examined more closely, it turns out that most cell lines have an abnormal karyotype, with chromosome rearrangements and nontypical numbers of chromosomes being common. By contrast, primary and secondary cultures generally have karyotypes that are typical of cells from the parent organism.

8–8 The two statements are not exactly the same. Both emphasize the essential feature of the hybridoma technique: its ability to generate antibodies of a single type in pure form. The two statements differ in exactly why this might be useful. The first statement stresses the possibility of obtaining a monoclonal antibody directed against a minor component of the mixture of molecules used to elicit an immune response. Thus, if a protein of interest were present as a minor component in an impure mixture, the hybridoma technique would allow specific monoclonal antibodies to be generated against it.

The second statement highlights another advantage of the hybridoma technique. Even when a pure protein is used to elicit an immune response, a variety of different antibodies are generated in different proportions. These antibodies are typically directed at different antigenic sites (epitopes) on the protein. In some cases, a minor type of antibody directed against a particular epitope can have very useful properties; the hybridoma technique allows such an antibody to be generated in a pure form as a monoclonal antibody.

8–9 Yes. In fact, it is common to raise antibodies against other antibodies. Usually, this is done by introducing antibodies from one species into a second species, for example, by injecting mouse antibodies into goats. In this example, the mouse antibodies are recognized as foreign proteins by the goat, which mounts an immune response and generates goat antibodies that bind to the mouse antibodies. It is also possible to raise antibodies against antibodies from the same species. In the same species, most parts of the injected antibody molecules will be indistinguishable from the host antibodies and thus will be treated as "self" and not elicit an immune response. But the portion of the antibody molecule that binds to an antigen (the so-called idiotype) can be recognized as foreign and elicit an immune response, generating antibodies directed against the antigen-combining site of the injected antibody. Idiotypes are not recognized as self because each individual species is present at too low a level.

CALCULATIONS

8–10 It will take 1000 seconds (about 17 minutes) to sort 10^6 cells, which on average would contain 10 of the rare cell type you are after. But because of the random distribution of the rare cells within the population, it could

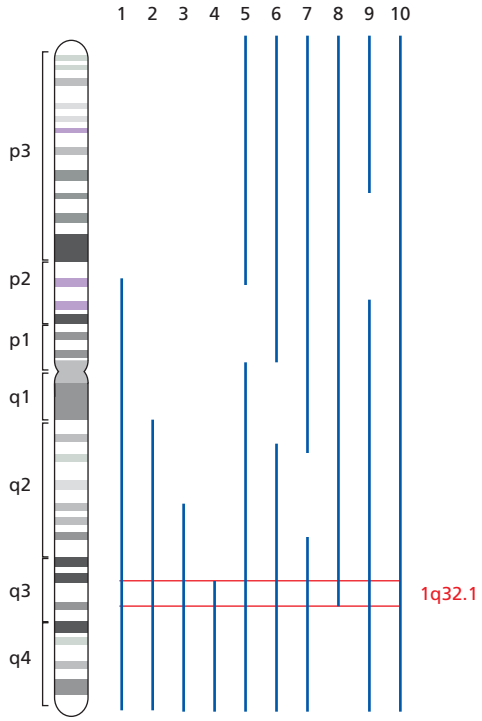


Figure 8–42 Location of the FeLV-C receptor to a segment of human chromosome 1 (Answer 8–11). The *red lines* encompass the portion of chromosome 1 that is common to all the hybrid cell lines.

either take somewhat more time or somewhat less time to sort exactly 10 rare cells.

DATA HANDLING

8–11 The FeLV-C receptor maps to that portion of human chromosome 1 (1q32.1) that is common to all of the infected hybrid cell lines, as illustrated in **Figure 8–42**. Surveying the known genes in this area of the chromosome, identifying likely candidates, and expressing their cDNAs in rodent cells to see whether they allowed infection by FeLV-C could identify the gene for the receptor.

Reference: Rasko JEJ, Battini J-L, Kruglyak L, Cox DR & Miller AD (2000) Precise gene localization by phenotypic assay of radiation hybrid cells. *Proc. Natl Acad. Sci. USA* 97, 7388–7392.

PURIFYING PROTEINS

DEFINITIONS

8–12 Column chromatography

8–13 High-performance liquid chromatography (HPLC)

8–14 Fusion protein

TRUE/FALSE

8–15 True. Present-day ultracentrifuges rotate at sufficiently high speed to generate forces up to 500,000 times gravity, which is more than adequate to drive hemoglobin through solution. Spun long enough, hemoglobin could be driven to the bottom of the tube and pelleted.

8–16 True. Gel-filtration columns exclude proteins that are too large to fit into the pores in the beads, but they fractionate the proteins that can enter the pores. Because the pores are not uniform in size, proteins experience the

internal volume of the beads to greater or lesser extents. Proteins that can enter only a small fraction of the pores will elute early from a gel-filtration column, while those that can enter all the pores will elute late. If all the pores were the same size, then a protein would either be excluded from the pores and elute in the external volume, or it would be included in the pores and elute with the external plus internal volume. This answer presupposes that the column is run slowly enough that equilibrium is achieved throughout and that the beads are inert so that there is no interaction between them and the proteins.

Reference: Cantor CR & Schimmel PR (1980) Biophysical Chemistry, pp. 670–675. New York: WH Freeman and Company.

THOUGHT PROBLEMS

8–17 The cell homogenate would first be centrifuged at a speed somewhat lower than that required to pellet mitochondria. At an appropriate rate of centrifugation, cell structures that are larger and denser than mitochondria will form a pellet, leaving the mitochondria in the supernatant. Centrifugation is then stopped and the supernatant is poured into a new centrifuge tube, leaving the pellet behind. The supernatant is then centrifuged at a rate that is sufficient to cause mitochondria to pellet, but which will leave smaller structures in the supernatant. Centrifugation is then stopped, the supernatant is discarded, and the pellet is resuspended in buffer, giving a solution enriched in mitochondria.

8–18 Velocity sedimentation is used to separate components that differ in size or shape, or both. It is carried out by layering a solution containing the components to be separated on top of a shallow density gradient formed by increasing concentrations—from top to bottom—of a small molecule such as sucrose. Upon centrifugation, individual components will move through the gradient according to their size and shape. Because identical components have the same properties, they move as a defined band, which can be collected.

Equilibrium sedimentation is used to separate components that differ in their buoyant density. Components to be separated are most often layered on top of a steep sucrose gradient and centrifuged until the components move to their equilibrium density. (The components can also be mixed into the gradient to start with, but for sucrose density gradients it is more common to establish the gradient and then layer the components on top.) Molecules with the same density will form defined bands, which can be collected.

Because most proteins have about the same density, velocity sedimentation would be preferred over equilibrium sedimentation for the separation of two proteins of different size.

8–19 The rate of sedimentation of a protein is based on size *and* shape. The nearly spherical hemoglobin will sediment faster than the more rod-shaped tropomyosin, even though tropomyosin is the larger protein. Shape comes into play because molecules that are driven through a solution by centrifugal force experience the equivalent of frictional drag. A spherical protein, with its smaller surface-to-volume ratio, will experience less drag than a rod, and therefore will sediment faster. You can demonstrate this difference using two sheets of paper. Crumple one into a sphere and roll the other into a tube. Now drop them. The crumpled ball will hit the ground faster than the tube. In this demonstration, the centrifugal force is replaced by gravity and the friction with molecules in solution is replaced by friction with air. The underlying principles are the same.

8–20 All these methods employ small beads that are packed into columns to which a solution of proteins is applied. Ion-exchange chromatography uses beads that carry positive charges (anion exchangers) or negative charges (cation exchangers). Proteins spend more or less time associated with the beads, depending on the arrangement of charged groups on their surfaces. Weaker-binding proteins elute from the column earlier and tighter-binding proteins elute later. Because the strength of association varies with pH and ionic strength of the solution that is passing down the column, the association between proteins and the beads can be varied systematically to find the best conditions for purification of a particular protein.

Hydrophobic chromatography uses beads that have hydrophobic groups protruding from their surfaces. These hydrophobic groups can interact with hydrophobic regions on the surfaces of proteins and delay their progress through the column. Once again, the stronger the interaction with the beads the longer the protein remains on the column.

Gel-filtration chromatography (also known as size-exclusion chromatography) uses beads with pores. Proteins that are too large to fit into the beads pass unretarded through the column, whereas proteins that can enter the beads are retarded by the time they spend inside the bead. For proteins that can enter the beads, larger proteins come off the column earlier than smaller proteins. Beads with a variety of pore sizes are available so that a gel-filtration column can be tailored to purification of a particular protein.

Affinity chromatography uses beads to which specific molecules, small or large, have been attached. The choice of molecule depends on the particular protein whose purification is desired. One common application, for example, uses glutathione Sepharose® (the small molecule glutathione attached to Sepharose beads) to capture proteins fused to GST (glutathione-*S*-transferase). Passing glutathione through the column, which displaces the GST-tagged protein, elutes the bound protein. Another common example attaches antibodies that are specific for a particular protein to the beads, allowing a specific protein to be bound to the column; these interactions can be disrupted with high salt or changes in pH to allow the protein to be eluted. Proteins that are known to associate with other proteins can also be used for affinity chromatography.

CALCULATIONS

8–21

A. The specific activity (units/mg) at each step in the purification is shown in **Table 8–7**. The increase in specific activity indicates that some degree of purification was achieved at each step.

B. The greatest increase in specific activity (17-fold) occurred with affinity chromatography. This increase indicates that the fraction of enzyme relative to total protein has increased most dramatically at this step. The

TABLE 8–7 Purification of an enzyme (Answer 8–21).

Procedure	Total volume (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude extract	2000	15,000	150,000	10
2. Ammonium sulfate precipitation	320	4000	140,000	35
3. Ion-exchange chromatography	100	550	125,000	227
4. Gel-filtration chromatography	85	120	105,000	875
5. Affinity chromatography	8	5	75,000	15,000

least effective step was ammonium sulfate precipitation, which gave only a 3.5-fold increase in specific activity. Ammonium sulfate precipitation is often used as the first step in purification because it is cheap, it reduces the total volume and protein, and cleans up the crude homogenate so that it can be run on subsequent columns.

C. With additional purification steps, the specific activity should rise until it reaches a value that doesn't change. Once the enzyme is pure, then its activity per mg of protein will remain constant. The specific activity increases with purification because irrelevant proteins are being discarded at each step. Purity of an enzyme preparation is routinely checked by gel electrophoresis. If the protein is pure, it will show up as a single band.

D. The overall purification is 1500-fold (from a specific activity of 10 at the start to 15,000 at the end). If the enzyme is pure at this stage, then it must have represented 1/1500 (0.07%) of the protein in the cell.

DATA HANDLING

8–22

A. In the beginning, the solution of CsCl is a uniform density throughout, 1.71 g/mL in these experiments, and the DNA is distributed evenly throughout (**Figure 8–43A**). Under the influence of the centrifugal force (70,000 times gravity), the CsCl is pushed toward the bottom of the tube. This downward force is counterbalanced by random diffusion of the ions. At equilibrium, a linear gradient forms that is typically about 7% denser at the bottom of the tube than at the top (**Figure 8–43B**). As the gradient of CsCl forms, the DNA floats to its density. The narrowing of the band with time shows how the gradient forms over time. The solution of CsCl becomes less dense at the top and denser at the bottom of the centrifuge tube, as is evident even at 4 hours in Figure 8–3.

B. The buoyant density of DNA—actually the cesium salt of DNA—is about 1.71 g/mL. At the center of the centrifuge tube, the density of the CsCl solution equals the average density, which is the starting density. Since the DNA forms a band near the center, it must have a density near the average. Using a refractometer (or other means), it is possible to determine the density of the gradient at any point, so that a material need not band at the center of the tube for its density to be deduced.

C. Three major factors contribute to the width of the band of DNA. One is diffusion of the DNA, which opposes the tendency of the DNA to focus at its density. Indeed, the diffusion coefficient of DNA (and other molecules) can be calculated from the width of the band in equilibrium centrifugation. The second factor is that the density of DNA depends on the nucleotide composition. Because the DNA in this experiment was derived by random fragmentation of the *E. coli* genome, the fragments have a range of nucleotide compositions, each with a slightly different density, which also contributes to the width of the band observed in Figure 8–3. The third factor is the speed of centrifugation; the higher the

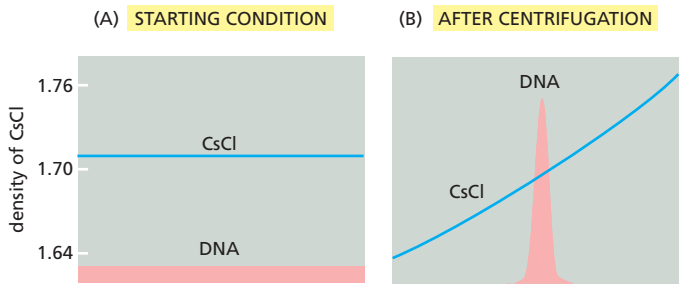


Figure 8–43 Equilibrium sedimentation of DNA in a solution of CsCl (Answer 8–22). (A) Density of CsCl and distribution of DNA at the beginning of the experiment. (B) Density distribution of CsCl and DNA at equilibrium.

speed, the greater the force times gravity. Because the diffusion coefficient does not change with speed, the higher the centrifugal force, the tighter the band.

References: Meselson M & Stahl FW (1958) The replication of DNA in *Escherichia coli. Proc. Natl Acad. Sci. USA* 44, 671–682.

Cantor CR & Schimmel PR (1980) Biophysical Chemistry, pp. 632–634. New York: WH Freeman and Company.

8–23

A. The smaller proteins come off the column later than the larger proteins because they experience a larger fraction of the internal volume of the column; that is, that portion of the column that is inside the beads. Smaller proteins can fit into more of the pores in the beads than can the larger proteins; hence their migration through the column will be retarded relative to larger proteins.

B. Plots of molecular mass and log of molecular mass versus elution volumes are shown in **Figure 8–44A and B**. It is clear that the plot of log of molecular mass gives a much closer approximation to a straight line than does the plot of molecular mass. The reason is that the time a protein spends on the column is related to its radius, which determines its ability to enter the pores. Since these proteins are roughly spherical, their radii will be related approximately to the cube roots of their volumes, which is equivalent to the cube roots of their molecular masses. A more rigorous treatment of this question shows that the Stokes radius, which is the effective hydrated radius of a protein and takes into account the shape of the protein, is the best predictor of elution volume.

Reference: Cantor CR & Schimmel PR (1980) Biophysical Chemistry, pp. 674–675. New York: WH Freeman and Company.

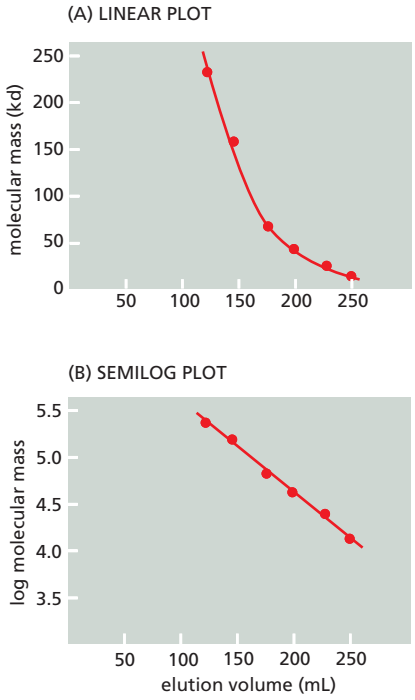


Figure 8–44 Plots of molecular mass against elution volumes (Answer 8–23). (A) Molecular mass versus elution volume. (B) Log molecular mass versus elution volume.

8–24

A. The protein will become more negative at higher pH values, as more ionizable groups give up their protons. Over the indicated range, the major effect will be on histidine residues, which have p*K* values around 6.5. These groups will be positively charged at pH 5.0 and uncharged at pH 7.5. This loss of positive charge means the protein will become more negative as the pH is raised.

B. You want to pick a pH at which your protein binds to the DEAE-Sepharose beads. If it does not bind to the beads, the protein will pass through the column with all the other proteins that don't bind. Ion-exchange chromatography is carried out under conditions in which the protein interacts with the beads. The nature of the interaction determines how the protein is fractionated relative to other proteins.

C. In general, you want to pick a pH at which the protein of interest binds to the column, but doesn't bind too strongly. Thus, pH 6.5 would be the best choice for your initial studies. If you pick too high a pH, the protein may bind to the column too strongly, requiring harsh conditions to remove it.

D. Your preliminary experiments have given you the proper pH to bind your protein to the column. Your first step will be to equilibrate the column with a buffer at that pH. Next, you will bring your protein sample to the same pH in the same buffer, and allow it to flow into the column. The column is then washed with one or two column volumes of buffer at the same pH, which washes out all of the protein that does not bind to the column. Finally, you elute your protein using a gradient of increasing ionic strength (salt concentration). At a characteristic ionic strength, the salt in the buffer will compete effectively with your protein for the charged groups on the column. The released protein will then flow out of the column in a relatively tight band.

ANALYZING PROTEINS

DEFINITIONS

8–25 Nuclear magnetic resonance (NMR) spectroscopy

8–26 Two-dimensional gel electrophoresis

8–27 SDS polyacrylamide-gel electrophoresis (SDS-PAGE)

8–28 X-ray crystallography

8–29 Western blotting (immunoblotting)

TRUE/FALSE

8–30 False. There are 6×10^{23} molecules per mole; hence, only 0.6 molecules in a yoctomole. The limit of detection is one molecule, or 1.7 yoctomole. No instrument can detect less than one molecule (it is either present in the instrument or it is not).

Reference: Castagnola M (1998) Sensitive to the yoctomole limit. *Trends Biochem. Sci.* 23, 283.

THOUGHT PROBLEMS

8–31 In gel-filtration chromatography, there are two different volumes to be concerned with: the volume outside the beads and the volume inside. Larger molecules spend less time in the internal volume and thus flow through the column more rapidly. By contrast, small molecules experience more of the internal volume (because they can enter the beads more easily). Because they are spread throughout a larger volume, it takes longer for them to be eluted from the column.

In SDS-PAGE, the entire gel is essentially one giant bead, and all molecules must find their way through the meshwork of holes in the cross-linked matrix. Because smaller molecules have more pathways available to them—more holes that can accommodate their passage—they traverse the gel more quickly.

8–32 The detergent SDS carries a negative charge so that when it binds, the proteins become highly negatively charged. Because they are negatively charged, the proteins will move toward the positive electrode (the anode). Thus, it is critical that you place the positive electrode at the bottom of the gel. (Nearly everyone who has ever run a gel has attached the electrodes incorrectly, but usually once is enough.)

8–33 The reducing environment inside cells keeps disulfide bonds from forming. Once the cells are broken open and exposed to the atmosphere (oxygen), their environment becomes oxidizing, which allows disulfide bonds to form. This situation is exacerbated during preparation of the sample for SDS-PAGE. SDS denatures the proteins, which exposes all the sulfhydryl groups, and the proteins are applied to the gel in a way that highly concentrates them as they enter the gel, dramatically increasing their opportunities to find inappropriate partners. In addition, the chemicals used to cross-link the polyacrylamide gel add to the oxidizing environment. NEM prevents formation of inappropriate disulfide bonds by reacting with the free sulfhydryl groups, thereby keeping them out of trouble.

8–34 Isoelectric focusing in the first dimension and SDS-PAGE in the second dimension are used to separate proteins by two-dimensional polyacrylamide-gel electrophoresis. Isoelectric focusing, which does not affect the

intrinsic charge of the proteins, must be carried out first. If SDS-PAGE were carried out first, all the proteins would be coated with negatively charged SDS, which would eliminate the effectiveness of isoelectric focusing.

8–35 It is difficult to overestimate the value of the current (and future) databases of protein structure and function. It seems entirely likely that such databases will yield increasingly detailed information about the basic properties of previously unknown proteins. Yet as valuable as such information is, it is unlikely to provide sufficient detail to permit one to know how the protein functions in its biological context. Knowing that a protein is a protein kinase, for example, does not define its targets of phosphorylation and the range of its biological effects. Far from putting biochemists out of work, such databases will allow them to tackle ever more interesting questions.

8–36 Although it is invaluable, hybridoma technology is labor intensive and time consuming, requiring several months to isolate a hybridoma cell line that produces a monoclonal antibody of interest. Also, there is no guarantee that the cell line will produce a monoclonal antibody with the specific properties you are after. It is much simpler—a few days' work—to add an epitope tag to your protein, using recombinant DNA technology, and use a commercially available, well-tested antibody to that epitope. The possibility that the tag may alter the function of the protein is a critical concern, but you can add the epitope easily to either the N- or C-terminus and test for the effect on the protein's function. In most cases, a tag at one or the other end of the molecule will be compatible with its function.

8–37 There is a good reason for the inverse relationship between half-life and maximum specific activity: the shorter the half-life, the greater the number of atoms that will decay per unit time, hence the greater the number of dpm or curies. If the radioactive atoms were present on an equimolar basis, then those with shorter half-lives would give more dpm; that is, they would have more Ci/mmol—a higher specific activity.

8–38 The half-life of ^{32}P is 14.3 days; thus, you had already exposed the film for one half-life in the first two weeks. If you could somehow wait long enough for all the remaining ^{32}P to decay, it would sum to the amount you detected in the first two weeks. You could expose the new film from now to the end of time and not have any brighter band than you had at the end of two weeks.

CALCULATIONS

8–39 You would need 10^5 copies of a 120-kd protein in a mammalian cell (and 100 copies in a bacterial cell) in order to be able to detect it on a gel. The calculation comes in two parts: how many cell-equivalents can be loaded onto the gel, and how many copies of a protein can be detected in the band. As shown below for mammalian cells, 100 μg corresponds to 5×10^5 mammalian cells and to 5×10^8 bacterial cells.

$$\frac{\text{cells}}{\text{gel}} = \frac{100\ \mu\text{g}}{\text{gel}} \times \frac{\text{mg}}{1000\ \mu\text{g}} \times \frac{\text{mL}}{200\ \text{mg}} \times \frac{\text{cm}^3}{\text{mL}} \times \frac{(10^4\ \mu\text{m})^3}{(\text{cm})^3} \times \frac{\text{cell}}{1000\ \mu\text{m}^3}$$

$$= 5 \times 10^5\ \text{cells/gel}$$

There are 5×10^{10} 120-kd proteins in a 10-ng band.

$$\frac{\text{molecules}}{\text{band}} = \frac{10\ \text{ng}}{\text{band}} \times \frac{\text{nmol}}{120{,}000\ \text{ng}} \times \frac{6 \times 10^{14}\ \text{molecules}}{\text{nmol}}$$

$$= 5 \times 10^{10}\ \text{molecules/band}$$

Thus, if you can detect 5×10^{10} proteins in a band and can load the equivalent of 5×10^5 mammalian cells per gel, there must be 10^5 copies of the protein per cell ($5 \times 10^{10}/5 \times 10^5$) in order for it to be detectable as a silver-stained band on a gel. For a bacterial cell, there need to be 100 copies of the protein per cell ($5 \times 10^{10}/5 \times 10^8$).

8–40 This problem involves a two-part calculation. If you know the specific activity of the labeled ATP (μCi/mmol), then you can use that value to convert 1 cpm per band (1 dpm/band for ^{32}P) into the number of proteins per band. The specific activity of ATP is 1.1×10^6 μCi/mmol. Since ATP is 0.9 mM in the extract after addition of the label,

$$\text{specific activity} = \frac{10\ \mu\text{Ci}}{10\ \mu\text{L}} \times \frac{\text{L}}{0.9\ \text{mmol ATP}} \times \frac{10^6\ \mu\text{L}}{\text{L}}$$

$$= 1.11 \times 10^6\ \mu\text{Ci/mmol ATP}$$

The specific activity of ATP is equal to the specific activity of phosphate on the labeled proteins. Using this specific activity (and various conversion factors given in Table 3 on page 964), you can calculate that 1 dpm/band corresponds to 2.4×10^8 protein molecules per band.

$$\frac{\text{proteins}}{\text{band}} = \frac{1\ \text{dpm}}{\text{band}} \times \frac{\mu\text{Ci}}{2.22 \times 10^6\ \text{dpm}} \times \frac{\text{mmol}}{1.11 \times 10^6\ \mu\text{Ci}} \times \frac{6 \times 10^{20}\ \text{molecules}}{\text{mmol}}$$

$$= 2.4 \times 10^8\ \text{molecules/band}$$

8–41

A. As indicated in Figure 8–6B, this technique can detect 5 fmol of myelin basic protein, which corresponds to 3×10^9 molecules [(5 fmol) × (6×10^8 molecules/fmole)].

B. Because monoclonal antibodies react with one antigenic site, the technique should not depend on the molecular mass of the protein. In practical terms, there are several possible sources of variation with different proteins: efficiency of transfer to the nitrocellulose filter, accessibility of the antigenic site to the primary antibody, and reactivity with the secondary antibody, to name a few.

DATA HANDLING

8–42

A. No, the standard proteins do not migrate as an inverse function of their molecular masses. It is clear in this set of proteins, for example, that the 67-kd protein has not migrated half as far as the 35-kd protein. A plot of log molecular mass versus migration does give a more linear relationship, although not a straight line (**Figure 8–45**).

B. There are two ways to estimate the molecular masses of proteins separated by SDS-PAGE. One is to guess at the mass, using the nearest standard protein as a guide. Thus, you might estimate cyclin B, for example, at about 58 kd. There are two problems with this approach. First, it is difficult to estimate masses with confidence directly from the gel in Figure 8–7 because there is not a linear relationship between molecular mass and distance of migration. Second, there is no guarantee that the standard protein runs exactly at its molecular mass.

The second approach is to plot migration of the standard proteins as a function of their molecular masses to give a line that can then be used to estimate the molecular masses of the unknown proteins. One such curve is shown in Figure 8–45, which plots the log of the molecular mass against the distance of migration. A plot using log values gives a much straighter line than a plot using molecular mass directly; this makes for

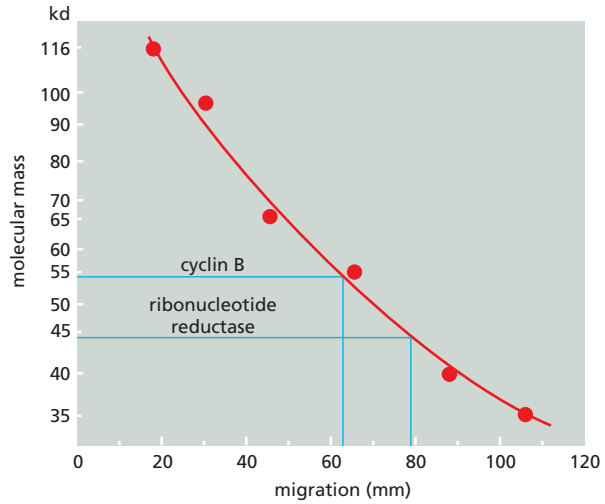


Figure 8–45 A plot of log molecular mass versus distance of migration (Answer 8–42).

more accurate estimates of the molecular masses. As shown in Figure 8–45, the estimated molecular mass of ribonucleotide reductase is 44 kd and that of cyclin B is 54 kd.

C. The molecular mass of the small subunit of ribonucleotide reductase, as estimated from the SDS gel, matches the calculated molecular mass very well. By contrast, the estimate for cyclin B is much higher than expected. Possible reasons for this large difference are post-translational modifications that are not accounted for by the calculated molecular mass, or peculiarities of cyclin B sequence that make it migrate anomalously. Examples of both effects are known (and not uncommon). In the case of cyclin B, the basis for its slow migration is its large number of positive charges, which partially offset the charges added by SDS. As a result, cyclin B carries less overall negative charge than would an average SDS-coated protein of its size. Histones, which are also highly positively charged, likewise run anomalously slowly on SDS gels.

8–43 An m/z difference of 80 corresponds to a phosphate. Addition of a phosphate to the hydroxyl of a serine, threonine, or tyrosine would add three O atoms (48), one P atom (31), and two H atoms (2), in place of one H atom (1) from the hydroxyl group, for a net addition of 80. Under conditions used in mass spectrometry analysis, there is no charge on the phosphate.

8–44

A. Since all four monoclonal antibodies were raised against Orc1, they should all bind to Orc1 at a minimum. If they can bind to Orc1 when it is a member of ORC, they should bring down a set of common proteins that represent the other members of ORC. As shown in **Figure 8–46**, several bands are common among the proteins precipitated by the antibodies. These proteins are candidates for components of ORC. As indicated in the figure, these common bands have been identified as members of ORC by other means.

B. Each of the monoclonal antibodies is presumably specific for a particular antigenic site (epitope) on Orc1. The antigenic sites recognized by TK37 and TK47 (or close relatives thereof) seem to be present on many other proteins (or proteins involved in many other complexes). TK1 recognized only a couple of additional proteins outside the common set, and TK15 seems specific for just the common set, suggesting that its target epitope is located only on Orc1.

C. The most specific monoclonal antibody is TK15. It would be the best one to use in future studies of this kind.

D. Since you have a monoclonal antibody specific for Orc1 (TK15), you could easily use it to identify Orc1 by immunoblotting. If you transferred

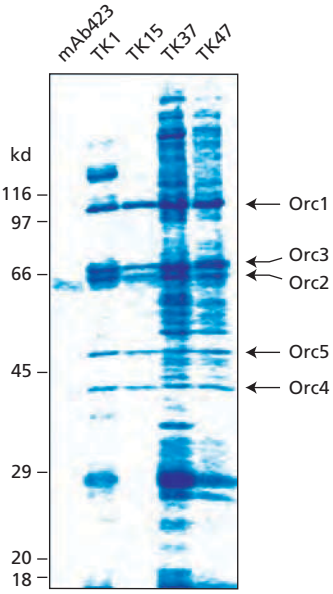


Figure 8–46 Identity of the common set of ORC proteins brought down by monoclonal antibodies to Orc1 (Answer 8–44). *Arrows* mark the bands that are common to all the monoclonal antibodies against Orc1. The identities of these common proteins are known, as indicated.

TABLE 8–8 Expected results of experiments to test the two-hybrid system (Answer 8–45).

Plasmid constructs		Growth on plates lacking histidine	Color on plates with XGAL
Bait	Prey		
LexA–Ras		–	white
LexA–lamin		–	white
	VP16	–	white
	VP16–CYR	–	white
LexA–Ras	VP16	–	white
LexA–Ras	VP16–CYR	+	blue
LexA–lamin	VP16	–	white
LexA–lamin	VP16–CYR	–	white

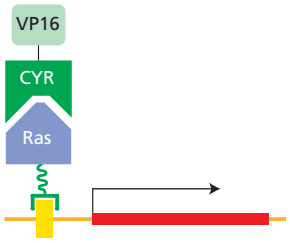


Figure 8–47 Structure of active transcription regulator in yeast transformed with the LexA–Ras and VP16–CYR fusion genes (Answer 8–45).

the proteins from the gel in Figure 8–9 onto a nitrocellulose filter, you could detect Orc1 by Western blotting using TK15 as the first antibody.

Reference: Tugal T, Zou-Yang XH, Gavin K, Pappin D, Canas B, Kobayashi R, Hunt T & Stillman B (1998) The Orc4p and Orc5p subunits of the *Xenopus* and human origin recognition complex are related to Orc1p and Cdc6p. *J. Biol. Chem.* 273, 32421–32429.

8–45

A. The expected results are indicated in **Table 8–8**.
B. The combination of LexA–Ras and VP16–CYR is the only one expected to grow in the absence of histidine and to give blue colonies in the presence of XGAL. The structure of the active transcription regulator is sketched in **Figure 8–47**.
C. In order for the two proteins of a fusion gene to be expressed as a single polypeptide chain, it is essential that there be no translational stop codons between the two coding segments and that the two genes be fused in the same translational reading frame.

References: Fields S & Song O (1989) A novel genetic system to detect protein–protein interactions. *Nature* 340, 245–246.

Vojtek AB, Hollenberg SM & Cooper JA (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74, 205–214.

ANALYZING AND MANIPULATING DNA

DEFINITIONS

8–46 Plasmid vector

8–47 Genomic library

8–48 Genome annotation

8–49 Restriction nuclease

8–50 cDNA clone

8–51 Polymerase chain reaction (PCR)

8–52 Deep RNA sequencing (RNA-seq)

8–53 Bacterial artificial chromosome (BAC)

8–54 Hybridization

TRUE/FALSE

8–55 False. The recognition sequences for the restriction nuclease, where they occur in the genome of the bacterium itself, are protected from cleavage by methylation at an A or a C residue.

8–56 False. When DNA molecules travel end-first through the gel in a snake-like configuration, their rates of movement are independent of length. In pulsed-field gel electrophoresis, the direction of the field is changed periodically, which forces the molecules to re-orient before continuing to move snakelike through the gel. This re-orientation takes more time for larger molecules, so that progressively larger molecules move more and more slowly.

8–57 True. Since eukaryotic coding sequences are usually in pieces in genomic DNA, cDNA clones allow expression of the protein directly and permit one to deduce the amino acid sequence of the protein.

8–58 True. If each cycle doubles the amount of DNA, then 10 cycles equal a 2^{10}-fold amplification (which is 1024), 20 cycles equal a 2^{20}-fold amplification (which is 1.05×10^6), and 30 cycles equal a 2^{30}-fold amplification (which is 1.07×10^9). (It is useful to remember that 2^{10} is roughly equal to 10^3 or 1000. This simple relationship allows you to estimate the answer to this problem rapidly without resorting to your calculator. It comes in handy in a variety of contexts.)

THOUGHT PROBLEMS

8–59 EcoRI will cleave the DNA once, generating two products; AluI will cleave the DNA twice, generating three products; and PstI will not cleave the DNA at all.

8–60

A. The 5′ and 3′ ends of the cut molecules are indicated in **Figure 8–48**. It is standard practice to represent DNA sequences so that the 5′ end of the top strand is on the left.

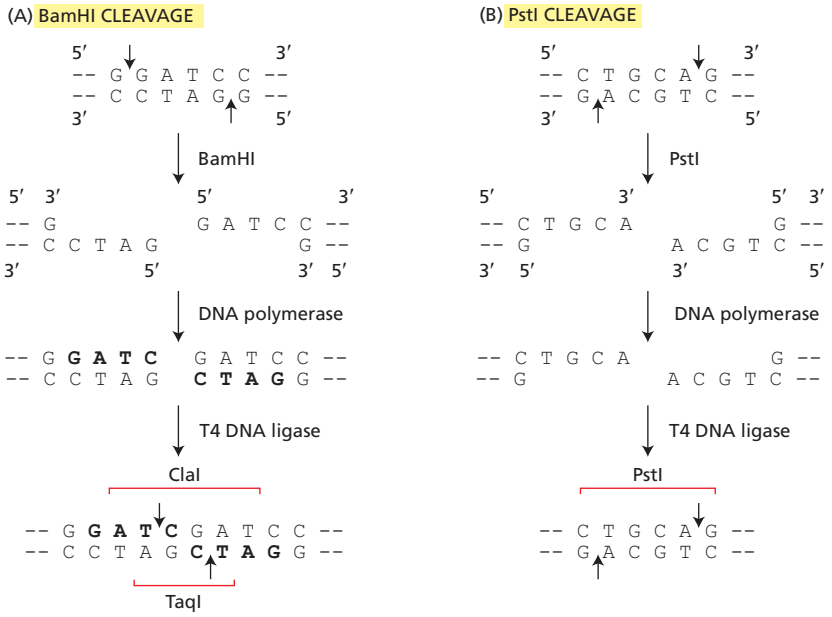


Figure 8–48 Cleavage, modification, and joining of DNA containing (A) a BamHI site or (B) a PstI site (Answer 8–60). *Brackets* indicate recognition sites for restriction nucleases.

B. As indicated in Figure 8–48, the BamHI ends can be filled in by DNA polymerase, but the PstI ends cannot. These different fates follow from the requirements of DNA polymerase: a primer with a 3′-OH to which dNTPs can be added and a template strand to specify correct addition. These requirements are met by the BamHI ends but not by the PstI ends, which have recessed 5′ ends that cannot serve as primers and, thus, cannot be filled in.

A standard technique in recombinant DNA technology is to use T4 DNA polymerase to blunt both types of ends. It will blunt BamHI ends by filling them in, as indicated in Figure 8–48A. It will also blunt PstI ends by virtue of an associated 3′-to-5′ exonuclease activity, which removes the 3′ extension, leaving a blunt end.

C. As indicated in Figure 8–48, the blunted BamHI ends and the unmodified PstI ends can both be joined by T4 DNA ligase.

D. Joining of the treated ends regenerates the PstI site but not the BamHI site. Joining of the filled-in BamHI ends generates two new restriction sites, as indicated in Figure 8–48A. Cleavage, filling in the ends, and rejoining often generates new restriction sites that sometimes are useful for further manipulation of the DNA.

8–61

A. The positions of the cleavage sites for EcoRI and PstI are shown on helical DNA in **Figure 8–49**.

B. The bonds cleaved by EcoRI are on the major-groove side of the DNA, suggesting that EcoRI approaches its recognition site from the major-groove side. This expectation has been confirmed by the three-dimensional structure of EcoRI complexed to its recognition site. The main part of the dimeric EcoRI molecule binds to the major-groove side of the recognition site, making specific contacts to the DNA in the major groove.

The bonds cleaved by PstI are on the minor-groove side of the DNA, suggesting that PstI approaches its recognition site from the minor-groove side. The structure of PstI complexed to its recognition site is not yet known. However, the structure of BglI, which also cleaves DNA to leave 3′ protruding single strands, is known; it binds to the minor-groove side of DNA. Thus, it is likely that restriction nucleases that leave 3′ protruding strands approach DNA from the minor-groove side.

References: Anderson JE (1993) Restriction endonucleases and modification methylases. *Curr. Opin. Struct. Biol.* 3, 24–30.

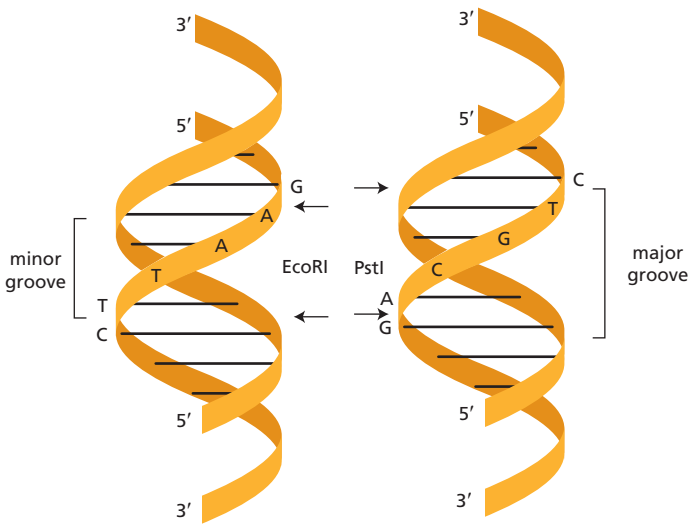


Figure 8–49 Cleavage sites for EcoRI and PstI on helical DNA (Answer 8–61).

Newman M, Lunnen K, Wilson G, Greci J, Schildkraut I & Phillips SEV (1998) Crystal structure of restriction endonuclease BglI bound to its interrupted DNA recognition sequence. *EMBO J.* 17, 5466–5476.

8–62 As indicated in **Figure 8–50**, the restriction nuclease Sau96I will definitely cleave the cDNA sequence at the segment corresponding to the adjacent codons for glycine and proline, regardless of the particular codon used for glycine. AluI might cut this DNA if the right codons were used for both proline and alanine. HindIII definitely will not cut this sequence.

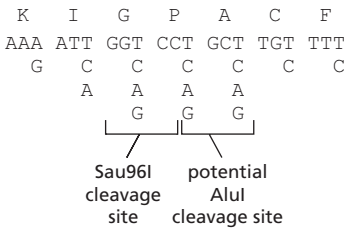


Figure 8–50 Representation of the nucleotide sequences that could encode the peptide KIGPACF (Answer 8–62). Only the DNA strand that corresponds to the mRNA sequence is shown.

8–63 The restriction map for the BamHI fragment is shown in **Figure 8–51**. A map that is flipped end for end is equally valid. One approach to deriving this map is as follows. Draw a line of appropriate length to represent the 3.0-kb BamHI fragment. Because HpaII cuts the fragment only once, the HpaII site can be located unequivocally; mark its position 1.6 kb from one end (either end) of the fragment. EcoRI cuts the fragment twice. If you position an EcoRI site 1.7 kb from either end of the fragment, you will find that its distance from the HpaII site cannot be reconciled with the sizes of bands from the double digest. Therefore, the 1.7-kb band must come from the middle of the 3.0-kb fragment and the two EcoRI sites must be 0.4 kb and 0.9 kb from the two ends. Only the arrangement shown in Figure 8–51 is consistent with the fragment sizes from the double digest.

8–64 The positive electrode should be placed at the bottom of the gel. DNA, which is negatively charged, will move toward the positive electrode in an electric field.

8–65

A. The KpnI–BamHI junction and its oligonucleotide splint are shown in **Figure 8–52A**. The oligonucleotide splint (5′-GATCGTAC) is identical to the one in Figure 8–16. Thus one splint works for both junctions.
B. Treatment of the mixture with DNA ligase produces the recombinant molecule illustrated in **Figure 8–52B.** As indicated by the arrows in the figure, two of the three nicks at each BamHI–KpnI junction will be ligated. This is sufficient to link the KpnI-cut fragment to the BamHI-cut vector.
C. Your friend's scheme works fine, in theory and in practice. The remaining nicks are repaired when the DNA is transformed into cells. Note that it is possible to add a phosphate to the 5′ end of the oligonucleotide using the enzyme polynucleotide kinase and ATP. Such a treated oligonucleotide would allow all nicks to be sealed by DNA ligase.

8–66 If the ratio of ddNTPs to dNTPs were increased, DNA polymerization would terminate more frequently and thus shorter DNA strands would be produced. Such conditions are favorable for determining short nucleotide sequences; that is, sequences that are close to the DNA primer used in the reaction. In contrast, decreasing the ratio of ddNTPs to dNTPs would cause less frequent termination of DNA polymerization, allowing one to determine nucleotide sequences more distant from the primer.

8–67 The presence of a mutation in a gene does not necessarily mean that the protein expressed from it will be defective. For example, the mutation could change one codon into another that still specifies the same amino acid, and thus not change the amino acid sequence of the protein. Or,

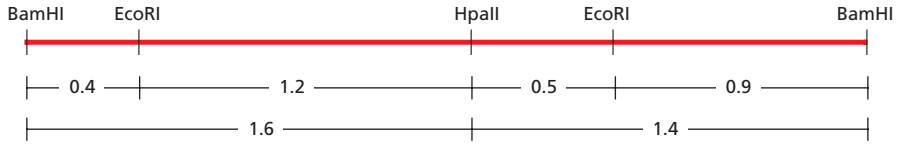


Figure 8–51 Restriction map of the 3.0-kb BamHI fragment (Answer 8–63). *Numbers* indicate fragment sizes in kilobases.

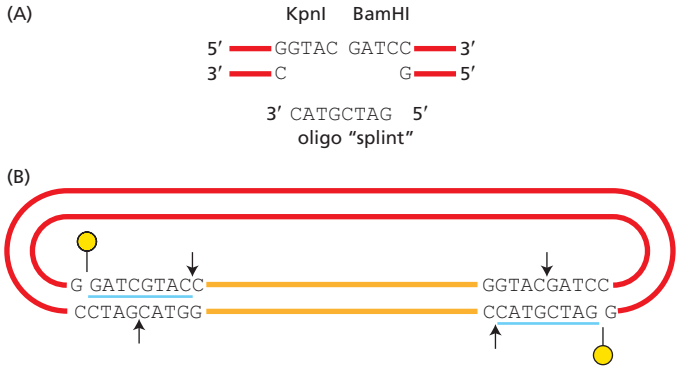


Figure 8–52 Scheme to splint BamHI–KpnI junctions (Answer 8–65). (A) Oligonucleotide splint required for KpnI–BamHI junction. It has the same sequence as the oligonucleotide splint shown in Figure 8–16. (B) Splint-mediated ligation of KpnI-cut fragment into a BamHI-cut vector. *Arrows* indicate nicks that can be ligated, and *yellow circles* indicate nicks that cannot be ligated without further enzymatic repair. The two oligonucleotide sequences are *underlined*.

the mutation could cause a change from one amino acid to another in the protein, but in a position that is not important for the folding or function of the protein. In assessing the likelihood that such a mutation might cause a defective protein, information on the known β-globin mutations that are found in humans would be essential. You would therefore want to know the precise nucleotide change in your mutant gene, and whether this change has any known or predictable consequences for the function of the encoded protein. If your mate has two normal copies of the globin gene, none of your children would manifest a disease arising from defective hemoglobin; however, on average, 50% of your children would be carriers of your one mutant gene.

8–68 The correct PCR primers are primer 1 (5′-GACCTGTGGAAGC) and primer 8 (5′-TCAATCCCGTATG). The first primer will hybridize to the bottom strand and prime synthesis in the rightward direction. The second primer will hybridize to the top strand and prime synthesis in the leftward direction. (Remember that complementary strands in DNA are antiparallel to one another.)

The middle two primers in each list (primers 2, 3, 6, and 7) would not hybridize to either strand. The remaining pair of primers (4 and 5) would hybridize, but would prime synthesis in the wrong direction—that is, outward, away from the central segment of DNA. Incorrect choices, like these, have been made at one time or another in most laboratories. The confusion generally arises because the conventions for writing nucleotide sequences have been ignored. By convention, nucleotide sequences are written 5′ to 3′ with the 5′ end on the left. For double-stranded DNA, the 5′ end of the top strand is on the left.

8–69 In PCR amplification, a double-stranded fragment of the correct size is first generated in the third cycle (**Figure 8–53**).

8–70 The pairs of PCR primers for adding a stretch of six histidines to either the N-terminus or the C-terminus of your protein are illustrated in **Figure 8–54**. In both cases, the primer on the left is complementary to the bottom strand of DNA (not shown) and primes synthesis in the rightward direction. The primer on the right is complementary to the top strand of DNA and primes synthesis in the leftward direction.

If you are set up to do database searches, you might try to find out what protein is being modified in this problem.

8–71

A. Imidazole is the ring of histidine. It competes with histidine for binding to the Ni^{2+} column, thereby releasing the protein.
B. It is not uncommon to detect short products when bacteria translate eukaryotic mRNA. They arise in two common ways: by proteolytic degradation of the full-length protein and by premature termination of

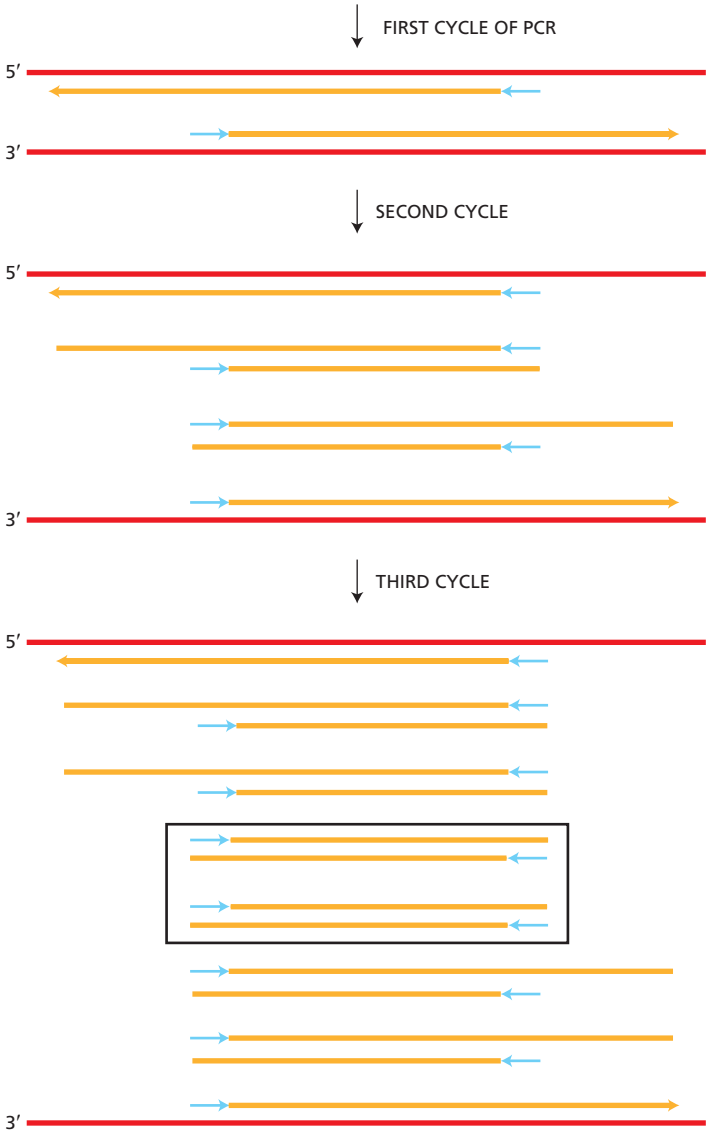


Figure 8–53 The PCR products generated during the first three cycles (Answer 8–69). DNA that has been synthesized during a cycle is shown as *orange lines*. The positions of the primers in new and old products are shown as *blue arrows*. The first products of the correct size are *boxed* in cycle 3.

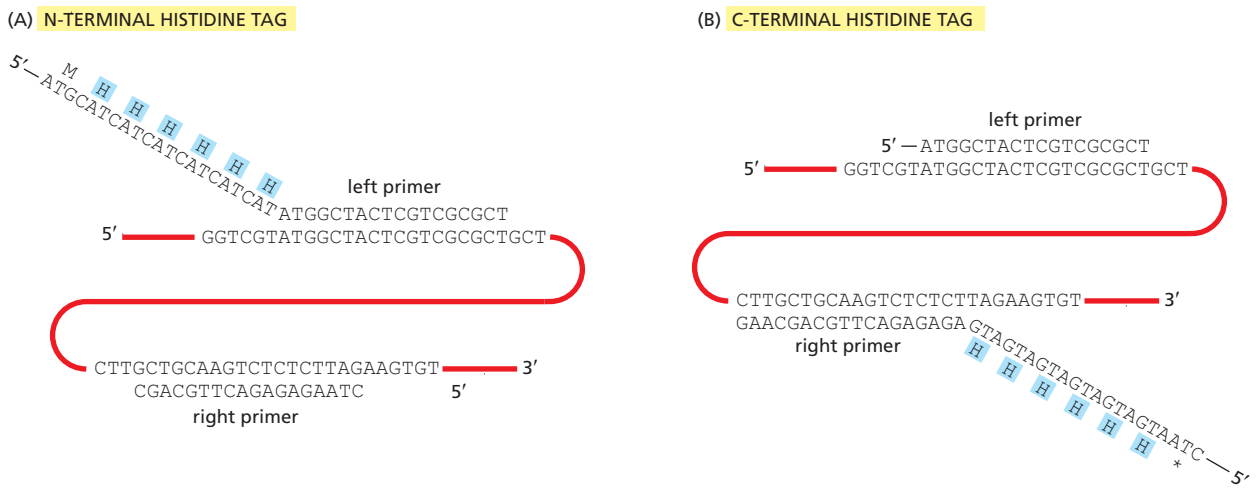


Figure 8–54 PCR primers to add hexahistidine tags to your protein (Answer 8–70). (A) Primers to add histidines to the N-terminus. (B) Primers to add histidines to the C-terminus. In these primers, only one codon for histidine is used, but the other histidine codon, or a mixture, could have been used. For the C-terminal histidine tag, the primer contains the complement of the histidine codon.

synthesis during translation. Either could explain the results with your construct.

For proteolytic degradation to be the explanation, you would need to account for the striking difference between your two constructs. One possibility is that the N-terminal histidine tag interferes with the folding of the protein, thereby rendering it more susceptible to proteases than its well-folded, C-terminally tagged counterpart.

The equal amounts of the full-length proteins produced by the two constructs suggest that the more likely explanation involves premature termination of translation. Premature termination generally occurs at codons that, while commonly used in eukaryotes, are rare in *E. coli*. At rare codons, the relevant tRNA is present at low concentration in the bacteria, and synthesis can terminate as a result. The low level of expression of your constructs is consistent with this sort of problem. But how can premature termination, which should affect both constructs equally, explain the difference between them? When the protein is tagged at the N-terminus, not only the complete protein but also all the partial products will be bound to the Ni^{2+} column. But when the protein is tagged at the C-terminus, only the full-length products will have the tag and thus be bound to the column.

8–72 The sequence of the cloned DNA and the amino acids encoded by the open reading frames are shown in **Figure 8–55**. Stop codons are underlined and labeled 2 or 3 to indicate their reading frame. As you can appreciate from this exercise, the sequencing gel must be read very carefully; omission of a single nucleotide would have disastrous consequences for determining the open reading frame. To minimize this problem, it is best to determine the sequence of both strands of DNA. Since the two strands are complementary, any mistakes will be readily apparent. This sequence encodes the canine receptor for the signal-recognition particle (SRP), which functions in the delivery of proteins to the ER.

CALCULATIONS

8–73

A. The central four nucleotides of a BamHI site constitute a Sau3A site. Since Sau3A recognizes only these four nucleotides, all BamHI sites will be cut by Sau3A. The converse is not true because BamHI recognizes a six-nucleotide site. Only that fraction of Sau3A sites with appropriate flanking nucleotides (specifically, a 5′ G and a 3′ C) will be cut by BamHI. Since on average 1 out of 4 of the flanking nucleotides on each side will be correct, only 1 of 16 possible arrangements of the two flanking nucleotides will be correct for BamHI cleavage. Thus, only about 1 of 16 Sau3A sites will be cut by BamHI.

B. Since Sau3A is indifferent to the flanking nucleotides, all Sau3A–BamHI hybrid sites can be cut with Sau3A. However, only 1 in 4 hybrid sites on average will be cut by BamHI. In a hybrid site, 5 of the 6 nucleotides will be correct for a BamHI site; the sixth nucleotide will be correct in 1 of 4 hybrid sites, on average.

C. The probability that any four-nucleotide sequence will be a Sau3A site is $(1/4)^4$, or 1 in 256, in a random DNA sequence with equal frequencies of all four nucleotides. Thus, the Sau3A recognition site will occur, on

```
  Y  K  L  D  N  Q  F  E  L  V  F  V  V  G  F  Q  K  I  L  T
TATAACTGGACAACCAGTTCGAGCTGGTGTTCGTGGTCGGTTTTCAGAAGATCCTAAC
   3                                                     2

  L  T  Y  V  D  K  L  I  D  D  V  H  R  L  F  R  D  K
GCTGACGTACGTAGACAAGTTGATAGATGATGTGCATCGGCTGTTTCGAGACAAGTA
  2        2        2  2     3
```

Figure 8–55 Nucleotide and amino acid sequence of a cloned gene (Answer 8–72). Stop codons are *underlined* and the reading frame they affect is indicated *below*. The amino acids encoded in frame 1 are *centered* over their codons.

average, once every 256 nucleotide pairs in a stretch of DNA. The BamHI recognition site, which is six nucleotide pairs, will occur, on average, once every 4096 nucleotide pairs, $(1/4)^6 = 4096$, in a random DNA sequence.

8–74

A. A restriction nuclease that recognizes a four-base-pair site will cut on average 1 out of 256 four-base-pair sites [$(1/4)^4$]. Thus, you would expect on the order of 1.3×10^7 fragments ($3.2 \times 10^9/256$) from a Sau3A digestion of human DNA. Similarly, you would expect on the order of 7.8×10^5 fragments ($3.2 \times 10^9/4096$) from an EcoRI digestion. These estimates are based on the assumptions that the genome is 50% GC and that the sequence is random: neither assumption is true, but they won't lead you far astray.

B. A set of overlapping DNA fragments will be generated by partial digestion. Libraries constructed from sets of overlapping fragments of a genome are valuable because they can be used to arrange cloned sequences in their original order on the chromosomes, and thus obtain the DNA sequence of a long stretch of DNA. Sequences from the end of one cloned DNA are used as hybridization probes to find other clones in the library that contain those sequences and, therefore, might overlap. By repeating this procedure, a long stretch of continuous DNA sequence can be gradually built up. Overlapping clones were crucial in the original sequencing of the human genome.

8–75 There are approximately 10^9 different possible sequences formed by 15 nucleotides ($4^{15} = 1.07 \times 10^9$). By chance, therefore, you would expect to find any given 15-nucleotide sequence an average of 3 times ($3.2 \times 10^9/1.07 \times 10^9$) in the human genome. Since the degenerate probe has two possibilities at each of four sites, it is a mixture of 16 (4^2) different 15-nucleotide sequences. Thus, you would expect to find about 48 matches (16×3) in the genome, of which only one will correspond to the Factor VIII gene.

To determine that a sequence identified by the probe indeed corresponds to the gene of interest—the Factor VIII gene in this case—one usually repeats the procedure using a second degenerate probe from elsewhere in the protein sequence. If both degenerate probes hybridize to one genomic DNA fragment, it is a strong indication that the target gene has been identified.

DATA HANDLING

8–76 The principle involved in this method of creating restriction maps is very similar to that used in chemical sequencing of DNA. In both cases, the molecule is labeled at one end and then partially fragmented by incomplete digestion at a set of specific sites. Finally, the fragments are separated on the basis of size. The length of a labeled fragment indicates the distance from the labeled end to the site of cleavage.

The restriction map derived from the autoradiograph in Figure 8–24 is shown in **Figure 8–56**, where it is related by vertical lines to the partial digestion products generated by one restriction nuclease. The sizes of the restriction fragments are estimated by comparison with the set of marker fragments in Figure 8–24. The 4-kb band in all three lanes represents the uncleaved fragment. (You will note in Figure 8–24 that the distance the fragments migrate during electrophoresis is not linearly related to their size—which may account for the differences between your estimates and those in Figure 8–56.)

8–77 The hybridization shows that the viral DNA is integrated into the cellular DNA in segment *b* on the viral genome, as shown in **Figure 8–57**.

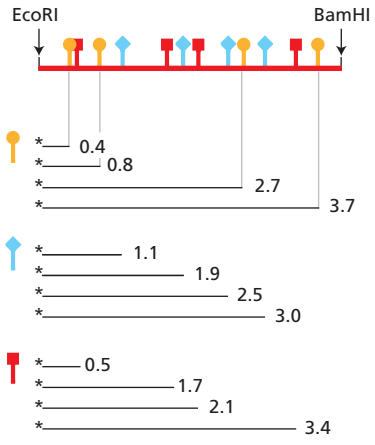


Figure 8–56 Restriction map of your cloned DNA segment and the partial digestion products from which it was deduced (Answer 8–76). *Numbers* beside the restriction fragments indicate their lengths in kilobases, as estimated from Figure 8–24.

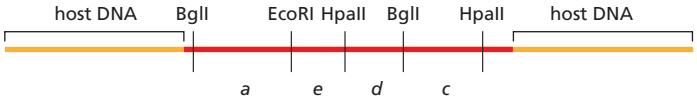


Figure 8–57 Structure of integrated viral DNA (Answer 8–77).

This integrated structure can be deduced as follows. Digests of cell DNA always contain one more band than the corresponding digests of viral DNA. EcoRI, for example, cuts the circular viral genome once, giving rise to a single fragment. EcoRI also cuts the integrated, linear viral genome once but gives rise to two fragments, which correspond to the two parts of the viral genome, each of which is linked to adjacent chromosomal DNA that extends to the first EcoRI site beyond the integrated genome.

The segment of the viral genome where the integration event occurred is always absent from the digests of the integrated genome. That segment is replaced by two other bands. (The sizes of the new bands depend on the proximity of the corresponding restriction sites in the adjacent cell DNA: their sizes can't be predicted.) The EcoRI digest is not very illuminating, but critical information is contained within the HpaII and BglI restriction digests. The small HpaII fragment and the large BglI fragment are present in the digest of the cell DNA. However, the large HpaII fragment and the small BglI fragment are not. These two missing fragments overlap on the viral genome in segment *b*, which therefore must contain the site of integration.

MEDICAL LINKS

8–78

A. Both oligonucleotides will hybridize to β^A and β^S DNA. The β^A oligo, for example, is a perfect (20-of-20) match for β^A DNA and a 19-of-20 match for β^S DNA. The situation is equivalent for the β^S oligo. The difference in hybridization between a 19-of-20 match and a 20-of-20 match is very small when the mismatch is at one end of the oligonucleotide. Under normal hybridization conditions, both oligonucleotides hybridize equally well to β^A and β^S DNA.

B. Although hybridization itself is not affected by the mismatch, the reaction catalyzed by DNA ligase is exquisitely sensitive to it. As shown in **Figure 8–58** for the β^S oligo, ligation can occur only if the bases on both sides of the nick are properly paired with the complementary bases in the target DNA. Ligation is essential if the radioactive oligo is to be linked to the biotin-labeled oligo and bound to the solid support where it can expose the x-ray film.

Reference: Landegren U, Kaiser R, Sanders J & Hood L (1988) A ligase-mediated gene detection technique. *Science* 241, 1077–1080.

8–79

A. The positions of the deletions in the DMD patients are shown in Figure 8–59. Patient A is the only one that has all nine PCR products. Thus,

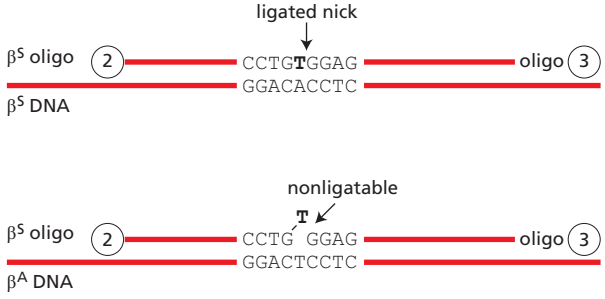


Figure 8–58 Sensitivity of ligation to terminal mismatches (Answer 8–78).

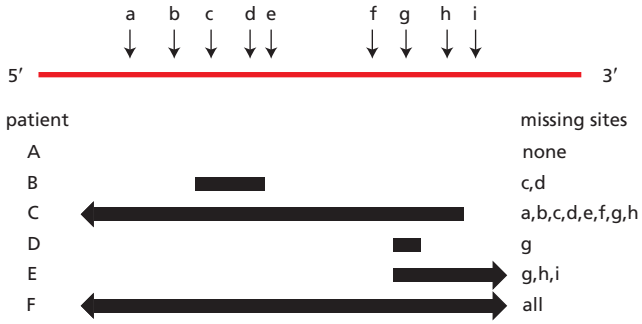


Figure 8–59 Positions of deletions in the DMD patients (Answer 8–79). *Bars* indicate the approximate extent of each deletion. A *flat end* indicates that the deletion end point lies within the interval between two PCR sites; an *arrowhead* indicates that the deletion extends for an unknown distance beyond a terminal PCR site.

patient A may not have a deletion, but this analysis does not prove that. It indicates only that the patient does not have a deletion that includes any of the sites of PCR amplification.

B. The analysis of patient F looks suspiciously like the negative control. This result could indicate that patient F has a large deletion that includes all nine PCR amplification sites. But it is also the expected result if something went wrong in the analysis—for example, the patient's DNA or some other critical component was left out of the reaction mixture. At a minimum, the analysis should be repeated. But additional controls should be used to verify the analysis. For example, one could include in the reaction mixture a pair of PCR primers that amplify some other segment of the genome, unrelated to the DMD gene. The presence of this control product in the otherwise blank gel would confirm the analysis. Alternatively, it would be possible to test for the deletion by Southern blotting using a radioactive probe from the DMD gene.

Reference: Chamberlain JS, Gibbs RA, Ranier JE & Caskey CT (1990) Multiplex PCR for the diagnosis of Duchenne muscular dystrophy. In PCR Protocols (MA Innis, DH Gelfand, JJ Sninsky & TJ White eds), 0pp. 272–281. San Diego, CA: Academic Press.

STUDYING GENE EXPRESSION AND FUNCTION

DEFINITIONS

8–80 Genetic screen

8–81 Polymorphism

8–82 Allele

8–83 Phenotype

8–84 Epistasis analysis

8–85 Haplotype block

8–86 Transgenic organism

8–87 Genotype

TRUE/FALSE

8–88 False. Even with a sequenced genome, it is still a laborious process to identify a gene that has been mutated by chemical mutagenesis. Such a mutation must be mapped to a chromosomal location (a time-consuming process) and then candidate genes in that region (many of which are known from genome sequencing efforts) can be screened for the

presence of the mutation. Insertional mutagenesis, by contrast, places a known sequence—often a transposable element—into the mutated gene. It is a simple process to obtain sequence information adjacent to a segment of known sequence. In a sequenced genome, a bit of sequence is all that is needed to identify the location of the inserted DNA and the mutated gene.

8–89 True. Mutations that eliminate the function of a protein are usually recessive. In a diploid organism, one copy of the wild-type allele can usually compensate for the loss-of-function allele. It is important to note, however, that in some cases half the normal amount of gene product is not sufficient to produce the normal phenotype. This situation, which is called haploinsufficiency, leads to a mutant phenotype for a loss-of-function mutation.

8–90 False. A synthetic phenotype usually means that the affected genes encode products that operate in different pathways. Two blocks in one pathway are usually no worse than either single block. Mutations that block two different pathways, however, can often generate a worse phenotype than either block can on its own.

THOUGHT PROBLEMS

8–91

A. The locus is the site of a gene, or genetic element, in the genome, more particularly its location in a chromosome. Each gene or genetic element has one defined locus in the genome. An allele is an alternative form of a gene at a locus. Within the population, there are often several "normal" alleles, whose functions are indistinguishable. In addition, there may be many rare alleles that are defective to varying degrees. An individual normally has a maximum of two alleles of a gene.

B. An individual is said to be homozygous if the two alleles at a locus are the same. An individual is said to be heterozygous if the two alleles at a locus are different.

C. Genotype is the specific set of alleles forming the genome of an individual; it is an enumeration of all the particular forms of each gene in the genome. In practice, for organisms studied in a laboratory, the genotype is usually specified as a list of the known differences between the individual and the wild type, which is the standard, naturally occurring type. Phenotype is a description of the visible characteristics of the individual. In practice, phenotype is usually a list of the differences in visible characteristics between the individual and the wild type.

D. An allele is dominant (relative to the second allele) if the phenotype is the same when the allele is homozygous and when it is heterozygous. In that case, the second allele, whose presence makes no difference to the phenotype, is said to be recessive (to the first allele). If the phenotype of the heterozygous individual differs from the phenotypes of individuals that are homozygous for either allele, the alleles are said to be co-dominant.

8–92 A gain-of-function mutation increases the activity of the protein product of the gene, makes it active in inappropriate circumstances, or gives it a novel activity. The change in activity often has a phenotypic consequence even when the normal protein is present, which is why such mutations are usually dominant. A dominant-negative mutation gives rise to a mutant gene product that interferes with the function of the normal gene product, causing a loss-of-function phenotype even in the presence of a normal copy of the gene. This ability of a single defective allele to determine the phenotype is the reason why such an allele is said to be dominant.

8–93 This statement is largely true. Diabetes is one of the oldest diseases described by humans, dating back at least to the time of the ancient Greeks. The term "diabetes" itself comes from the Greek word for siphon, which was used to describe one of the main symptoms—increased production of urine: "The disease was called diabetes, as though it were a siphon, because it converts the human body into a pipe for the transflux of liquid humors." If there were no human disease, the role of insulin would not have come to our attention as soon as it did. It is difficult to overstate the case for the role of disease in focusing our efforts toward a molecular understanding. Even today, the quest to understand and alleviate human disease is a principal driving force in biomedical research.

8–94 SNPs are single-nucleotide differences between individuals, which occur roughly once per 1000 nucleotides of sequence. Many have been collected and mapped in various organisms, including several million in the human genome. SNPs, which can be detected by oligonucleotide hybridization, serve as physical markers whose genomic locations are known. By tracking a mutant gene through different matings, and correlating the presence of the gene with the co-inheritance of particular SNPs, one can narrow down the potential location of a gene to a chromosomal region that may contain only a few genes. These candidate genes can then be tested for the presence of a mutation that could serve as the basis for the original mutant phenotype.

8–95 Knowledge that the seven proteins in *E. coli* correspond to seven domains of the mammalian fatty acid synthase means that they are likely to be functionally associated, and probably work together in a protein complex.

8–96 Standard genetics traditionally begins with the isolation of mutants and characterization of their phenotypes, and then proceeds toward identification of the affected gene and protein. Reverse genetics moves in the opposite direction: from genes and proteins to mutants and phenotypes.

8–97 Although several explanations are possible, the simplest is that the DNA probe has hybridized predominantly with its corresponding mRNA. When a gene is expressed, its mRNA is present in many more copies than the gene. The strongly hybridizing cells probably express the gene at high levels and therefore have high levels of the mRNA.

8–98 Three oligonucleotides that could be used to effect the desired changes in the protein are illustrated in **Figure 8–60**. As a general rule, it is best to center the mismatch within the oligonucleotide. To carry out the site-directed mutagenesis, the oligonucleotides would be hybridized to

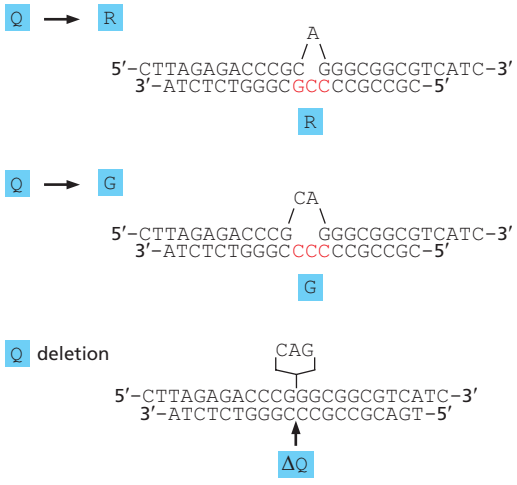


Figure 8–60 Three oligonucleotides for site-directed mutagenesis (Answer 8–98).

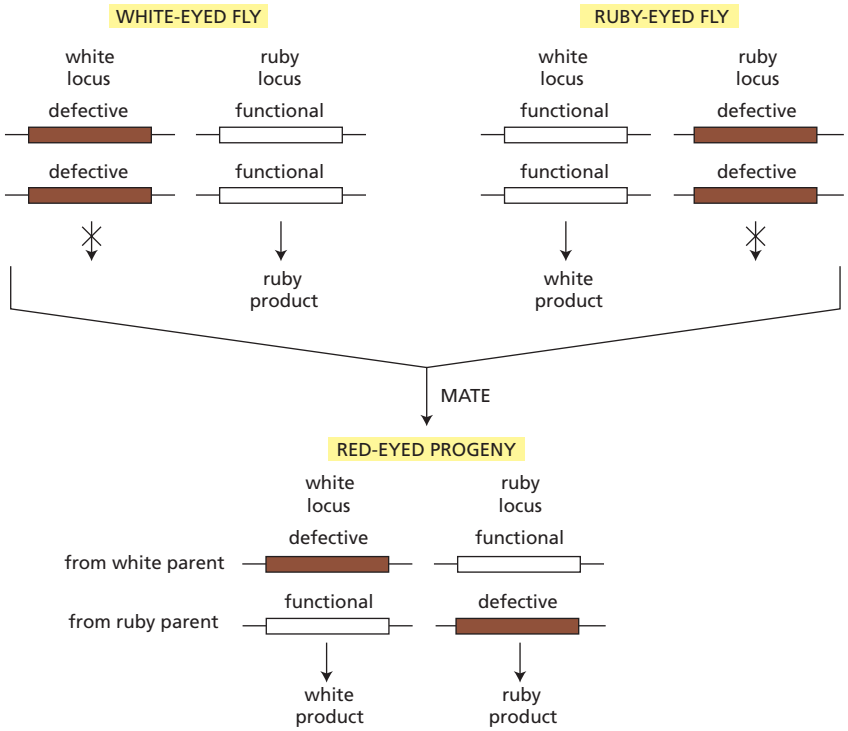


Figure 8–61 Complementation between white and ruby (Answer 8–100).

the circular, single-stranded DNA from the recombinant vector (Figure 8–29B) and then extended with DNA polymerase to complete the second strand. The double-stranded DNA would be transfected into bacterial cells, where replication would generate the desired mutant.

8–99 These results are what you would expect if the mRNA were alternatively spliced. The numbers of reads for exon 4 versus the rest of the mRNA suggest that about half the mRNA was spliced to include all five exons, whereas the rest was spliced to skip exon 4. Thus the mRNA produced from this gene includes two relatively abundant species.

DATA HANDLING

8–100

A. As outlined in **Figure 8–61**, if flies are defective in different genes, their progeny will have one normal gene at each locus. In the case of a mating between a ruby-eyed fly and a white-eyed fly, for example, every progeny fly will inherit one functional copy of the white gene from one parent and one functional copy of the ruby gene from the other parent. Since each of the mutant alleles is recessive to the corresponding wild-type allele, the progeny will have the wild-type phenotype—brick-red eyes.

B. Garnet, ruby, vermilion, and carnation complement one another and the various alleles of the white gene; thus they define separate genes. White, cherry, coral, apricot, and buff do not complement each other; thus, they must be alleles of the same gene, which has been named the *White* gene. Thus, these nine different eye-color mutants define five different genes.

C. Different alleles of the same gene, like the five alleles of the *White* gene, often have different phenotypes. Different mutations compromise the function of the gene product to different extents, depending on the location of the mutation. Different null alleles of the same gene, which have no function at all, do have the same phenotype.

Reference: Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM & Veres RC (2000) Genetics: From Genes to Genomes, pp 191–195. Boston, MA: The McGraw Hill Companies, Inc.

8–101 The patterns of hybridization are shown in **Figure 8–62**. DNA from the individual homozygous for the wild-type allele will hybridize only to the upper spot, the position of $ASO^{\beta A}$. DNA from the individual homozygous for the sickle-cell allele will hybridize only to the lower spot, the location of $ASO^{\beta S}$. DNA from a heterozygous individual will hybridize to both spots.

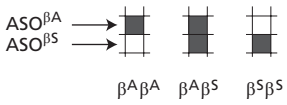


Figure 8–62 Expected pattern of hybridization of genomic DNA to sites on a DNA microarray to which ASOs are attached (Answer 8–101).

8–102 The sample marked JC has two copies of mutant allele 3 and thus will have the disease. The sample marked BF has a single copy of mutant allele 2 and thus is heterozygous; this individual should not have the disease. BF will, however, be a carrier of the mutation and should be apprised of that fact eventually. The sample marked HK has one copy of mutant allele 2 and one copy of mutant allele 3. Since both copies of the gene are defective, this individual will have the disease. (You may have considered the possibility that a recombination event in one of the parental lineages placed both mutations on one chromosome, generating a new allele that carries both mutations. For mutations in the same gene, the frequency of recombination is so low that this possibility is extremely remote in humans.) The sample marked TW has no mutant alleles; thus this individual will not have the disease and will not be a carrier of the mutation.

8–103

A. The three possible products of a Cre-mediated site-specific recombination are shown in **Figure 8–63**.
B. Products I and II would both be null alleles. Product I would be missing the *Neo* gene.
C. Product III has a normal gene structure except for the presence of lox sites in two of the introns.
D. Product III can be used as a conditional allele. The gene can be knocked out in a tissue-specific (conditional) manner. For example, if the ES cells containing product III were introduced into the germ line of a mouse, mice could be bred that carried the floxed (conditional) allele in all their cells. If such mice were bred to transgenic mice that expressed Cre recombinase only in liver (by having the *Cre* gene under control of a liver-specific promoter), then the floxed allele would be specifically deleted in liver cells, but continue to function normally in the rest of the animal.

8–104

A. In the experiment in which you incubated the Cas9–guide RNA with the DNA curtain, you see a single site—a line of fluorescent dots across the curtain at the same level—which corresponds to the expected site of Cas9–guide RNA binding. Importantly, that line of fluorescent dots is missing from the curtain incubated with Cas9 alone.
B. The main concern for off-target binding is that the Cas9–guide RNA complex will bind to additional sites that are related to the primary target. That sort of off-target site would show up in this experiment as a second line of fluorescent dots, perhaps fainter than the primary site. There is no evidence for that kind of off-target site in this experiment. There is,

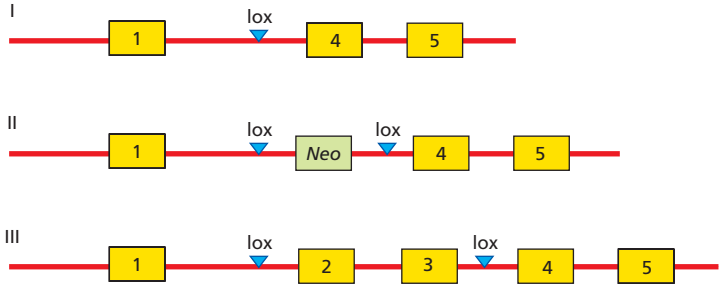


Figure 8–63 Products of Cre-mediated site-specific recombination of a floxed allele (Answer 8–103).

however, another aspect of the data in Figure 8–35 that raises some concern; namely, the apparently random background of dots elsewhere on the DNA curtain. Since the dots look more or less the same whether the guide RNA is present or not, it is likely they are meaningless background noise. But further experiments would need to be done.

Finding one site in lambda DNA, with no off-target binding, does not guarantee that you would get the same result in human chromosome 1, which is 5000 times larger (or in the human genome, which is bigger still).

Reference: Sternberg SH, Redding S, Jinek M, Greene EC & Doudna JA (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 507, 62–67.

MATHEMATICAL ANALYSIS OF CELL FUNCTIONS

DEFINITIONS

8–105 Robustness

8–106 Stochastic

TRUE/FALSE

8–107 True. Without quantitative details, it would be impossible to know if the interaction is likely to occur in cells at all, and if it does, whether it is a stable interaction with a long half-life or a dynamic interaction with rapid binding and dissociation.

8–108 False. The association constant is the reciprocal of the dissociation constant; thus, $K_a = 1/K_d$.

8–109 True. This is a fundamental premise in the analysis of biochemical reactions. It applies equally to molecular complexes (such as *AB*, which forms when proteins *A* and *B* bind to each other, and disappears when *AB* dissociates) and metabolic reactions (for example, when metabolite B forms by chemical modification of metabolite A, and disappears when it is converted to metabolite C).

8–110 False. A protein with a rapid degradation rate will reach its new steady-state concentration more quickly. The rate of approach to the new steady state is inversely related to the protein's half-life.

8–111 True. The *Lac* operon is turned on maximally when the activator concentration, [*A*], is high and the repressor concentration, [*R*], is zero. Thus, the *Lac* operon displays AND NOT logic ([*A*] and not [*R*]).

THOUGHT PROBLEMS

8–112 The network in Figure 8–36A is a positive feedback loop. When expression of gene *X* is activated, protein *X* represses gene *Y*, eliminating expression of protein *Y*, which relieves repression of gene *X*, thereby increasing its expression. The network in Figure 8–36B is a negative feedback loop. Its behavior is the same up to the repression of gene *Y*; however, in the absence of protein *Y*, gene *Z* is turned on, producing protein *Z*, which represses gene *X*.

In network motifs, as in the English language, two negatives make a positive, but an odd number of negatives is still negative.

8–113 Of the network motifs in Figure 8–37, A, B, and C are positive feedback loops, whereas the motif in D is a negative feedback loop. You can

analyze these motifs step by step. For motif A, when expression of gene *X* is activated, repressor *X* turns off gene *Y*, which eliminates expression of repressor *Y*, thereby turning on gene *Z*, which activates expression of activator *Z*, which enhances expression of gene *X*, completing the positive feedback loop. For motif B, when expression of gene *X* is activated, repressor *X* turns off gene *Y*, which eliminates expression of activator *Y*, thereby turning off gene *Z*, which eliminates expression of repressor *Z*, which enhances expression of gene *X*, completing the positive feedback loop. The other motifs can be analyzed in the same way, or you can apply the quick rule that two negatives make a positive (see Answer 8–112). Motifs A, B, and C each contain two inhibitory steps; hence, they are positive feedback loops. Motif D contains a single negative step; hence, it is a negative feedback loop.

8–114 If the perturbed system were exactly at the boundary between the two regions of attraction—the two stable states—it would be balanced on a knife edge. The slightest random fluctuation would drive the system to one or the other of the two stable states.

8–115 To get a pulse of protein *X*, K_A must be much less than K_R. Under these conditions, *R* binds more tightly than *A*, overriding any activation caused by the weaker binding of *A*. The delay in activation of *R* gives rise to an initial burst of protein *X* that is turned off when *R* is generated.

CALCULATIONS

8–116 The fraction of promoters bound is 99% when [*R*] is 100 times $1/K$; 50% when [*R*] equals $1/K$; and about 1% when [*R*] is 1/100 times $1/K$. For the situation in which [*R*] is 100-fold greater than $1/K$,

$$\begin{aligned}\text{bound fraction} &= [R{:}p_X]/[p_X^T] = K[R]/(1 + K[R])\\ &= K(100/K)/(1 + K\{100/K\})\\ &= 100/101 = 99\%\end{aligned}$$

8–117 The molar concentration of a single promoter sequence in *E. coli* is 1.1 nM. The volume of *E. coli* is 1.5 μm^3. [Volume = (3.14)(0.4 $\mu m)^2$ 3 μm = 1.5 μm^3.] One promoter per 1.5 μm^3 equals 1.1 nM. [(1 promoter/1.5 μm^3)(10^4 $\mu m/cm)^3$(1000 cm^3/L)(mole/6 × 10^{23}) = 1.1 × 10^{-9} M = 1.1 nM.]

8–118 Under these conditions, *K* would equal 9.9 × 10^8 M^{-1}. The concentration of the repressor would be 111 nM. As calculated in Problem 8–117, the volume of *E. coli* is 1.5 μm^3. [Volume = (3.14)(0.4 $\mu m)^2$ 3 μm = 1.5 μm^3.] 100 molecules of a transcription regulator per 1.5 μm^3 equals 111 nM. [(100 regulators/1.5 μm^3)(10^4 $\mu m/cm)^3$(1000 cm^3/L)(mole/6 × 10^{23}) = 111 × 10^{-9} M = 111 nM.] (100 times the concentration calculated in Problem 8–117 for one promoter per cell).

For the binding site for the transcription regulator to be occupied 99% of the time, the equilibrium constant must be 9.9 × 10^8 M^{-1}. As discussed in Problem 8–116,

$$\begin{aligned}\text{bound fraction} &= K[R]/(1 + K[R])\\ 0.99 &= K(111\text{ nM})/(1 + K\{111\text{ nM}\})\\ 0.99 + (110\text{ nM})K &= (111\text{ nM})K\\ 0.99 &= (1\text{ nM})K\\ K &= 0.99/10^{-9}\text{ M} = 9.9 \times 10^8\text{ M}^{-1}\end{aligned}$$

8–119

A. Since the concentration of *Lac* repressor (10^{-8} M) is 10^5 times the $1/K$ ($1/10^{13}$ M^{-1}), you would expect 99.999% of the sites in a bacterial population to be occupied by the *Lac* repressor.

$$\text{bound fraction} = [R{:}p_X]/[p_X^T] = K[R]/(1 + K[R])$$
$$= K(10^5/K)/(1 + K\{10^5/K\})$$
$$= 100{,}000/100{,}001 = 99.999\%$$

B. When inducer is present, the concentration of *Lac* repressor will be only 100 times more than the $1/K$, but you would still expect 99% of the sites to be occupied.

C. If 99% of the binding sites were occupied by the repressor even in the presence of the inducer, you would expect that the genes would still be very effectively turned off. This sort of straightforward calculation, and its nonbiological answer—after all, the genes are known to be turned on by the inducer—tells you that some critical information is missing.

D. Low-affinity, nonspecific binding of the *Lac* repressor is the missing information suggested by the calculation in part C. Since there are 4×10^6 nonspecific binding sites in the genome (a number equal to the size of the genome), there is a competition for repressor between the multitude of low-affinity sites and the single high-affinity site. This competition reduces the effective concentration of the repressor. As can be calculated, the competition reduces repressor occupancy at the specific site to about 96% in the absence of lactose, and to about 3% in the presence of lactose. These numbers account nicely for the genes being turned off in the absence of lactose and turned on in its presence.

8–120

A. The slopes ($-1/K_d$) of the lines in Figure 8–40 can be estimated by taking the difference between two points on the *y* axis divided by the difference between the corresponding points on the *x* axis. The points where the lines intersect the axes are the most convenient. Thus, the slope of line A is

$$-1/K_d = (0.43 - 0)/(0\text{ M} - 6.0 \times 10^{-7}\text{ M})$$
$$= 0.43/(-6.0 \times 10^{-7}\text{ M})$$
$$0.43\,K_d = 6.0 \times 10^{-7}\text{ M}$$
$$K_d = 1.4 \times 10^{-6}\text{ M}$$

For line B,

$$-1/K_d = (1.1 - 0)/(0\text{ M} - 6 \times 10^{-7}\text{ M})$$
$$= (1.1)/(-6 \times 10^{-7}\text{ M})$$
$$K_d = 5.5 \times 10^{-7}\text{ M}$$

B. The lower the K_d, the tighter the binding; thus, the tighter IPTG-binding mutant of the *Lac* repressor corresponds to line B ($K_d = 5.5 \times 10^{-7}$ M) and the wild-type *Lac* repressor corresponds to line A ($K_d = 1.4 \times 10^{-6}$ M). That a lower value corresponds to tighter binding is apparent from the definition of K_d in the problem. Tighter binding will give more complex ($Pr{-}L$) and fewer free components ($Pr + L$); thus, the ratio of concentrations, K_d, will be smaller.

References: Gilbert W & Müller-Hill B (1966) Isolation of the Lac repressor. *Proc. Natl Acad. Sci. USA* 56, 1891–1898.

Kyte J (1995) Mechanism in Protein Chemistry, pp. 175–177. New York: Garland Publishing.

DATA HANDLING

8–121

A. Comparison of repression levels with each individual operator (Figure 8–41, constructs 4, 6, and 7) shows that only O_1 gives rise to a significant level of repression. O_2 and O_3 give the same level of expression (no repression) as a construct with no operators (construct 8).

B. So long as the combination of operators contains O_1, the dimeric operator causes significant repression; however, repression is only slightly elevated (less than 2-fold) relative to construct 4, which contains only operator O_1. Thus with a dimeric repressor, the activity of O_1 is not substantially enhanced by the presence of O_2 or O_3. By contrast, additional operators greatly increase repression by the tetrameric repressor by 10-fold (construct 3 versus construct 4) to 50-fold (construct 1 versus construct 4). These results suggest that the presence of multiple binding sites allows the tetrameric repressor—but not the dimeric repressor—to bind two sites at the same time, creating a loop in the DNA. Such a loop might be a more effective way to exclude RNA polymerase from the promoter, thereby increasing repression.

C. The ability of the tetrameric repressor to bind O_3 when it is in the presence of O_1 is an example of cooperative binding. The tight binding of the repressor to O_1 increases the local concentration in the neighborhood of O_3, which increases the effectiveness with which the repressor can bind O_3.

Reference: Oehler S & Müller-Hill B (2010) High local concentration: a fundamental strategy of life. J. Mol. Biol. 395, 242–253.

MCAT STYLE

8–122

C. PCR analysis offers the most sensitive test for the presence of the *Bcr-Abl* fusion gene in circulating cells. By designing the reaction with one primer in the *Bcr* gene and the other in the *Abl* gene, the reaction will produce a product only if the *Bcr-Abl* fusion is present. Moreover, since PCR is highly sensitive, it can detect very small amounts of the *Bcr-Abl* fusion, hence, very small numbers of cancer cells in the bloodstream. Choice A is not correct because DNA sequencing is not rapid enough or sensitive enough (or cheap enough) to be used as a blood test. DNA sequencing is more appropriate for analysis of the fusion gene in more detail once it has been amplified by PCR. Choice B is not correct because there is no way to fluorescently mark cells that carry the *Bcr-Abl* fusion, and flow cytometry would be less sensitive than PCR. Choice D is incorrect because Western blotting, although it could detect the fusion protein using antibodies that bind to the Bcr-Abl protein, lacks the sensitivity needed to detect the minute quantities of the fusion protein generated by the rare circulating cancer cells.

8–123

A. Amplification and sequencing of the *Bcr-Abl* gene is the most simple and direct way to test whether there are mutations in *Bcr-Abl*, which is the key element of your hypothesis. Choice B might be something worth trying if you already knew that your hypothesis was true. Indeed, second-generation drugs related to imatinib have been shown to kill imatinib-resistant cells. Choice C would be a good control, just to make sure that the imatinib-resistant cancer cells really do possess the *Bcr-Abl* fusion gene. These resistant cells typically retain the Philadelphia chromosome; thus, this analysis would not test your hypothesis. Choice D might be the next step you would pursue after confirming that *Bcr-Abl* is mutated in imatinib-resistant cells, but it is too laborious to carry out as the first step.

8–124

D. Expression of the *Bcr-Abl* gene in bacteria would be the quickest way to obtain large amounts of protein for such tests. Since one can express ample amounts of protein in bacteria, purification is relatively easy. In addition, a genetically engineered affinity tag can be added to allow one-step affinity purification. It is crucial to amplify the *Bcr-Abl* fusion from cDNA, which is copied from mature mRNA, so that it does not contain introns, which would prevent expression in bacteria. Expression of foreign proteins in bacteria does not always work because the protein sometimes does not fold properly and forms large aggregates in the cell. Nevertheless, it is almost always the first approach that is tried. Choice A is not correct because *Bcr-Abl* cloned from genomic DNA would contain introns, which would block expression in bacteria. Choice B is incorrect, not only because it is difficult (and expensive) to isolate large amounts of a protein from cultured cells, but also because SDS denatures the protein, which would render it inactive, therefore useless for your experiments. Choice C would be difficult because of the labor and expense of culturing the large amount of cells necessary for isolating a protein that is normally expressed at low levels. You would also need to develop an assay to detect the Bcr-Abl fusion protein during purification, and go through extensive trial and error to optimize each step.

8–125

C. Quantitative RT-PCR would provide a simple and rapid way to assay levels of the *Bcr-Abl* mRNA. Choices A and B are not correct because, although they can detect *Bcr-Abl* mRNA, they would not provide a way to accurately measure its levels. Choice D is not correct because Western blotting, although it allows a quantitative measure of protein levels, does not provide a direct readout of how much *Bcr-Abl* mRNA is produced. Increased levels of protein could arise for a variety of reasons other than increased transcription. For example, a mutation in Bcr-Abl that slowed its degradation would lead to higher levels of protein, with no increase in mRNA.

8–126

B. If resistance is not caused by *Bcr-Abl* mutations or overexpression, it must be caused by mutations elsewhere in the genome, but it is impossible to know where. Thus, sequencing the entire genome and looking for additional mutations is the best choice. Ideally, one would compare the genomes of drug-sensitive and drug-resistant cells from the same patient, which would eliminate genetic variability between patients that would complicate the analysis. Alternatively, one could sequence the genomes of drug-resistant cells from many different patients and search for common mutations that could explain resistance. Choice A is not correct because it would be extremely fortuitous for a gene that affects imatinib resistance to be located near the *Bcr-Abl* gene. Choices C and D are not correct because they involve classical genetic analysis techniques that cannot be carried out with cancer cells.

8–127

B. The most likely explanation for the loss of activity is that multiple components are required for activity, and they were separated in the two fractions. Thus, a common procedure in protein purification is to combine fractions to see if you can reconstitute the activity. The other choices do not test the simplest hypothesis that could explain the loss of activity.

8–128

B. The investigators attached ubiquitin to beads and used affinity chromatography to identify proteins that interact with ubiquitin and were retained on the column. This approach led to the discovery of the key components of the pathway for regulated proteolysis. Choice A—a

BLAST search—would not identify proteins that interacted with ubiquitin, but rather, proteins that were related to ubiquitin. Choice C is not correct because separation of proteins by size would not lead directly to discovery of additional components of the pathway. Choice D is incorrect because it is used to separate proteins from one another, not to uncover protein–protein associations; it would not reveal additional components of the pathway.

8–129

A. The complete loss of function of a gene that controls cell division—an essential cell function—would be lethal; hence, such mutants could not be isolated for study. To study genes that control the cell cycle, the investigators needed to isolate conditional mutations that are active in one set of conditions but inactive in another. The most commonly used conditional mutations are temperature-sensitive mutations; this means that they encode proteins that are active at low temperatures but inactive at higher temperatures. Temperature-sensitive mutations thus provide a powerful tool for studying the effects of inactivating specific gene products. The other choices do not allow retention of the essential function of the genes; hence, these would not be useful for isolating mutants to use to study the cell cycle.

8–130

A. In a co-immunoprecipitation experiment, one would immunoprecipitate one of the proteins and then test whether the other proteins are also in the precipitate. This technique is relatively rapid because it only requires antibodies against the proteins. This is often simplified by attaching genetically engineered tags to the proteins, so that well-tested, commercially available antibodies can be used to precipitate specific proteins via their tags. The other choices do not describe methods that could be used to rapidly test for associations between proteins.

8–131

D. Western blotting allows one to detect a single protein in a complex mixture rapidly and directly. To test whether securin is destroyed during the cell cycle, one would periodically take samples of the synchronized cells as they go through the cell cycle. All of the samples would then be resolved by SDS polyacrylamide-gel electrophoresis and analyzed by Western blotting to determine whether securin decreases in amount during the cell cycle. Choice A is not correct because hybridization is a technique to analyze DNA and RNA. Choice B is more complicated than necessary, because the precipitated protein would then have to be analyzed by Western blotting. Choice C could be used to assay for loss of a specific protein, but it is more complicated and expensive than necessary.

CHAPTER 9

Visualizing Cells

IN THIS CHAPTER

LOOKING AT CELLS IN THE LIGHT MICROSCOPE

DEFINITIONS

9–1 Green fluorescent protein (GFP)

9–2 Limit of resolution

9–3 Bright-field microscope

9–4 Image processing

9–5 Fluorescence microscope

9–6 Confocal microscope

9–7 Fluorescence resonance energy transfer (FRET)

9–8 Microelectrode

TRUE/FALSE

9–9 False. Although it is not possible to see DNA by light microscopy in the absence of a stain, chromosomes are clearly visible under phase-contrast or Nomarski differential-interference-contrast microscopy when they condense during mitosis. Condensed human chromosomes are more than 1 μm in width—well above the resolution limit of 0.2 μm.

9–10 False. Monoclonal antibodies are homogeneous and recognize the particular protein against which they were raised, but they may still react with other unrelated proteins. In general, the specificity of a monoclonal antibody depends on whether the segment of protein it binds (the epitope, which is usually just a few amino acids) is present in identical or similar form on another protein. Establishing the specificity of an antibody probe (whether it is monoclonal or a mixture) is a critical element in determining its practical usefulness for experimental applications.

9–11 True. Caged molecules are photosensitive precursors of biologically active substances such as Ca^{2+}, cyclic AMP, and inositol trisphosphate. They are designed to carry an inactivating moiety attached by a photosensitive linkage. When exposed to intense light of the correct wavelength, the inactivating group is split off and the active small molecule is released. Because laser beams can be tightly focused, caged molecules can be activated at defined locations in a cell. Thus, the time and location of activation are under the experimenter's control.

9–12 True. Superresolution techniques allow localization of fluorescent molecules to accuracies much below the diffraction limit to resolution, which is about 200 nm. Current superresolution techniques such as STED, PALM, and STORM use optical methods to illuminate a sparse set of

fluorescent tags, whose precise mathematical centers can then be calculated from the point spread function of the blurred image representing the tag. By combining multiple such images, these superresolution techniques build up a high-resolution image of the object that was tagged.

THOUGHT PROBLEMS

9–13 The components of the light microscope are labeled in **Figure 9–17**. Magnification of the specimen occurs at two points: in the objective and in the eyepiece.

9–14 All such imperfections scatter, refract, and reflect light, reducing the amount of light that goes through the specimen and adding spurious light rays that add a background haze to the image. As a result, both contrast and resolution are reduced.

9–15 The parallel light rays will converge (be focused) by passing through the lens, as shown in **Figure 9–18A**. The rays will also be focused by the inverted lens (**Figure 9–18B**).

9–16 In a dry lens, a portion of the illuminating light is internally reflected at the interface between the cover slip and the air. By contrast, in an oil-immersion lens, there is no interface because glass and immersion oil have the same refractive index; hence, no light is lost to internal reflection. In essence, the oil-immersion lens increases the width of the cone of light that reaches the objective, which is a key limitation on resolution.

9–17 The main refraction in the human eye occurs at the interface between air (refractive index 1.00) and the cornea (refractive index 1.38). Because of the small differences in refractive index between the cornea and the lens and between the lens and the vitreous humor, the lens serves to fine-tune the focus in the human eye.

9–18 Humans see poorly under water because the refractive index of water (1.33) is very close to that of the cornea (1.38), thus eliminating the main refractive power of the cornea. Goggles improve underwater vision by placing air in front of the cornea, which restores the normal difference in refractive indices at this interface. The image is still distorted by the refractive index changes at the water–glass and glass–air interfaces of the goggles, but the distortion is small enough that the image can still be focused onto the retina, allowing us to see clearly.

9–19 Viewed through a beaker of clear glass beads, the eye chart will be illegible because the light will be refracted and reflected at every glass–air interface. Filling the beaker with water would help somewhat by reducing the refractive index difference (1.33 for water and 1.51 for glass), but there would still be considerable distortion due to refraction and reflection. Filling the beaker with immersion oil would eliminate the refractive

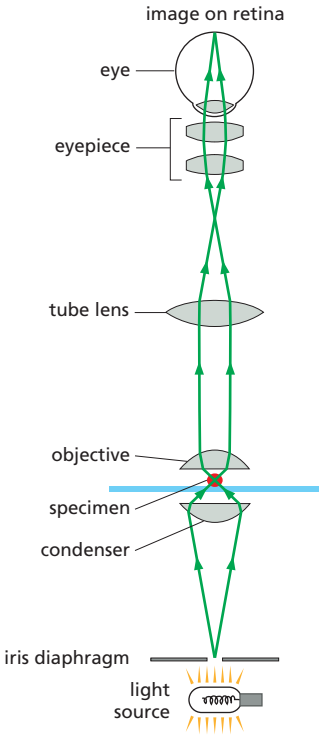


Figure 9–17 Schematic diagram of a light microscope with components labeled (Answer 9–13).

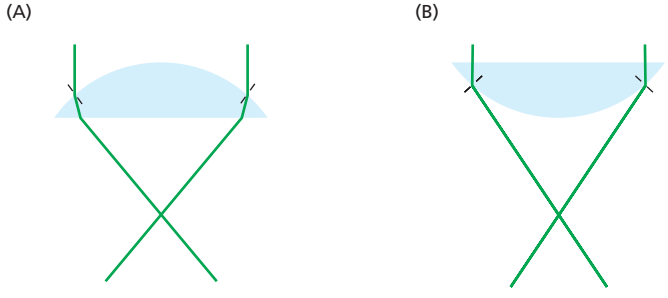


Figure 9–18 Focusing of parallel rays by a glass lens (Answer 9–15). (A) Lens with curved surface facing incident light. (B) Lens with flat surface toward incident light. Lines normal to the curved surface of the lens are shown by *black lines*.

index difference since glass and immersion oil have the same refractive index. Thus, the beaker would appear transparent and the eye chart would be fully legible.

9–20 Image A was taken by bright-field microscopy, image B by phase-contrast microscopy, image C by Nomarski differential-interference-contrast microscopy, and image D by dark-field microscopy.

9–21 Resolution refers to the ability to see two small objects as separate entities, which is limited ultimately by the wavelength of light used to view the objects. Magnification refers to the size of the image relative to the size of the object. It is possible to magnify an image to an arbitrarily large size. It is important to remember that magnification does not change the limit of resolution.

9–22 Ultraviolet light has the potential to damage DNA, whose bases absorb maximally at around 260 nm. By confining the illuminating beam to wavelengths well above 260 nm, or by using filters that block the damaging wavelengths, living cells can be viewed using ultraviolet light without significant threat to the DNA.

9–23 Longer wavelengths correspond to lower energies. Because some energy is lost during absorption and re-emission, the emitted photon is always of a lower energy (longer wavelength) than the absorbed photon.

9–24

A. The general idea is to filter the light from the source so that it carries wavelengths that can excite Hoechst 33342 but will not allow passage of longer wavelengths of light that overlap the emission spectrum. Among the listed filters the only choice is filter 1. Similarly, the filter between the sample and the eyepiece should block out wavelengths that are passed by the first filter, but not block wavelengths corresponding to the emission spectrum. This discrimination could be accomplished by two of the listed filters. Filter 2 would capture virtually the entire emission spectrum, whereas filter 3 would capture the main part of the emission spectrum and would likely work just fine, although with lower sensitivity. In practice, filters 1 and 2 are combined in a set for use with Hoechst 33342 (**Figure 9–19**).

B. For the microscope to work properly, you would like to have the beam-splitter reflect the wavelengths from the source and transmit the fluorescent light emitted by the sample. For Hoechst 33342, a mirror that reflected light below 400 nm and transmitted light above 400 nm would be ideal (Figure 9–19).

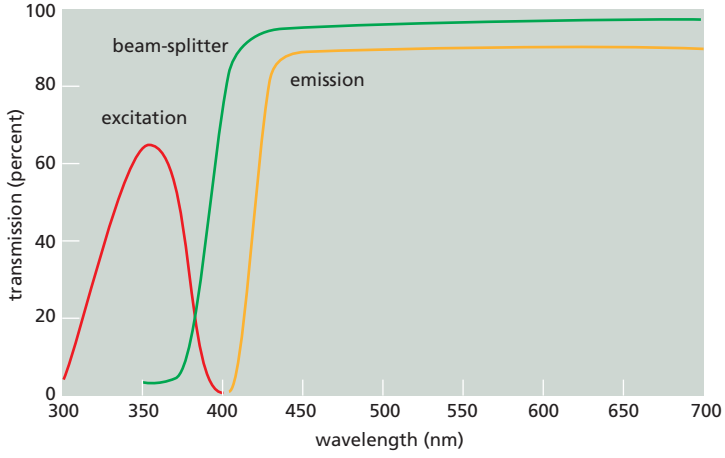


Figure 9–19 Transmission by a commercially available filter set with a dichroic mirror, which is suitable for viewing Hoechst 33342 (Answer 9–24). The first (excitation) filter is optimal for transmission of light in the range that would be absorbed by Hoechst 33342. The second (emission) filter would transmit all the fluorescent light emitted by Hoechst 33342. The beam-splitting mirror would not transmit (would reflect) light that passes through the first filter, but would transmit the light that passes through the second filter.

9–25 Fluorescently tagged antibodies and enzyme-tagged antibodies each have the advantage of amplifying the initial signal provided by the binding of the primary antibody. For fluorescently tagged secondary antibodies, the amplification is usually several-fold; for enzyme-linked antibodies, amplification can be more than 1000-fold. Although the extensive amplification makes enzyme-linked methods very sensitive, diffusion of the reaction product (often a colored precipitate) away from the enzyme limits the spatial resolution.

9–26 In the absence of oxygen, GFP does not become fluorescent, an observation that suggests oxygen is required for GFP fluorescence. The absence of fluorescence in inclusion bodies, which contain denatured protein, indicates that native protein structure is required for fluorescence. The first-order kinetics of the development of fluorescence and its lack of dependence on GFP concentration imply that fluorescence depends only on GFP and oxygen: no other proteins or small molecules are needed. The ability of minor changes in protein sequence to influence brightness and color indicates that the chromophore is very sensitive to the molecular environment provided by the protein.

The chromophore of GFP is formed from a stretch of three adjacent amino acids—serine, tyrosine, and glycine. In the presence of oxygen, the backbone cyclizes to form a five-membered ring whose double bonds are conjugated with those of the phenol ring of tyrosine (**Figure 9–20**). The ability of this chromophore to interact with light is sensitive to the surrounding molecular environment of the protein.

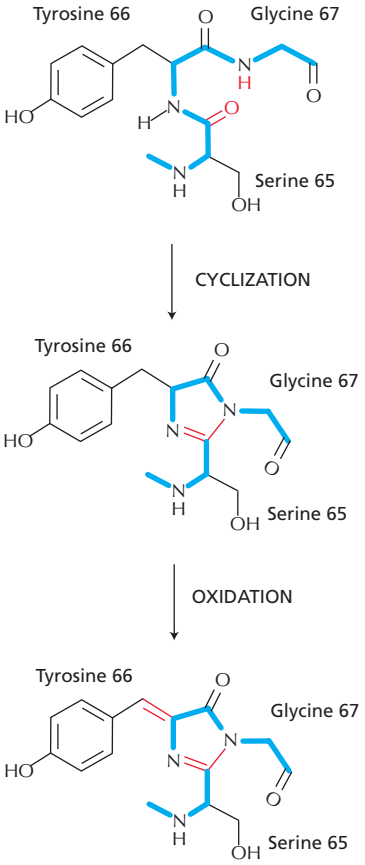


Figure 9–20 Formation of the chromophore of GFP (Answer 9–26). The bonds in the backbone of the protein are shown as *blue lines.*

References: Chalfie M, Tu Y, Euskirchen G, Ward WW & Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805.

Heim R, Prasher DC & Tsien RY (1994) Wavelength mutations and post-translational autoxidation of green fluorescent protein. *Proc. Natl Acad. Sci. USA* 91, 12501–12504.

9–27 The wavelengths at which the chromophore is excited and at which it emits fluorescent light depend critically on its molecular environment. Using a variety of mutagenic and selective procedures, investigators have generated mutant GFPs that fluoresce throughout the visible range. These modified GFPs have a variety of different amino acids around the chromophore, which subtly influence its ability to interact with light.

Reference: Service RF (2004) Immune cells speed the evolution of novel proteins. *Science* 306, 1457.

CALCULATIONS

9–28 Substituting values into the equation, the resolution for violet light in air is 0.28 μm and in oil is 0.19 μm. The resolution for red light in air is 0.49 μm and in oil is 0.33 μm. Clearly the best resolution is obtained with violet light (0.4 μm) using oil immersion ($n = 1.51$), as calculated below.

$$\text{resolution} = \frac{0.61\,\lambda}{n \sin\theta}$$

$$= (0.61)(0.4\ \mu\text{m}) / (1.51)(\sin 60°)$$

$$= 0.19\ \mu\text{m}$$

9–29 When parallel to the interface, the angle of the transmitted light (θ_t) is 90°. Substituting this angle and the refractive indices into the equation gives an incident angle of 41.5°.

$$n_i \sin \theta_i = n_t \sin \theta_t$$

$$1.51 \sin \theta_i = 1.00 \sin 90°$$

$$\sin \theta_i = 1.00/1.51 = 0.66$$

$$\theta_i = \arcsin 0.66 = 41.5°$$

9–30 Fluorescence occurs when a molecule absorbs a photon within a narrow energy range (a narrow range of wavelengths), so that an electron is boosted to an allowable higher energy level. When that electron decays back to a lower energy state, it emits a photon that is less energetic (that is, at a longer wavelength). An electron can be boosted to the higher energy state by two lower-energy photons, so long as the second one is absorbed before the partially activated electron decays [within a femtosecond (10^{-15} sec) or so]. The rule for multiphoton activation is that the total energy of absorbed photons must add up to the amount needed to boost the electron into its allowable higher electronic state. Photons have half the energy at twice the wavelength. Thus, if the sample is illuminated with light of a narrow wavelength, so that all the photons have about the same energy, then the molecule will be activated maximally by two photons when the energy of the photons is about half that required for maximum activation by a single photon.

Reference: Bestvater F, Spiess E, Stobrawa G, Hacker M, Feurer T, Porwol T, Berchner-Pfannschmidt U, Wotzlaw C & Acker H (2002) Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging. *J. Microsc.* 208, 108–115.

9–31 In order to follow the fluorescent proteins independently, you would ideally like to excite them separately and detect their emissions separately. Thus, the best pairs are those with as little overlap in their excitation and emission spectra as possible. Clearly, of the three possible pairs, CFP and YFP have the smallest overlap in their excitation and emission spectra.

9–32 The increase in FRET depends on phosphorylation of the protein, since no increase occurs in the absence of Abl protein or ATP, or when the phosphate is removed by a tyrosine phosphatase (see Figure 9–11B). Thus, phosphorylation must cause CFP and YFP to be brought closer together. A reasonable explanation is that addition of phosphate to the tyrosine in the substrate peptide allows that segment of the protein to fold back to bind to the adjacent phosphotyrosine-binding domain, thereby decreasing the separation of the CFP and YFP domains (**Figure 9–21**).

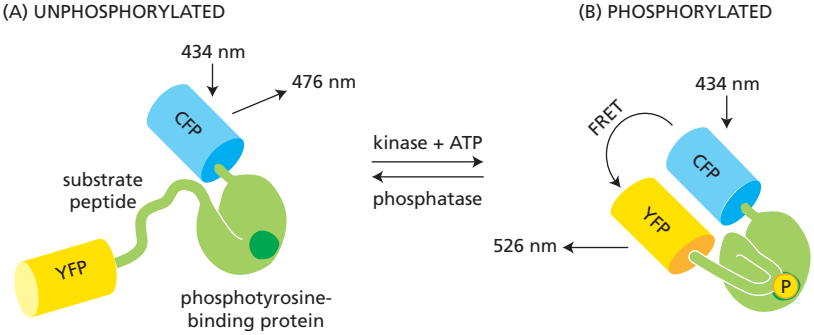


Figure 9–21 Conformational change in FRET reporter protein upon tyrosine phosphorylation (Answer 9–32).

9–33 The data in Figure 9–12 indicate the location of the reporter protein in the cell, and by inference the location of active Abl. The reporter is not phosphorylated (activated) in the nucleus. It is activated in the cytoplasm, but is most highly activated in membrane ruffles. These results are most simply consistent with the idea that active Abl is most prevalent in the membrane ruffles. (You can watch this process in a color movie. Go to the PNAS website, www.pnas.org, type in the volume and first page number of the article referenced below, and select "supporting movies." Movie 2 is spectacular.)

Reference: Ting AY, Kain KH, Klemke RL & Tsien RY (2001) Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc. Natl Acad. Sci. USA* 98, 15003–15008.

9–34 There are two keys to how the indicator works. First, emission at 535 nm after excitation at 440 nm means that the indicator depends on fluorescence resonance energy transfer (FRET) between CFP and YFP. As shown in Figure 9–10, CFP is efficiently excited at 440 nm and its emission spectrum overlaps with the excitation spectrum of YFP, which in turn emits light near maximally at 535 nm. Second, the efficiency of FRET depends on the distance between the chromophores in the two fluorescent proteins. In the absence of Ca^{2+}, calmodulin is in its extended form so that the CFP and YFP domains are maximally separated; hence, FRET should be inefficient and emission at 535 nm will be low. In the presence of Ca^{2+}, calmodulin will be folded much more compactly and CFP and YFP will be brought closer together; thus, FRET will occur much more efficiently and emission at 535 nm will increase.

References: Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M & Tsien RY (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388, 882–887.

Nagai T, Yamada S, Tominaga T, Ichikawa M & Miyawaki A (2004) Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc. Natl Acad. Sci. USA* 101, 10554–10559.

9–35

A. If these green dots represent clathrin-coated structures, the static ones likely represent clathrin-coated pits, which form on the surface of the cell and wait to be filled. The dots that disappear are likely to represent clathrin-coated vesicles, which have pinched off from the cell surface and moved into the cytosol, outside of the narrow range of viewing by TIRF microscopy.

B. You are right to be concerned about whether the dots represent functional structures; the dynamics you observed could have arisen in any number of artifactual (and uninteresting) ways. Your colleague's suggestion would allow you to test one aspect of the behavior expected from clathrin-coated pits and vesicles. If the green dots represent functional clathrin-coated structures, then the red transferrin, once bound by its receptor, should accumulate in the green dots, and the dots that disappear might be expected to contain transferrin. Thus, if you carried out this experiment, you would expect to see the red and green fluorescence colocalize. The authors of this study showed that these and other functional tests confirmed that the dots do indeed behave like clathrin-coated pits and vesicles.

Reference: Rappoport JZ & Simon SM (2003) Real-time analysis of clathrin-mediated endocytosis during cell migration. *J. Cell Sci.* 116, 847–855.

LOOKING AT CELLS AND MOLECULES IN THE ELECTRON MICROSCOPE

DEFINITIONS

9–36 Negative staining

9–37 Electron microscope (EM)

9–38 Cryoelectron microscopy

9–39 Scanning electron microscope (SEM)

9–40 Immunogold electron microscopy

TRUE/FALSE

9–41 False. Although the indicated statements are true with regard to TEM, they are incorrect for SEM. SEM gathers and analyzes electrons that are scattered from the surface of the object being viewed, which in this case is the thin section itself. SEM is not useful for examining internal structures.

THOUGHT PROBLEMS

9–42 The best current approach to preserving the original structures in the living cell is to freeze the sample rapidly before the components have a chance to rearrange themselves and before water can form crystals. The water can then be removed using organic solvents, and the sample can be embedded in plastic resin, cut into thin sections, stained to provide contrast, and viewed.

9–43 Biological structures are composed of atoms with similar, low atomic numbers; thus, most structures are marginally different from their surroundings in terms of their ability to scatter electrons. Because electron-scattering is proportional to the square of the number of electrons in the atom (which equals the atomic number), heavy metals scatter electrons much more efficiently than biological atoms, enormously enhancing the contrast in electron micrographs. Negative staining enhances the ability to see biological structures by providing contrast based on how the structure stands relative to the surface. A solution of uranyl acetate is typically used to coat the low-lying areas adjacent to biological molecules or complexes, providing a dark (electron-dense) background against which the less-electron-dense biological structure stands out. The characteristic features of the biological structure are rendered visible by the adjacent heavy-metal atoms.

9–44 Electron microscopists can be sure whether a structure is a pit or a bump. Shadowed structures are unlike shaded circles in a key way: structures that stand above the surface cast a shadow beyond themselves, whereas pits do not (**Figure 9–22**). In everyday experience, shadows cast by the sun are dark, but in the world of microscopy, where platinum atoms replace sunlight, the shadow is the absence of metal, hence bright. If you examine the micrographs in Figure 9–14, especially in the orientation that looks like pits (look at the lower left-hand corner in Figure 9–14D), you can see that most of the pits are elongated toward the lower left of the micrograph. Thus, these structures are casting shadows; hence, they are bumps. To have the structures perceived as bumps, microscopists arrange the micrographs as shown in Figure 9–14C, so that the dark portion of each bump is at the bottom. Evidently, this arrangement fits with

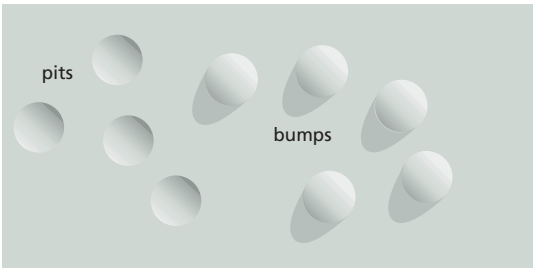


Figure 9–22 Bumps and pits (Answer 9–44). Bumps and pits can be distinguished because bumps cast shadows.

our hardwired perceptions of the world around us, with the sun coming from above. Note also that we naturally interpret the light areas of such images as structures that reflect light; in reality, they are simply the absence of electron-dense material and give us no more information about the structure than does a shadow cast by sunlight.

9–45 Averaging structures that are not in the same state will tend to emphasize those parts of the structure that don't vary. Parts that are moving (that is, in different locations in different images) would be de-emphasized or even eliminated. By way of analogy, imagine combining a series of low-quality snapshots of a cyclist in motion, in order to get a better picture. The frame of the bike and the cyclist's torso would be clear, but the spokes of the wheels would be invisible and the cyclist's legs would be a blur.

One way to improve the image of the nuclear pore complex is to try to pre-classify individual images into similar types, and then combine only those in the same class. This was in fact done for the nuclear pore complexes according to the distribution of mass in the central cavity. Those images with the central mass displaced toward the cytoplasm gave a somewhat different combined image from those that had the central mass more on the nuclear side. The advantages and potential pitfalls of this process can be appreciated by again considering the analogy of the cyclist. In the low-quality images, you might notice that the "legs" (of course, you wouldn't know they were legs) were together in some images and separated in others. Grouping images according to that criterion and combining images within each class would generate two final pictures that each showed a blurred image of "legs" in two positions. In the case of the bicycle, we know that the final image would be crude, at best, because legs occupy a continuum of positions and our classification would combine left and right legs.

Reference: Beck M, Förster F, Ecke M, Plitzko JM, Melchior F, Gerisch G, Baumeister W & Medalia O (2004) Nuclear pore complex structure and dynamics revealed by cryoelectron tomography. *Science* 306, 1387–1390.

CALCULATIONS

9–46 Substituting numbers into the equation gives a value for θ of 1.4°, which is about 43 times (60°/1.4°) smaller than θ for a typical light microscope.

$$\text{resolution} = \frac{0.61\,\lambda}{n \sin\theta}$$

$$\sin\theta = \frac{0.61\,(0.004\text{ nm})}{(0.1\text{ nm})}$$

$$\theta = \arcsin 0.0244 = 1.4°$$

If the electron microscope had a comparable θ (60°), its resolution would be about 0.003 nm.

DATA HANDLING

9–47 The micrograph in Figure 9–15 shows clearly that both proteins localize to the gap junction. Black dots (gold particles) of two different sizes are apparent in the gap junction. And all the black dots except one (near the top right-hand corner) are present in the gap junction. Close examination of the single outlier shows that it is also associated with a small patch of membrane that looks exactly like the gap junction. In reality, the two proteins labeled in the micrograph are connexins, which are components of the membrane channels that make up gap junctions.

Reference: Fujimoto K (1995) Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins. *J. Cell Sci.* 108, 3443–3449.

9–48

A. The gold particles in Figure 9–16 are consistently associated with an obvious structure in the membrane, termed square arrays. It is thought that these square arrays represent aggregates of aquaporin water channels.

B. There are a few black dots that are not obviously associated with square arrays. These may arise from antibodies that were unbound but not washed off. They may also represent antibodies that are bound to square arrays that are poorly defined in the micrograph or that are small enough to be obscured by the black dot. So long as the proportion of nonassociated dots is reasonably small, it does not affect the principal conclusion. The second issue—some square arrays that were not labeled—may indicate that the antibody is not saturating, or that the denatured aquaporins (denaturation is a consequence of the preparation technique) do not all present appropriate sites for the antibody to recognize. The absence of labeling of all such structures also does not detract from the primary conclusion.

Reference: Rash JE, Yasumura T, Hudson CS, Agre P & Nielsen S (1998) Direct immunogold labeling of aquaporin-4 in square arrays of astrocyte and ependymocyte plasma membranes in rat brain and spinal cord. *Proc. Natl Acad. Sci. USA* 95, 11981–11986.

MCAT STYLE

9–49

A. A long polymer assembled from many fluorescently labeled subunits can be detected by standard fluorescence microscopy. Although the diameter of actin is below the limit of resolution for light microscopy, the bright fluorescent signal from the actin filament will be easily detectable. Approach II is not correct because the diameter of actin filaments is well below the resolving power of any standard type of light microscopy, even using contrast-enhancement techniques. Approach III is not correct because digital image processing techniques can improve image quality and contrast, but they cannot overcome the resolution limit of standard light microscopy techniques.

9–50

D. The limit of resolution of standard light microscopy techniques is 200 nm. Thus, even though an actin filament can be detected using fluorescence microscopy, it will appear as a blurred image with a diameter of approximately 200 nm.

9–51

A. A 2 μm long actin filament assembled from many fluorescently labeled subunits would be easily detectable by standard fluorescence microscopy, and it is long enough to be visible as a line in the microscope. In addition, it should be easy to detect rotation of the long actin filament,

much like the rotation of a blade of a fan. This approach was actually used to directly detect rotation of the central stalk protein (see Problem 14–31). All the other choices, while making the position of the ring or stalk visible, would not provide sufficient resolution to see rotation of the 10 nm ring or the stalk inside it.

CHAPTER 10

Membrane Structure

IN THIS CHAPTER

THE LIPID BILAYER

DEFINITIONS

10–1 Liposome

10–2 Lipid raft

10–3 Ganglioside

10–4 Amphiphilic

10–5 Phosphoglyceride

10–6 Cholesterol

TRUE/FALSE

10–7 True. The hydrophobic interior of the lipid bilayer acts as a barrier to the passage of the hydrophilic lipid head groups that must occur during flip-flop. The energetic cost of this movement effectively prevents spontaneous flip-flop of lipids, so that it occurs extremely rarely in the absence of specific catalysts known as phospholipid translocators.

10–8 True. The positively charged moieties in all cases are balanced by the negative charge on the phosphate group; thus, none of the common phospholipids carries a net positive charge.

10–9 True. Glycolipids are synthesized in the lumen of the Golgi apparatus, which is topologically equivalent to the outside of the cell, and cannot flip-flop across the bilayer.

THOUGHT PROBLEMS

10–10 Water is a liquid, and thus hydrogen bonds between water molecules are not static; they are continually formed and broken again by thermal motion. When a water molecule happens to be next to a hydrophobic solute, it is more restricted in motion and has fewer neighbors with which it can interact because it cannot form any hydrogen bonds in the direction of the hydrophobic solute. It will therefore form hydrogen bonds to the more limited number of water molecules in its proximity. Bonding to fewer partners results in a more ordered water structure, which constitutes the icelike cage in Figure 10–1. The true cage of water molecules exists in three dimensions, forming a pentagonal dodecahedron (like a soccer ball) or clusters of them that enclose the hydrophobic solute. The structure is similar to ice, although it is a more transient, less organized, and less extensive network than even a tiny ice crystal. The formation of any ordered structure decreases the entropy of the system, which is energetically unfavorable.

10–11 The same forces that dictate that certain lipids will form a bilayer, as opposed to micelles, operate in the repair of a tear in the bilayer. The tear will heal spontaneously because a bilayer is the most energetically favorable arrangement. The lipids that make up a bilayer are cylindrical in shape and therefore do not readily form a micelle (or a hemi-micelle), which would require cone-shaped lipids.

10–12 Lipid bilayers assemble because the surrounding water molecules exclude the component lipids; thus, analogy (2) is the correct one. If bilayers formed because of attractive forces among the lipids—analogy (1)—the properties of the bilayer would likely be quite different. Molecules "attract" one another by forming specific bonds that hold them together. Such bonding among lipids would make the bilayer less fluid, perhaps even rigid, depending on the strength of the interactions.

10–13 Bilayers formed by lipids with saturated hydrocarbon tails would be much less fluid. Whereas a normal lipid bilayer has the viscosity of olive oil, a bilayer made of lipids with saturated hydrocarbon tails would have the consistency of bacon fat. In contrast, bilayers formed by lipids with unsaturated hydrocarbon tails would be much more fluid. Also, because the lipids would pack together less well, there would be more gaps and the bilayer would be more permeable to small water-soluble molecules.

10–14 In a two-dimensional fluid, the molecules are constrained to move in a plane; the molecules in a normal fluid can move in three dimensions.

10–15 Vegetable oil is converted to margarine by reduction of double bonds (by hydrogenation), which converts unsaturated fatty acids to saturated ones. This change allows the fatty acid chains in the lipid molecules to pack more tightly against one another, increasing the viscosity, turning oil into margarine.

10–16

C. Phosphatidylinositol is a minor component of the phospholipids in the plasma membrane, yet it plays a very important role in cell signaling. All the other phospholipids are common components of plasma membranes and play important structural roles in membrane integrity.

10–17 Phosphatidylcholine is the phospholipid that is cleaved by your snake venom enzyme.

10–18

A. Antarctic fish, which are cold-blooded, live in freezing waters. In order to maintain an appropriate fluidity of their membranes under such extreme conditions, they require a higher proportion of unsaturated fatty acid chains in their membranes to keep them from freezing solid like a stick of margarine. Polar bears also live in extreme cold, but they are warm-blooded and maintain a high internal temperature; thus, they have no special requirement for unsaturated fatty acids in their membranes.

10–19 The size of a lipid raft depends on the affinity of its components for one another. If sphingolipids and cholesterol molecules, for example, bound one another sufficiently tightly, they would aggregate into a single domain in the membrane. If they bound one another with the same affinity as they bind to other species of lipid molecules, they would remain dispersed. The small size of the lipid rafts indicates that sphingolipids and cholesterol molecules, for example, have only a slightly higher affinity for one another than for other lipids. Presumably, at the typical size of a raft, the aggregated lipid components, including sphingolipids and cholesterol molecules, are in equilibrium with their free forms, so that they are added to and leave a raft at equal rates.

10–20 It is not a paradox. The fluidity of the bilayer is strictly confined to one plane. The lipid molecules can diffuse laterally, but do not readily flip from one monolayer to the other. Specific types of lipid molecule remain in the monolayer they are inserted into, unless they are actively transferred by an enzyme—a phospholipid translocator (a flippase).

10–21 The redistribution of phosphatidylserine from the cytoplasmic to the outer monolayer of the plasma membrane occurs by two mechanisms: (1) the phospholipid translocators that normally transport this lipid from the noncytoplasmic monolayer to the cytoplasmic monolayer are inactivated in apoptotic cells; and (2) a "scramblase" that transfers phospholipid nonspecifically in both directions between the two monolayers is activated.

CALCULATIONS

10–22 A raft 70 nm in diameter would have an area of 3.8×10^3 nm^2 (3.14×35^2), and a lipid molecule 0.5 nm in diameter would have an area of 0.20 nm^2 (3.14×0.25^2). Thus, there would be about 19,000 lipid molecules per monolayer of raft ($3.8 \times 10^3/0.20 = 19{,}000$), and about 38,000 molecules in the raft bilayer. At a ratio of 50 lipids per protein, a raft would accommodate about 760 protein molecules. The true ratio of lipids to proteins in a raft is unknown.

DATA HANDLING

10–23

A. The difference in rate of loss of the ESR signals is due to the location of the nitroxide radical on the two phospholipids. The nitroxide radical in phospholipid 1 is on the head group and is therefore in direct contact with the external medium. Thus, it can react quickly with ascorbate. The nitroxide radical in phospholipid 2 is attached to a fatty acid chain and is therefore partially buried in the interior of the membrane. As a consequence, it is less accessible to ascorbate and is reduced more slowly.

B. The key observation is that the extent of loss of ESR signal in the presence and absence of ascorbate is the same for phospholipids 1 and 2 in resealed red cell ghosts, but different in red cells. These results suggest that there is an undefined reducing agent in the cytoplasm of red cells (which is absent from red cell ghosts). Like ascorbate, this cytoplasmic agent can reduce the more exposed phospholipid 1, but not the less exposed phospholipid 2. Thus, in red cells, phospholipid 2 is stable in the absence of ascorbate; in the presence of ascorbate, the spin-labeled phospholipids in the outer monolayer are reduced, causing loss of half the ESR signal. Phospholipid 1, on the other hand, is not stable in red cells in the absence of ascorbate because the phospholipids in the cytoplasmic monolayer are exposed to the cytoplasmic reducing agent, which destroys half the ESR signal. When ascorbate is added, labeled phospholipids in the outer monolayer are also reduced, causing loss of the remaining ESR signal.

C. The results in Figure 10–4 indicate that the labeled phospholipids were introduced equally into the two monolayers of the red cell plasma membrane. Phospholipid 2 was 50% sensitive to ascorbate, indicating that half the label was present in the outer monolayer, and 50% insensitive to ascorbate, indicating that half was present in the cytoplasmic monolayer. Similarly, phospholipid 1 was 50% sensitive to the cytoplasmic reducing agent and 50% sensitive to ascorbate, indicating an even distribution between the cytoplasmic and outer monolayers.

Reference: Rousselet A, Guthmann C, Matricon J, Bienvenue A & Devaux PF (1976) Study of the transverse diffusion of spin labeled phospholipids

in biological membranes. I. Human red blood cells. *Biochim. Biophys. Acta* 426, 357–371.

10–24

A. For randomly dispersed receptors (see Figure 10–5A), FRET will depend critically on the concentration of the receptors in the membrane. At high density, there will be efficient FRET, but low FRET at low density. For receptors that are confined to microdomains such as lipid rafts (see Figure p10–5B), the overall fluorescence intensity will decrease with decreasing density of the rafts, but FRET, as a fraction of direct fluorescence, will remain constant.

B. The results suggest that transmembrane-anchored folate receptors are randomly dispersed in the membrane, while GPI-anchored receptors are clustered in microdomains. Although such microdomains are likely to be lipid rafts, these experiments do not prove that point.

Reference: Varma R & Mayor S (1998) GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* 394, 798–801.

10–25

A. The half-time for flip-flop in these experiments is the point at which 50% of the ESR signal is lost. For cells labeled in the cytoplasmic monolayer, the curve in Figure 10–6 suggests a half-time for flip-flop of about 7 hours. For cells labeled in the outer monolayer, the half-time of flip-flop is much longer but cannot be estimated reliably. These data indicate that the rate of flip-flop of phospholipids between the two monolayers of the plasma membrane in red cells is extremely low. Similar experiments using synthetic bilayers have given even longer times; in fact, in the best experiments, when great care was taken not to allow oxidation or other damage to the lipids, the rate of flip-flop was immeasurably low (less than once per month).

B. Phospholipid 2 was used to label the cytoplasmic monolayer, and phospholipid 1 was used to label the outer monolayer. As shown by the experiments in Figure 10–4B, phospholipid 2 in the cytoplasmic monolayer is not reduced by the cytoplasm of red cells; when it is present in the outer monolayer, it can be reduced by ascorbate. Thus, phospholipid 2 is appropriate for measuring the rate of flip-flop from the cytoplasmic to the outer monolayer. As shown by the experiments in Figure 10–4A, phospholipid 1 in the cytoplasmic monolayer is reduced by red cell cytoplasm, but it is stable in the outer monolayer in the absence of ascorbate. Thus, phospholipid 1 is appropriate for measuring the rate of flip-flop from the outer to the cytoplasmic monolayer.

C. One can make intact red cells with spin-labeled phospholipids exclusively in the cytoplasmic monolayer by introducing phospholipid 2 into the membrane and then incubating the red cells for 1 hour in the presence of ascorbate. Ascorbate reduces the lipids in the outer monolayer, leaving red cells that are labeled only in the cytoplasmic monolayer. Similarly, one can make intact red cells with spin-labeled phospholipids exclusively in the outer monolayer by introducing phospholipid 1 into the membrane and then incubating the red cells for 15 minutes in the absence of ascorbate. In this case, the spin-labeled lipids in the cytoplasmic monolayer are reduced by agents in the cytoplasm, leaving red cells that are labeled only in the outer monolayer.

Reference: Rousselet A, Guthmann C, Matricon J, Bienvenue A & Devaux PF (1976) Study of the transverse diffusion of spin labeled phospholipids in biological membranes. I. Human red blood cells. *Biochim. Biophys. Acta* 426, 357–371.

MEMBRANE PROTEINS

DEFINITIONS

10–26 Lectin

10–27 Carbohydrate layer

10–28 Spectrin

10–29 Multipass transmembrane protein

10–30 Bacteriorhodopsin

10–31 Cortex

10–32 Glycosylphosphatidylinositol (GPI) anchor

TRUE/FALSE

10–33 False. The carbohydrate on internal membranes is directed away from the cytosol toward the lumen of an internal membrane-enclosed compartment. Remember that the lumen of an internal compartment is topologically equivalent to the outside of the cell.

10–34 False. Human red blood cells contain no internal membranes at all; at an early stage in their development, they extrude their nuclei. The lack of any internal membranes is the principal reason they have been used so extensively to investigate the structure of the plasma membrane.

10–35 False. In addition to lipid rafts, which are microdomains with distinct lipid compositions, the apical and basolateral surfaces of epithelial cells, which are separated by intercellular tight junctions, also have different lipid compositions.

THOUGHT PROBLEMS

10–36 Thus far, arrangements 1, 2, 4, 5, 6, and 7 have been found in biological membranes. Arrangement 3, which shows the protein attached by a fatty acid on the external surface, is unlikely because the enzymes responsible for linking fatty acids to proteins are located in the cytosol. Arrangement 8, which shows a protein with just its tip embedded in the membrane, is unlikely to occur on theoretical grounds since it would position the charged N- or C-terminus in the middle of the membrane.

10–37

D. The mass ratio depends on the membrane. In the myelin membrane around nerve-cell axons, proteins account for less than 25% of the mass. In the mitochondrial inner membrane, proteins account for about 75% of the total mass. In typical plasma membranes, the masses of proteins and lipids are about the same.

10–38 Fatty acid chains, prenyl groups, and glycosylphosphatidylinositol (GPI) anchors are the three common lipid anchors for membrane proteins.

10–39

A. Sequence A is the actual membrane-spanning α-helical segment of glycophorin, a transmembrane protein from red blood cells. It is composed predominantly of hydrophobic amino acids, although it does contain the uncharged polar amino acids threonine (T) and serine (S), which are not uncommon in membrane-spanning α helices.

Sequence B is unlikely to be a membrane-spanning segment because it contains three prolines (P), which would disrupt an α helix and thereby expose polar groups to the hydrophobic environment of the lipid bilayer.

Sequence C is also unlikely to be a transmembrane segment because it contains three charged amino acids, glutamic acid (E), arginine (R), and aspartic acid (D), whose presence in the hydrophobic lipid bilayer would be energetically unfavorable.

10–40 The hydrophilic faces of the five membrane-spanning α helices, each contributed by a different subunit, can come together to form a hydrophilic pore across the lipid bilayer that is lined with the hydrophilic amino acid side chains (**Figure 10–12**). The hydrophobic amino acid side chains on the opposite sides of the α helices can then interact with the hydrophobic lipid tails in the bilayer.

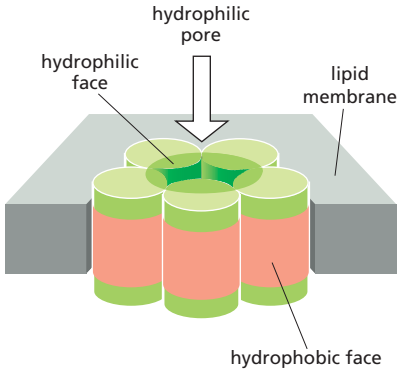


Figure 10–12 Proposed structure for a hydrophilic pore formed by five membrane-spanning α helices (Answer 10–40).

10–41 In both an α helix and a β barrel, the polar hydrogen-bonding groups in the peptide bond are fully satisfied by internal hydrogen bonds with groups in other peptide bonds. These internal hydrogen bonds dictate the secondary structures known as α helices and β sheets (or β barrels when the edges of a sheet pair to complete the cylinder). By contrast, in a disordered chain, the polar groups in the peptide bonds are not involved in bonding to one another. Such disordered segments can exist in proteins because hydrogen bonds can be made with water molecules or to other polar groups in the protein. In a membrane, however, the hydrophobic hydrocarbon chains of the bilayer provide no hydrogen-bonding partners. As a result, a disordered peptide chain in a membrane is energetically very unfavorable.

10–42 Your friend's suggestion is based on an important difference between inside-out and right-side-out vesicles. The contaminating right-side-out vesicles will carry carbohydrate on their exposed surface and, therefore, should be retained on a lectin affinity column. Inside-out vesicles, by contrast, will lack carbohydrate on their exposed surface and, therefore, should pass through the column.

10–43 The sulfate group in SDS is charged and therefore hydrophilic. The OH group and the C–O–C groups in Triton X-100 are polar; they can form hydrogen bonds with water and are therefore hydrophilic. The gray portions of these detergents are either hydrocarbon chains or aromatic rings, neither of which have polar groups that can hydrogen-bond to water molecules; they are therefore hydrophobic.

10–44 Membrane proteins anchor the lipid bilayer to the cytoskeleton, which strengthens the plasma membrane so that it can withstand the shear forces red blood cells are subjected to when they are pumped through small blood vessels.

10–45 Transmembrane domains that are composed entirely of hydrophobic amino acid side chains obviously cannot interact with one another via hydrogen bonds or electrostatic attractions, two of the more important ways to link proteins together noncovalently. Nevertheless, they can interact specifically via van der Waals attractions. If their surfaces are complementary, they can fit together well enough to make a large number of van der Waals contacts, which can hold them together. It should be noted, however, that the transmembrane segment of glycophorin contains a few polar amino acids that may participate in the dimerization process.

10–46 Proteins can be restricted to specific regions of the plasma membrane in several ways: by attachment to extracellular or intracellular proteins, by attachment to proteins in other cells, and by molecular fences that corral proteins in specific membrane domains. The fluidity of the lipid bilayer is not significantly affected by the anchoring of membrane proteins; the

lipid molecules flow around anchored proteins like water around rocks in a stream.

10–47 Cytosolic membrane-binding proteins could induce protrusion of the membrane in several ways. For example, a protein that bound to a concave surface of the membrane, instead of the convex surface shown in Figure 10–9B, would bend the membrane to induce a protrusion. Alternatively, a protein that bound phospholipids with small head groups in the cytosolic leaflet of the membrane, instead of large head groups as shown in Figure 10–9C, or removed head groups from the phospholipids, would induce a concave curvature of the membrane, giving rise to a protrusion. For the third method of membrane-bending shown in Figure 10–9A—inserting a segment of protein into the cytosolic leaflet—it is difficult to see how such a mechanism could be used to induce a protrusion.

Reference: Prinz WA & Hinshaw JE (2009) Membrane-bending proteins. *Crit. Rev. Biochem. Mol. Biol.* 44, 278–291.

CALCULATIONS

10–48 Using these average molecular weights, there are 96 lipid molecules (phospholipid + cholesterol) for every protein molecule {[(50,000/800) + (50,000/386)]/2 = 96}. A similar lipid-to-protein ratio is present in many cell membranes.

10–49

A. The calculation for the number of spectrin molecules per red blood cell is shown in detail below. In essence, one first calculates the fraction of total protein that is spectrin and then converts that number into the number of spectrin molecules using the molecular weight of spectrin and Avogadro's number:

$$\frac{\text{spectrin}}{\text{cell}} = \frac{5\text{ mg protein}}{10^{10}\text{ cells}} \times \frac{0.25\text{ spectrin}}{\text{total protein}} \times \frac{\text{mmol spectrin}}{250{,}000\text{ mg}} \times \frac{6 \times 10^{20}\text{ molecules}}{\text{mmol spectrin}}$$

$$= 3 \times 10^5\text{ spectrin molecules/cell}$$

The analogous calculations give values of 9×10^5 molecules of AE1 and 2.3×10^5 molecules of glycophorin per red cell. The calculated number of glycophorin molecules per cell is too low by a factor of 2.5 because about 60% of the molecular weight of glycophorin is carbohydrate, which is not stained by Coomassie Blue.

B. The fraction of the plasma membrane that is occupied by AE1 is the area of the face of a single AE1 molecule (πr^2), times the total number of AE1 molecules per cell (9×10^5), divided by the total area of the red blood cell (10^8 nm^2). Note that the height of the molecule is irrelevant to the calculation.

$$\frac{\text{AE1}}{\text{plasma membrane}} = \frac{3.14 \times (3\text{ nm})^2}{\text{molecule}} \times \frac{9 \times 10^5\text{ molecules}}{\text{cell}} \times \frac{1\text{ cell}}{10^8\text{ nm}^2}$$

$$= 0.25$$

Thus, AE1 occupies about 25% of the surface area of a red blood cell. This result is consistent with freeze-fracture electron micrographs of red blood cells, which show a high density of intramembrane particles that are thought to be dimers of AE1.

DATA HANDLING

10–50 There are two populations of AE1 in the red cell membrane: one population is immobilized by attachment to the spectrin-based cytoskeleton;

the other is freely mobile. Only the freely mobile population will be able to diffuse into the bleached spot and contribute to recovery of fluorescence. Thus, the curve for recovery of fluorescence will reach a plateau below the original level of fluorescence (**Figure 10–13**). The extent of recovery will correspond to the proportion of AE1 proteins that are freely mobile.

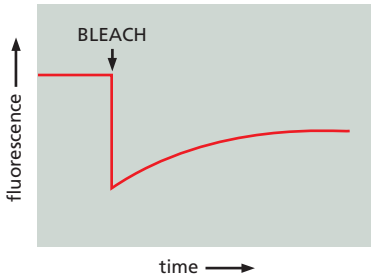


Figure 10–13 Recovery of fluorescence after photobleaching of AE1 (Answer 10–50).

MEDICAL LINKS

10–51 Normally, the cytosol is sufficiently reducing that cytosolic proteins contain no disulfide bonds, even in G6PD-deficient individuals. However, in G6PD-deficient individuals who eat fava beans, the cytosol of red cells can become sufficiently oxidizing that disulfide bonds form. Since all such bonds are inappropriate in the cytosol, they link proteins in ways that were never intended, leading to clumps and aggregates that stick to the cell membrane. The resulting distorted shape of the cell serves as a signal to the spleen to remove the damaged cells from circulation, leading to the severe anemia.

Although this question was framed in an ancient context, the problem is ongoing. For centuries, schoolteachers on the Mediterranean island of Sardinia have witnessed a curious phenomenon. Every April as Spring arrives—and fresh fava beans become available—some of their students (mostly boys—the G6PD gene is on the X chromosome) suddenly seem drained of energy. For the next three months, their schoolwork suffers, and they complain of dizziness and nausea and fall asleep at their desks. Then, just as suddenly, they return to normal and remain healthy and active until the next April rolls around. In children it might be brushed off as "spring fever," but Sardinian adults (mostly males) suffer the same symptoms.

It was during the Korean War that the connection was made between the Mediterranean form of G6PD deficiency and the hemolytic effects of antimalarial drugs, which, like the substance in fava beans, are oxidizing agents. The collapse of some soldiers, who were given such prophylactic drugs, led to a detailed investigation of the problem.

MCAT STYLE

10–52

B. Cholesterol is a hydrophobic lipid molecule with low solubility in aqueous solutions. Cholesterol must therefore be transported in the blood in a form that masks its hydrophobic nature. All the other choices are incorrect.

10–53

A. Apolipoprotein must have hydrophilic surfaces that interact with the aqueous environment, as well as hydrophobic surfaces that interact with the lipid core of the LDL particle. It is therefore an amphiphilic protein. The other classes of protein listed in the question are found on cells, rather than on LDL particles. In addition, LDL particles are not surrounded by a lipid bilayer, so apolipoprotein cannot be a transmembrane protein.

10–54

C. Phospholipids contain a polar head group that must interact with a polar environment and a hydrophobic tail that must interact with a hydrophobic environment. Thus, the most reasonable position for the phospholipid is at the surface of the LDL particle, with the polar head group facing the polar aqueous environment and the hydrophobic tail buried in the hydrophobic core of the particle. Thus, the answer is C. Choice A is not correct because the hydrophilic domains of apolipoprotein face the aqueous environment. Choice B is incorrect because there is no lipid

bilyer surrounding an LDL particle. LDL particles are enclosed in a lipid monolayer, with one side exposed to the hydrophilic surrounding and the other to the hydrophobic core. Choice D is wrong because location of the phospholipid completely within the core of the particle would be energetically unfavorable since the polar head group would be in a hydrophobic environment.

10–55

D. The LDL receptor on the cell surface must bind to LDL particles with high specificity and affinity, and then initiate a series of events that brings the LDL particle into the cell. Integral membrane proteins are the most likely candidates to serve this function and the LDL receptor is known to be a single-pass transmembrane protein. Lipids such as cholesterol, phospholipids, and glycolipids most commonly serve as structural—rather than specificity—components of the membrane. A cholesterol-binding protein is an unlikely choice because cholesterol is buried in the core of the LDL particle and, therefore, unavailable for binding by a cell-surface protein.

10–56

B. Cholesterol is an important component of the plasma membrane. Thus, membrane growth, as occurs during cell proliferation, requires cholesterol, which is acquired largely by uptake of LDL particles. The other choices—glycolipid synthesis, protein secretion, and protein synthesis—are unrelated to LDL particles.

Membrane Transport of Small Molecules and the Electrical Properties of Membranes

CHAPTER 11

IN THIS CHAPTER

PRINCIPLES OF MEMBRANE TRANSPORT

DEFINITIONS

11–1 Channel

11–2 Passive transport

11–3 Membrane transport protein

11–4 Active transport

11–5 Electrochemical gradient

TRUE/FALSE

11–6 False. Lipid bilayers are impermeable to ions, but the plasma membrane contains specific ion channels and transporters that make it very permeable to particular ions and charged solutes under certain circumstances.

11–7 True. Transporters bind specific molecules and undergo a series of conformational changes to move the bound molecule across a membrane. They can transport passively down the electrochemical gradient, or the transporters can link the conformational changes to a source of metabolic energy such as ATP hydrolysis to drive active transport. By contrast, channels form aqueous pores that can be open or shut, but always transport downhill; that is, passively. Channels interact much more weakly with the solute to be transported, and they do not undergo conformational changes to accomplish transport. As a consequence, transport through channels cannot be linked to an energy source and is always passive.

THOUGHT PROBLEMS

11–8 The order is CO_2 (small and nonpolar) > ethanol (small and slightly polar) > H_2O (small and polar) > glucose (large and polar) > Ca^{2+} (small and charged) > RNA (very large and highly charged). This list nicely illustrates the two basic properties that govern the capacity of molecules to diffuse through a lipid bilayer: size (small > large) and polarity (nonpolar > polar > charged).

11–9 Specific solutes move through the membrane much more slowly via transporters than by channels because transporters must bind the solute and undergo a series of conformational changes to transfer the solute across the membrane. Transport through channels is much faster because they are ion-specific pores that neither bind the ion nor undergo any conformational changes in order to move it across the membrane.

11–10

A. Transport by a transporter can be described by an equation that is strictly analogous to the one for a simple enzyme reaction:

$$T + S_{high} \rightleftharpoons TS \rightarrow T + S_{low}$$

where S_{high} is the solute on the high side of the concentration gradient and S_{low} is the solute on the low side. For a transport process, the solute remains unchanged—unlike the substrate in an enzyme reaction—but it is moved to the other side of the membrane.

B. The Michaelis–Menten equation for transport by a transporter is also strictly analogous to that for a simple enzyme reaction:

$$\text{rate} = V_{max} \frac{[S_{high}]}{[S_{high}] + K_m}$$

The terms also have analogous meanings. The "rate" is the initial rate of transport; V_{max} is the maximum rate of transport; and K_m is the concentration of solute at which the rate of transport is half-maximal. The accuracy of the analogy allows one to apply classical enzyme "thinking" to transporters.

C. These equations do not describe the behavior of channels, because solutes passing through channels do not bind to them in the same way a substrate binds to an enzyme (or a solute to a transporter).

11–11 The transporter would work well at the low concentrations, which are well below its K_m, rapidly equilibrating the concentrations across the membrane. At the higher concentrations, however, the transporter would not work well, at all. At such high concentrations, the transporter would always be saturated on both sides of the membrane and, hence, no net flux would occur: every time it transferred a solute outside, it would transfer one inside.

11–12 The equilibrium distribution of a molecule across a membrane depends on the chemical gradient (concentration) and on the electrical gradient (membrane potential). An uncharged molecule does not experience the electrical gradient and, thus, will be at equilibrium when it is at the same concentration on both sides of the membrane. A charged molecule responds to both components of the electrochemical gradient and will distribute accordingly. K^+ ions, for example, are nearly at their equilibrium distribution across the plasma membrane even though they are about 30-fold more concentrated inside the cell. The difference in concentration is balanced by the membrane potential (negative inside), which opposes the movement of cations to the outside of the cell.

CALCULATIONS

11–13

A. The rates of glucose uptake in brain and liver cells at various concentrations of glucose are shown in **Table 11–4**.

TABLE 11–4 Uptake of glucose as a percentage of V_{max} (Answer 11–13).

Glucose concentration (mM)	Rate/V_{max} = [S]/([S] + K_m)	
	Brain cells GLUT3 (K_m = 1.5 mM)	Liver cells GLUT2 (K_m = 15 mM)
3	67%	17%
5	77%	25%
7	82%	32%
15	91%	50%

B. At 15 mM glucose in the portal circulation, the liver transports glucose at 50% of the maximum rate.

C. These calculations fit with the physiological functions of brain and liver. Brain cells depend on glucose as their primary energy source and take it up at about the same rate over the normal range of circulating glucose concentrations. Only during starvation does the brain derive a significant fraction of its energy from another source, namely, ketone bodies. Thus, brain cells have to be able to take up glucose efficiently to meet their energy needs, and the low-K_m transporter, GLUT3, seems well matched to the brain's physiological role as a glucose consumer.

By contrast, the physiological role of the liver is more complex. The liver serves as the body's storehouse for glucose (in the form of glycogen), which is built up after a meal and then doled out between meals to meet the rest of the body's needs for glucose. The high-K_m transporter, GLUT2, is matched to these needs. It helps to ensure that at low circulating glucose concentration the liver does not compete with the rest of the body for glucose; its role is to supply glucose, not consume it, under these conditions. At higher glucose concentration, however, it can take up glucose to build its glycogen reserves. In liver, these physiological functions are enforced by hormone-induced changes to key regulatory enzymes in the pathways of glucose metabolism.

11–14

A. The concentration of circulating glucose, 5 mM, equals 5×10^{-3} mole/L, which is 5×10^{-3} mole/1000 cm^3. The surface area of a spherical cell 20 μm in diameter is 1.26×10^{-5} cm^2 [$4 \times 3.14 \times (10\ \mu m)^2 \times (cm/10^4\ \mu m)^2$]. Using these values, the rate of diffusion into a cell is

$$\text{rate} = \frac{5 \times 10^{-3}\ \text{mole}}{1000\ \text{cm}^3} \times 1.26 \times 10^{-5}\ \text{cm}^2 \times \frac{3 \times 10^{-8}\ \text{cm}}{\text{sec}} \times \frac{6 \times 10^{23}\ \text{molecules}}{\text{mole}}$$

$$\text{rate} = 1.1 \times 10^6\ \text{molecules/sec}$$

B. At 5 mM glucose, GLUT3 will transport glucose at 77% of its maximum rate {rate/V_{max} = [S]/([S] + K_m)}. Since the maximum rate is 10^4 molecules/sec, each GLUT3 will transport 7.7×10^3 molecules/sec. With 10^5 GLUT3 molecules per cell, glucose will enter the cell under these conditions at 7.7×10^8 molecules/sec. This rate is 700 times faster than simple diffusion ($7.7 \times 10^8 / 1.1 \times 10^6$).

DATA HANDLING

11–15 Cytochalasin B inhibits glucose transport competitively, suggesting that it binds at or near the site of D-glucose binding on GLUT1. If an excess of D-glucose is present, the binding site on GLUT1 will be occupied by D-glucose, preventing cytochalasin from binding and thereby interfering with cross-linking. On the other hand, L-glucose does not interfere with cross-linking because it does not bind to the transporter and protect it from the binding of cytochalasin.

Reference: Allard WJ & Lienhard GE (1985) Monoclonal antibodies to the glucose transporter from human erythrocytes: identification of the transporter as a M_r = 55,000 protein. *J. Biol. Chem.* 260, 8668–8675.

11–16

A. These data indicate that insulin stimulation of glucose uptake results from a redistribution of preexisting GLUT4 molecules from an internal pool to the plasma membrane. The 5-fold increase in the rate of glucose uptake in insulin-treated cells (see Figure 11–1) is accompanied by a 5-fold increase in the number of GLUT4 molecules in the plasma membrane (see Table 11–1). Furthermore, the increase in GLUT4 molecules

in the plasma membrane is accompanied by a corresponding decrease in the number of GLUT4 molecules present in the internal membrane fraction. Fat cells and muscle cells are unique in expressing the insulin-dependent glucose transporter GLUT4, which fits with their role in storage of energy after a meal (when insulin levels increase).

B. K_m and V_{max} do not change. The K_m for glucose transport in the untreated cells and in the insulin-treated cells is about 2 mM, which is the concentration of glucose under both conditions at which the rate of transport is half-maximal (see Figure 11–1). The 5-fold increase in the rate of glucose transport can be fully accounted for by the 5-fold increase in the number of GLUT4 molecules in the plasma membrane. It may seem confusing that this 5-fold increase in rate of transport does not mean that the V_{max} has increased; however, V_{max} refers to the maximum rate *for a specified quantity of enzyme*. When the observed experimental rates are adjusted for the 5-fold difference in number of transporters, the maximum rates of transport are identical; therefore, V_{max} has not changed.

Reference: Oka Y & Czech MP (1984) Photoaffinity labeling of insulin-sensitive hexose transporters in intact rat adipocytes: direct evidence that latent transporters become exposed to the extracellular space in response to insulin. *J. Biol. Chem.* 259, 8125–8133.

TRANSPORTERS AND ACTIVE MEMBRANE TRANSPORT

DEFINITIONS

11–17 ABC transporter

11–18 Multidrug resistance (MDR) protein

11–19 Antiporter

11–20 Transcellular transport

11–21 Symporter

TRUE/FALSE

11–22 False. A symporter binds two different solutes on the *same side* of the membrane. Turning it around would not change it into an antiporter, which must bind two different solutes on *opposite sides* of the membrane.

11–23 False. Primary active transport is mediated by carriers that are driven by ATP hydrolysis. In co-transport of Na^+ and a solute into a cell, the energy in the Na^+ gradient is used to drive uptake of the solute. The Na^+ that enters the cell is then pumped back out by an ATP-dependent Na^+ pump. Because the Na^+ gradient must be restored by ATP hydrolysis in a subsequent event, co-transport of Na^+ and a solute is termed secondary active transport.

11–24 False. The Ca^{2+}-pumps in the SR use the energy of ATP hydrolysis to move Ca^{2+} from the cytosol, where its concentration is low, into the SR lumen, where its concentration is high. The energy of ATP hydrolysis is required to move Ca^{2+} against its concentration gradient. When the plasma membrane of a muscle cell depolarizes, a Ca^{2+} channel in the SR opens, allowing Ca^{2+} to flow spontaneously down its concentration gradient into the cytosol, where it initiates muscle contraction.

THOUGHT PROBLEMS

11–25 Of the ions listed in Table 11–2, Na^+, K^+, Ca^{2+}, and Cl^- all have large enough

concentration differences inside and outside the cell to drive a coupled transport. Mg^{2+} and H^+ differ only slightly in their concentrations across the membrane and therefore would be unlikely candidates to drive coupled transport. Na^+, Ca^{2+}, and Cl^- would move inward, whereas K^+ would move outward. To maintain electrical neutrality, Na^+, K^+, and Ca^{2+} would symport negatively charged ions, or antiport positively charged ions. Cl^- would symport positively charged ions, or antiport negatively charged ions. To maintain neutrality, the numbers of charges transported in a single event would need to sum to zero.

A variety of negatively charged molecules are inside cells; indeed, most cellular constituents are negatively charged. These ions include HCO_3^-, HPO_4^{2-}, metabolites carrying phosphate groups (such as ATP) or carboxyl groups, and polyanions such as proteins, RNA, and DNA.

11–26

A. These properties define a symporter.

B. No additional properties need to be specified. The important feature that provides the coupling of the two solutes is that the protein cannot switch its conformation if only one of the two solutes is bound. Solute B, which is driving the transport of solute A, is in excess on the side of the membrane from which transport initiates and occupies its binding site most of the time. In this state, the carrier protein, prevented from switching its conformation, waits until a solute A molecule binds. With both binding sites occupied, the carrier protein switches conformation. Now exposed to the other side of the membrane, the binding site for solute B is mostly empty because there is little of it in the solution on this side of the membrane. Although the binding site for A is now more frequently occupied, the carrier can switch back only after solute A is unloaded as well.

C. An antiporter analogous to the symporter described in the problem could be defined using a similar set of rules:

1. It has two binding sites, one for solute A and one for solute B.
2. The protein can undergo a conformational change to switch between two states, with both binding sites exposed on one side of the membrane, or with both binding sites exposed on the other side of the membrane.
3. The protein can switch between the two conformational states only if one binding site is occupied, but cannot switch if both binding sites are occupied or if both binding sites are empty.

You will note that only the third rule, which governs the relationship between binding-site occupancy and conformational switching, differs from those for a symporter.

11–27 One model for incorporating ATP into the cycle of conformational changes necessary to drive glucose transport against its concentration gradient is shown in **Figure 11–16**. ATP donates a phosphate group to the transporter when—and only when—it has glucose bound on the inside face of the membrane. The binding of glucose signals to the kinase that the transporter is ready to be phosphorylated (step 1 → 2). The attachment of the phosphate would trigger an immediate conformational change, thereby capturing the glucose and exposing it to the outside

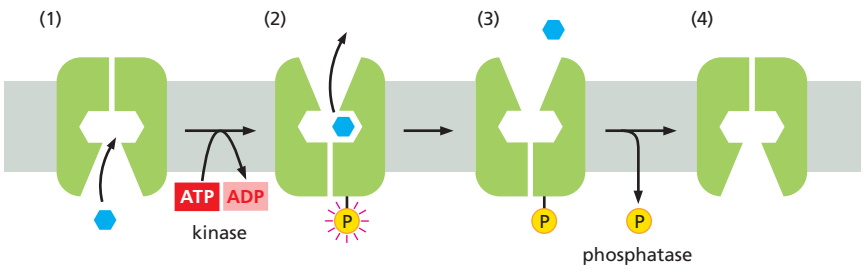


Figure 11–16 Coupling ATP hydrolysis to a hypothetical transporter to convert it from passive to active transport (Answer 11–27).

(step 2 → 3). The phosphate would be removed from the protein when—and only when—the solute has dissociated, and the now empty, non-phosphorylated transporter would switch back to the starting position (step 3 → 4).

11–28

A. Yes, they can normalize both the H^+ and Na^+ concentrations. For every three cycles of the Na^+–H^+ antiporter, which imports one Na^+ and exports one H^+, the Na^+-K^+ pump cycles once, exporting three Na^+ ions with each operation.

(You may have wondered how to deal with the hydrolysis of ATP that occurs with each cycle of the Na^+-K^+ pump. When ATP is hydrolyzed by H_2O, the products are ADP and $H_2PO_4^-$. $H_2PO_4^-$ has a pK of 6.86, which means that it is about 70% ionized into HPO_4^{2-} and H^+ at the intracellular pH of 7.2. It turns out that you don't need to worry about this H^+ because elsewhere in the cell other processes reconvert the products of hydrolysis back into ATP to maintain a steady-state concentration.)

B. The linked action of these two pumps moves $3H^+$ out for every $2K^+$ that are brought into the cell, thereby increasing both the internal K^+ concentration and the membrane potential.

11–29

A. In the absence of ATP no ions will be pumped.

B. The pumps will use ATP hydrolysis to transport Na^+ into the vesicles and K^+ out, generating oppositely oriented concentration gradients of Na^+ and K^+ across the membrane. The pump will stop when the ATP runs out or when the K^+ inside the vesicle is exhausted.

C. Under these conditions, the pump will proceed through the first three steps (see Figure 11–3) and then stop. Because the conformational changes are strictly sequential, the subsequent steps cannot occur and the pump will be stuck.

Similar experiments, leaving out individual ions and analyzing the consequences, were used to determine the sequence of steps by which the Na^+-K^+ pump works.

D. If the pumps were randomly oriented in the membrane, the result would be the same as in B. The pumps oriented with the ATP hydrolysis site exposed to the inside of the vesicle would be inactive because they would not have access to ATP.

CALCULATIONS

11–30 Based on the scale bars in Figure 11–4, each microvillus approximates a cylinder 0.1 μm in diameter and 1.0 μm in height. The ratio of the area of the sides of a cylinder, which represent new membrane (new surface area), to the top of a cylinder (which is equivalent to the plasma membrane that would have been present anyway, had the microvillus not been extruded) gives the increase in surface area due to an individual microvillus. The area of the sides of a cylinder ($2\pi rh$, where r is the radius and h is the height) is 0.31 μm^2 (2 × 3.14 × 0.05 μm × 1.0 μm); the area of the top of the cylinder (πr^2) is 0.0079 μm^2 [3.14 × $(0.05)^2$]. Thus, the increase in surface area for one microvillus is 0.31 μm^2/0.0079 μm^2 or 40. This value overestimates the increase for the entire plasma membrane, since the microvilli occupy only a portion of the surface. The fraction of plasma membrane occupied by microvilli can be estimated from the cross section in Figure 11–4. A conservative estimate is that about half the plasma membrane is covered by microvilli. Thus, microvilli increase the surface area in contact with the lumen of the gut by approximately 40/2 or 20-fold.

Reference: Krstić RV (1979) Ultrastructure of the Mammalian Cell, p. 207. Berlin, Germany: Springer-Verlag.

11–31

A. If the entire free-energy change due to ATP hydrolysis ($\Delta G = -50$ kJ/mole) could be used to drive transport, then the maximum concentration gradient that could be achieved by ATP hydrolysis would have a free-energy change of +50 kJ/mole.

$$\Delta G_{in} = -2.3RT \log \frac{C_o}{C_i} + zFV$$

Rearranging the equation gives

$$\log \frac{C_o}{C_i} = \frac{-\Delta G_{in} + zFV}{2.3RT}$$

For an uncharged solute, the electrical term (zFV) drops to zero. Thus,

$$\log \frac{C_o}{C_i} = \frac{-\Delta G_{in}}{2.3RT}$$

Substituting for ΔG_{in}, R, and T gives

$$\log \frac{C_o}{C_i} = \frac{-50 \text{ kJ/mole}}{2.3 \times (8.3 \times 10^{-3} \text{ kJ/K mole}) \times 310 \text{ K}}$$

$$\log \frac{C_o}{C_i} = -8.45$$

$$\log \frac{C_i}{C_o} = 8.45$$

$$\frac{C_i}{C_o} = 2.8 \times 10^8$$

Thus, for an uncharged solute, a transport system that couples the hydrolysis of 1 ATP to the transport of 1 solute molecule could, in principle, drive a concentration difference across the membrane of more than eight orders of magnitude. Amazing!

B. If the entire free-energy change due to ATP hydrolysis ($\Delta G = -50$ kJ/mole) could be used to drive transport of Ca^{2+} out of the cell, then the maximum concentration gradient would yield a free-energy change of +50 kJ/mole.

$$\Delta G_{out} = 2.3RT \log \frac{C_o}{C_i} - zFV$$

Rearranging the equation gives

$$\log \frac{C_o}{C_i} = \frac{\Delta G_{out} + zFV}{2.3RT}$$

Since Ca^{2+} is charged, the electrical term must be included. Substituting for ΔG_{out}, R, T, z, F, and V, gives

$$\log \frac{C_o}{C_i} = \frac{50 \text{ kJ/mole} + (2 \times 96 \text{ kJ/V mole} \times -0.06 \text{ V})}{2.3 \times (8.3 \times 10^{-3} \text{ kJ/K mole}) \times 310 \text{ K}}$$

$$\log \frac{C_o}{C_i} = 6.50$$

$$\frac{C_o}{C_i} = 3.2 \times 10^6$$

Thus, a transport system that couples the hydrolysis of 1 ATP to the transport of 1 Ca^{2+} ion to the outside of the cell could, in principle, drive a concentration difference across the membrane of more than six orders of magnitude. Note, by comparison with an uncharged solute, that pumping against the membrane potential reduces the theoretical limit by two orders of magnitude. The difference in Ca^{2+} concentration across a typical mammalian plasma membrane is more than four orders of magnitude, but well within the theoretical limit (see Table 11–2).

C. The free-energy change for transporting Na^+ out of the cell is

$$\Delta G_{out} = 2.3RT \log \frac{C_o}{C_i} - zFV$$

Substituting (with $2.3RT = 5.9$ kJ/mole),

$$\Delta G_{out} = \left(5.9 \frac{\text{kJ}}{\text{mole}} \times \log \frac{145 \text{ mM}}{10 \text{ mM}}\right) - \left(1 \times \frac{96 \text{ kJ}}{\text{V mole}} \times -0.06 \text{ V}\right)$$

$$\Delta G_{out} = 12.6 \text{ kJ/mole } Na^+$$

$$\Delta G_{out} = 37.8 \text{ kJ/3 mole } Na^+$$

The free-energy change for transporting K^+ into the cell is

$$\Delta G_{in} = -2.3RT \log \frac{C_o}{C_i} + zFV$$

Substituting (with $2.3RT = 5.9$ kJ/mole),

$$\Delta G_{in} = -\left(5.9 \frac{\text{kJ}}{\text{mole}} \times \log \frac{5 \text{ mM}}{140 \text{ mM}}\right) + \left(1 \times \frac{96 \text{ kJ}}{\text{V mole}} \times -0.06 \text{ V}\right)$$

$$\Delta G_{in} = 2.8 \text{ kJ/mole } K^+$$

$$\Delta G_{in} = 5.6 \text{ kJ/2 mole } K^+$$

The overall free-energy change for the Na^+-K^+ pump is

$$\Delta G = \Delta G_{out} + \Delta G_{in}$$
$$= 37.8 \text{ kJ/3 mole } Na^+ + 5.6 \text{ kJ/2 mole } K^+$$
$$= 43 \text{ kJ/(3 mole } Na^+ \text{ and 2 mole } K^+)$$

D. Since the hydrolysis of ATP provides 50 kJ/mole and the pump requires 43 kJ to transport 3 Na^+ out and 2 K^+ in, the efficiency of the Na^+-K^+ pump is

$$\text{eff} = \frac{43}{50}$$
$$= 86\%$$

Even with this remarkable efficiency, the Na^+-K^+ pump typically accounts for a third of a mammalian cell's energy requirements and thus, presumably, a corresponding fraction of a mammal's total caloric intake.

DATA HANDLING

11–32

A. The ester form of SNARF-1 readily diffuses across membranes because it is an uncharged, hydrophobic molecule. Once the blocking ester groups are removed, SNARF-1 acquires a negative charge due to a carboxyl group, whose proton dissociates at intracellular pH. The charge dramatically decreases its ability to diffuse through a membrane.

B. The two peaks of fluorescence arise because of the very different electronic structures of the acid (HA) and salt (A^-) forms of SNARF-1 (see Figure 11–6). The acid form has three distinct sets of conjugated bonds, whereas the resonance structure for the salt form (the dominant structure) has a fully conjugated set of double bonds. The change from isolated sets of double bonds to a fully conjugated system dramatically alters the molecule's fluorescence properties. At pH 6.0, the acid form predominates and thus its fluorescence dominates the emission spectrum. At pH 9.0, the salt form predominates and thus its fluorescence properties dominate the emission spectrum.

C. From the Henderson–Hasselbalch equation you can calculate that at pH 6.0, 97% of SNARF-1 will be in the acid form (HA) and 3% will be in the salt form (A^-). At pH 9.0, 97% of SNARF-1 will be in the salt form and 3% will be in the acid form. Because the pHs are equidistant from the p*K*, you really only need to do one calculation.

$$\text{pH} = \text{p}K + \log([\text{salt}]/[\text{acid}])$$

$$6.0 = 7.5 + \log([\text{salt}]/[\text{acid}])$$

$$\log([\text{salt}]/[\text{acid}]) = -1.5$$

$$([\text{salt}]/[\text{acid}]) = 0.032, \text{ or } [\text{salt}] = 0.032\,[\text{acid}]$$

Since the concentration of the salt form of SNARF-1 plus the concentration of the acid form must sum to 100% (the total SNARF-1 present),

$$[\text{salt}] + [\text{acid}] = 100\%$$

Substituting,

$$0.032\,[\text{acid}] + [\text{acid}] = 100\%$$

$$1.032\,[\text{acid}] = 100\%$$

$$[\text{acid}] = 96.9\% \text{ and thus } [\text{salt}] = 3.1\%$$

D. Emission spectra for SNARF-1 at several different wavelengths, including pH 7.2, are shown in **Figure 11–17**. At pH 7.2, just below the p*K*, about two-thirds of SNARF-1 will be in the acid (HA) form. Thus, you would expect the emission peak at 580 nm to be above the halfway point between the high value at pH 6.0 and the low value at pH 9.0. Likewise, at 640 nm, the emission peak should be below the halfway point between the high value at pH 9.0 and the low value at pH 6.0. The ratio of the intensities at the two wavelengths is a very sensitive measure of pH.

E. Two peaks, corresponding to the acid and salt forms, are better than either single peak because the two peaks act as internal controls for each other. Indicators with a single peak are subject to several troublesome artifacts, including fluorescence bleaching, cell thickness, instrument stability, and non-uniform loading into cells. To calculate the pH from the intensity of a single peak assumes that you know the total concentration of indicator in the cell. By using a ratio of two peaks, however, you avoid this requirement since the intensities at the two peaks give you the total concentration.

Reference: Molecular Probes Handbook (www.probes.com/handbook).

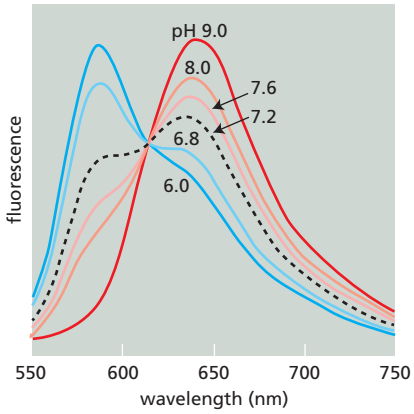


Figure 11–17 Emission spectra for SNARF-1 at a variety of pHs (Answer 11–32). The spectrum at pH 7.2 is shown as a *dashed line.*

MEDICAL LINKS

11–33

A. The intracellular pH of the red blood cell changes very little because the histidine group on hemoglobin buffers it effectively.

B. CO_2 enters the red cell in the tissues and is immediately converted to HCO_3^-, which is transported out of the cell by the Cl^-–HCO_3^- exchanger. Thus, CO_2 is carried from the tissues to the lungs as HCO_3^- in the plasma outside the red cell.

C. In both the tissues and the lungs, the Cl^-–HCO_3^- exchanger moves HCO_3^- down its concentration gradient. In the tissues, the hydration of CO_2 by carbonic anhydrase increases the intracellular concentration of HCO_3^-, allowing the exchanger to transport it out of the cell down its concentration gradient. In the lungs, the removal of CO_2 by exhalation lowers the CO_2 concentration in the cell, pulling the carbonic anhydrase reaction ($CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$) to the left. The resultant lowering of the HCO_3^- concentration allows the exchanger to transport HCO_3^- into the cell down its concentration gradient.

11–34 If the Na^+-K^+ pump is not working at full capacity because it is partially inhibited by ouabain or digitalis, it generates an electrochemical gradient of Na^+ that is less steep than normal. Consequently, the Ca^{2+}-Na^+ antiporter works less efficiently, and Ca^{2+} is removed from the cell more slowly. When the next cycle of muscle contraction begins, there is still an elevated level of Ca^{2+} in the cytosol. The entry of the same number of Ca^{2+} ions into the cell leads therefore to a higher Ca^{2+} concentration than in untreated cells, which in turn leads to a stronger and longer-lasting contraction. Because the Na^+-K^+ pump fulfills essential functions in all animal cells, both to maintain osmotic balance and to generate the Na^+ gradient used to power many transporters, the drugs are deadly poisons at higher concentrations.

CHANNELS AND THE ELECTRICAL PROPERTIES OF MEMBRANES

DEFINITIONS

11–35 Adaptation

11–36 Long-term potentiation (LTP)

11–37 Action potential

11–38 Nernst equation

11–39 Channelrhodopsin

11–40 Membrane potential

11–41 Voltage-gated cation channel

11–42 Selectivity filter

11–43 Myelin sheath

11–44 K^+ leak channel

11–45 Axon

11–46 Ion channel

11–47 Synapse

11–48 Neurotransmitter

11–49 Patch-clamp recording

TRUE/FALSE

11–50 False. Transporters *and* channels saturate. It is thought that permeating ions have to shed most of their associated water molecules in order to pass, in single file, through the narrowest part—the selectivity filter—of the channel. This requirement limits their rate of passage. Thus, as ion concentrations increase, the flux of ions through a channel increases proportionally, but then levels off (saturates) at a maximum rate.

11–51 True. It takes a difference of only a minute number of ions to set up the membrane potential.

11–52 False. Channels open in an all-or-nothing fashion. Thus, the aggregate current does not indicate the degree to which individual channels are open, but rather the total number of channels in the membrane that are open at any one time.

11–53 True. In the absence of a specific ligand, such ion channels will remain closed, preventing them from generating an action potential.

11–54 False. Voltage-gated Ca^{2+} channels and Ca^{2+}-release channels in the sarcoplasmic reticulum control the entry of Ca^{2+} into the cytoplasm when the plasma membrane is depolarized. The Ca^{2+}-pump, which requires ATP, is responsible for the reverse process: pumping cytosolic Ca^{2+} back up its concentration gradient into the sarcoplasmic reticulum.

THOUGHT PROBLEMS

11–55 Just as a falling body in air reaches a terminal velocity due to friction, an ion in water also reaches a terminal velocity due to friction with water molecules. An ion in water will accelerate for less than 10 nanoseconds before it reaches terminal velocity.

11–56 Ion channels are ion-selective and they are gated, whereas simple aqueous pores allow movement of many different ions and they are open all the time.

11–57

A. With equal concentrations of K^+ ions on both sides of the vesicle there will be no net movement of K^+ ions, although they will move in both directions. Also, no membrane potential will develop.
B. K^+ ions will move out of the vesicle through the K^+ leak channel down their concentration gradient. This movement will continue until the membrane potential (negative inside) exactly matches the concentration difference (higher K^+ inside).
C. K^+ ions will move into the vesicle through the K^+ leak channel down their concentration gradient. This movement will continue until the membrane potential (negative outside) exactly matches the concentration difference (higher K^+ outside). You will note that the K^+ leak channel works in the same way regardless of its orientation in the membrane; it always moves K^+ down its electrochemical gradient.

11–58 Red blood cells have water channels—aquaporins—that make them about 10-fold more permeable to water than a lipid bilayer. Frog eggs do not express aquaporins and thus their permeability to water is roughly that of a lipid bilayer. If there were only a 10-fold difference in permeability to water, wouldn't a frog egg still burst, but just take ten times as long to do so? A part of the answer lies in the enormous volume difference between a red cell and a frog egg, more specifically in the surface-to-volume ratio. Assuming that both are spheres, which of course red cells are not, the 10^6-fold difference in volume translates into a 100-fold lower surface-to-volume ratio in the egg. (This is an underestimate of the difference in the surface-to-volume ratio because the red cell biconcave disc has a larger surface-to-volume ratio than does a sphere of the same volume.) Thus, in the absence of aquaporins, the egg should take up water at more than a 1000-fold lower rate than a red cell. If aquaporins are engineered to be expressed into frog eggs, the eggs also swell and burst when placed in water.

The deeper question of why red blood cells express aquaporins remains unanswered. It has been argued that red blood cells need aquaporins to maintain hemoglobin concentration at 33 g/dL; however, humans with null mutations in the aquaporin gene have red blood cells that appear to be normal in every respect.

Reference: Abrami L, Simon M, Rousselet G, Berthonaud V, Buhler JM & Ripoche P (1994) Sequence and functional expression of an amphibian water channel, FA-CHIP: a new member of the MIP family. *Biochim. Biophys. Acta* 1192, 147–151.

Preston GM, Smith BL, Zeidel ML, Moulds JJ & Agre P (1994) Mutations in aquaporin-1 in phenotypically normal humans without functional CHIP water channels. *Science* 265, 1585–1587.

11–59 The narrow pore in aquaporins is lined with hydrophobic amino acids on one side and a string of carbonyl oxygens on the other, which forms a path that water molecules follow. The narrowness of the pore does not allow passage of hydrated ions of K^+, Na^+, Ca^+, and Cl^-, nor does the channel provide enough polar groups to balance the charge on the ions.

H^+ ions, which are present in cells as H_3O^+, offer a special challenge because they normally "move" through solution by relay along a chain of hydrogen-bonded water molecules. If such a chain of water molecules existed in the aquaporin pore, then H^+ ions would whiz through membranes unobstructed. Aquaporins prevent this eventuality by positioning two asparagines in the middle of the pore, thereby tying up both free valences of the central water molecule in the string (**Figure 11–18**). Without a free valence for hydrogen-binding, the central water molecule cannot participate in the relay of the H^+ ion.

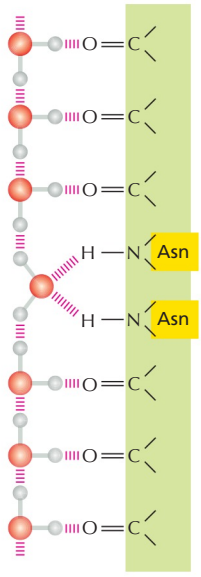


Figure 11–18 Interruption of H^+ relay by central asparagines in the aquaporin channel (Problem 11–59).

11–60 When the resting membrane potential of an axon drops below a threshold value, voltage-gated Na^+ channels in the immediate neighborhood open, allowing an influx of Na^+. This influx depolarizes the membrane further, causing more distant voltage-gated Na^+ channels to open as well. The resulting wave of depolarization—the action potential—spreads rapidly along the axon. Because Na^+ channels become inactivated soon after they open, the flow of K^+ through voltage-gated K^+ channels and K^+ leak channels quickly restores the original resting membrane potential after the action potential has passed. (89 words)

11–61 An H^+–acetylcholine antiporter in the vesicle membrane transports acetylcholine into the vesicles. The H^+ gradient that drives the uptake is generated by an ATP-driven H^+ pump in the vesicle membrane, which pumps H^+ into the vesicle (hence the dependence of the reaction on ATP). Raising the pH of the solution (lowering the H^+ concentration) increases the H^+ gradient. This mechanism explains the observed enhanced rate of acetylcholine uptake. Although this is the actual mechanism, other possibilities fit the data. For example, an H^+–acetylcholine antiporter could be coupled with an ATP-dependent acetylcholine pump.

11–62 Opening Na^+ channels allows an influx of Na^+ ions that depolarizes the membrane toward the threshold potential for firing an action potential. By contrast, opening either Cl^- or K^+ channels opposes membrane depolarization. Both Cl^- and K^+ ions are near their equilibrium distribution across the membrane: the resting membrane potential (negative inside) balances their concentration differences across the membrane (Cl^- high outside and K^+ high inside). As the membrane begins to depolarize (that is, as the membrane potential becomes more positive), both ions will tend to move down their concentration gradients (Cl^- ions into the cell, K^+ ions out of the cell). If a channel for either ion is opened, its movement across the membrane will make the inside of the cell more negative, tending to reestablish the original membrane potential and suppressing the firing of an action potential.

11–63 There is little net movement of K^+ because it is nearly at its equilibrium distribution; the membrane potential opposes movement out of the cell down its concentration gradient. By contrast, Na^+ is not at its equilibrium distribution; both the concentration difference and the membrane potential tend to push it into the cell. The same is true for Ca^{2+}; however, its external concentration is only about 1 mM versus about 145 mM for Na^+. Thus, when an acetylcholine-gated channel opens, Na^+ ions constitute the great majority of the cations that enter.

CALCULATIONS

11–64 The volume of the hemisphere explored by the ball is 2.05×10^4 nm^3 [$(2/3) \times 3.14 \times (21.4 \text{ nm})^3$]. This volume corresponds to 2.05×10^{-20} liters [$(2.05 \times 10^4 \text{ nm}^3) \times (\text{cm}/10^7 \text{ nm})^3 \times (\text{liter}/1000 \text{ cm}^3)$]. One ball in this volume corresponds to 8.13×10^{-5} M or 81.3 μM [$(1 \text{ molecule}/2.05 \times 10^{-20} \text{ liters}) \times (\text{mole}/6 \times 10^{23} \text{ molecules})$]. Thus, the local concentration of a tethered ball is about the same as the concentration of free peptide needed to inactivate the channel.

Reference: Zagotta WN, Hoshi T & Aldrich RW (1990) Restoration of inactivation in mutants of *shaker* potassium channels by a peptide derived from ShB. *Science* 250, 568–571.

11–65 The voltage gradient across the membrane is 156,000 V/cm [$(70 \times 10^{-3} \text{ V})/(4.5 \times 10^{-7} \text{ cm})$]. This extremely powerful electric field is close to the limit at which insulating materials—such as the lipid bilayer—break down and cease to act as insulators. The large field corresponds to the large amount of energy that can be stored in electrical gradients across the membrane, as well as to the extreme electrical forces that proteins can experience in a membrane. A voltage of this magnitude would instantly discharge in an arc across a 1-cm wide gap (that is, air would be an insufficient insulator for this strength of field).

11–66

A. The expected membrane potential due to differences in K^+ concentration across the resting membrane is

$$V = 58 \text{ mV} \times \log \frac{C_o}{C_i}$$

$$V = 58 \text{ mV} \times \log \frac{9 \text{ mM}}{344 \text{ mM}}$$

$$V = -92 \text{ mV}$$

For Na^+, the equivalent calculation gives a value of +48 mV.

The assumption that the membrane potential is due solely to K^+ leads to a value near that of the resting potential. The assumption that the membrane potential is due solely to Na^+ leads to a value near that of the action potential.

These assumptions approximate the resting potential and action potential because K^+ *is* primarily responsible for the resting potential and Na^+ *is* responsible for the action potential. A resting membrane is 100-fold more permeable to K^+ than it is to Na^+ because of the presence of K^+ leak channels. The leak channel allows K^+ to leave the cell until the membrane potential rises sufficiently to oppose the K^+ concentration gradient. The theoretical maximum gradient (based on calculations like those above) is lowered somewhat by the entrance of Na^+, which carries positive charge into the cell (compensating for the positive charges on the exiting K^+). Were it not for the Na^+-K^+ pump, which continually removes Na^+, the resting membrane potential would be dissipated completely.

The action potential is due to a different channel, a voltage-gated Na^+ channel. These channels open when the membrane is stimulated, allowing Na^+ ions to enter the cell. The magnitude of the resulting membrane potential is limited by the difference in the Na^+ concentrations across the membrane. The influx of Na^+ reverses the membrane potential locally, which opens adjacent Na^+ channels and ultimately causes an action potential to propagate away from the site of the original stimulation.

B. The substitution of choline chloride for sodium chloride eliminates the action potential, as expected, since the action potential is due to specific Na^+ channels. As illustrated in the calculation above, the difference in concentrations of Na^+ across a membrane determines the magnitude of

the action potential that results from Na^+ influx. Thus, if the external Na^+ concentration were reduced to half or one-quarter the normal value, the calculated membrane potentials would be reduced to 30 mV and 13 mV, respectively. Measurements of the action potential for various mixtures of choline chloride and sodium chloride match these expectations.

Reference: Hille B (1992) Ionic Channels of Excitable Membranes, 2nd ed., pp. 23–58. Sunderland, MA: Sinauer.

11–67

A. During a single action potential, 340 cpm of radioactive Na^+ entered the axon. At a specific activity of 2×10^{14} cpm/mole, this incorporation corresponds to 1.7×10^{-12} moles of entering Na^+.

$$\text{entering } Na^+ = \frac{340 \text{ cpm}}{\text{axon}} \times \frac{\text{mole}}{2 \times 10^{-14} \text{ cpm}}$$

$$= 1.7 \times 10^{-12} \text{ mole/axon}$$

The area of membrane for the giant axon used in the experiment is 1.6 cm^2.

$$\text{area} = 2\pi rh = 2 \times 3.14 \times 0.05 \text{ cm} \times 5 \text{ cm}$$
$$= 1.6 \text{ cm}^2$$

Thus, the amount of Na^+ entering per square centimeter is

$$\text{entering } Na^+/\text{cm}^2 = \frac{1.7 \times 10^{-12} \text{ mole}}{\text{axon}} \times \frac{\text{axon}}{1.6 \text{ cm}^2}$$

$$= 1.1 \times 10^{-12} \text{ mole/cm}^2$$

Thus, the experimental measurement matches the theoretical calculation.

B. The amount of K^+ that must leave the axon to reestablish the resting potential is exactly the same as the amount of Na^+ that entered during the action potential. Thus, 1.7×10^{-12} moles of K^+ must leave the cell.

C. The volume of this giant axon is 39 μL.

$$\text{volume} = \pi r^2 h = 3.14 \times (0.05 \text{ cm})^2 \times 5 \text{ cm}$$
$$= 0.039 \text{ cm}^3 = 0.039 \text{ mL}$$
$$= 39 \text{ μL}$$

At a Na^+ concentration of 65 mM, the axon contains 1.5×10^{18} Na^+ ions.

$$\text{number of } Na^+ = \frac{65 \text{ mmol } Na^+}{\text{L}} \times \frac{39 \times 10^{-6} \text{ L}}{\text{axon}} \times \frac{6.0 \times 10^{20} \text{ } Na^+}{\text{mmol } Na^+}$$

$$= 1.5 \times 10^{18} \text{ } Na^+/\text{axon}$$

During an action potential, 1×10^{12} Na^+ ions enter the axon.

$$\text{entering } Na^+ = \frac{1.7 \times 10^{-12} \text{ mole}}{\text{axon}} \times \frac{6.0 \times 10^{23} \text{ } Na^+}{\text{mole}}$$

$$= 1.0 \times 10^{12} \text{ } Na^+/\text{axon}$$

Thus, a single action potential in a squid giant axon raises the internal Na^+ concentration by less than one part per million.

$$\text{rise in } [Na^+] = \frac{1.0 \times 10^{12}}{1.5 \times 10^{18}}$$

$$= 6.7 \times 10^{-7}$$

D. One approach to this question is to recalculate the necessary values after substituting for the radius of the dendrite (0.05 μm). However, there is a shortcut, which depends on realizing that for nerves of equal length, the

number of entering Na^+ ions depends on r (surface area = $2\pi rh$), whereas the number of Na^+ ions in the axon depends on r^2 (volume = $\pi r^2 h$). Since the radius of the dendrite is $1/10^4$ that of the giant axon, its surface area is also $1/10^4$ that of the giant axon; thus, 10^4 fewer Na^+ ions (1.0×10^8) will enter the dendrite during an action potential. The volume of the dendrite is $1/10^8$ that of the giant axon and thus contains $1/10^8$ the number of Na^+ ions (1.5×10^{10}). Consequently, the fractional increase in internal Na^+ ion concentration in the dendrite (6.7×10^{-3}) is 10^4-fold higher than in the squid axon [$(1.0 \times 10^8)/(1.5 \times 10^{10})$].

E. Since the internal Na^+ concentration in a dendrite increases by nearly 1% with each action potential (as compared to 0.0001% for a giant axon), the removal of Na^+ ions by the Na^+-K^+ pump is much more crucial to the continuing performance of a small dendrite than it is to that of the squid giant axon.

Reference: Hille B (1992) Ionic Channels of Excitable Membranes, 2nd ed., pp. 1–20. Sunderland, MA: Sinauer.

11–68

A. There are two kinds of cation channel in the rat muscle membrane—a 4-pA channel and a 6-pA channel. You can tell that there are two different channels by the characteristic amount of current they carry. Note that the 6-pA channel cannot be confused with the simultaneous opening of two 4-pA channels, which would give a current of 8 pA.

B. The number of Na^+ ions flowing through the 4-pA channel each millisecond is 2.5×10^4. The 6-pA channel carries 1.5 times as many (3.8×10^4 Na^+ ions) each millisecond.

$$\frac{Na^+}{msec} = 4\ pA \times \frac{A}{10^{12}\ pA} \times \frac{C/sec}{A} \times \frac{1\ Na^+}{1.6 \times 10^{-19}\ C} \times \frac{sec}{10^3\ msec}$$

$$= 2.5 \times 10^4$$

Reference: Sakmann B (1992) Elementary steps in synaptic transmission revealed by currents through single ion channels. *Science* 256, 503–512.

DATA HANDLING

11–69

A. K^+ channels composed of toxin-resistant subunits without balls will open and stay open in the presence or absence of toxin (**Figure 11–19A**).

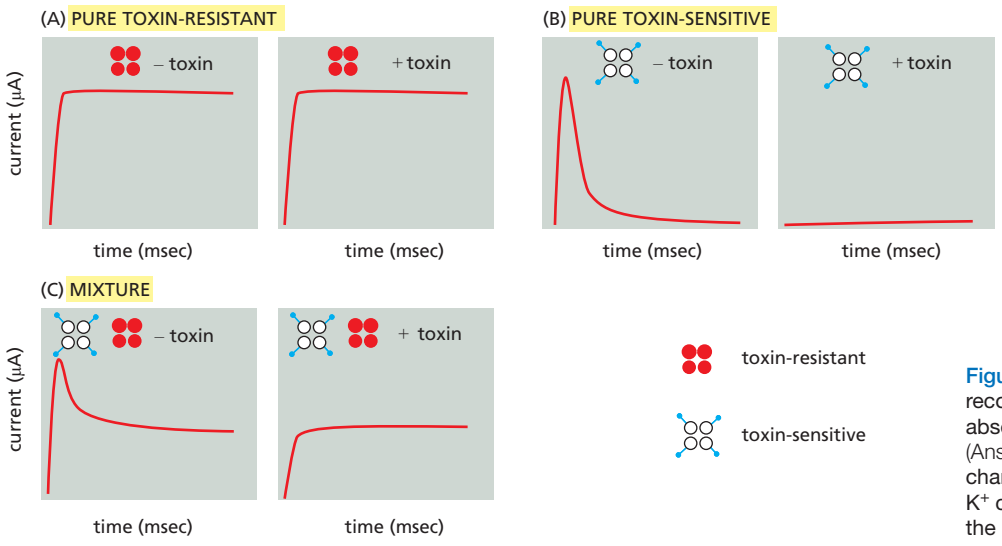


Figure 11–19 Expected patch-clamp recordings for mutant K^+ channels in the absence and presence of scorpion toxin (Answer 11–69). (A) Toxin-resistant K^+ channels without balls. (B) Toxin-sensitive K^+ channels with balls. (C) A 50:50 mix of the K^+ channels in A and B.

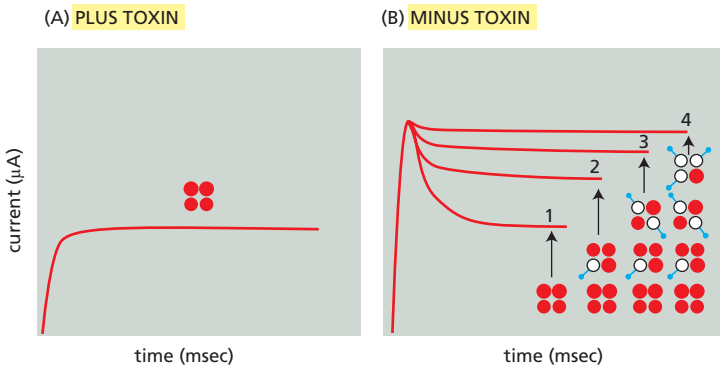


Figure 11–20 Results in the presence of toxin compared to those expected in the absence of toxin if 1, 2, 3, or 4 balls were required to close a channel (Answer 11–69). Pictures illustrate the types of channels that would remain open if 1, 2, 3, or 4 balls were required.

B. K^+ channels composed of toxin-sensitive subunits with balls will open and then inactivate normally in the absence of toxin, but they will not open at all in the presence of toxin (Figure 11–19B).

C. A 50:50 mixture of K^+ channels composed of toxin-resistant subunits without balls or toxin-sensitive subunits with balls will give a mixture of the curves shown in Figure 11–19A and B. In the absence of toxin, all the channels will open; the 50% with balls will close and the 50% without balls will remain open (Figure 11–19C). In the presence of toxin, the 50% composed of toxin-sensitive subunits will not open at all; the 50% composed of toxin-resistant subunits without balls will open and stay open (Figure 11–19C).

D. The patch-clamp recording in Figure 11–12B indicates that a single ball is sufficient to close a channel. The reasoning is as follows. When the mixture of K^+ channels in the oocyte membrane was subjected to membrane depolarization in the absence of toxin, all the channels opened and then those without a sufficient number of balls remained opened to give a plateau current. Thus, the plateau current in the absence of toxin is due to all forms of the channel that cannot close—but those forms are undefined. In the presence of toxin, only those channels composed of four toxin-resistant subunits can open and those stay open because they have no balls. Since the plateau current in the presence of toxin, which is entirely due to channels without balls, exactly equals the plateau current in the absence of toxin, channels with a single ball must be able to close. If a channel required two, three, or four balls to close, the plateau current in the absence of toxin would be higher than in the presence of toxin (Figure 11–20).

Reference: MacKinnon R, Aldrich RW & Lee AW (1993) Functional stoichiometry of *shaker* potassium channel inactivation. *Science* 262, 757–759.

11–70 Each of the rectangular peaks corresponds to the opening of a single channel, which allows a small current to pass. Individual channels in the patch of membrane open and close frequently, remaining open for a very short, somewhat variable time, averaging about 5 milliseconds. When open, the channels allow a small current with a unique amplitude (4 pA) to pass. In two instances, the current doubles, indicating that two channels in the same membrane patch were open at the same time.

If acetylcholine were omitted or added to the solution outside the micropipette, no peaks of current would be seen: only the baseline would be observed. Acetylcholine must bind to the extracellular portion of the acetylcholine receptor to allow the channels to open. In the membrane patch in Figure 11–13A, the binding sites for acetylcholine are exposed only to the solution in the micropipette.

11–71

A. Nonsaturation of toxin binding indicates that it is binding to something else in the preparation in addition to the target receptor. These additional sites are not specific for the toxin or they would eventually saturate, which they did not. The point of the control experiment is to estimate the degree of nonspecific binding. In this case, the excess of nonradioactive toxin swamps out binding of the labeled toxin to the specific target sites. In the presence of a large molar excess of unlabeled toxin, most of the toxin that is bound to the receptor will be unlabeled; that is, the unlabeled toxin *competes* with the labeled toxin for specific binding. By contrast, the number of nonspecific sites is virtually unlimited; thus, the unlabeled toxin does not interfere with nonspecific binding by the labeled toxin. The unlabeled toxin does not compete with the labeled toxin for the nonspecific sites. Thus, in the presence of unlabeled toxin, the nerves behave toward the labeled ligand as though they had no Na^+ channels. As a result, the radioactive toxin binds only nonspecifically. By subtracting the nonspecific component from the total binding, a specific binding curve can be generated; it shows saturation as expected (**Figure 11–21**).

B. The corrected binding curve indicates that the amount of specific binding is 100 pmol of toxin per gram of nerve. The 100 pmol corresponds to 6.0×10^{13} molecules of toxin.

$$\text{bound toxin} = 100 \text{ pmol} \times \frac{6.0 \times 10^{23} \text{ molecules}}{\text{mole}} \times \frac{\text{mole}}{10^{12} \text{ pmol}}$$

$$= 6.0 \times 10^{13} \text{ molecules}$$

Assuming one toxin-binding site per channel, there are also 6.0×10^{13} Na^+ channels per gram of nerve. Since 1 gram of nerve contains 6000 cm^2 of membrane, there are 1.0×10^{10} channels per square centimeter of membrane, which corresponds to 100 Na^+ channels per square micrometer (1 cm = 10^4 μm).

With a radius of 3 nm, each channel has a cross-sectional area of 28 nm^2 (area = πr^2). Thus, for each 1 μm^2 of membrane, the Na^+ channels cover a total area of 2.8×10^3 nm^2 (100×28 nm^2). Since there are 10^6 nm^2 in 1 μm^2, the Na^+ channels occupy about 0.3% of the membrane. This result emphasizes how small a fraction of the cell membrane needs to be occupied by gated ion channels in order to display excitability.

Reference: Hille B (1992) Ionic Channels of Excitable Membranes, 2nd ed., pp. 315–336. Sunderland, MA: Sinauer.

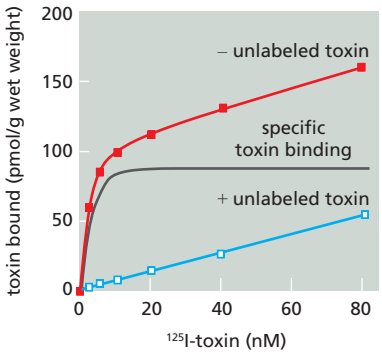


Figure 11–21 Specific binding curve for saxitoxin (Answer 11–71).

MEDICAL LINKS

11–72 When acetylcholine is released from the synaptic vesicles of neurons, some of the acetylcholine finds target receptors, some diffuses away, but most is rapidly hydrolyzed to acetate and choline, which are taken up by the nerve terminal. When the density of receptors is reduced by reaction with antibodies, the probability diminishes that an acetylcholine molecule will find its receptor before it is hydrolyzed. The suboptimal transmission of the signal is responsible for the muscular weakness in myasthenic patients. One way to overcome their muscular weakness is to increase the concentration of acetylcholine to compensate for the reduced number of receptors. By inhibiting acetylcholinesterase, neostigmine increases the effective concentration of acetylcholine, thereby increasing the efficiency of signal transmission across the synapse.

11–73 Each of the diverse subtypes of these channels is expressed in a narrow set of neurons. A narrow range of expression of individual subtypes gives rise to the hope that drugs can be discovered or designed to affect

specific subtypes in a selected set of neurons, thus influencing particular brain functions specifically.

MCAT STYLE

11–74

A. Acetylcholine triggers muscle contraction by opening a ligand-gated channel at synapses, leading to Na^+ influx and depolarization of the muscle cell. Thus, scopolamine could block depolarization by blocking the opening of the acetylcholine-gated Na^+ channel. Choice B is not correct because Ca^{2+} channels in the sarcoplasmic reticulum open in *response* to membrane depolarization, releasing Ca^{2+} into the cytosol, which triggers muscle contraction. If scopolamine blocked the sarcoplasmic reticulum Ca^+ channels, acetylcholine-induced membrane depolarization would still occur in the presence of scopolamine. Choice C is not correct because the sodium enters cells during an action potential via channel proteins, not transporters. Choice D is not correct because voltage-gated potassium channels play a role in reversing the effects of an action potential; they are not involved in triggering the action potential.

11–75

D. Figure 11–15 shows that the α-toxin-treated membrane remains depolarized for a much longer time than normal, which could be explained by a delay in closing the sodium channel. Choice A is incorrect because premature opening of voltage-gated K^+ channels would return the membrane potential to normal more quickly rather than more slowly. Choice B could accelerate the opening of the sodium channel, but would not be expected to prolong depolarization of the membrane. Choice C is not correct because ligand-gated Na^+ channels, specifically acetylcholine-gated Na^+ channels, initiate action potentials, but are not involved in propagating them.

11–76

D. The active ingredient in extracts of *Stropthantus gratus* is oubain, which inhibits the Na^+-K^+ pump. Inhibiting the pump causes the concentration of sodium within the cell to rise gradually, upsetting the osmotic balance and allowing water to enter the cell until it swells and bursts. Choice A is not correct because closing potassium leak channels would interfere only with the cell's ability to establish the resting membrane potential. Choices B and C are not correct because the concentrations of sodium and glucose are higher outside the cell than inside. Thus, shutting down movement of these molecules would not alter the osmotic balance and would not cause the cells to burst.

11–77

C. A robust Cl^- current across the membrane suggests that CFTR behaves as an ion channel. The other members of the family of ABC transporters act as normal transporters; they bind to the transported molecule and undergo complex conformational changes to move the molecule across a membrane. These conformational changes limit the rate at which they can transport molecules. Thus, they could not generate a robust ion current across the membrane. Choice A is incorrect because ion channels, CFTR included, do not bind their ions; they provide a preferred path through the membrane. Choices B and D describe normal behaviors expected for members of the ABC transporter family.

11–78

A. The co-transport of glucose and Na^+ into the intestinal cells increases the osmolarity inside the cells, which drives the uptake of water and reverses the effects of the cholera toxin. Choice B is not correct because the Na^+-K^+ pump pumps Na^+ out of the cell. Choice C is not correct because the

rehydration solution is about the same osmolarity as inside the cells. Choice D is incorrect because this mild solution of salt and glucose will have no effect at all on *Vibrio cholerae*.

Intracellular Compartments and Protein Sorting

CHAPTER 12

IN THIS CHAPTER

THE COMPARTMENTALIZATION OF CELLS

DEFINITIONS

12–1 Cytoplasm

12–2 Signal patch

12–3 Cytosol

12–4 Gated transport

12–5 Organelle

12–6 Signal sequence

TRUE/FALSE

12–7 False. Lipid bilayers by themselves are impermeable to hydrophilic molecules, but biological membranes, which contain proteins in addition to the bilayer, are not. Cellular membranes contain various transport proteins that make them selectively permeable, allowing certain small molecules and particular proteins to cross. It is this selective permeability that establishes the unique chemical identity of each compartment.

12–8 False. The interior of the nucleus and the cytosol communicate through the nuclear pore complexes, which allow free passage of ions and small molecules. The cytoplasm and the nucleus are said to be topologically equivalent because the outer and inner nuclear membranes are continuous with one another, so that the flow of material between the nucleus and cytosol occurs without crossing a lipid bilayer. By contrast, the lumen of the ER and the outside of the cell are each separated from the cytosol by a layer of membrane and thus are topologically distinct from the cytosol, but they are topologically equivalent to each other.

12–9 True. Ribosomes all begin translating mRNAs in the cytosol. The mRNAs for certain proteins encode a signal sequence for the ER membrane. After this sequence has been synthesized, it directs the nascent protein, along with the ribosome and the mRNA, to the ER membrane. Ribosomes translating mRNAs that do not encode such a sequence remain free in the cytosol.

12–10 True. Stretches of amino acids, typically 15–60 residues long, serve as sorting signals for most proteins in the cell. Signal sequences that specify particular cellular destinations—import into ER, import into nucleus, etc.—have characteristic features that allow their interaction with appropriate sorting receptors, which guide the proteins to their correct compartment.

THOUGHT PROBLEMS

12–11 In terms of its functional importance to a cell, the plasma membrane is anything but minor. It is the boundary that separates the cell from the outside world, it controls selective entry and exit of molecules, and it is the principal site at which intercellular communications are received. Only in terms of its surface area and mass is it a minor component, accounting for 2–5% of all the membranes in a eukaryotic cell.

12–12 While the vast majority of cells in the human body do have a complete set of membrane-enclosed organelles, certain specialized cells do not. A prime example is the red blood cell. At a late stage in its development, the precursor of the red blood cell—the reticulocyte—jettisons all of its internal membrane-enclosed organelles, leaving just the plasma-membrane-enclosed cytosol. The cells that make up the lens of the eye, which lack mitochondria, are similar. But in a way, these are exceptions that prove the rule; these cells are derived from cells that do carry the complete set of membrane-enclosed organelles.

12–13 Cells do not synthesize the Golgi apparatus from scratch after the drugs that caused its fragmentation are removed. The Golgi apparatus reassembles itself from the dispersed vesicles. Once the microtubule skeleton of the cell has been reestablished, the vesicles apparently use it to track back to their normal location in the cell, where they fuse with one another to re-form the Golgi apparatus. This same process of fragmentation and reassembly occurs in normal cells at each cell division, when the cytoskeleton breaks down in preparation for mitosis.

12–14 Eukaryotic gene expression is more complicated than prokaryotic gene expression. In particular, prokaryotic cells do not have introns that interrupt the coding sequences of their genes, so that an mRNA can be translated immediately after it is transcribed, without further processing. In fact, in prokaryotic cells, ribosomes start translating most mRNAs before transcription is finished. This would have disastrous consequences in eukaryotic cells, because most RNA transcripts have to be spliced before they can be translated. The nuclear envelope separates the transcription and translation processes in space and time. A primary RNA transcript is held in the nucleus until it is properly processed to form an mRNA, and only then is it allowed to leave the nucleus so that ribosomes can translate it.

12–15 In the absence of a sorting signal, a protein will remain in the cytosol.

12–16 One way to approach this problem is to compare the relative volumes of the compartments that are served by cytosolic and ER protein synthesis. Assuming that the average density and lifetimes of proteins are about the same in all compartments—a reasonable first approximation—their relative volumes would provide a rough estimate of the amount of protein synthesis. The compartments served by cytosolic protein synthesis, which include the cytosol, nucleus, mitochondria, and peroxisomes, account for more than 80% of the cell volume. The compartments that depend on ER protein synthesis—the ER, Golgi apparatus, endosomes, and lysosomes—account for less than 20% of the cell volume. On this basis, then, one would conclude that cytosolic protein synthesis is responsible for the majority of cellular protein synthesis.

In cells that do not secrete large amounts of protein, the majority of protein synthesis is likely to occur in the cytosol. One of the main functions of liver cells, however, is to export proteins such as albumin, which makes up about half of the total serum protein. The fraction of liver ribosomes engaged in synthesizing albumin is probably less than 10%. At this

level, a liver cell would still be carrying out the majority of its protein synthesis on cytosolic ribosomes, but in other, specialized secretory cells (like those of the pancreas), ER protein synthesis may exceed cytosolic protein synthesis.

12–17 The nucleus is the only compartment that receives its proteins by gated transport, which is carried out by nuclear pore complexes. The ER, mitochondria, and peroxisomes all receive their proteins by transmembrane transport, which is mediated by specific protein translocators that reside in the membrane of the target organelle. The Golgi apparatus, secretory vesicles, early and late endosomes, and lysosomes all obtain their proteins via vesicular transport, in which small vesicles ferry proteins from one compartment to another.

12–18

A. The protein would enter the ER. The signal for import into the ER is located at the N-terminus of the protein and functions before the internal signal for nuclear import is synthesized. Once the protein entered the ER, the signal sequence for nuclear import could not function because it would be prevented from interacting with cytosolic nuclear import receptors.
B. The protein would enter the ER. Once again, the N-terminal signal for ER import would function before the internal signal for peroxisome import is synthesized. The peroxisome import signal could not function once the protein was sequestered in the ER.
C. The protein would enter the mitochondria. In order to be retained in the ER, the protein must first be imported into the ER. Without a signal for ER import, the ER retention signal could not function.
D. A protein with signals for both nuclear import and nuclear export would shuttle between the cytosol and the nucleus. Unlike the other pairs of signals, these signals are not necessarily in conflict. A number of cellular proteins, whose function requires shuttling in and out of the nucleus, are designed in just this way.

12–19 A taxi is the closest analogy. Anyone who has the fare—the sorting signal—is taken on the journey. A private car implies a specific relationship between the traveler (the sorted protein) and the vehicle (the sorting receptor); namely, that there are specific sorting receptors for each different kind of protein. In reality, all the proteins destined for the ER, for example, use the same sorting receptor. A bus would imply that travelers—sorted proteins—are carried in groups. Sorting receptors handle proteins one at a time.

CALCULATIONS

12–20 Ten billion average proteins would occupy a volume of 589 μm^3:

$$V = 10^{10}\ \text{proteins} \times \frac{450\ \text{aa}}{\text{protein}} \times \frac{110\ \text{d}}{\text{aa}} \times \frac{\text{g}}{6 \times 10^{23}\ \text{d}} \times \frac{\text{cm}^3}{1.4\ \text{g}} \times \frac{(10^4\ \mu\text{m})^3}{(\text{cm})^3}$$

$$V = 589\ \mu\text{m}^3$$

where aa is amino acid.

This value represents about 12% of the volume of a liver cell (589/5000) and about 59% of the volume of a pancreatic exocrine cell (589/1000). Thus, 10 billion protein molecules would fit in a typical animal cell.

This calculation obviously gives only the crudest of estimates. The number of protein molecules expressed in a cell is not constant. On average, smaller cells would be expected to make fewer proteins than larger cells; thus, the volume occupied by proteins would be roughly constant. Some specialized cells might be very different. For example, a large

droplet of triglycerides takes up most of the volume of a fat cell, which therefore has a lower fraction of its total cell volume occupied by protein (although if one excludes the volume of the triglyceride droplet from the calculation, a fat cell is probably not different from other cells). The values calculated here bracket the accepted value of about 20%.

12–21 The lipid bilayers in all the membranes in liver and pancreatic exocrine cells would have a volume of 330 μm^3 and 39 μm^3, respectively, as calculated below for a liver cell:

$$V = 110{,}000\ \mu m^2 \times 5\ nm \times \frac{\mu m}{10^3\ nm} \times 0.60$$

$$V = 330\ \mu m^3$$

Thus, lipid bilayers would account for 6.6% (330/5000) of the volume of a liver cell, and 3.9% (39/1000) of the volume of a pancreatic exocrine cell. For typical cells, then, about 5% of their volume is occupied by lipid bilayers.

12–22

A. If the equivalent of one plasma membrane transits the ER every 24 hours and individual membrane proteins remain in the ER for 30 minutes (0.5 hr), then at any one time, 0.021 (0.5 hr/24 hr) plasma membrane equivalents are present in the ER. Since the area of the ER membrane is 20 times greater than the area of the plasma membrane, the fraction of plasma membrane proteins in the ER is 0.021/20 = 0.001. Thus, the ratio of plasma membrane proteins to other membrane proteins in the ER is 1 to 1000. Out of every 1000 proteins in the ER membrane, only 1 is in transit to the plasma membrane.

B. In a cell that is dividing once per day, the equivalent of one Golgi apparatus also must transit the ER every 24 hours. If the membrane of the Golgi apparatus is three times the area of the plasma membrane, three times as many Golgi apparatus membrane proteins will be present in the ER. Therefore, the ratio of Golgi apparatus membrane proteins to other membrane proteins in the ER is 3 to 1000.

C. If the areas of the membranes of all the rest of the compartments are equal to the area of the plasma membrane, then the ratio of membrane proteins bound for these compartments to the membrane proteins in the ER is 1 to 1000. Summing the contributions from all compartments, the ratio of membrane proteins, in transit, to proteins that are permanent residents of the ER membrane is 5 to 1000. Thus, 99.5% of the membrane proteins in the ER are permanent residents.

As this problem illustrates, the sorting of proteins to various cellular membranes represents a substantial purification from the mix of proteins in the ER.

DATA HANDLING

12–23

A. The negative charge nearest to the transmembrane segment is the more important. In the presence of a normal-length hydrophobic segment, neither N-terminal negative charge is essential for membrane insertion, as shown by the results with constructs 1 and 2. Proteins with shortened hydrophobic segments, however, depend on the negative charges for proper insertion, as shown by comparison of constructs 4 through 7. Construct 5 with the single negative charge adjacent to the hydrophobic segment is inserted nearly as efficiently as construct 4, which has both negative charges. By contrast, construct 6 with the single negative charge near the N-terminus is not inserted at all.

B. In the presence of the membrane potential (minus CCCP), the hydrophobic segment is more important than the N-terminal negative charge.

Construct 2, which has neither negative charge, is inserted as efficiently as construct 1, which has both negative charges. In addition, when both negative charges are present, the amount of inserted protein decreases as the length of the hydrophobic segment is reduced (compare constructs 1, 3, and 4).

C. In the absence of the membrane potential (plus CCCP), the hydrophobic segment is still the most important determinant of insertion efficiency: construct 2 with no negative charges is inserted as efficiently as construct 1. As the hydrophobic helix is made shorter, insertion comes to depend much more on the presence of the negative charges. Construct 3 is only about half as efficient at insertion when CCCP is added, and construct 4, with a slightly shorter hydrophobic sequence, is absolutely reliant on the membrane potential for insertion.

Thus, in the absence of a membrane potential—presumably the case in the earliest cells—a sufficiently long hydrophobic segment may have been adequate to accomplish insertion of a protein into a membrane. In the presence of a membrane potential, a second feature—the distribution of charges around a transmembrane segment—would have been available for translocator-independent insertion of membrane proteins.

Reference: Delgado-Partin VM & Dalbey RE (1998) The proton motive force, acting on acidic residues, promotes translocation of amino-terminal domains of membrane proteins when the hydrophobicity of the translocation signal is low. *J. Biol. Chem.* 273, 9927–9934.

THE TRANSPORT OF MOLECULES BETWEEN THE NUCLEUS AND THE CYTOSOL

DEFINITIONS

12–24 Nuclear export signal

12–25 Nuclear pore complex (NPC)

12–26 Ran

12–27 Nuclear lamina

12–28 Nuclear import receptor

12–29 Nuclear localization signal

12–30 Outer nuclear membrane

TRUE/FALSE

12–31 True. The nuclear membrane allows free passage of ions and small molecules because it is perforated with numerous nuclear pore complexes—3000–4000 in a typical mammalian cell—each of which has one or more open aqueous channels through which small water-soluble molecules can passively diffuse.

12–32 False. Individual nuclear pores mediate transport in both directions. It is unclear how pores coordinate this two-way traffic so as to avoid head-on collisions.

12–33 True. Gene regulatory proteins in particular are subject to this kind of regulation, as a way of preventing gene activation (or repression) until the proper time.

12–34 False. Resident proteins of the cytosol do not have nuclear export signals. They are efficiently excluded from reassembling nuclei by the mechanism

of reassembly. The nuclear envelope is initially closely applied to the surface of the chromosomes, excluding all proteins except those bound to the mitotic chromosomes. Once the envelope is complete, other residents of the nucleus are imported via their nuclear localization signals.

THOUGHT PROBLEMS

12–35 Two aspects of protein function may contribute to the difference in the protein compositions of the inner and outer nuclear membranes. First, proteins that function in the inner membrane are usually anchored by their interactions with components of the nucleus such as chromosomes and the nuclear lamina, which is a protein meshwork underlying the inner nuclear membrane. Freely diffusing proteins that are anchored once they reach the inner membrane would accumulate there. Second, proteins that form the nuclear pore itself may restrict the free diffusion of other membrane proteins by virtue of their insertion into the lipid bilayer at the boundary between the inner and outer membranes. Any membrane protein that cannot pass through the ring of nuclear pore proteins would be restricted to the outer membrane.

12–36 A single nuclear pore complex can transport proteins with quite different kinds of nuclear localization signal because transport is mediated by a variety of nuclear import receptors that are encoded by a family of related genes. Each family member—each gene product—is specialized for transport of a group of nuclear proteins that share structurally similar nuclear localization signals. At the same time, all family members share common features that allow them to interact with nuclear pore complexes. Thus, nuclear import receptors act as adaptors between proteins with diverse nuclear localization signals and the uniform population of nuclear pore complexes.

12–37 Proteins with a nuclear export signal also have a nuclear localization signal. Thus, such proteins typically shuttle between the nucleus and cytoplasm.

12–38

A. In a heterokaryon with two nuclei, one expressing the GFP-tagged protein and the other not, it is possible to decide whether the protein is a true nuclear protein or a shuttling protein. If it is a nuclear protein, the GFP should remain associated with a single nucleus. If it is a shuttling protein, the GFP will redistribute to both nuclei.

B. It is critical in this experiment to block synthesis of new GFP-tagged protein. If new protein were made, it would enter both nuclei, regardless of whether it was a nuclear protein or a shuttling protein.

12–39 At each mitosis, the contents of the nucleus and the cytosol mix when the nuclear envelope disassembles. When the nucleus reassembles, the nuclear proteins must be selectively re-imported. If the nuclear localization signals were removed upon import, the proteins would be trapped in the cytosol after the next mitosis. By contrast, the contents of other organelles never mix with the cytosol. At mitosis, organelles such as the Golgi apparatus and the ER break up into vesicles, which retain the luminal contents of their larger parents. Because of this, their resident proteins have to be imported only once, and their signal sequences are therefore dispensable.

12–40

A. Fluorescent NES-BSA does not accumulate in the nucleus in these experiments because no gradient of Ran-GTP can be established. Since RanQ69L does not hydrolyze its bound GTP, it will ultimately be present in the same concentration on both sides of the nuclear membrane. Once

the concentration of NES-BSA in the nucleus becomes equal to that outside the nucleus, RanQ69L-GTP/ Crm1 will transport NES-BSA at equal rates in both directions.

B. In the standard nuclear import assay, proteins enter the nucleus and cannot readily escape. Because of its high concentration inside the nucleus, Ran-GTP binds to the nuclear import receptors and displaces the transport substrate. Ran-GTP then accompanies the import receptor back outside the nucleus, where a Ran-GAP (Ran-GTPase-activating protein) rapidly converts Ran-GTP to Ran-GDP. Ran-GDP is then specifically transported back into the nucleus, where it is rapidly converted to Ran-GTP by a Ran-GEF (Ran-guanine exchange factor). Maintenance of nuclear Ran in its Ran-GTP form ensures that no import receptors will be available to carry the transport substrate back out of the nucleus, thereby allowing essentially 100% of the transport substrate to accumulate in the nucleus.

Reference: Nachury MV & Weis K (1999) The direction of transport through the nuclear pore can be inverted. *Proc. Natl Acad. Sci. USA* 96, 9622–9627.

CALCULATIONS

12–41 In this experiment, NES-BSA was imported at a rate of 0.25 molecules/pore/sec. The number of molecules of NES-BSA in a nucleus after 60 seconds is 4.5×10^4.

$$\frac{\text{molecules}}{\text{nucleus}} = \frac{0.15\ \mu\text{mol}}{\text{L}} \times \frac{500 \times 10^{-15}\ \text{L}}{\text{nucleus}} \times \frac{6 \times 10^{17}\ \text{molecules}}{\mu\text{mol}}$$

$$= 4.5 \times 10^4\ \text{NES-BSA/nucleus}$$

Since this number of molecules entered through 3000 pores in 60 seconds, the rate of entry was 0.25 molecules/pore/sec [$(4.5 \times 10^4$ NES-BSA/nucleus) × (1 nucleus/3000 pores) × (1/60 seconds)]. This rate is well within the physiological range.

Reference: Nachury MV & Weis K (1999) The direction of transport through the nuclear pore can be inverted. *Proc. Natl Acad. Sci. USA* 96, 9622–9627.

12–42 The volume of a spherical protein 26 nm in diameter is 9200 nm^3 [$(4/3) \times 3.14 \times (13\ nm)^3 = 9198\ nm^3$]. The molecular mass of a protein with this volume is 7.7×10^6 g/mole.

$$\text{molecular mass} = \frac{9200\ \text{nm}^3}{\text{molecule}} \times \frac{6 \times 10^{23}\ \text{molecules}}{\text{mole}} \times \frac{\text{cm}^3}{10^{21}\ \text{nm}^3} \times \frac{1.4\ \text{g}}{\text{cm}^3}$$

$$= 7.7 \times 10^6\ \text{g/mole (equivalent to about 70,000 amino acids of average molecular mass, 110 g/mole)}$$

As this calculation indicates, a nuclear pore can dilate to accommodate very large proteins.

12–43 Each nuclear pore complex must transport about 1 histone molecule per second, on average, throughout a day:

$$\text{transport} = \frac{32 \times 10^6\ \text{octamers}}{\text{day}} \times \frac{8\ \text{histones}}{\text{octamer}} \times \frac{\text{day}}{8.64 \times 10^4\ \text{sec}} \times \frac{1}{3000\ \text{pores}}$$

$$= 0.99\ \text{histones/second/pore}$$

Because histones are synthesized and imported into nuclei only during S phase, which is typically about 8 hours long, the transport rate is about 3 histones per second per pore during S phase (and none during the rest of the cell cycle).

12–44

A. The concentration of FG-repeats in yeast nuclear pores is about 287 mM, almost 6 times higher than the concentration used *in vitro*. Thus, the concentration inside the pore is certainly sufficient to allow gel formation. The volume of a yeast nuclear pore ($v = \pi r^2 h$) is 28.8×10^3 nm^3 [$3.14 \times (35 \text{ nm}/2)^2 (30 \text{ nm}) = 28{,}849$ nm^3]; 5000 FG-repeats in this volume corresponds to a concentration of 287 mM.

$$\text{concentration} = \frac{5000 \text{ FG}}{28.8 \times 10^3 \text{ nm}^3} \times \frac{\text{mmole}}{6 \times 10^{20} \text{ FG}} \times \frac{10^{21} \text{ nm}^3}{\text{cm}^3} \times \frac{1000 \text{ cm}^3}{\text{L}}$$

$$= \frac{289 \text{ mmol}}{\text{L}} = 289 \text{ mM}$$

B. With a diffusion coefficient of 0.1 μm^2/sec, it would take importin-MBP-GFP about 4.5 msec to traverse a yeast nuclear pore.

$$t = \frac{x^2}{2D}$$

$$= (30 \text{ nm})^2 \times \frac{\text{sec}}{2(0.1 \text{ μm}^2)} \times \frac{\text{μm}^2}{10^6 \text{ nm}^2} \times \frac{10^3 \text{ msec}}{\text{sec}}$$

$$= 4.5 \text{ msec}$$

This rate of diffusion appears fast enough to meet biological needs and matches reasonably well with the rates of 5 to 10 msec measured for the import of various proteins through nuclear pores.

References: Frey S & Görlich D (2007) A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes. *Cell* 130, 512–523.

Frey S & Görlich D (2009) FG/FxFG as well as GLFG repeats form a selective permeability barrier with self-healing properties *EMBO J.* 28, 2554–2567.

DATA HANDLING

12–45

A. The portion of nucleoplasmin responsible for localization to the nucleus must reside in the tail. The nucleoplasmin head does not localize to the nucleus when injected into the cytoplasm, and it is the only injected component that is missing a tail.

B. These experiments suggest that the nucleoplasmin tail carries a nuclear localization signal and that accumulation in the nucleus is not the result of passive diffusion. The observations involving complete nucleoplasmin or fragments that retain the tail do not distinguish between passive diffusion and active import; they say only that the tail carries the important part of nucleoplasmin—be it a localization signal or a binding site. The key observations that argue against passive diffusion are the results with the nucleoplasmin heads. They do not diffuse into the cytoplasm when they are injected into the nucleus, nor do they diffuse into the nucleus when injected into the cytoplasm, suggesting that the heads are too large to pass through the nuclear pores. Since the more massive forms of nucleoplasmin with tails do pass through the nuclear pores, passive diffusion of nucleoplasmin is ruled out.

Reference: Dingwall C, Sharnick SV & Laskey RA (1982) A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* 30, 449–458.

12–46

A. The rationale for the experiment is that the restriction nuclease EcoRI will cleave the cell's DNA into pieces if it gains access to the nucleus,

thereby killing the cell. In glucose-containing medium, the hybrid gene is not transcribed and the fusion protein is not made; thus, both types of yeast proliferate perfectly well. In the presence of galactose, the hybrid gene is expressed. In yeasts carrying the pNL^- plasmid, the hybrid protein, although expressed, cannot enter the nucleus because it lacks an NLS and thus does no harm to the cell. By contrast, in yeasts carrying the pNL^+ plasmid, the hybrid protein, which has a functional NLS, enters the nucleus and cuts up the cell's DNA; thus, the cells die.

B. The pNL^+ plasmid possesses the basic features needed for a selection assay to isolate cells defective in nuclear transport. In the presence of galactose, yeast cells will express the hybrid protein. A normal cell will transport the protein into the nucleus where it will kill the cell by cleaving its DNA. By contrast, in a mutant cell defective for nuclear transport, the killer protein will be confined to the cytosol, where it can do no harm. Thus, in a culture of yeast cells carrying the plasmid, individual cells defective for nuclear transport would be expected to survive transfer to a galactose-containing medium.

Actually building a working selection assay requires dealing with several additional considerations. For example, a cell that is permanently defective for nuclear transport would not be viable. Therefore, the mutants would need to be conditionally lethal; that is, with functional nuclear transport at low temperature, for instance, so you can grow the cells, and defective transport at high temperature, where you apply selection. After sufficient exposure to the hybrid protein to kill normal cells, the culture could then be shifted to a glucose medium at low temperature to permit the mutant cells to grow.

An additional complication is that the killer protein, which was made at the high temperature but denied access to the nucleus in the translocation mutants, will still be present when the cells are shifted to low temperature. When nuclear translocation resumes at the low temperature, the killer protein will be imported and the mutant cells will die. Thus, you would need to modify the assay so that the previously made killer protein is rendered inactive before nuclear import is allowed to resume. There are many possible ways to accomplish this. For example, you might try leaving the cells at high temperature in the presence of glucose (so there is no new synthesis) for increasing periods of time to allow the previously made killer protein to be inactivated by normal degradation processes. You might also try to increase its rate of degradation by engineering its N-terminus so that it carries a destabilizing amino acid (see Problem 6–87). Such a modification could turn the killer protein into a very short-acting molecule, which would disappear very rapidly in the absence of new synthesis. Alternatively, you might try to make a mutant of EcoRI that is active at the high temperature and inactive at the low temperature. Such a *cold-sensitive* protein would be active when nuclear transport was blocked in the import mutant and inactive when nuclear transport resumed.

Reference: Barnes G & Rine J (1985) Regulated expression of endonuclease EcoRI in *Saccharomyces cerevisiae*: nuclear entry and biological consequences. *Proc. Natl Acad. Sci. USA* 82, 1354–1358.

12–47

A. Figure 12–7A shows that in the absence of GTP, but in the presence of importin, labeled substrate accumulates at the nuclear periphery. A reasonable inference is that importin facilitates the binding of the substrate to the nuclear pore complexes and that this binding is independent of GTP.

B. These data, especially the two-step incubation in Figure 12–7B, suggest that importin first binds the substrate to the nuclear periphery (presumably the nuclear pore complexes). Bound substrate is then acted on by

Ran-GTP to promote uptake into the nucleus. These data do not define how Ran-GTP promotes uptake. Additional experiments have led to the current view that Ran-GTP in the nucleus binds to importin, displacing the substrate and thereby accomplishing the final step of nuclear uptake: release into the nucleus. Ran-GTP then normally accompanies the receptor out of the nucleus, where it is converted by a Ran-GAP to Ran-GDP.

References: Görlich D, Prehn S, Laskey RA & Hartmann E (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* 79, 767–778.

Moore MS & Blobel G (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* 365, 661–663.

Moore MS & Blobel G (1994) Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl Acad. Sci. USA* 91, 10212–10216.

12–48

A. Ran is a GTPase and will slowly convert GTP to GDP. Thus, if you had prepared Ran-GTP to start with, by the time you did the experiments you would have had an undefined mixture of Ran-GTP and Ran-GDP, which would have confused the results. By using a form of Ran that cannot hydrolyze GTP, you guaranteed that Ran was in its Ran-GTP conformation. Either RanQ69L-GTP, as was used here, or Ran-GppNp would have served equally well in the experiments described in this problem.
B. Since the Ran-GDP column removed the nuclear import factor, whereas the RanQ69L-GTP column did not, you are looking for a protein that is present in lane 1 but not in lane 2 (see Figure 12–9). One such protein is present, between the 7 kd and 14 kd markers. Note that the RanQ69L-GTP column binds a set of proteins between the 97 kd and 116 kd markers that the Ran-GDP column does not. They are members of the importin family of nuclear import receptors, which are evidently not required for the nuclear uptake of Ran-GDP.
C. The small protein—the presumptive import factor—that binds to Ran-GDP is known as NTF2. In addition to binding tightly to Ran-GDP, NTF2 binds to the FG-repeats present in the nucleoporins of the nuclear pore complex. It is the progressive movement of the NTF2–Ran-GDP complex through the FG-repeat gel in the nuclear pore that allows Ran-GDP to be delivered to the nucleus. In the nucleus, the Ran-GEF converts Ran-GDP to Ran-GTP, causing it to dissociate from NTF2. NTF2 then recycles to the cytoplasm to bring in another Ran-GDP.
D. The information in the problem says only that cytoplasm passed over a Ran-GDP column is depleted of some factor that is essential for nuclear uptake, and the experimental results shown in Figure 12–9 indicate that the small protein later identified as NTF2 binds to Ran-GDP. The inference is that NTF2 is the critical factor necessary for nuclear uptake of Ran-GDP. To prove that NTF2 is the import factor, you would need to show that purified or recombinant NTF2 can promote uptake of Ran-GDP into nuclei. The authors of this study went even further. Using information from the crystal structure of the NTF2–Ran-GDP complex, they mutated the glutamate at position 42 in NTF2 to lysine, thereby disrupting a key salt bridge between the two proteins. This NTF2E42K mutant no longer promoted nuclear uptake of Ran-GDP. These additional experiments demonstrate that NTF2 is necessary for nuclear uptake of Ran-GDP.

Reference: Ribbeck K, Lipowsky G, Kent HM, Stewart M & Görlich D (1998) NTF2 mediates nuclear import of Ran. *EMBO J.* 17, 6587–6598.

12–49 These results are more or less what you would expect if leptomycin B blocked nuclear export. In the absence of leptomycin B, NES-GFP is

excluded from the nuclei, as shown by the dark areas that correspond to the positions of the nuclei in the DNA panels (see Figure 12–10). This result indicates that NES-GFP is efficiently exported from nuclei. (Of course, it could also mean that NES-GFP never entered the nuclei in the first place.) The same result is observed in leptomycin B-resistant cells in the presence of leptomycin B, as expected if it is without effect in the mutant cells. The presence of NES-GFP in the nuclei of wild-type cells treated with leptomycin B confirms that NES-GFP can enter the nucleus and that leptomycin B prevents its export. The presence of NES-GFP in the cytoplasm, as well, indicates either that NES-GFP doesn't enter the nucleus very well or that leptomycin B doesn't completely block nuclear export.

Reference: Kudo N, Matsumori N, Taoka H, Fujiwara D, Schreiner EP, Wolff B, Yoshida M & Horinouchi S (1999) Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc. Natl Acad. Sci. USA* 96, 9112–9117.

12–50

A. Your technique is excellent, as judged by the lack of labeled RNA in the cytoplasm immediately after injection (see Figure 12–11). Thus, you must have hit the nucleus and not done irreparable harm to it.
B. Both mRNA and tRNA are fully exported from the nucleus, whereas U1 snRNA and U5 snRNA are only partially exported. U6 snRNA is retained within the nucleus.
C. Leptomycin B decreases the export of U1 snRNA and blocks the export of U5 snRNA from the nucleus, but has no effect on mRNA or tRNA export. Thus, mRNA and tRNA must be exported by a mechanism that is not affected by leptomycin B. This result implies that there are multiple export pathways (multiple nuclear export receptors) and that leptomycin B affects only one or a subset of them.

Reference: Fornerod M, Ohno M, Yoshida M & Mattaj IW (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 90, 1051–1060.

THE TRANSPORT OF PROTEINS INTO MITOCHONDRIA AND CHLOROPLASTS

DEFINITIONS

12–51 Mitochondria

12–52 Mitochondrial hsp70

12–53 TOM complex

12–54 Stroma

12–55 Inner membrane

12–56 Matrix space

12–57 Thylakoid

12–58 Mitochondrial precursor protein

TRUE/FALSE

12–59 True. Regardless of their final destination in the mitochondrion, all proteins that are synthesized in the cytosol (that is, all nucleus-encoded mitochondrial proteins) must first enter the TOM complex. After the

TOM complex, the pathways of import diverge as proteins are sorted to their appropriate mitochondrial compartment.

12–60 False. Only one of the two signal sequences is cleaved. The N-terminal signal is cleaved off the imported protein when it reaches the mitochondrial matrix. The second signal—a very hydrophobic sequence at the new N-terminus—directs the protein to the inner membrane through either the TIM23 complex or the OXA complex. The second signal is not cleaved; it anchors the protein in the inner membrane.

12–61 False. Although import of proteins is similar, the components of the import machinery in chloroplasts and mitochondria are not related. The functional similarities appear to have arisen by convergent evolution, reflecting the common requirements for translocation across a double-membrane system.

THOUGHT PROBLEMS

12–62 Import of mitochondrial proteins occurs post-translationally. Normally, translation is much faster than mitochondrial import, so that proteins completely clear the ribosome before interacting with the mitochondrial membrane. By blocking protein synthesis with cycloheximide, you have made the rate of translation artificially slower than the rate of import. Since the signal peptide for protein import into mitochondria resides at the N-terminus, some of the partially synthesized mitochondrial proteins, which are still attached to ribosomes, will be able to interact with the mitochondrial membrane. The attempted import of even one such protein will attach the ribosome and the mRNA (and all other ribosomes translating the same mRNA molecule) to the mitochondrial membrane.

Reference: Kellems RE, Allison VF & Butow RA (1975) Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. *J. Cell Biol.* 65, 1–14.

12–63 Peptides with mitochondrial import signals would be expected to compete with mitochondrial proteins for binding to the translocation machinery. Thus, an excess of such peptides should reduce or abolish import of mitochondrial proteins.

12–64 Normal cells that carry the modified *Ura3* gene make Ura3 that gets imported into mitochondria. It is therefore unavailable to carry out an essential reaction in the metabolic pathway for uracil synthesis. These cells might as well not have the enzyme at all, and they will grow only when uracil is supplied in the medium. By contrast, in cells that are defective for mitochondrial import, Ura3 is prevented from entering mitochondria and remains in the cytosol where it can function normally in the pathway for uracil synthesis. Thus, cells with defects in import into the mitochondrial matrix can grow in the absence of added uracil because they can make their own.

Reference: Maarse AC, Blom J, Grivell LA & Meijer M (1992) MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. *EMBO J.* 11, 3619–3628.

12–65 Although it is true that mitochondria normally generate the majority of the cell's ATP, they are not the sole source. Two steps in the glycolytic pathway for glucose catabolism, which occurs in the cytosol, also generate ATP. If glucose is provided, yeast (and many other cells) can survive

on the ATP they generate by glucose metabolism. It is this process that allows cells to survive in the absence of oxygen, which is required for ATP production by mitochondria. In the absence of oxygen, or when mitochondria are defective, the end product of glucose metabolism in yeast is ethanol.

12–66 Incubate the radiolabeled proteins with isolated mitochondria under conditions you wish to test, allow a sufficient time for import, and then treat the mixture with a protease. Proteins that are not imported will be digested by the protease. Proteins that have been imported will be resistant to the protease. Protease-resistant proteins could be assayed by re-isolating the mitochondria and measuring the counts associated with them. Alternatively, they could be assayed by solubilizing the entire mixture and separating the proteins by gel electrophoresis. Protease-resistant proteins would run at the same position as untreated proteins.

These analyses assume that proteins are protease-resistant because they are sequestered inside mitochondria, meaning they have been imported. You would need to include several controls before you could make this conclusion. You would need to know that the protease is working, which could be measured by leaving the mitochondria out of the incubation mixture. You would need to know that the protein is stable in the absence of the protease, which you could assay by leaving the protease out of the incubation mixture. You would need to know that protease-resistant proteins are in the mitochondria, which could be assayed by solubilizing the mitochondria with a detergent to show that protease-resistant proteins now become protease-sensitive. Appropriate controls are essential for informative research into any biological problem.

12–67 The binding of methotrexate to the active site prevents the enzyme from unfolding, which is necessary for import into mitochondria. Evidently, methotrexate binds so tightly that it locks the enzyme into its folded conformation and prevents chaperone proteins from unfolding it.

Reference: Eilers M & Schatz G (1986) Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* 322, 228–232.

12–68 The pores formed by porins are large enough for all ions and metabolic intermediates, but not large enough for most proteins. The size cutoff for free passage through the pores of mitochondrial porins is roughly 10 kilodaltons.

CALCULATIONS

12–69 At a rate of 3 amino acids per second, a single TOM complex would import 5.9×10^{-15} mg of protein per generation [(3 amino acids/sec) × (3600 sec/hr) × (3 hr/generation) × (110 d/amino acid) × (mg/6×10^{20} d)]. In 1 mg of mitochondrial protein there are 10 pmol or 6×10^{12} TOM complexes [(10 pmol) × (mole/10^{12} pmol) × (6×10^{23} TOM complexes/mole)]. Thus, 10 pmol of TOM complexes could co-translationally import 0.035 mg of protein per generation [(6×10^{12}) × (5.9×10^{-15})]. This is nearly a factor of 30 less than the required amount. Even adjusting for the doubling of TOM complexes that would occur each generation, co-translational import of mitochondrial proteins is much too slow to account for the bulk of mitochondrial protein import.

Reference: Neupert W (1997) Protein import into mitochondria. *Annu. Rev. Biochem.* 66, 863–917.

DATA HANDLING

12–70 Since each modified barnase includes an import signal and the length of the N-terminal extension does not affect the stability of the barnase domain, the dependence of import on the length of the extension presumably reflects some process inside mitochondria. The most likely possibility is that only the longer extensions can span both mitochondrial membranes and project into the matrix. There they can be bound by the mitochondrial hsp70, which can use the hydrolysis of ATP to help drive import. Presumably, the 95-amino-acid extension is long enough to be efficiently engaged by hsp70, whereas the 65-amino-acid extension must be less efficiently bound. Hsp70 and the energy of ATP hydrolysis are required for import of barnase because of its extremely stable folded structure. If the protein is first denatured, all three N-terminal extensions can facilitate its import at the same high rate because the unfolded protein does not hinder entry into the matrix.

Reference: Huang S, Ratliff KS, Schwartz MP, Spenner JM & Matouschek A (1999) Mitochondria unfold precursor proteins by unraveling them from their N-termini. *Nat. Struct. Biol.* 6, 1132–1138.

12–71 If barnase could be imported in its native folded configuration, the cross-links should have no effect on import; however, import of N65-barnase was blocked by the C5–C78 cross-link. On the other hand, if import required a completely unfolded, extended polypeptide chain, the presence of either cross-link should have blocked import of both N65-barnase and N95-barnase. Thus, it seems that the mitochondrial import machinery cannot import completely folded structures, but it doesn't require that the protein be in a fully extended configuration either. The ability of the import machinery to accommodate cross-links indicates that it can pass at least two, side-by-side polypeptide chains.

The authors of the original study point out that a key event in mitochondrial import is destabilization of the N-terminus of the imported protein. Thus, import of N65-barnase, which occurs at a low rate, is completely blocked when the N-terminus is stabilized by a cross-link. They also show that while the N95 extension attached to dihydrofolate reductase (DHFR) allows efficient import, import of N95-DHFR is blocked by methotrexate (see Problem 12–67).

Reference: Huang S, Ratliff KS, Schwartz MP, Spenner JM & Matouschek A (1999) Mitochondria unfold precursor proteins by unraveling them from their N-termini. *Nat. Struct. Biol.* 6, 1132–1138.

12–72

A. Tim23 appears to be an integral component of both mitochondrial membranes. In intact mitochondria, a small portion of Tim23 is digested by the protease, indicating that a segment of Tim23 is exposed outside mitochondria. This result implies that a portion of Tim23 extends through the outer mitochondrial membrane. The digested segment of Tim23 must be at the N-terminus because the remaining portion is still recognized by antibodies specific for the C-terminus (see Figure 12–13B, lane 2). In mitoplasts, a larger N-terminal segment of Tim23 is digested by the protease but the C-terminal portion is still protected, indicating that it is in the inner membrane or inside the mitoplasts (see Figure 12–13B, lane 3). In combination, these results indicate that Tim23 must extend through both mitochondrial membranes.

B. The pattern of protease sensitivity of Tim23 in mitochondria and mitoplasts suggests that Tim23 is arranged as shown in **Figure 12–24**.

You may have noticed that the hydropathy plot does not predict a membrane-spanning segment at the N-terminus. The authors of the

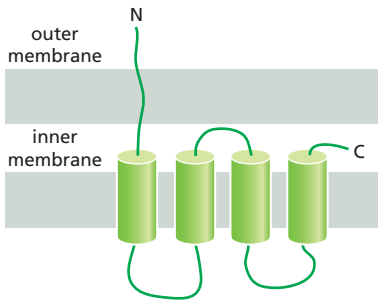


Figure 12–24 Arrangement of Tim23 in the inner and outer mitochondrial membranes (Answer 12–72).

original study noticed the same thing. There is, however, a predicted propensity for β-sheet formation in the N-terminus (not shown). Thus, the authors suggest that Tim23 may span the outer membrane in a β-strand conformation, which is typical of certain outer membrane proteins such as porins.

References: Donzeau M, Káldi K, Adam A, Paschen S, Wanner G, Guiard B, Bauer MF, Neupert W & Brunner M (2000) Tim23 links the inner and outer mitochondrial membranes. *Cell* 101, 401–412.

Paschen SA, Rothbauer U, Káldi K, Bauer MF, Neupert W & Brunner M (2000) The role of the TIM8-13 complex in the import of Tim23 into mitochondria. *EMBO J.* 19, 6392–6400.

PEROXISOMES

DEFINITIONS

12–73 Peroxisome

12–74 Peroxin

TRUE/FALSE

12–75 False. All eukaryotic cells contain peroxisomes.

THOUGHT PROBLEMS

12–76 Catalase is located in the cytosol of peroxisome-deficient cells, as shown by the uniform staining outside the nuclei in Figure 12–14B. Catalase appears as small dots (punctate staining) in normal cells because it is located in peroxisomes, which are small and distributed throughout the cytosol.

Reference: Kinoshita N, Ghaedi K, Shimozawa N, Wanders RJA, Matsuzono Y, Imanaka T, Okumoto K, Suzuki Y, Kondo N & Fujiki Y (1998) Newly identified Chinese hamster ovary cell mutants are defective in biogenesis of peroxisomal membrane vesicles (peroxisomal ghosts), representing a novel complementation group in mammals. *J. Biol. Chem.* 273, 24122–24130.

12–77 If a population of cells was grown in the presence of P9OH and irradiated with UV light, most of the normal cells would be killed. Cells that are defective for peroxisome assembly should be preferentially enriched among the survivors of such a treatment. Screening the survivors for peroxisome functions would identify those that are missing their peroxisomes.

Reference: Morand OH, Allen LA, Zoeller RA & Raetz CR (1990) A rapid selection for animal cell mutants with defective peroxisomes. *Biochim. Biophys. Acta* 1034, 132–141.

DATA HANDLING

12–78

A. Translation of mRNA from normal cells and each of the mutant cell lines yielded equal amounts of the 75 kd form of acyl CoA oxidase, but none of the 53 kd form. Since the 53 kd form is present only in the normal cells, it is likely that it arises from the 75 kd form during the process of import into peroxisomes. This reasoning suggests that the 53 kd form is the active

form of the enzyme. The observation that the mutant cells, which have no acyl CoA oxidase activity, also have none of the 53 kd form supports this conclusion.

B. The mutant cells have only the 75 kd form of the enzyme because their defective peroxisomes cannot import and process it to the active 53 kd form. Because the 75 kd form disappears so quickly in the pulse-chase experiments in the mutant cells (without giving rise to the 53 kd form), the 75 kd form must be unstable in the cytosol and rapidly degraded. A similar experiment performed with catalase would be expected to show no degradation since catalase activity is normal in the mutant cells.

Reference: Tsukamoto T, Yokota S & Fujiki Y (1990) Isolation and characterization of Chinese hamster ovary cell mutants defective in assembly of peroxisomes. *J. Cell Biol.* 110, 651–660.

12–79

A. If the modified Pex5 remains in the cytosol, it will not be cleaved by the peroxisomal protease. Thus, it will be recognized only by mAb2, which will detect it only in the whole-cell extract and in the supernatant.

If the modified Pex5 can be imported into peroxisomes but not exported, the cleaved form (recognized by mAb1) will be found in the whole-cell extract and in the pellet, which contain peroxisomes, but not in the supernatant, which lacks them. Any modified Pex5 that has not entered peroxisomes, and thus has not been cleaved, will be detected by mAb2 in the whole-cell extract and in the supernatant. The cleaved Pex5 in the whole-cell extract and in the pellet will also be recognized by mAb2.

If the modified Pex5 can cycle between the peroxisomal matrix and the cytosol, the cleaved form will be detected by mAb1 in the whole-cell extract and pellet, as expected for imported Pex5. The cleaved form will also be detected in the supernatant, indicating that it was exported from the peroxisome. Any modified Pex5 that has not entered peroxisomes will be detected by mAb2 in the whole-cell extract and in the supernatant; mAb2 will also detect cleaved Pex5 in all three fractions.

B. The results in Figure 12–16C match the expectations for the cycling mechanism for Pex5-mediated import of proteins into peroxisomes. The critical observation is that the cleaved form of Pex5, which is detected by mAb1, is found in the whole-cell extract, in the pellet, and in the supernatant. Modified Pex5 must have entered the peroxisome to be cleaved, and it must also exit the peroxisome since it is found in the supernatant. The absence of uncleaved Pex5 in the pellet (peroxisomes) indicates that cleavage must occur rapidly upon entry. The larger amount of Pex5 in the supernatant (cytosol) than in the pellet (peroxisomes) suggests that Pex5 is rapidly recycled to the cytosol.

C. Pex5-mediated import into peroxisomes resembles import into the nucleus, which is mediated by nuclear import receptors. In both cases, a cytosolic receptor binds to the cargo, accompanies it through a complex of membrane proteins to the interior of the compartment, drops off the cargo, and then returns to the cytosol. The results in Figure 12–16C do not directly answer the question of whether cleaved Pex5 continues to cycle between the cytosol and peroxisome matrix, but other experiments in the original paper suggest that Pex5 does function by such a cycling mechanism.

Reference: Dammai V & Subramani S (2001) The human peroxisomal targeting signal receptor, Pex5p, is translocated into the peroxisomal matrix and recycled to the cytosol. *Cell* 105, 187–196.

MEDICAL LINKS

12–80 The AGT enzyme is found predominantly (95%) in the mitochondria of these patients. A positively charged amphiphilic α helix is a signal for mitochondrial import. The amphiphilic helix formed by the substitution of a leucine for a proline at position 11 is a weak mitochondrial targeting signal. In fact, this particular mutation exists as a polymorphism in the human population with an allelic frequency of about 10%. By itself, it mistargets about 10% of AGT to mitochondria, with the rest being accurately targeted to peroxisomes. Combined with a second mutation that inhibits peroxisomal targeting, this weak mitochondrial targeting signal misdirects about 95% of AGT to mitochondria.

Reference: Purdue PE, Allsop J, Isaya G, Rosenberg LE & Danpure CJ (1991) Mistargeting of peroxisomal L-alanine:glyoxylate aminotransferase to mitochondria in primary hyperoxaluria patients depends upon activation of a cryptic mitochondrial targeting sequence by a point mutation. *Proc. Natl Acad. Sci. USA* 88, 10900–10904.

12–81

A. The hybridization results in Figure 12–17 indicate that mRNAs for *PGK* genes 1 and 3 are expressed in humans and that mRNAs for *PGK* genes 1 and 2 are expressed in tsetse flies.
B. The glycosomal form of PGK is expressed only in trypanosomes from humans. Since the oligonucleotide probe for *PGK* gene 3 hybridizes to an mRNA that is expressed uniquely in trypanosomes from humans, *PGK* gene 3 probably encodes the glycosomal form of PGK.
C. The results in Figure 12–17 suggest that the low level of cytosolic PGK activity in trypanosomes from humans is probably not due to inaccurate sorting into glycosomes. *PGK* gene 1 is expressed at low levels in trypanosomes from both humans and tsetse flies. Since all the PGK activity in trypanosomes from tsetse flies is present in the cytosol, *PGK* gene 1 must encode a cytosolic form of PGK. Therefore, since *PGK* gene 1 is expressed in trypanosomes from humans, it is probably responsible for the low level of cytosolic PGK activity. Thus, there is no indication that import into glycosomes in trypanosomes from humans is inaccurate.

Reference: Osinga KA, Swinkels BW, Gibson WC, Borst P, Veeneman GH, Van Boom JH, Michels PAM & Opperdoes FR (1985) Topogenesis of microbody enzymes: a sequence comparison of the genes for the glycosomal (microbody) and cytosolic phosphoglycerate kinases of *Trypanosoma brucei*. *EMBO J*. 4, 3811–3817.

THE ENDOPLASMIC RETICULUM

DEFINITIONS

12–82 Smooth ER

12–83 GPI anchor

12–84 Endoplasmic reticulum (ER)

12–85 Signal-recognition particle (SRP)

12–86 Stop-transfer signal

12–87 Co-translational

12–88 ER retention signal

12–89 Unfolded protein response

12–90 Sec61 complex

12–91 Glycoprotein

12–92 Free ribosome

TRUE/FALSE

12–93 False. When the hydrophobic signal peptide emerges from the ribosome, it is bound by SRP, which causes a pause in protein synthesis. Synthesis resumes when the ribosome with a bound SRP then binds to the SRP receptor on the cytosolic surface of the rough ER.

12–94 True. The Sec61 complex is composed of several proteins that assemble into a doughnutlike structure. The central pore in the complex lines up with a tunnel in the large ribosomal subunit through which the growing polypeptide chain exits from the ribosome.

12–95 False. The first (most N-terminal) transmembrane segment that exits the ribosome initiates translocation (acts as a start-transfer signal). Its orientation in the ER membrane fixes the reading frame for insertion of subsequent transmembrane segments. If the first transmembrane segment is oriented with its N-terminus in the cytosol, even-numbered segments will act as stop-transfer signals, and odd-numbered segments will act as start-transfer signals. If the first segment is oriented with its N-terminus in the lumen, then the second segment and subsequent even-numbered segments will act as start-transfer signals. Subsequent odd-numbered segments will act as stop-transfer signals.

12–96 False. The ER lumen does not contain reducing agents (they are in the cytosol) and therefore S–S bonds can form in the ER.

THOUGHT PROBLEMS

12–97 An mRNA molecule is attached to the ER membrane by the ribosomes translating it. This ribosome population, however, is not static. As the mRNA moves through the ribosomes, those ribosomes that have finished translation dissociate from the 3′ end of the mRNA and from the ER membrane. But the mRNA remains attached to the ER by other ribosomes that are still in the process of translating it. As ribosomes move along the mRNA, its 5′ end becomes exposed and new ribosomes, recruited from the cytosolic pool, bind to it and begin translation. Depending on its length, there are some 10–20 ribosomes attached to each membrane-bound mRNA molecule, forming what is known as a polyribosome.

12–98 Protein import into mitochondria and chloroplasts occurs as a post-translational event, and cytosolic hsp70 chaperones are required to keep the newly made proteins in an unfolded conformation so they can be imported. By contrast, during co-translational import into the ER, the protein is imported as it is being made; thus, there is no possibility that it will fold and no need to involve hsp70 proteins to keep it unfolded.

12–99 You would not see microsomes in any cell. Microsomes are generated when cells are disrupted by homogenization. Microsomes are fragments of cellular membrane that have closed to form small vesicles.

12–100 Most proteins are translocated into the ER as they are being made by ribosomes; however, some proteins are translocated after they have been completely synthesized. In all cases, the proteins are translocated across the membrane as unfolded polypeptide chains. In contrast, proteins can be imported into the nucleus after they have been synthesized,

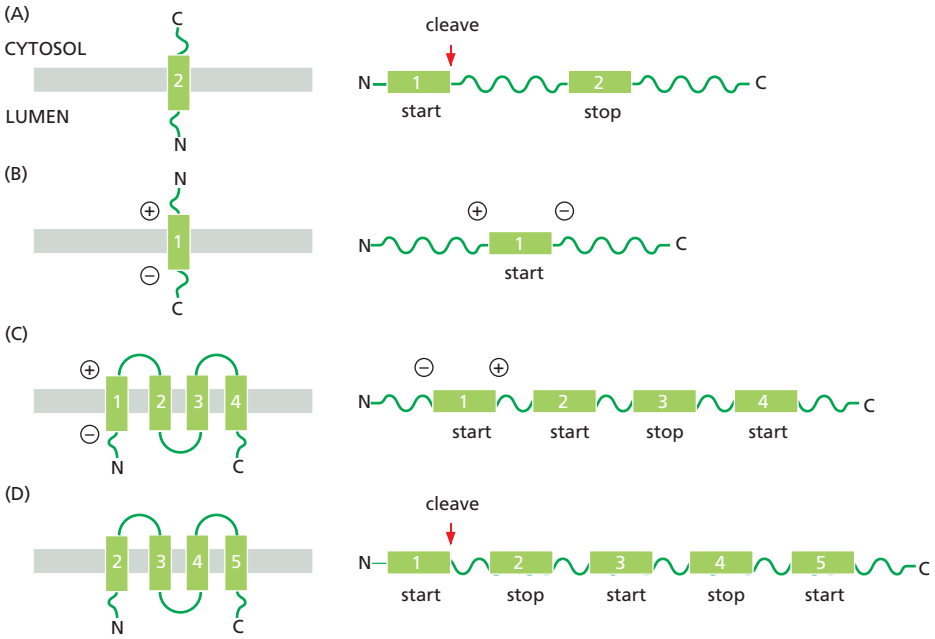


Figure 12–25 Arrangements of proteins across the ER membrane (Answer 12–101).

folded, and assembled into complexes. Many of the proteins imported into nuclei shuttle between the nucleus and cytosol and have functions in both places. ER proteins do not pass back into the cytosol, except to be degraded in proteasomes.

Translocation channels in the ER membrane are normally closed. They open only after the ribosome attaches to the membrane and the translocating polypeptide chain seals the channel from the cytosol. By contrast, nuclear pores are gates, which are always open to small molecules. It is important that the ER membrane remains impermeable to small molecules during the translocation process. The ER is a major store for Ca^{2+} in the cell and Ca^{2+} release into the cytosol must be tightly controlled.

Signal sequences for ER import are often cleaved off after import, whereas nuclear localization signals are not. Translocation into the ER is a one-time process, so that signals are dispensable. In contrast, nuclear localization signals are needed to re-import nuclear proteins repeatedly after the nuclear membrane reassembles at each mitosis.

12–101 The predicted arrangements of the proteins in the membrane of the ER and the identity of the membrane-spanning segments as start- or stop-transfer signals are illustrated in **Figure 12–25**. The arrangement of the membrane-spanning segments for all these proteins is fixed by the orientation of the initial start-transfer signal. For proteins A and D, which have cleavable signal sequences, the start-transfer peptide is oriented with its N-terminal end toward the cytosol. Cleavage of the signal peptide on the luminal side of the ER exposes the new N-terminus of the protein in the lumen (Figure 12–25A and D). The initial start-transfer signals in proteins B and C are oriented so that the positively charged end faces the cytosol. This orientation for protein C means that membrane-spanning segment 2 must be a start-transfer signal.

12–102 As shown in **Figure 12–26**, elimination of the first transmembrane segment (by making it hydrophilic) would be expected to give rise to a protein with the N-terminal segment in the cytosol (unglycosylated), but with all other membrane-spanning segments in their original orientation. In the unmodified protein, the first transmembrane segment served as a

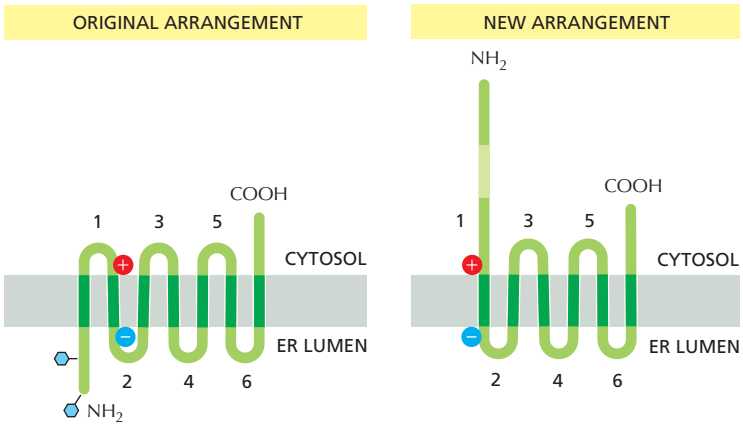


Figure 12–26 Arrangement of the original multipass transmembrane protein and of the new protein after the first hydrophobic segment was converted to a hydrophilic segment (Answer 12–102).

start-transfer signal, oriented so that it caused the N-terminal segment to pass across the ER membrane. The next transmembrane segment is also a start-transfer signal, but oriented so that it passes C-terminal protein across the membrane until it reaches the next transmembrane segment, which serves as a stop-transfer signal. Two more pairs of similarly orientated start- and stop-transfer signals give rise to the final arrangement.

Eliminating the first start-transfer signal would permit the second start-transfer signal to initiate transfer. The arrangement of its flanking charged amino acids would orient it in the membrane so that its positively charged end faces the cytosol, just as it did in the original protein. It would then initiate transfer of C-terminal segments just as it did in the unmodified, original protein.

12–103 The preassembled sugar chain allows for better quality control. The assembled oligosaccharide chains can be checked for accuracy before they are added to the proteins; if a mistake were made in adding sugars individually to the proteins, the whole protein might have to be discarded. Since far more energy is used in building a protein than in building a short oligosaccharide chain, this is a much more economical strategy. Also, once a sugar tree is added to a protein, it is more difficult for enzymes to modify its branches, compared with modifying them on the free sugar tree. This difficulty becomes apparent as the protein moves to the cell surface: although sugar chains are continually modified by enzymes in various compartments of the secretory pathway, these modifications are often incomplete and result in considerable heterogeneity of the glycoproteins that leave the cell. The heterogeneity is largely due to the restricted access that the enzymes have to the sugar trees attached to the surface of proteins. The heterogeneity also explains why glycoproteins are more difficult to study and purify than nonglycosylated proteins.

12–104 Misfolded proteins in the ER bind to and activate a transmembrane kinase in the ER, which activates its own endoribonuclease domain, causing it to remove an intron from a specific mRNA. This "spliced" mRNA encodes a gene regulatory protein that enters the nucleus and activates the gene for an ER chaperone protein. This chaperone enters the ER and aids in the correct folding of misfolded proteins. This portion of the unfolded protein response is beneficial to the cell because it keeps misfolded proteins from building up in the ER and interfering with the processing of other, correctly folded proteins. Other parts of this response lead to activation of genes for ER protein degradation, which helps to unclog the ER.

12–105 Symmetry of phospholipids in the two leaflets of the ER membrane is generated by a phospholipid translocator, called a scramblase, that rapidly

flips phospholipids of all types back and forth between the monolayers of the bilayer. Because it flips phospholipids indiscriminately, the different types of phospholipid become equally represented in the inner and outer leaflets of the bilayer; that is, they become symmetrically distributed. The plasma membrane contains a different kind of phospholipid translocator, which is specific for phospholipids containing free amino groups (phosphatidylserine and phosphatidylethanolamine). These flippases remove these specific phospholipids from the external leaflet and transfer them to the internal leaflet of the plasma membrane, thereby generating an asymmetrical distribution.

DATA HANDLING

12–106

A. In the absence of microsomes, a unique protein is synthesized (see Figure 12–20, lane 1). This protein is accessible to protease digestion whether or not detergent is present (lanes 2 and 3), indicating that the protein is not protected by a membrane bilayer. Finally, treatment of the protein with endo H does not alter its mobility (lane 4), indicating that the protein carries no *N*-linked sugars of the type added in the ER.

B. By the three criteria outlined in the problem (protease protection, *N*-linked sugars, and cleaved signal peptide), this protein is translocated across the microsomal membrane. (1) The protein is fully sensitive to protease in the presence of detergent (see Figure 12-20, lane 7), but only a segment of it is sensitive to protease in the absence of detergent (lane 6). Thus, the protein is partially protected from protease in the presence of microsomes. (2) The rate of migration of the protein increases after treatment with endo H (compare lanes 5 and 8), indicating that sugars are attached to the protein when it is translated in the presence of microsomes. (3) When the sugars are removed, the protein migrates faster than the precursor protein (compare lanes 1 and 8), indicating that a portion of the protein—presumably the signal peptide—is removed from the precursor when the protein is translated in the presence of microsomes.

C. The reduced size of the protein after protease treatment (see Figure 12–20, compare lanes 5 and 6) indicates that a portion of the protein remains on the outside of the microsomes, accessible to protease. In combination with the results indicating cleavage of the signal peptide and addition of *N*-linked sugars, this result shows that the protein spans the membrane. Thus, the protein is inserted only partway through the membrane and is presumably anchored in the membrane by a stop-transfer segment.

12–107

A. This scheme is analogous to that described in Problem 12–64 for isolating mitochondrial import mutants. Normal cells that carry the modified *His4* gene make His4 that gets incorporated into the ER and is therefore unavailable to convert histidinol to histidine; hence, such cells will not be able to survive if they are required to convert added histidinol (which is taken up into the cytosol) to histidine. By contrast, in cells that are defective for ER import, the modified His4 protein will remain in the cytosol, where it can convert histidinol to histidine, allowing the cells to survive.

B. The difficulty in using such a selection scheme to find ER translocation mutants is that ER import is essential for the cell's survival (in contrast to the situation in mitochondria, where mitochondrial function can be made nonessential by providing a fermentable carbon source.) Temperature-sensitive mutants are one way to get around this problem, allowing cells to be grown at 24°C, where ER import is functional, and then studied at 37°C, where import is defective. The selection scheme would not be expected to work at either of these temperatures, however. At 24°C, both normal and mutant cells would import the modified His4 protein into

the ER and therefore would not survive on added histidinol. At 37°C, the normal cells would import modified His4 into the ER and die, but mutant cells, which would not import the modified His4, would also die because of the defect in ER import, which is essential. The idea of selecting for survival at an intermediate temperature reflects a balance between the extremes. At the intermediate temperature it was hoped that mutant cells would have adequate ER function to survive, but would incorporate His4 inefficiently enough to leave some in the cytosol so the cells could grow on added histidinol. The scheme worked!

Reference: Deshaies RJ & Schekman R (1987) A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105, 633–645.

12–108

A. The hydropathy plot shows three hydrophobic segments from about amino acids 1–25, 75–100, and 185–210. Results with the fusion proteins indicate that each of these segments is a membrane-spanning segment and define their organization in the membrane (**Figure 12–27A**). Fusions between segments 1 and 2 (constructs 1 and 2) have high phosphatase activity, indicating that alkaline phosphatase is in the periplasmic space. These results show that segment 1 serves as a start-transfer signal with its N-terminus facing the cytoplasm and its C-terminus (alkaline phosphatase) in the periplasm. Fusions between segments 2 and 3 (constructs 3 and 4) have low activity, indicating that alkaline phosphatase is in the cytoplasm; thus, segment 2 must serve as a stop-transfer signal. Fusions after segment 3 have high activity, indicating that alkaline phosphatase is in the periplasm; thus, segment 3 must serve as a start-transfer signal.

B. Deletion of codons 68–103 removes the second membrane-spanning segment. The protein domain between the remaining two membrane-spanning segments (segments 1 and 3) should now be in the periplasm and the region beyond the last membrane-spanning segment should be in the cytoplasm (**Figure 12–27B**). Once again, the activities of the hybrid proteins agree with this expectation: constructs 3* and 4*, which should have periplasmic alkaline phosphatase, have high activity, and constructs 5* and 6*, which should have cytoplasmic alkaline phosphatase, have low activity.

Reference: Manoil C & Beckwith J (1986) A genetic approach to analyzing membrane protein topology. *Science* 233, 1403–1408.

12–109

A. Experiment 1 tests whether the acceptor membranes (red cell ghosts) are in excess. Since the PC exchange protein catalyzes an *exchange* reaction, there is a simple theoretical limit to how much transfer can occur at equilibrium. If the amount of donor and acceptor membranes were equal, for example, the limit of possible transfer would be 50%. Doubling the amount of acceptor membrane would raise the limit to 67% (a 2 to 1 ratio of donor to acceptor); tripling the acceptor membranes would raise the limit to 75% (a 3 to 1 ratio); and so on. Since adding more acceptor membranes made no difference, the red cell membranes must be in excess. Thus, the 70% limit is not an equilibrium point for the exchange.

Experiment 2 rules out the possibility that the enzyme is inactivated during the reaction, since addition of fresh exchange protein caused no further exchange.

Experiment 3 eliminates the possibility that the starting labeled material was impure (that is, untransferable by the PC exchange protein, which is specific for PC) or was somehow altered during the course of the incubation.

B. The apparent explanation for the 70% limit is that the PC exchange protein transfers PC only from the outer monolayer of the vesicle bilayer. The

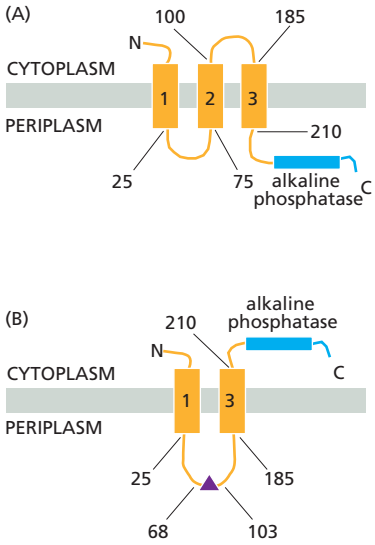


Figure 12–27 Organization of hybrid proteins in the bacterial membrane (Answer 12–108). (A) Intact transmembrane protein fused to alkaline phosphatase at its C-terminus. (B) Internally deleted transmembrane protein fused to alkaline phosphatase. Membrane-spanning segments are indicated as *orange boxes* and numbered to correspond to the hydrophobic stretches of the hydropathy plot in Figure 12–21. Numbers outside the boxes refer to amino acids.

area of the outside face of the donor vesicles is about 2.6 times the area of the inner face. The area of the surface of a sphere is $4\pi r^2$. Thus, the ratio of the areas of the outer and inner faces of the donor vesicle is the ratio of squares of their radii, which is $10.5^2/(10.5 - 4.0)^2$, or 2.6. Since the outer surface area is 2.6 times larger than the inner surface area, 74% (2.6/3.5) of the lipid is in the outer monolayer. Thus, 70% transfer is close to the expected limit if the exchange protein can only exchange PC from the outer leaflet and PC does not flip-flop between the outer and inner leaflets.

C. If the exchange protein exchanges PC only between outer leaflets, the label in the acceptor red cell membranes will all be in the outer leaflet and therefore all available for transfer. This result supports the idea that the PC exchange protein only transfers PC between outer monolayers.

Reference: Rothman JE & Dawidowicz EA (1975) Asymmetric exchange of vesicle phospholipids catalyzed by the phosphatidylcholine exchange protein. Measurement of inside–outside transitions. *Biochemistry* 14, 2809– 2816.

MCAT STYLE

12–110

A. Binding to a nuclear import receptor is required for import into the nucleus. Ran-GDP is generated in the cytoplasm when Ran-GTP is exported from the nucleus, but it is not required for nuclear import. The Sec61 complex is required for transport of proteins into the endoplasmic reticulum.

12–111

B. Phosphorylation near a nuclear export signal could block binding of a nuclear export receptor, thereby causing the transcription factor to accumulate in the nucleus. In this case, the absence of phosphorylation in the mutant transcription factor should cause it to be transported out of the nucleus as fast as it is transported in, preventing its accumulation in the nucleus. Hypotheses A and C are incorrect because they would lead to accumulation of the mutant transcription factor in the nucleus. Hypothesis D is incorrect because Ran-GTP binds to nuclear import and export receptors, not to their cargoes.

12–112

B. If phosphorylation blocks export of the normal transcription factor from the nucleus, and absence of phosphorylation in the mutant protein allows export—the best hypothesis from Problem 12–111—then deletion of the nuclear export signal from the mutant protein should cause it to accumulate in the nucleus. This result would also rule out an alternative hypothesis; namely, that phosphorylation activates nuclear import, and that the absence of phosphorylation in the mutant protein prevents it from entering the nucleus. If this alternative hypothesis were true, deletion of the nuclear export signal would not lead to accumulation of the mutant transcription factor in the nucleus, because its nuclear import signal would not be active. Choice A would not test the hypothesis, since the transcription factor presumably already contains a nuclear localization signal; otherwise it would not be able to enter the nucleus. Choice C is incorrect because Ran-GDP does not bind to cargo proteins. Choice D, inactivating Ran-GTPase, would not be informative: it would shut down the nuclear import/export system.

12–113

B. Signal-recognition particle is required because it binds to signal sequences, halts translation, and brings the ribosome to the surface of the ER. Protein translation is required to generate the signal sequence

that is recognized by the signal-recognition particle. Chaperones are not required for ribosomes to associate with the membrane; they help with proper protein folding at a later stage.

12–114

B. Newly synthesized peptides are translocated through the membrane via a pore formed by the Sec61 complex. When the rough microsomes are isolated, many proteins are in the process of translocation and block movement of ions through the pores. When the peptides are released by puromycin, the pore stays open and ions are able to pass through it. This classic experiment demonstrated that peptides are translocated through a water-filled pore. The other choices are either irrelevant (A) or made up (C and D).

12–115

C. When ribosomes bind to the protein-translocation pore and begin translation, the pore opens to allow the nascent peptide to pass through. Washing the membrane with a buffer containing a high salt concentration causes the ribosomes to dissociate, permitting the pore to close. Choice A is irrelevant since the conductance occurs through the water-filled protein translocation channels. Choice B is not correct because a high-salt wash does not alter the resistance of the membrane, which is an inherent property of the lipid components of the membrane. Choice D is irrelevant because the unfolded protein response is unrelated to the conductance properties of the membrane.

CHAPTER 13

Intracellular Membrane Traffic

IN THIS CHAPTER

THE MOLECULAR MECHANISMS OF MEMBRANE TRANSPORT AND THE MAINTENANCE OF COMPARTMENT DIVERSITY

DEFINITIONS

13–1 Transport vesicle

13–2 Rab protein

13–3 Adaptor protein

13–4 Dynamin

13–5 NSF

13–6 Clathrin-coated vesicle

13–7 ARF protein

13–8 Rab effector

13–9 SNARE protein (SNARE)

13–10 Lumen

13–11 Coated vesicle

13–12 Sar1 protein

TRUE/FALSE

13–13 True. The cytosolic leaflets of the two membrane bilayers are the first to come into contact and fuse, followed by the noncytosolic leaflets. It is this pattern of leaflet fusion that maintains the topology of membrane proteins, so that protein domains that face the cytosol always do so, regardless of what compartment they occupy.

13–14 False. Although the organelle-specific distribution of Rab proteins made such a hypothesis attractive at one time, it is now clear that the Rab proteins do not bind to complementary Rab proteins. Instead, Rab proteins bind to specific Rab effectors to accomplish the docking of appropriate vesicles to the target membrane.

THOUGHT PROBLEMS

13–15 If the flow of membrane between cellular compartments were not balanced in a nondividing liver cell, some compartments would grow in size and others would shrink (in the absence of new membrane synthesis). Keeping all the membrane compartments the same relative size is

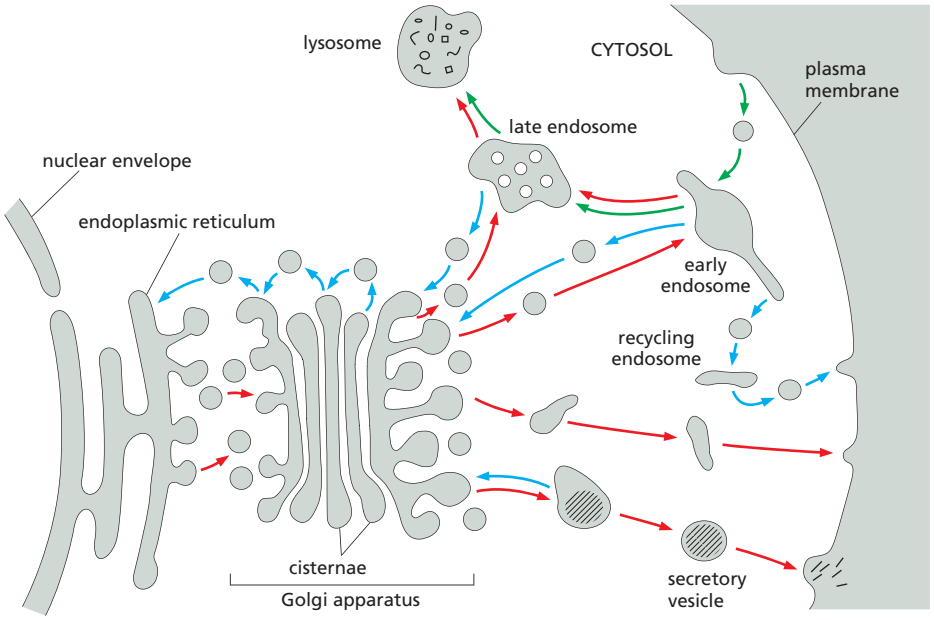


Figure 13–19 Identity of compartments and pathways involved in the biosynthetic-secretory, endocytic, and retrieval pathways (Answer 13–16). Biosynthetic-secretory pathways are indicated with *red arrows*; endocytic pathways are indicated with *green arrows*; retrieval pathways are indicated with *blue arrows*.

essential for proper functioning of a liver cell. The situation is different in a growing cell such as a gut epithelial cell. Over the course of a single cell cycle, all the compartments must double in size to generate two daughter cells. Thus, there will be an imbalance in favor of the outward flow, which will be supported by new membrane synthesis equal to the sum total of all the cell's membrane.

13–16

A. The intracellular compartments involved in the biosynthetic-secretory pathway and endocytic pathway are labeled in **Figure 13–19**.

B. The biosynthetic-secretory pathway (red arrows), endocytic pathway (green arrows), and retrieval pathway (blue arrows) are shown in Figure 13–19.

13–17 This is an apt analogy. Cargo receptors can be incorporated into a coated vesicle only if they can bind to adaptor proteins—"have a ticket for a particular ride"—which allows them to enter a coated pit and be incorporated into a vesicle—"the cable car." Cargo receptors—"like the skiers"—are mixed together without any guaranteed traveling companions, but all get to the next compartment—"station."

Reference: Pearse BMF, Smith CJ & Owen DJ (2000) Clathrin coat construction in endocytosis. *Curr. Opin. Struct. Biol.* 10, 220–228.

13–18 The position of one triskelion is shown in **Figure 13–20A**. A triskelion must be flexible at its vertex to be able to accommodate different sizes of coated vesicles (**Figure 13–20B**). As the size of the coat increases, its radius of curvature decreases, requiring individual triskelions to flatten out slightly. To accommodate the different angles required to fit into a pentagon and a hexagon, a triskelion needs to be flexible at its "knees" (Figure 13–20B).

References: Kirchhausen T (2000) Clathrin. *Annu. Rev. Biochem.* 69, 699–727.

Ybe JA, Brodsky FM, Hofmann K, Lin K, Liu SH, Chen L, Earnest TN, Fletterick RJ & Hwang RK (1999) Clathrin self-assembly is mediated by a tandemly repeated superhelix. *Nature* 399, 371–375.

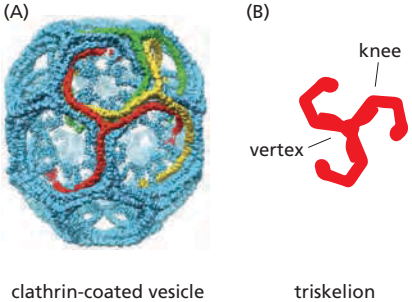


Figure 13–20 Formation of a clathrin coat (Answer 13–18). (A) The location of a single triskelion in a coated vesicle. (B) The sites of maximum flexibility of a triskelion.

13–19 The specificity for both the transport pathway and the transported cargo comes not from the clathrin coat, but from the adaptor proteins that link the clathrin to the transmembrane receptors for specific cargo proteins. The several varieties of adaptor proteins allow different cargo receptors, hence different cargo proteins, to be transported along specific transport pathways.

Incidentally, humans are different from most other organisms in that they have two heavy-chain genes. Like other mammals, they also have two light-chain genes. In addition, in the neurons of mammals, the light-chain transcripts are alternatively spliced. Thus, there exists the potential in humans for additional complexity of clathrin coats; the functional consequences of this potential variability are not clear.

References: Kirchhausen T (2000) Clathrin. *Annu. Rev. Biochem.* 69, 699–727.

Pearse BMF, Smith CJ & Owen DJ (2000) Clathrin coat construction in endocytosis. *Curr. Opin. Struct. Biol.* 10, 220–228.

13–20 If ARF1 were mutated so that it could not hydrolyze GTP, ARF1 would exist in a cell as ARF1-GTP. Since ARF1-GTP promotes assembly of COPI-coated vesicles, you would expect such vesicles to form readily, but they might not form at the right place in the cell. Normally, ARF1 is delivered specifically to the Golgi membrane by a Golgi-bound ARF1-GEF, which converts the cytosolic ARF1-GDP to ARF1-GTP, exposing a fatty acid tail that allows it to bind to the membrane. The mutant ARF1, which would always have GTP bound and its fatty acid tail exposed, might bind inappropriately to other cell membranes, thus promoting COPI-coated vesicle formation at inappropriate places in a cell.

Disassembly of the COPI coat requires hydrolysis of GTP by ARF1. Thus, in the presence of the mutant form of ARF1, the COPI coat would not be able to disassemble. Since the uncoated vesicle is the substrate for the fusion reaction with the target membrane, the mutant ARF1-GTP would be expected to block ARF1-mediated transport.

If the mutant ARF1 were the only form of ARF1 in the cell, it would likely prove lethal. Under these conditions, all ARF1-mediated transport involving COPI-coated vesicles should be blocked. Furthermore, there should be an accumulation of COPI-coated vesicles that cannot be uncoated, which might reduce the availability of free COPI subunits necessary for other transport pathways. What is unclear is the extent to which other members of the ARF family of proteins might substitute for ARF1. It would be necessary to do the experiment to know the result for certain.

13–21 There will always be some v-SNAREs in the target membrane. Immediately after fusion, the v-SNAREs will be in inactive complexes with t-SNAREs. Once NSF pries the complexes apart, v-SNAREs may be kept inactive by binding to inhibitory proteins. Accumulation of v-SNAREs in the target membrane beyond some minimal population is thought to be prevented by active retrieval pathways that incorporate v-SNAREs into vesicles for redelivery to the original donor membrane.

13–22 The cell's SNAREs are all bound to the cytosolic surface of whatever membrane they are in. They function by juxtaposing the cytosolic surfaces of the two membranes to be fused. By contrast, an enveloped virus must fuse with a cell membrane by bringing together its external surface with an external surface of a cell membrane. Thus, enveloped viruses cannot make use of a cell's SNAREs because they are located on the wrong side of the membrane. It is for this reason that enveloped viruses make their own fusion proteins, which are properly situated on their external surface.

CALCULATIONS

13–23 The volume of a cylinder 1.5 nm in diameter and 1.5 nm in height is 2.65 nm^3 [$3.14 \times (0.75\ nm)^2 \times 1.5\ nm$], which equals 2.65×10^{-24} L [$2.65\ nm^3 \times (cm/10^7\ nm)^3 \times (L/10^3\ cm^3)$]. There are about 88 water molecules in this volume.

$$\frac{\text{water molecules}}{\text{cylinder}} = \frac{2.65 \times 10^{-24}\ \text{L}}{\text{cylinder}} \times \frac{55.5\ \text{mole}}{\text{L}} \times \frac{6 \times 10^{23}\ \text{molecules}}{\text{mole}}$$

$$= 88.2$$

In each monolayer in a circle of membrane 1.5 nm in diameter, there are about 9 phospholipids (PL) [$3.14 \times (0.75\ nm)^2 \times (PL/0.2\ nm^2) = 8.8$ PL]. Thus, there are about 5 water molecules per phospholipid in the area of close approach of the two membranes. This number is slightly less than half the number (10–12) estimated to be associated with phospholipid head groups under normal circumstances. This means that when a vesicle and its target membrane are drawn together in preparation for fusion, somewhat more than half of the water molecules that would normally bind to the membranes must be squeezed out.

Reference: Meuse CW, Krueger S, Majkrzak CF, Dura JA, Fu J, Connor JT & Plant AL (1998) Hybrid bilayer membranes in air and water: infrared spectroscopy and neutron reflectivity studies. *Biophys. J.* 74, 1388–1398.

DATA HANDLING

13–24 These observations indicate that brefeldin A blocks COPI-coated vesicle formation by interfering with the exchange of GTP for GDP, which is essential for ARF to bind to the Golgi membrane and initiate formation of coated vesicles. Brefeldin A does not affect Arf-GTP-mediated formation of COPI-coated vesicles.

Observation 1 shows that brefeldin A does not block coated vesicle formation if ARF is first locked into its active form by GTPγS. Thus, the assembly of the COPI coats and formation of vesicles are not affected by brefeldin A, if the active membrane-bound form of ARF is present.

Observation 2 shows that a protein in the Golgi membrane—a GEF—catalyzes the exchange of GTP for GDP. This exchange reaction is blocked by brefeldin A.

Observation 2, by itself, does not distinguish whether the effect of brefeldin A is on the GEF or on ARF: binding of brefeldin A to either protein could interfere with the exchange reaction. In fact, recent experiments show that brefeldin A binds to the complex of ARF and the GEF, locking ARF into a nonproductive GDP-bound conformation.

References: Chardin P & McCormick F (1999) Brefeldin A: the advantage of being uncompetitive. *Cell* 97, 153–155.

Donaldson JG, Finazzi D & Klausner RD (1992) Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide into ARF protein. *Nature* 360, 350–352.

Helms JB & Rothman JE (1992) Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. *Nature* 360, 352–354.

13–25 These results indicate that all three components—ARF1, ARF1-GAP, and COPI subunits—are required for efficient hydrolysis of GTP. Based on the effects of other GAPs on small GTPases, it is tempting to speculate that COPI subunits bind to the complex of ARF1 and ARF1-GAP and provide the arginine "finger" that seems to be critical for GTP hydrolysis.

It would be very informative to have a crystal structure of the assembly; however, COPI is too complex to make this a simple task. You might test your conclusion in other ways. If COPI subunits provide the catalytic arginine, then site-directed mutagenesis of arginines in COPI might identify one that is essential for GTP hydrolysis.

Reference: Goldberg J (1999) Structural and functional analysis of the ARF1–ARFGAP complex reveals a role for coatomer in GTP hydrolysis. *Cell* 96, 893–902.

13–26 To generate maximal alkaline phosphatase activity, vesicles from each strain must carry both v-SNAREs and t-SNAREs (see Figure 13–5B, experiment 1). If either vesicle is lacking v-SNAREs or t-SNAREs, phosphatase activity is reduced to 30–60% of the maximum (see experiments 3, 4, 6, 7, 8, and 9). If both vesicles are missing either v-SNAREs (see experiment 2) or t-SNAREs (see experiment 5), phosphatase activity is very low, as it is if one vesicle is missing both SNAREs (see experiments 10 and 11). For a reasonable level of fusion, complementary SNAREs must be present on the vesicles. It does not matter which kind of SNARE is on vesicles from strain A so long as vesicles from strain B carry a complementary SNARE (compare experiments 3 and 4, experiments 6 and 7, and experiments 8 and 9).

You might have wondered why there is a low background of phosphatase activity, even where no fusion is expected (see experiments 2, 5, 10, and 11). If a few vesicles were to break, releasing small amounts of pro-Pase and protease, then a small amount of active alkaline phosphatase could be generated in the absence of vesicle fusion.

Reference: Nichols BJ, Ungermann C, Pelham HRB, Wickner WT & Haas A (1997) Homotypic vacuolar fusion mediated by t- and v-SNAREs. *Nature* 387, 199–202.

13–27 Syntaxin and SNAP25 are t-SNAREs, and synaptobrevin is a v-SNARE. NSF and its accessory proteins recognize complexes of t- and v-SNAREs, and use the energy of ATP hydrolysis to pry them apart. Binding of NSF and its accessory proteins to a SNARE complex depends on the presence of ATP. In the absence of ATP, the complex does not form; thus, the beads did not bring down anything other than NSF. In the presence of ATP, the complex forms, but NSF then hydrolyzes ATP to separate the SNAREs and release them. Thus, in the presence of ATP, only NSF is attached to the beads. In the presence of ATPγS, the complex can form, but it cannot dissociate because NSF cannot hydrolyze ATPγS. As a result, all members of the complex remain attached to NSF and are brought down by the beads.

Reference: Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P & Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318–324.

13–28

A. Since vesicles form and accumulate when the function of Sec4 is impaired (as in *Sec4*^{ts} and *Sec4N133I*), Sec4 cannot be involved in vesicle formation. Accumulation of vesicles in these mutants suggests that the vesicles can no longer deliver their cargo to the growing bud when Sec4 is not working properly. Thus Sec4 seems to be involved in vesicle targeting and fusion.

Functionally, Sec4 resembles mammalian Rab proteins, which are also required for proper delivery of transport vesicles to their target membrane. Indeed, Sec4 was the first identified member of the Rab family of proteins. Sec4 is unlike mammalian Sar1 and ARF proteins, which are required for formation of coated vesicles.

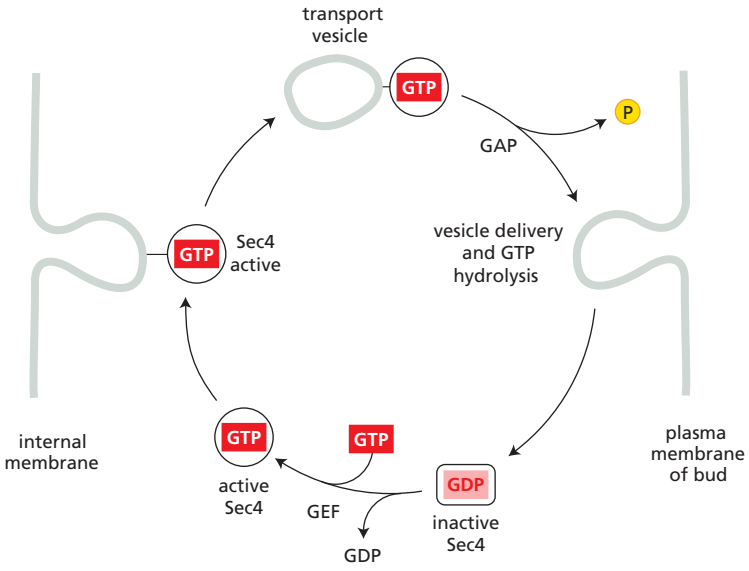


Figure 13–21 Outline of Sec4 function in delivery of transport vesicles from an internal membrane to the bud membrane (Answer 13–28). Additional proteins that are involved are not shown.

B. From the description of the defects in the presence of the mutant Sec4 proteins, and by analogy to Rab proteins, it is possible to outline the way normal Sec4 functions in delivery of vesicles to the bud membrane (**Figure 13–21**).

The presence of some Sec4 (20% of total) in the cytosol of wild-type cells represents Sec4 that is recycling after delivery of vesicles to the bud membrane. Removal of the C-terminal cysteines prevents attachment of a lipid that is essential for the binding of Sec4 to the forming vesicle. If Sec4 does not bind to the vesicle, it cannot carry out its function.

C. The inhibitory properties of Sec4N133I are very interesting and not altogether easy to interpret. Since Rab proteins function as monomers, it is unlikely that there is a direct effect of Sec4N133I on normal Sec4. More likely, there is an indirect effect that prevents normal Sec4 from carrying out its function. For example, if a vesicle component such as a v-SNARE were present in limiting amounts, the accumulation of vesicles carrying Sec4N133I might deplete the supply, and thereby interfere with the proper fusion of vesicles carrying normal Sec4. Alternatively, Sec4N133I may bind too tightly to its Rab-like effector on the target membrane, preventing normal Sec4 from gaining access to the docking machinery.

References: Walworth NC, Goud B, Kabcenell AK & Novick PJ (1989) Mutational analysis of *SEC4* suggests a cyclical mechanism for the regulation of vesicular traffic. *EMBO J.* 8, 1685–1693.

Guo W, Roth D, Walch-Solimena C & Novick P (1999) The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *EMBO J.* 18, 1071–1080.

TRANSPORT FROM THE ER THROUGH THE GOLGI APPARATUS

DEFINITIONS

13–29 Cisternal maturation model

13–30 Proteoglycan

13–31 *Cis* face

13–32 Complex oligosaccharide

13–33 Golgi apparatus (Golgi complex)

13–34 High-mannose oligosaccharide

13–35 *Trans* Golgi network (TGN)

TRUE/FALSE

13–36 True. A misfolded protein is selectively retained in the ER by binding to chaperone proteins such as BiP and calnexin. Only after it has been released from such a chaperone protein—and thus approved as properly folded—does a protein become a substrate for exit from the ER.

13–37 True. The oligosaccharide chains are added in the lumens of the ER and Golgi apparatus, which are topologically equivalent to the outside of the cell. This basic topology is conserved in all membrane budding and fusion events. Thus, oligosaccharide chains are always topologically outside the cell, whether they are in a lumen or on the cell surface.

13–38 True. Addition of very long, unbranched chains of repeating disaccharide units in the Golgi apparatus can create proteoglycans in which the mass of sugar far exceeds that of protein.

THOUGHT PROBLEMS

13–39 Soluble ER proteins that are destined to reside in other membrane-enclosed organelles or to be secreted are bound by transmembrane cargo receptors. The cytosolic domains of these cargo receptors bind to the COPII coats on the vesicles that form on the ER membrane, incorporating the cargo receptors, along with their cargo, into COPII-coated vesicles.

13–40 The gene that is mutated in patients with cystic fibrosis encodes a protein that functions as a Cl^- channel in the plasma membrane. Many of the mutations that cause cystic fibrosis produce a protein that is only slightly misfolded. Although the protein would function perfectly normally—and would prevent the disease phenotype—if it reached the plasma membrane, it is retained in the ER and degraded.

13–41 Calnexin and HMG CoA reductase are transmembrane proteins and calreticulin is soluble. You can deduce this by examining the C-termini of the proteins for the ER retrieval signal for soluble proteins. Calreticulin has at its C-terminus the classic ER retrieval signal, KDEL. The KDEL receptor binds the retrieval signal and returns calreticulin to the ER whenever it escapes to the Golgi apparatus. Although not discussed in *MBoC*, HMG CoA reductase and calnexin each bear a C-terminal ER retrieval signal for a transmembrane protein: KKXX for HMG CoA reductase and KXRXX for calnexin.

Reference: Zerangue N, Malan MJ, Fried SR, Dazin PF, Jan YN, Jan LY & Schwappach B (2001) Analysis of endoplasmic reticulum trafficking signals by combinatorial screening in mammalian cells. *Proc. Natl Acad. Sci. USA* 98, 2431–2436.

13–42 The modified PDI would be located outside the cell. If PDI were missing the ER retrieval signal, its gradual flow out of the ER to the Golgi apparatus would not be countered by its capture and return to the ER, as normally occurs. Similarly, it would be expected to leave the Golgi apparatus by the default pathway, mixed with the other proteins that the cell is secreting. It would not be expected to be retained anywhere else along the secretory pathway because it presumably has no signals to promote such localization.

Reference: Munro S & Pelham HR (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899–907.

13–43 The KDEL receptor binds its ligands more tightly in the Golgi apparatus, where it captures proteins that have escaped the ER, so it can return them. The receptor binds its ligands more weakly in the ER, so that those proteins that have been captured in the Golgi apparatus can be released upon their return to the ER. The basis for the different binding affinities is thought to be the slight difference in pH; the lumen of the Golgi apparatus is slightly more acidic than that of the ER, which is neutral.

Since the primary job of the KDEL receptor is to capture proteins that have escaped from the ER, it would be reasonable to design the system so that the receptors are found in the highest concentration in the Golgi apparatus. This is, in fact, the way it is in the cell. You would be correct if you predicted that the KDEL receptor does not have a classic ER retrieval signal; after all, the receptor is designed to spend most of its time in the Golgi apparatus, and a classic signal would ensure its efficient return to the ER. It does, however, have a "conditional" retrieval signal; upon binding to an ER protein in the Golgi apparatus, its conformation is altered so that a binding site for COPI subunits is exposed. That signal allows it to be incorporated into COPI-coated vesicles, which are destined to return to the ER.

Reference: Teasdale RD & Jackson MR (1996) Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* 12, 27–54.

13–44 If the KDEL signal and the KDEL receptor were all that was required to retain a protein in the ER, then addition of KDEL to a secreted protein should result in its retention in the ER. Clearly, addition of KDEL to rat growth hormone or human chorionic gonadotropin did not result in their efficient retention in the ER. Presumably, their slower rate of secretion was due to the KDEL system, since changing KDEL to KDEV abolished the effect. A comparable effect is also seen for ER residents that have had their KDEL signals removed; they are secreted, but at significantly slower rates than true secreted proteins. One explanation that might account for both these effects is kin recognition, which embodies the idea that residents of the ER might have a general affinity for one another, making it more difficult for any of them to leave the compartment. According to this idea, ER proteins that are missing their KDEL signal are secreted slowly because they still retain their affinity for other ER residents. Similarly, secreted proteins with an added KDEL signal would not be expected to have a general affinity for ER residents, and thus would escape the ER at a higher rate than true ER residents.

Reference: Zagouras P & Rose JK (1989) Carboxy-terminal SEKDEL sequences retard but do not retain two secretory proteins in the endoplasmic reticulum. *J. Cell Biol.* 109, 2633–2640.

13–45 (1) Attached carbohydrates promote protein folding by making the intermediates more soluble and mediating their binding to chaperones. (2) Attached carbohydrates serve as recognition markers for transport from the ER and for protein sorting in the *trans* Golgi network. (3) Oligosaccharides on proteins provide protection against proteases. (4) Oligosaccharides on cell-surface proteins can function in cell–cell adhesion. (5) Oligosaccharides on the cell surface provide a protective coat against pathogens. (6) Attached carbohydrates can play a regulatory role by modulating protein–protein interactions.

DATA HANDLING

13–46

A. The altered VSV G proteins with "membrane-spanning" segments that are 12, 8, or 0 amino acids long do not make it to the plasma membrane; they remain in an intracellular location (see Table 13–2). The presence of oligosaccharides (endo H sensitivity) indicates that each of these proteins was inserted into the ER membrane, as expected since the signal peptide was not altered, and was subsequently modified by addition of oligosaccharide chains. The presence of the small C-terminal domain (protease sensitivity) on the proteins with segments 12 and 8 amino acids long indicates that these proteins are anchored in the membrane much like the normal G protein. By contrast, the complete protease resistance of the G protein with a zero amino acid transmembrane segment indicates that it passed all the way through the ER membrane into the lumen. Thus, the VSV G proteins with segments 12 and 8 amino acids long are in an internal membrane, but the one that is missing the membrane-spanning segment entirely is in an internal lumen.

   The partial endo H resistance of the G protein with a membrane-spanning segment of 12 amino acids suggests that some fraction of this G protein makes it as far as the medial compartment of the Golgi, which is where the relevant sugar modification occurs. The remainder of this protein is either in the membrane of the ER or the *cis* compartment of the Golgi. The endo H sensitivities of the G proteins with 8- and 0-amino-acid segments indicate that they do not make it past the *cis* compartment of the Golgi and may not make it out of the ER.

B. For the VSV G protein, the minimum length of the membrane-spanning segment appears to be 8 amino acids or less. G proteins with modified membrane-spanning segments only 8 amino acids long are anchored in the membrane much like the normal G protein. This result is surprising since 8 amino acids arranged in an α helix are not thought to be long enough to span the membrane. There are several possibilities: the short membrane-spanning segments may be arranged as extended chains rather than as α helices; the membrane may be less than 3 nm thick at the point where these segments penetrate the membrane; or adjacent portions of the G protein, including at least one basic amino acid (K or R), may be pulled into the membrane.

C. The minimum length of a membrane-spanning segment consistent with proper sorting of the VSV G protein is 13 or 14 amino acids. VSV G proteins with segments 14 amino acids long are sorted to the plasma membrane like normal G proteins, whereas those with segments 12 amino acids long are not (see Table 13–2). It is curious that shorter membrane-spanning segments anchor the protein in the membrane perfectly well but interfere with sorting. This is thought to be the case because the vesicles that leave the Golgi apparatus have thicker membranes than those that come from the ER. The difference in thickness of vesicle membranes is due to the high concentration of cholesterol in Golgi-derived vesicles.

Reference: Adams GA & Rose JK (1985) Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell* 41, 1007–1015.

13–47

A. The mutant cell lines are arranged in **Table 13–8** in the order that corresponds to the steps in the processing pathway for *N*-linked oligosaccharides. The numbers and kinds of sugars in *N*-linked oligosaccharides from the mutant cells define their position in the processing pathway by reference to Figure 13–8. Mutant C, for example, has lost no glucoses,

TABLE 13–8 Mutant and wild-type cell lines arranged in the order corresponding to the steps in the pathway for oligosaccharide processing (Answer 13–47).

Cell line	Man	GlcNAc	Gal	NANA	Glc	Site	Enzyme
Mutant C	9	2	0	0	3	ER	glucosidase I
Mutant H	9	2	0	0	2	ER	glucosidase II
Mutant D	9	2	0	0	0	ER	ER mannosidase
Mutant G	8	2	0	0	0	Golgi	Golgi mannosidase I
Mutant E	5	2	0	0	0	Golgi	GlcNAc transferase I
Mutant B	5	3	0	0	0	Golgi	Golgi mannosidase II
Mutant F	3	3	0	0	0	Golgi	GlcNAc transferase II
Mutant A	3	5	0	0	0	Golgi	galactose transferase
Mutant I	3	5	3	0	0	Golgi	NANA transferase
Wild type	3	5	3	3	0		

"Site" indicates the location of the processing step that is defective. The listed enzymes are the ones that are directly responsible for adding or removing sugars at the steps that are blocked. As indicated in the answer, some mutations in these cells might be in other enzymes.

therefore it must carry a defect in the first processing enzyme; namely, glucosidase I. Mutant H has lost one glucose, but retains the other two, therefore it must be defective in the second step of the pathway; namely, the one controlled by glucosidase II. Similar reasoning allows all the mutants to be identified with individual steps in the pathway in Figure 13–8, and thus ordered as shown in Table 13–8.

A more straightforward way to approach this problem is to begin by writing out the numbers of each kind of sugar that are present at each step in the pathway. You will find that the distribution of sugars at a step will match the distribution in a mutant, thereby allowing you to order the mutants in the pathway.

B. The site of oligosaccharide processing at which each of the mutants is defective is indicated in Table 13–8. Note that the oligosaccharide in mutant G was generated in the ER, but the step at which processing is blocked (that is, the next step) occurs in the Golgi.

C. The processing enzymes modify the *N*-linked oligosaccharide in one of two ways: they remove sugars or they add them. Mutants C, H, D, G, and B are defective in steps at which carbohydrate is removed; they are likely to be defective in the processing enzymes themselves. Mutants E, F, A, and I are defective in steps at which carbohydrate is added. These mutants may be defective in the processing enzymes; however, they could be defective in one of the enzymes responsible for synthesizing the sugar monomer, or the enzyme responsible for activating the sugar in preparation for addition (for example, synthesizing UDP-GlcNAc, the activated form of GlcNAc), or in the proteins responsible for transporting the sugar monomers into the lumen of the ER or Golgi.

13–48 In the vesicle transport model, vesicles carry proteins across the stack by budding from one cisterna and fusing with the next. It is this role in the forward movement of proteins that is the critical difference between the two models. Vesicles are also required to maintain the identity of each cisterna by capturing resident proteins that have escaped and returning them to the appropriate cisterna. This retrograde flow is also used to capture ER resident proteins that have escaped into the Golgi apparatus and return them to the ER.

In the cisternal maturation model, vesicles are not required to move proteins across the Golgi apparatus. Movement of the stacks themselves accomplishes the forward movement of proteins. Vesicles are still required to maintain the identity of individual cisternae, but in this model they are not returning escaped proteins, but rather are transferring proteins in a retrograde direction to a new residence because their old residence has changed identities, from a *cis* cisterna to a medial cisterna, for example. In this model, as in the vesicle transport model, vesicles are responsible for returning escaped ER proteins to the ER.

The critical difference between the two models is that the forward movement of proteins is accomplished by vesicles in the vesicle transport model and by movement of the cisternae themselves in the cisternal maturation model.

13–49

A. The radioactive label (GlcNAc) is added in the medial compartment, and the lectin precipitation depends on the presence of galactose, which is added in the *trans* compartment. Therefore, this experiment follows the movement of material between the medial and the *trans* compartments of the Golgi apparatus.

B. If proteins moved through the Golgi apparatus by cisternal maturation, then a protein that entered the Golgi in a mutant cell should remain with that stack and mature as the newly formed cisterna moves through the stack. Thus, the cisternal maturation model predicts that none of the labeled G protein (which was labeled in the medial compartment of the Golgi apparatus in the mutant cell) should have galactose attached to it (which could only have been added in the Golgi apparatus from the wild-type cell). For this model, the fusion of the infected mutant cells to uninfected wild-type cells (see Table 13–4, line 1) should be the same as the fusion of infected mutant cells to uninfected mutant cells (line 2).

   By contrast, if material moved through the Golgi apparatus by vesicular transport, there is the possibility that proteins might move between separated Golgi stacks inside transport vesicles. The vesicle transport model predicts that some labeled G protein may acquire galactose in this way. For this model, the fusion of infected mutant cells to uninfected wild-type cells (line 1) should yield more radioactive precipitate than fusion of infected mutant cells to uninfected mutant cells (line 2) but less than fusion of infected wild-type cells to uninfected wild-type cells (line 3).

C. The results in Table 13–4 support the vesicle transport model, since nearly half the labeled G protein acquired galactose. The extent of galactose addition is surprising because it suggests that once a vesicle leaves a cisterna, it has roughly an equal chance of fusing with a cisterna in the same or different Golgi stack. A number of other control experiments showed that the morphology of the Golgi stacks was unaltered by the fusion procedure, that the mutant and wild-type Golgi stacks remained distinct from one another, and that G protein did move into the wild-type Golgi stack.

Reference: Rothman JE, Miller RL & Urbani LJ (1984) Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane protein between two Golgi populations. *J. Cell Biol.* 99, 260–271.

MEDICAL LINKS

13–50 If therapeutic proteins with *N*-linked oligosaccharides were produced in nonprimate cells, they would carry occasional oligosaccharides with Gal(α1–3)Gal linkages. Since such linkages are not present on normal human proteins, the protein might be recognized as foreign by the

immune system, triggering production of antibodies against the protein. Such antibodies would eliminate the protein, along with any potential therapeutic benefit. Humans, who are periodically infected with microorganisms that contain Gal(α1–3)Gal linkages, already have circulating antibodies to this disaccharide, and are thus likely to eliminate the protein even more quickly.

Reference: Takeuchi Y, Porter CD, Strahan KM, Preece AF, Gustafsson K, Cosset FL, Weiss RA & Collins MK (1996) Sensitization of cells and retroviruses to human serum by (α1-3) galactosyltransferase. *Nature* 379, 85–88.

TRANSPORT FROM THE *TRANS* GOLGI NETWORK TO LYSOSOMES

DEFINITIONS

13–51 Autophagy

13–52 Vacuole

13–53 Lysosome

TRUE/FALSE

13–54 False. The proton pump in lysosomes pumps protons *into* the lysosome to maintain a low pH.

13–55 True. Endosomal membrane proteins are selectively retrieved from late endosomes by transport vesicles that deliver the proteins back to endosomes or to the *trans* Golgi network. The interior of late endosomes is mildly acidic (about pH 6), and as they mature into lysosomes the pH drops to the lysosomal value of pH 5.0.

13–56 False. Addition of a weak base would cause M6P receptors to accumulate in late endosomes. M6P receptors, which bind lysosomal enzymes quite well at neutral pH, normally release bound enzymes at the lower pH of the late endosome and are then recycled to the Golgi. If the pH of the late endosome were raised, M6P receptors could not release their bound enzymes, and because they could not be recycled, they would become trapped in the late endosome.

THOUGHT PROBLEMS

13–57 The lysosomal enzymes are all acid hydrolases, which have optimal activity at the low pH (about 5.0) in the interior of lysosomes. If a lysosome were to break, the acid hydrolases would find themselves at pH 7.2, the pH of the cytosol, and would therefore do little damage to cellular constituents.

13–58 As shown in **Figure 13–22**, an autophagosome formed by engulfment of a mitochondrion by the ER membrane will have four layers of membrane that separate the matrix of the mitochondrion from the cytosol. From outside to inside, the sources of membranes and spaces are ER membrane, ER lumen, ER membrane, cytosol, outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and matrix.

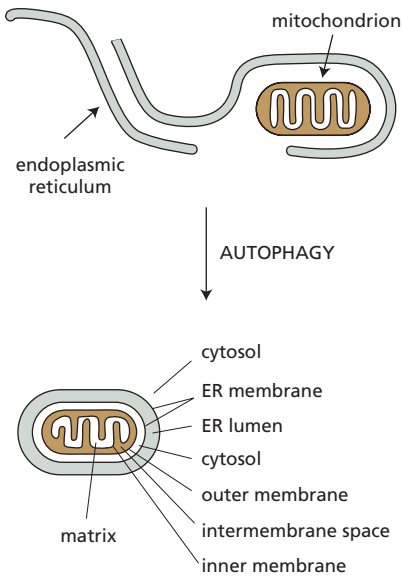


Figure 13–22 Engulfment of a mitochondrion by ER membrane to form an autophagosome (Answer 13–58).

13–59

A. If the pH in late endosomes were raised to pH 6.6, the M6P receptor would bind hydrolases in the normal way in the *trans* Golgi network

and transport them to late endosomes. At the higher endosomal pH, the receptor would not release the hydrolases and thus could not be recycled back to the *trans* Golgi network. Under these circumstances, the receptor might be dragged into mature lysosomes and destroyed.

B. If the pH in the *trans* Golgi network were lowered to pH 6, the M6P receptor would not bind to the lysosomal hydrolases, and thus could not deliver them to late endosomes via the principal transport pathway. Under these conditions, the hydrolases would exit the cell via the default pathway. Once outside the cell, where the pH is around 7, some hydrolases would bind to M6P receptors that tour through the plasma membrane and then be delivered to late endosomes, via endocytosis to early endosomes. In the late endosomes, the M6P receptors would release the bound hydrolases and recycle to the *trans* Golgi network in the normal way.

13–60 Adaptor proteins in general mediate the incorporation of specific cargo proteins into clathrin-coated vesicles by linking the clathrin coat to specific cargo receptors. Because melanosomes are specialized lysosomes, it would seem reasonable that the defect in AP3 affects the pathway for delivery of pigment granules from the *trans* Golgi network, which involves clathrin-coated vesicles. AP3 localizes to coated vesicles budding from the *trans* Golgi network, which is consistent with a function in transport from the Golgi to lysosomes. Interestingly, humans with the genetic disorder Hermansky–Pudlak syndrome have similar pigmentation changes, and they also have bleeding problems and pulmonary fibrosis. These symptoms are all thought to reflect deficiencies in the production of specialized lysosomes, which result from just a single biochemical defect.

References: Kantheti P, Qiao X, Diaz ME, Peden AA, Meyer GE, Carskadon SI, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL & Burmeister M (1998) Mutation in AP-3 delta in the *mocha* mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. *Neuron* 21, 111–122.

Zhen L, Jiang S, Feng L, Bright NA, Peden AA, Seymour AB, Novak EK, Elliott R, Gorin MB, Robinson MS & Swank RT (1999) Abnormal expression and subcellular distribution of subunit proteins of the AP-3 adaptor complex lead to platelet storage pool deficiency in the *pearl* mouse. *Blood* 94, 146–155.

DATA HANDLING

13–61 From the position of the melanosomes in *Ashen*, *Dilute*, and *Leaden* melanocytes, it appears that these mice have defects in transporting melanosomes to the tips of the branches so that the pigment can be properly released. The proteins that are missing in these mice normally form a complex, Rab27a/Mlph/MyoVa, which links melanosomes (via Rab27a) to a microtubule-based motor (MyoVa). That linkage allows melanosomes to be transported along microtubule tracks to the tips of the melanocyte branches. Once delivered, and prior to release, the melanosomes may be anchored to the cell cortex by the binding of Mlph to actin filaments. Defects in any of the individual proteins in the Rab27a/Mlph/MyoVa complex would prevent melanosome transport to the cell extremities, leaving them clustered together in the interior of the cell.

References: Wilson SM, Yip R, Swing DA, O'Sullivan TN, Zhang Y, Novak EK, Swank RT, Russell LB, Copeland NG & Jenkins NA (2000) A mutation in *Rab27a* causes the vesicle transport defects observed in *ashen* mice. *Proc. Natl Acad. Sci. USA* 97, 7933–7938.

Hume AN, Tarafder AK, Ramalho JS, Sviderskaya EV & Seabra MC (2006) A coiled-coil domain of melanophilin is essential for myosin Va recruitment and melanosome transport in melanocytes. *Mol. Biol. Cell* 17, 4720–4735.

MEDICAL LINKS

13–62 This striking result indicates that there must be a lysosomal delivery pathway that is independent of M6P and the M6P receptor. The M6P-independent pathway might operate inside the cell to accomplish sorting—presumably—from the *trans* Golgi to lysosomes, or as a scavenger pathway that picks up lysosomal enzymes from outside the cell and delivers them to lysosomes, where they are perfectly happy. Studies with M6P-receptor-deficient mice indicate that both types of pathways may operate. In thymocytes from such mice, lysosomal enzymes appear to be delivered via an intracellular route, whereas liver and skin cells can pick them up via an extracellular route.

Reference: Dittmer F, Ulbrich EJ, Hafner A, Schmahl W, Meister T, Pohlmann R & von Figura K (1999) Alternative mechanisms for trafficking of lysosomal enzymes in mannose 6-phosphate receptor-deficient mice are cell type-specific. *J. Cell Sci.* 112, 1591–1597.

13–63

A. The corrective factors are the lysosomal enzymes themselves. Hurler's cells supply the enzyme missing from Hunter's cells, and Hunter's cells supply the enzyme missing from Hurler's cells. These enzymes are present in the medium because of inefficiency in the sorting process. Since they carry M6P, which normally should direct them to lysosomes, they presumably escaped capture by the lysosomal pathway and were secreted. They are taken into cells and delivered to lysosomes by receptor-mediated endocytosis, which operates due to a small number of M6P receptors on the cell surface. The degradative enzymes, bound to receptors, are taken up through coated pits into endosomes and are eventually delivered to lysosomes. Since lysosomes are the normal site of action for these degradative enzymes, the defect is thereby corrected.
B. Protease treatment destroys the lysosomal enzymes themselves. Periodate treatment and alkaline phosphatase treatment both remove the M6P signal that is required for binding to the receptor, thus preventing the enzymes (which are still active) from entering the cell.
C. Such a scheme is unlikely to work for defects in cytosolic enzymes. External proteins normally do not cross membranes; thus, even when they are taken into cells, they remain in the lumen of a membrane-enclosed compartment. In addition, foreign proteins are usually delivered to lysosomes and degraded.

Reference: Kaplan A, Achord DT & Sly WS (1977) Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc. Natl Acad. Sci. USA* 74, 2026–2030.

13–64 The results with the hypothetical I-cell mutants indicate that cells from mutant A are defective in the receptor for M6P, that cells from mutant B are defective in GlcNAc phosphotransferase, and that cells from mutant C are defective in GlcNAc phosphoglycosidase. These defects can be deduced from the experimental observations as indicated below.

Cells from mutant A were unable to take up lysosomal enzymes from wild-type cells, indicating that they have defective M6P receptors (observation 1). They are not defective in the M6P marker, since the lysosomal enzymes they secrete can correct the defect in Hurler's cells (observation 2).

Cells from mutant B were able to take up lysosomal enzymes from wild-type cells (observation 1), indicating that they have functional M6P receptors. Since their secreted enzymes could not correct the defect in Hurler's cells (observation 2), they were not properly modified. The improper modification was not corrected by treatment with the enzyme that removes GlcNAc (observation 3), suggesting (by elimination) that the defect is in GlcNAc phosphotransferase.

Cells from mutant C were able to take up lysosomal enzymes from wild-type cells (observation 1), indicating that they have a functional M6P receptor. Since their secreted enzymes could not correct the defect in Hurler's cells (observation 2), they were not properly modified. The improper modification was corrected by treatment with the enzyme that removes GlcNAc (observation 3), indicating that the cells are defective in GlcNAc phosphoglycosidase.

TRANSPORT INTO THE CELL FROM THE PLASMA MEMBRANE: ENDOCYTOSIS

DEFINITIONS

13–65 Endocytosis

13–66 Multivesicular body

13–67 Macrophage

13–68 Pinocytosis

13–69 Caveola

13–70 Clathrin-coated pit

13–71 Receptor-mediated endocytosis

13–72 Caveolin

13–73 Early endosome

13–74 Phagocytosis

TRUE/FALSE

13–75 False. Not all particles that bind are ingested. Phagocytes have a variety of specialized surface receptors that are functionally linked to the phagocytic machinery of the cell. Only those particles that bind to these specialized receptors can be phagocytosed.

13–76 False. The LDL receptor and many other receptors enter coated pits irrespective of whether they have bound their specific ligands.

13–77 False. Many molecules that enter early endosomes are specifically diverted from the journey to late endosomes and lysosomes; they are recycled instead from early endosomes back to the plasma membrane via transport vesicles. Only those molecules that are not retrieved from endosomes are delivered to lysosomes for degradation.

13–78 False. During transcytosis, vesicles that form from either the apical or basolateral surface first fuse with early endosomes, then move to recycling endosomes, where they are sorted into transport vesicles bound for the opposite surface.

THOUGHT PROBLEMS

13–79 Since the surface area and volume of a macrophage do not change significantly over this time, the rate of exocytosis must also equal 100% of the plasma membrane each half hour.

13–80

A. Extracellular space
B. Cytosol
C. Plasma membrane
D. Clathrin coat
E. Membrane of deeply invaginated clathrin-coated pit
F. Captured cargo particles
G. Lumen of deeply invaginated clathrin-coated pit

13–81 Because lipid rafts are thicker than other areas of the plasma membrane, the membranes inside caveolae, which form from lipid rafts, are presumably also thicker. Thus, transmembrane proteins that collect passively in caveolae might be expected to have longer transmembrane segments than normal.

13–82 In the absence of bound Fe, transferrin does not interact with its receptor and circulates in the bloodstream until it catches an Fe ion. Once iron is bound, the iron–transferrin complex can bind to the transferrin receptor on the surface of a cell and be endocytosed. Under the acidic conditions of the endosome, the transferrin releases its iron, but the transferrin itself remains bound to the transferrin receptor, which is recycled back to the cell surface. The neutral pH of the blood causes the receptor to release the transferrin into the circulation, where it can pick up another Fe ion to repeat the cycle. (The iron released in the endosome moves on to lysosomes, and from there it is transported into the cytosol.)

This system allows cells to take up iron efficiently, even though the concentration of iron in the blood is extremely low. The iron bound to transferrin is concentrated at the cell surface by binding to transferrin receptors; it becomes further concentrated in clathrin-coated pits, which collect the transferrin receptors. In this way, transferrin cycles between the blood and endosomes, delivering the iron that cells need to grow.

CALCULATIONS

13–83

A. HRP does not bind to a specific cellular receptor and is taken up only by fluid-phase endocytosis. Since endocytosis is a continuous process, HRP gets taken up steadily at a rate that depends only on its concentration in the medium; thus, its uptake rate does not saturate. By contrast, EGF binds to a specific EGF receptor and is internalized by receptor-mediated endocytosis. The limit to the amount of EGF that gets taken up is set by the number of EGF receptors on the cells; when the receptors are saturated, no further increase in uptake occurs (except at enormously high concentrations, where fluid-phase endocytosis becomes significant).
B. At 40 nM, EGF is taken up at a rate of 16 pmol/hr, while at a 1000-fold higher concentration (40 μM), HRP is taken up at 2 pmol/hr. Since the uptake of HRP is linear, the rate at a 1000-fold lower concentration is expected to be 2×10^{-3} pmol/hr. Thus, at equal concentrations of 40 nM, EGF should be taken up 8000 times faster than HRP [(16 pmol/hr)/(2×10^{-3} pmol/hr)].

If EGF and HRP were present at 40 μM, both would be taken up by endocytosis at the same rate (2 pmol/hr). EGF, however, would also be taken up by receptor-mediated endocytosis at the saturation rate of 16 pmol/hr. Thus, EGF would be taken up 9 times faster than HRP [(2 pmol/hr + 16 pmol/hr)/(2 pmol/hr)].

C. An endocytic vesicle 20 nm (2×10^{-6} cm) in radius contains 3.4×10^{-17} mL of fluid.

$$\text{vesicle volume} = \frac{40\ \pi r^3}{3}$$

$$= (4/3) \times 3.14 \times (2 \times 10^{-6}\ \text{cm})^3$$

$$= 3.4 \times 10^{-17}\ \text{cm}^3 = 3.4 \times 10^{-17}\ \text{mL}$$

A solution of 40 μM HRP contains 2.4×10^{16} molecules/mL of HRP.

$$\text{HRP} = \frac{40\ \mu\text{mol HRP}}{\text{L}} \times \frac{\text{L}}{1000\ \text{mL}} \times \frac{6 \times 10^{17}\ \text{molecules}}{\mu\text{mol}}$$

$$= 2.4 \times 10^{16}\ \text{molecules/mL}$$

Hence each vesicle contains, on average, 0.8 molecule of HRP [$(2.4 \times 10^{16}$ molecules/mL$)(3.4 \times 10^{-17}$ mL/vesicle)].

D. These calculations, as alluded to by the authors, make the point that by having specific tight-binding receptors on the cell surface, cells can take up molecules from their surroundings at much higher rates—several orders of magnitude higher—than they could simply by taking in fluid, especially at the low concentrations that are typical in biology. Fishing provides an analogy. You could fish by taking random netfuls from a stream, and occasionally you might catch a fish. But if you put bait where you cast your net, you increase your chances of success enormously. Each time a molecule of EGF hits a receptor, it sticks and subsequently makes its way to a coated pit to be internalized. If the EGF were simply trapped like HRP, its rate of uptake would be infinitesimal at the usual *in vivo* concentrations.

Reference: Haigler HT, McKanna JA & Cohen S (1979) Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J. Cell Biol.* 83, 82–90.

13–84 If all the receptors were bound to ligand, there would be 10 ligands in a vesicle with a volume of 1.66×10^{-18} L, which is 10 μM [(10 ligands/1.66×10^{-18} L) × (mole/6×10^{23} ligand) = 10^{-5} mole/L or 10 μM]. This concentration is 10,000 times higher than the circulating concentration (1 nM) in the extracellular fluid.

In order to concentrate the ligand 1000-fold in the vesicle, only 1 in 10 of the receptors would need to carry a bound ligand. If the K_d were 1 nM (10^{-9} M), half the receptors would be occupied by ligand. From the rule-of-thumb relationships worked out in Problem 3–86 (see Table 3–5), if the K_d were 10-fold higher than the ligand concentration, only 10% of the ligand would be bound to the receptor, which in this case corresponds to 1 in 10 of the receptors in the vesicle having a bound ligand. Thus, the K_d for the receptor–ligand binding would need to be 10 nM (10^{-8} M) to concentrate the ligand 1000-fold above the ligand concentration in the extracellular fluid.

13–85

A. At 0°C, endocytosis is blocked and the labeled transferrin receptors are trapped on the cell surface and accessible to trypsin treatment. After 1 hour at 37°C, most of the receptors in intact cells (≈70%) are not sensitive to trypsin because they are inside the cell (presumably in endosomes) and, therefore, are not accessible. When cells are incubated at 37°C, the labeled receptors are endocytosed and cycle through the endosomal compartment of the cell, thereby becoming inaccessible to trypsin.

B. Both trypsin treatment and antibody binding indicate that 30% of the total transferrin receptor is on the cell surface after 1 hour at 37°C. When the transferrin receptors are allowed to recycle by incubation at 37°C, 30% is accessible to trypsin treatment of intact cells; therefore, 30% is on the surface. Similarly, antibody binds to 30% of the total receptor in the absence of detergent (0.54%/1.76% = 30%). Recycling of transferrin receptors is very fast, and this distribution between the surface and internal compartments turns out to be the equilibrium distribution for transferrin receptors.

Reference: Bleil JD & Bretscher MS (1982) Transferrin receptor and its recycling in HeLa cells. *EMBO J.* 1, 351–355.

MEDICAL LINKS

13–86

A. Binding of LDL by normal cells and JD's cells reaches a plateau because there are a limited number of LDL receptors per cell and they become saturated at high levels of LDL. The slope of the binding curve gives a measure of the binding affinity and the plateau gives a measure of the total number of binding sites (about 20,000 to 50,000, though you could not calculate this from the data shown here). JD has slightly fewer receptors on his cells, but they have an affinity similar to those in normal cells.

Cells from patient FH bind essentially no LDL, even at saturating external LDL levels. Either these cells completely lack the LDL receptor, or the receptor is defective so that its affinity for LDL is drastically reduced. It could also be that the cells do contain receptors, but for some reason they fail to appear on the surface of the cell.

B. Cells from the hypercholesterolemic patients take up LDL at a very low rate. Lack of entry is readily explained for patient FH because no LDL bound to the cells. This result indicates that the receptor is crucial for LDL cholesterol to enter cells. Since LDL is not taken up by JD's cells, his LDL receptors must also be defective, but in a different way from FH's LDL receptors. JD's cells bind LDL with the same affinity as normal and almost to the same level. Although his receptors are normal as far as LDL binding is concerned, the bound LDL does not get in at the normal rate. Thus, mere possession of a receptor on the cell surface is no guarantee of entry.

C. LDL must enter cells in order for the cholesterol esters to be released and hydrolyzed to cholesterol, which causes inhibition of cholesterol synthesis. In the affected patients, LDL enters the cells very slowly and, therefore, inhibits cholesterol synthesis only slightly.

D. If the defects in the hypercholesterolemic patients are due to defects in their LDL receptors, then free cholesterol should inhibit cholesterol synthesis in their cells as well as in normal cells. Free cholesterol does inhibit cholesterol synthesis in all these cells, strongly supporting the idea that the defects in the patients are due solely to problems with their LDL receptors.

Reference: Brown MS & Goldstein JL (1979) Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc. Natl Acad. Sci. USA* 76, 3330–3337.

13–87

A. JD's mother evidently has one normal gene and one defective gene that encodes a receptor that cannot bind LDL (and hence does not "show up" in any of the assays). With a single good gene, her cells synthesize half the usual number of functional LDL receptors, as confirmed by electron microscopy. These receptors are distributed about 50% in and

50% outside of coated pits, as are the receptors in a normal person, and they can internalize bound LDL. JD's mother has a mild hypercholesterolemia because, with only half the usual number of functional receptors, she cannot clear LDL from the circulation as efficiently as normal individuals. Since JD can internalize none of his LDL receptors, she must have passed the defective gene to her son.

B. Several peculiar observations must be accounted for to understand the behavior of JD's father's LDL receptors: (1) his cells bind more LDL than normal cells, but internalize less than half the bound LDL; (2) his cells carry about 50% more LDL receptors on their surface than normal cells; and (3) only about 20% of the receptors are associated with coated pits. These observations can be understood if JD's father carries one normal gene for the LDL receptor and one gene that encodes an LDL receptor that cannot be internalized. The one normal copy of the gene allows JD's father to clear some circulating LDL, but not as efficiently as a person with two functional genes, accounting for his mild hypercholesterolemia.

   The father's defective gene, unlike the mother's, encodes an LDL receptor that can bind LDL, but cannot internalize it. This defective receptor is more numerous on the cell surface precisely because it cannot be internalized. A normal LDL receptor is constantly cycling from the cell surface to the interior and back, carrying any bound LDL inside. Thus, at any given time, a portion of the normal LDL receptor population is in the interior and unavailable for surface binding. By contrast, the entire population of internalization-defective LDL receptors will be on the cell surface, available for LDL binding. This explains why the father's cells have more LDL receptors on their surface and bind more LDL than normal cells, but internalize less than half. Because the excess LDL receptors on the cell surface are located outside of coated pits, the defective receptors must not be able to enter coated pits and bind there.

C. JD's inability to metabolize LDL is a direct consequence of acquiring two defective forms of the LDL receptor gene from his parents. JD received one binding-defective LDL receptor gene from his mother and one internalization-defective LDL receptor gene from his father. Since both genes are defective, JD has severe hypercholesterolemia. The behavior of the receptors on JD's cells is like his father's defective population of receptors. The near-normal number of receptors and amount of LDL binding occur because greater numbers of the internalization-deficient receptors are present on the cell surface.

   The inability of these receptors to associate with coated pits, which was inferred from the behavior of the mixture of receptors in his father, is clear in JD. In JD's cells, only 2.8% of his LDL receptors are found in coated pits, close to what might be expected from a purely random distribution (coated pits occupy about 2% of the cell surface). This distribution suggests that JD's LDL receptors are defective in the domain that is necessary to localize the receptor in coated pits.

D. These studies show clearly that JD's defective LDL metabolism is due to defective receptors, not to defective internalization machinery. The critical observations are the ones with JD's father. The behavior of the defective population of receptors in the father is very much like the behavior of JD's receptors. The defect in this population of receptors in JD's father cannot be due to a defect in the internalization machinery. If the defect were in the internalization machinery, all of the bound LDL in JD's father would behave the same. Instead, half behaves normally, and half never seems to get in.

Reference: Brown MS & Goldstein JL (1979) Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc. Natl Acad. Sci. USA* 76, 3330–3337.

TRANSPORT FROM THE *TRANS* GOLGI NETWORK TO THE CELL EXTERIOR: EXOCYTOSIS

DEFINITIONS

13–88 Synaptic vesicle

13–89 Constitutive secretory pathway (or default pathway)

13–90 Secretory vesicle

13–91 Exocytosis

13–92 Regulated secretory pathway

TRUE/FALSE

13–93 False. Secretory proteins, even those that are not normally expressed in a given secretory cell, are appropriately packaged into secretory vesicles. For this reason, it is thought that the sorting signal is common to proteins in this class.

13–94 False. Once positioned beneath the plasma membrane, a secretory vesicle waits until the cell receives an appropriate signal—often a rise in Ca^{2+} concentration—before fusing with the membrane and releasing its contents.

THOUGHT PROBLEMS

13–95 In a cell capable of regulated secretion, the three main classes of protein that must be sorted before they leave the *trans* Golgi network are (1) those destined for lysosomes, (2) those destined for secretory vesicles, and (3) those destined for immediate delivery to the cell surface.

13–96

A. Vesicles on the endocytic pathway will contain transferrin, and thus be labeled with colloidal gold; vesicles on the exocytic pathway will contain albumin, and thus be labeled with ferritin.

B. Clathrin-coated vesicles are rapidly uncoated after they pinch off from the plasma membrane, so some will be caught with their coats off, while others will still have their coats on.

13–97 Aggregates of the secretory proteins would be expected to form in the ER, just as they do in the *trans* Golgi network. Because the aggregation is specific for secretory proteins, ER proteins would be largely excluded from the aggregates. It is likely that such aggregates would eventually be degraded by the quality control mechanisms that operate in the ER.

13–98 Synaptic transmission involves the release of neurotransmitters by exocytosis. During this event, the membranes of the synaptic vesicles fuse with the plasma membrane of the nerve terminals. To make new synaptic vesicles, membrane must be retrieved from the plasma membrane by endocytosis. This endocytosis step is blocked if dynamin is defective, as expected since dynamin is required to pinch off the clathrin-coated endocytic vesicles. Thus, the *shibire* mutant flies are paralyzed at the elevated temperature because they cannot recycle their synaptic vesicle membranes.

Reference: Koenig JH & Ikeda K (1999) Contribution of active zone subpopulation of vesicles to evoked and spontaneous release. *J. Neurophysiol.* 81, 1495–1505.

DATA HANDLING

13–99 Your experiments show that vesicles transport G protein without concentrating their contents. The concentration of G protein in the cisternal space was actually slightly higher than in the vesicles and vesicle buds, as measured by linear and surface density of labeled G proteins (see Table 13–6). If the vesicles were transporting G protein in a selective way (like clathrin-coated vesicles), the concentration of G protein in the vesicles should have been substantially higher than in the Golgi cisternae.

Reference: Orci L, Glick BS & Rothman JE (1986) A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* 46, 171–184.

13–100 The pro-peptide is removed from pro-insulin in immature secretory vesicles. The red fluorescence in compartments from the ER through the *trans* Golgi network indicates that they contain only pro-insulin. The green fluorescence in mature secretory vesicles indicates that they contain insulin. The yellow fluorescence, which arises when both the red and green fluorophores are excited in the same place—the combination of red and green light is yellow—indicates that pro-insulin and insulin are both present in immature secretory vesicles. Thus, immature secretory vesicles must be where the pro-peptide is removed. The absence of label in lysosomes, mitochondria, and nuclei (and other compartments) provides assurance that you are indeed following the secretory pathway.

13–101

A. If the mechanism of sorting in polarized cells involved a signal-dependent pathway to one domain of the plasma membrane and a default pathway to the other, a foreign protein would be expected to follow one or the other pathway. Since foreign proteins would not be expected to contain the signal responsible for specific sorting, they would be more likely to follow the default pathway.
B. The equal delivery of foreign proteins to the apical and the basolateral surfaces of polarized MDCK cells is not in agreement with the simplest expectations of the proposed sorting mechanism. Thus, the hypothesis that there is one signal-dependent pathway and one default pathway, as formulated, seems to be incorrect for polarized MDCK cells. Whatever pathway is being followed by the foreign proteins in these experiments is indifferent to which part of the cell surface it delivers its cargo.

References: Gottlieb TA, Beaudry G, Rizzolo L, Colman A, Rindler MJ, Adesnik M & Sabatini DD (1986) Secretion of endogenous and exogenous proteins from polarized MDCK cell monolayers. *Proc. Natl Acad. Sci. USA* 83, 2100–2104.

Kondor-Koch C, Bravo R, Fuller SD, Cutler D & Garoff H (1985) Exocytotic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. *Cell* 43, 297–306.

13–102 Antibodies specific for the cytoplasmic domain of synaptotagmin do not stain the nerve terminals because the cytoplasmic domain is never exposed on the outside of the cell. By contrast, the lumenal domain is exposed to the outside of the cell when the synaptic vesicle fuses with the plasma membrane to release neurotransmitter molecules into the synaptic cleft. At that time, the antibody can bind to the lumenal domain of synaptotagmin. The membrane of the synaptic vesicle is quickly retrieved from the plasma membrane and reused to form new synaptic vesicles that contain bound antibodies within them. When the fusion of synaptic vesicles with the plasma membrane is stopped by lowering the temperature to 0°C, no labeling is observed.

Reference: Matteoli M, Takei K, Perin MS, Südhof TC & De Camilli P (1992) Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell Biol.* 117, 849–861.

13–103

A. The rapid component of the fusion response is due to vesicles that are already docked at the membrane and waiting for the signal to fuse and release their contents. The slow component of the fusion process is due to those vesicles that are not already docked and waiting; they are in various states of preparation for the "ready-to-go" state.
B. NSF in some way must be required for a step in the preparation for the ready-to-go state. Thus, when NSF is inhibited, the slow component of the fusion process is also inhibited. Interference with a preparation step would also explain why inhibition of NSF blocks the rapid component only after the second flash. Nearly all the ready-to-go vesicles fuse after the first flash. In the absence of NEM (active NSF), the pool of ready-to-go vesicles is repopulated in the 2 minutes between flashes. But when NSF is inhibited, the pool remains depleted, giving rise to a much reduced rapid component in response to the second flash.
C. These results show very clearly that NSF does not control the final step in fusion, otherwise its inhibition would have blocked the initial rapid fusion in response to the first flash (see Figure 13–18B, +NEM). They also point to a role of NSF in a preparatory (early) step by showing that the slow component is inhibited after both flashes, and that the rapid component is inhibited after the second flash.
D. NSF-mediated ATP hydrolysis is required to disentangle v- and t-SNAREs after the fusion event. Vesicles that are docked and waiting already have their SNAREs paired, but are probably kept from fusing by a regulatory protein. In response to Ca^{2+}, the regulatory protein is thought to release the brake on the paired SNAREs so they can complete the fusion event. For a vesicle to re-form so it can dock and fuse again, its SNAREs must be pried apart. If they are not, the vesicle cannot dock with the membrane. It is this step in the recycling of SNAREs that requires NSF.

Reference: Xu T, Ashery U, Burgoyne RD & Neher E (1999) Early requirement for α-SNAP and NSF in the secretory cascade in chromaffin cells. *EMBO J.* 18, 3293–3304.

MEDICAL LINKS

13–104 The actual explanation is that the single amino acid change causes the protein to misfold slightly so that, although it is still active as a protease inhibitor, it is prevented by chaperone proteins in the ER from exiting the cell. It therefore accumulates in the ER lumen and is eventually degraded. Alternative interpretations might have been: (1) the mutation affects the stability of the protein in the bloodstream so that it is degraded faster than the normal protein; (2) the mutation inactivates the ER signal sequence and prevents the protein from entering the ER; or (3) the mutation created an ER (or Golgi) retrieval signal so that the protein was continually returned to the ER (or Golgi). One could distinguish among these possibilities by using fluorescently tagged antibodies against the protein to follow its transport in the cells.

MCAT STYLE

13–105

D. Ricin is a large protein, so it must be taken into the cell via endocytosis. Choices A and C are not correct because clathrin and SNARE proteins work in the cytoplasm, so they would not be accessible to ricin outside the cell. Choice B is incorrect because there are no known pore complexes in the plasma membrane.

13–106

B. Once ricin enters the cell via endocytosis, it makes its way from endosomes to the ER via the Golgi apparatus. Retrograde transport from the Golgi to the ER uses COPI-coated vesicles. Choice A is not directly involved in the fusion of vesicles with their target membranes, a process that involves SNAREs. After fusion, however, the SNAREs need to be pried apart by NSF before they can be used again. Thus, NSF would be required for a continuous flow of ricin to the ER, but would not be required for the initial delivery. Choice C is incorrect because M6P receptors are used to concentrate various lysosomal hydrolases into clathrin-coated vesicles for antegrade transport from the *trans* Golgi network to lysosomes. Choice D is not correct because the Sar1 protein is required for assembly of COPII coats at the ER membrane and those vesicles are used for antegrade transport to the Golgi apparatus.

13–107

D. To get from the ER to the cytosol, ricin must cross the ER membrane, and the only known route is the pathway that translocates unfolded proteins into the cytosol for degradation. Ricin triggers this pathway by partially unfolding in the lumen of the ER. It avoids degradation by refolding correctly in the cytosol. Choices A, B, and C are not correct because they do not describe known pathways.

13–108

A. If the viral protein moves as a free protein—not enclosed in a vesicle—it will be exposed to the protease and degraded. If the viral protein moves from stack to stack inside a vesicle, however, it will be protected from the protease and remain intact. The experimental results showed that addition of a protease did not interfere with transport of viral protein, eliminating the possibility that the protein moves between Golgi apparatuses as a free protein. Choice B is not correct because clathrin is not used for transport between Golgi stacks. Choice C is not correct because it could only show that the modified viral protein is associated with membranes, which is the expected result for either hypothesis. Choice D is not correct because detergent would solubilize all membranes, which would not provide any relevant information.

13–109

C. Because all components—except the acceptor membranes—were present initially, donor vesicles are expected to form normally. When NSF is inactivated prior to addition of the acceptor Golgi membranes, however, no vesicle transport was detected. Thus, NSF must be required in some fashion for fusion of donor vesicles to the acceptor Golgi apparatus. Choices A and B are not correct because the experimental design does not provide any information about the possible requirement for NSF in these processes. Because NSF was present during formation of the donor vesicles, it is not possible to conclude whether NSF is (or is not) required for donor vesicle formation. Choice D is not correct because the viral protein is a membrane protein; it is not released into the acceptor Golgi.

13–110

C. Statements I and III are correct. Statement I states that cleavage of synaptobrevin leads to muscle paralysis. If synaptobrevin activity is blocked, synaptic vesicles will not be able to fuse with the presynaptic plasma membrane to release the neurotransmitter that triggers muscle contraction. Statement III is correct because the location of synaptobrevin on vesicles and syntaxin on the plasma membrane was a crucial piece of evidence that the specificity of fusion events could be dictated by pairs of v-SNAREs and t-SNAREs that bind to one another. Statement II is not correct. The energy for membrane fusion does not derive from ATP; it

comes from the strong binding of SNAREs to one another. The energy of ATP is required to pry the SNAREs apart.

13–111

D. NSF and ATP are required for fusion of transport vesicles with natural membranes because the resident v-SNAREs and t-SNAREs are tightly bound to one another as a result of previous fusion events. They must be separated—a process that requires ATP hydrolysis—before they can carry out another fusion event. In the system using lipid vesicles and purified v-SNARES and t-SNAREs, the SNAREs are already separated; thus, no energy is required. Choice A is not correct because the binding of v-SNAREs and t-SNAREs to one another provides the energy for fusion between natural membranes; high concentration is not necessary. Choice B is not correct because there is no cytosolic inhibitor that binds to SNARE proteins to prevent fusion. Choice C is incorrect because the extraneous proteins in natural vesicles and their target membranes do not require any additional energy to move out of the way. The fluid mosaic structure of the membrane ensures free diffusion of proteins in the plane of the membrane (unless they are anchored in place).

References: Balch WE, Dunphy WG, Braell WA & Rothman JE (1984) Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* 39, 405–416.

Malhotra V, Orci L, Glick BS, Block MR & Rothman JE (1988) Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell* 54, 221–227.

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P & Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318–324.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner T & Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. *Cell* 92, 759–772.

Energy Conversion: Mitochondria and Chloroplasts

CHAPTER 14

IN THIS CHAPTER

THE MITOCHONDRION

DEFINITIONS

14–1 Intermembrane space

14–2 Respiratory chain

14–3 Chemiosmotic coupling

14–4 Oxidative phosphorylation

14–5 Electrochemical gradient

14–6 Outer mitochondrial membrane

TRUE/FALSE

14–7 False. The intermembrane space is chemically equivalent to the cytosol with respect to small molecules, not because of the presence of any specialized transport proteins in the mitochondrial outer membrane, but because this membrane contains many copies of the channel-forming protein porin, which forms large aqueous channels. These channels convert the outer membrane into a sieve that allows free passage of all molecules of molecular mass less than 5000 daltons, which includes all the ions and metabolites in a cell and some small proteins.

14–8 True. When the citric acid cycle is operating as a cycle of reactions, its sole products are CO_2 and reduced forms of the electron carriers NADH and $FADH_2$. The electrons in NADH and $FADH_2$ are passed into the electron-transport chain to generate ATP via oxidative phosphorylation.

14–9 True. The natural (thermodynamic) tendency of electrons is to move from low-affinity to high-affinity carriers.

THOUGHT PROBLEMS

14–10 The characteristic organization of mitochondria in liver cells and cardiac muscle cells is likely to reflect functional differences in these cells. Most of the energy needs of liver cells derive from metabolic processes in the cytosol, whereas the energy needs of cardiac muscle cells result primarily from contractions of the myofibrils. Mitochondria are thus positioned in these cells at the sites where energy, in the form of ATP, is most required.

14–11 The cristae membranes, which contain the complexes of the electron-transport chain and ATP synthase, are the sites of oxidative phosphorylation and produce most of the cell's ATP. Mitochondria that have a higher density of cristae therefore have a greater capacity to carry out oxidative

phosphorylation. Heart muscle expends a lot of energy during its continuous contractions, whereas skin cells have a lesser energy demand. An increased density of cristae therefore increases the ATP-production capacity of the heart muscle cell. This is a remarkable example of how cells adjust the abundance of their individual components according to need.

14–12 In the presence of oxygen, yeast can generate about 15 times more ATP from each glucose molecule than they can in the absence of oxygen. Thus, to meet their energy needs, they need to process about 15-fold fewer glucose molecules; hence the dramatic drop in glucose consumption when O_2 is introduced.

14–13 The citric acid cycle uses NAD^+ and FAD to make NADH and $FADH_2$. Oxidative phosphorylation converts NADH and $FADH_2$ back to NAD^+ and FAD. The cycling of these cofactors between the citric acid cycle and oxidative phosphorylation is critical because they are present in very small amounts relative to the quantity of acetyl CoA that is metabolized in the cycle. In the absence of oxygen, electron transport and ATP synthesis do not occur; thus, NADH and $FADH_2$ cannot be converted back to NAD^+ and FAD. As a result, the citric acid cycle quickly converts the available NAD^+ and FAD into NADH and $FADH_2$ and then grinds to a halt. The requirement for cycling of cofactors is a general principle of metabolic regulation: it provides a rough coordination of flow through pathways that are not otherwise connected.

14–14

A. DNP will collapse the electrochemical gradient completely. Protons that are pumped across the inner membrane will be carried back across the membrane by DNP, and therefore no energy can be stored across the membrane.

B. The electrochemical gradient is made up of two components: an H^+ concentration difference and an electrical potential (the membrane potential). If the membrane is made permeable to K^+ with valinomycin, K^+ ions will be driven into the matrix by the membrane potential of the inner membrane (negative inside, positive outside). The influx of positively charged K^+ ions will eliminate the membrane potential. By contrast, the concentration component of the electrochemical gradient (the pH difference) is unaffected by valinomycin. As a result, only part of the driving force that makes it energetically favorable for protons to flow back into the matrix will be lost.

14–15 The antiport of the amino acids Asp and Glu (V) is electrically neutral and doesn't involve a proton; thus, it is unaffected by the electrochemical gradient. All the rest are affected by the gradient. The symport of pyruvate and H^+ (I) is electrically neutral, but because a proton is transported into the matrix, it is driven by the ΔpH component of the electrochemical gradient. The antiport of citrulline and ornithine (II), which are components of the urea cycle, is driven by the membrane-potential component of the electrochemical gradient. The symport of phosphate with two protons (III) is driven by the ΔpH component of the electrochemical gradient. The antiport of citrate plus a proton with malate (IV) occurs against the ΔpH component of the electrochemical gradient.

CALCULATIONS

14–16 It would take the heart 6 seconds to consume its steady-state levels of ATP. Because each pair of electrons reduces one atom of oxygen, the 12 pairs of electrons generated by oxidation of one glucose molecule would reduce 6 O_2. Thus, 30 ATP are generated per 6 O_2 consumed. At steady

state, the rate of ATP production equals its rate of consumption. The time in seconds required to consume the steady-state level of ATP is

$$\text{time} = \frac{5\ \mu\text{mol ATP}}{\text{g}} \times \frac{6\ O_2}{30\ \text{ATP}} \times \frac{\text{min g}}{10\ \mu\text{mol } O_2} \times \frac{60\ \text{sec}}{\text{min}}$$
$$= 6\ \text{sec}$$

THE PROTON PUMPS OF THE ELECTRON-TRANSPORT CHAIN

DEFINITIONS

14–17 Cytochrome *c* oxidase complex

14–18 Cytochrome

14–19 Iron–sulfur center

14–20 Redox reaction

14–21 Redox potential

TRUE/FALSE

14–22 True. The flow of electrons down the respiratory chain moves from carriers with lower affinity for electrons to ones with higher affinity and finally to oxygen, which has the highest affinity of all. Thus, the relative positions of cytochromes and iron–sulfur centers make sense in terms of the natural flow of electrons to oxygen.

14–23 True. Although the three respiratory enzyme complexes can exist as independent entities in the mitochondrial inner membrane, the transfers of electrons between the complexes mediated by the two mobile carriers—ubiquinone and cytochrome *c*—are facilitated by the formation of a larger structure.

THOUGHT PROBLEMS

14–24 H^+ ions move much faster than Ca^{2+} ions through an aqueous solution because their "movement" is virtual; the H^+ ion that appears on one side of the cell is not the same one that started on the other side. H^+ ion movement, instead, depends on exchanges of hydrogen bonds for covalent bonds in a chain of water molecules. By contrast, a Ca^{2+} ion must actually diffuse through the medium.

The difference in the nature of the movements of these two ions is nowhere better illustrated than by their behavior in ice. As expected, the rate of diffusion of Ca^{2+} ions decreases considerably. Surprisingly, H^+ ions move even more rapidly. That is because H^+ movement depends on chains of water molecules. In ice, most of the water molecules are linked into chains, allowing H^+ ions to move very rapidly over long distances. In liquid water, the chains involve only a few water molecules, which means that there are periodic delays as H_3O^+ ions connect up with a new chain.

14–25 A hydrogen atom is a proton plus one electron; it is neutral. A proton is a hydrogen atom that has lost its single electron and thus is positively charged. A hydride ion is a hydrogen atom that has gained an extra electron and thus is negatively charged. A hydrogen molecule is a pair of

hydrogen atoms that share their two electrons in a covalent bond; it is neutral.

14–26 The E_0' values for the electron carriers in Table 14–1 suggest that they appear in the respiratory chain in the same order as they appear (top to bottom) in the table. Remember that E_0' values refer to standard conditions (reactants and products at 1 M). You would really need to know the E values to know the order of the carriers in the chain. The concentration terms might be especially important in distinguishing the order of ubiquinone and cytochrome *b*, and the orders of cytochromes c_1, *c*, and *a*. Despite these caveats, the order of carriers in the respiratory chain is as listed in Table 14–1.

14–27 Treatment with sodium nitrite oxidizes the Fe^{2+} in a proportion of hemoglobin molecules to Fe^{3+}. Because there is so much more hemoglobin than the cytochrome *c* oxidase complex, the Fe^{3+} form of hemoglobin competes effectively with the cytochrome *c* oxidase for the binding of cyanide. Treatment with sodium nitrite also impairs the oxygen-carrying function of hemoglobin, which will bind oxygen only when the heme group carries an Fe^{2+} ion. The treatment is therapeutically effective because there is a useful middle range where sufficient hemoglobin has been converted to the Fe^{3+} form to bind up the cyanide, but adequate Fe^{2+} hemoglobin remains to carry oxygen.

14–28 It is unlikely that the same diffusible carrier could be used productively for both steps. Such a carrier would need to have a redox potential and a binding specificity that would allow it to accept electrons from the NADH dehydrogenase complex and pass them on to cytochrome *c* reductase, and to accept electrons from cytochrome *c* reductase and pass them on to the cytochrome *c* oxidase complex. Leaving aside the tricky problem of donating and receiving electrons from cytochrome *c* reductase, such an arrangement would be counterproductive because it would allow frequent, if not inevitable, bypass of cytochrome *c* reductase: electrons could be accepted from the NADH dehydrogenase complex and passed directly to the cytochrome *c* oxidase complex. This short-circuit of the standard flow would waste the energy normally harvested by cytochrome *c* reductase, releasing it as heat instead.

14–29 In the presence of an artificially large electrochemical gradient, you would expect electrons to flow in the reverse of their normal direction. This is a straightforward expectation of thermodynamics and the principle of microscopic reversibility. The imposed large electrochemical gradient provides the thermodynamic driving force; that is, the free-energy change (ΔG) of the gradient is sufficiently negative to overcome the normally positive free-energy change of moving electrons from carriers with high affinity to carriers with lower affinity (the reverse of normal). The principle of microscopic reversibility provides a mechanism for doing so. If electron flow down the respiratory chain is coupled to proton pumping out of the matrix, then the same mechanism must be able to operate in reverse to couple proton movement into the matrix (backward through the proton pumps) to electron flow up the respiratory chain.

14–30 For all ATP synthases that use a proton-motive force to generate ATP, the flow of protons is to the same side of the membrane on which ATP is made. For a bacterium that lives in an alkaline environment, the natural flow of protons would be from the inside of the cell to the outside. Thus, the proton flow is in the wrong direction to allow a normally oriented ATP synthase to make ATP; in fact, outward proton flow would cause ATP hydrolysis. Even if the ATP synthase were reversed in the membrane, so that it could use the proton flow, it would then make ATP on the outside of the cell—not a very useful arrangement.

Bacteria of this kind have adapted to use a gradient of another ion such as Na^+ to drive their ATP synthases, suggesting that using another ion probably is the simplest adaptation accessible to evolution. As a purely theoretical engineering problem, other solutions can be imagined. Structurally, the ATP synthase consists of a proton-powered motor embedded in the membrane, an ADP to ATP converter, and a drive shaft that connects the two. Normally, an *inward* proton flow through the motor drives a *counterclockwise* rotation of the drive shaft inside the converter to cause the *normal* sequence of conformational changes that converts ADP to ATP. (An *outward* flow drives a *clockwise* rotation of the shaft that causes the *reverse* sequence of conformational changes, converting ATP to ADP.) With this scheme in mind, you might consider re-engineering the motor so that an *outward* flow drives a *counterclockwise* rotation of the drive shaft. Alternatively, you might re-engineer the converter so that a *clockwise* rotation of the drive shaft inside it would drive the *normal* sequence of conformational changes. Either of these changes would allow the re-engineered ATP synthase to make ATP from the proton gradient in an alkaline environment.

CALCULATIONS

14–31

A. With all reactants and products at 1 M, the concentration term reduces to zero; thus, ΔE is equal to $\Delta E_0'$, and ΔG is

$$\begin{aligned}\Delta G &= -nF\,\Delta E \\ &= -2 \times \frac{96\ \text{kJ}}{\text{V mole}} \times 0.13\ \text{V} \\ &= -25\ \text{kJ/mole}\end{aligned}$$

B. When the ratio of lactate to pyruvate equals 1 and the ratio of NAD^+ to NADH equals 1, the overall concentration term reduces to zero; thus, ΔE is equal to $\Delta E_0'$, and ΔG equals –25 kJ/mole.

C. In order for ΔG to be zero, the concentration term must equal 15,600. When ΔG is zero, ΔE is also zero, thus

$$\Delta E_0' = \frac{2.3\,RT}{nF} \log \frac{[\text{lactate}][\text{NAD}^+]}{[\text{pyruvate}][\text{NADH}]}$$

$$0.13\ \text{V} = 0.031\ \text{V} \log \frac{[\text{lactate}][\text{NAD}^+]}{[\text{pyruvate}][\text{NADH}]}$$

$$4.19 = \log \frac{[\text{lactate}][\text{NAD}^+]}{[\text{pyruvate}][\text{NADH}]}$$

$$15{,}600 = \frac{[\text{lactate}][\text{NAD}^+]}{[\text{pyruvate}][\text{NADH}]}$$

D. At an NAD^+ to NADH ratio of 1000, and a lactate to pyruvate ratio of 5.1 (0.77/0.15), ΔE is

$$\begin{aligned}\Delta E &= \Delta E_0' - \frac{2.3\,RT}{nF} \log(5.1 \times 1000) \\ &= 0.13\ \text{V} - 0.031\ \text{V} \log(5100) \\ &= 0.13\ \text{V} - 0.115\ \text{V} = 0.015\ \text{V}\end{aligned}$$

And ΔG is

$$\begin{aligned}\Delta G &= -nF\,\Delta E \\ &= -2 \times \frac{96\ \text{kJ}}{\text{V mole}} \times 0.015\ \text{V} \\ &= -2.9\ \text{kJ/mole}\end{aligned}$$

Thus, under normal conditions in vascular smooth muscle, the reaction is slightly in favor of reduction of pyruvate to lactate.

Reference: Barron JT, Gu L & Parrillo JE (2000) NADH/NAD redox state of cytoplasmic glycolytic compartments in vascular smooth muscle. *Am. J. Physiol. Heart Circ. Physiol.* 279, H2872–H2878.

14–32

A. The balanced equation for the reduction of O_2 by Fe^{2+} is

$$4\ Fe^{2+} + O_2 + 4\ H^+ \longrightarrow 4\ Fe^{3+} + 2\ H_2O$$

Reversing the half reaction for Fe^{3+}/Fe^{2+} and summing gives a $\Delta E_0'$ value of 0.05 V (–0.77 V + 0.82 V).

Under standard conditions, $\Delta E = \Delta E_0'$ and ΔG is

$$\Delta G = -nF\Delta E$$

$$= -4 \times \frac{96\ \text{kJ}}{\text{V mole}} \times 0.05\ \text{V}$$

$$\Delta G = -19.2\ \text{kJ/mole}$$

or as sometimes stated, since this is a four-electron reaction,

$$\Delta G = -4.8\ \text{kJ/mole for each electron}$$

Although the flow of electrons from Fe^{2+} to O_2 is thermodynamically favorable, the free-energy change for each electron is fairly small. Fortunately, *T. ferrooxidans* does not depend on this redox reaction as a source of energy, but rather as a way of detoxifying entering protons (from which it makes ATP) and as a source of electrons for reducing $NADP^+$.

B. The balanced reaction for reduction of $NADP^+ + H^+$ by Fe^{2+} is

$$NADP^+ + H^+ + 2\ Fe^{2+} \longrightarrow NADPH + 2\ Fe^{3+}$$

Reversing the half reaction for Fe^{3+}/Fe^{2+} and summing gives a $\Delta E_0'$ value of –1.09 V (–0.77 V – 0.32 V). Because $\Delta E = \Delta E_0'$ under standard conditions, ΔG is

$$\Delta G = -nF\Delta E$$

$$= -2 \times \frac{96\ \text{kJ}}{\text{V mole}} \times -1.09\ \text{V}$$

$$\Delta G = 209\ \text{kJ/mole (or 105 kJ/mole for each electron)}$$

At the concentrations specified in the problem,

$$\Delta E = \Delta E_0' - \frac{2.3\ RT}{nF} \log \frac{[\text{NADPH}][Fe^{2+}]^2}{[NADP^+][Fe^{3+}]^2}$$

Note the use of exponents for Fe^{2+} and Fe^{3+}, in accord with the balanced equation. Since the concentrations of Fe^{2+} and Fe^{3+} are equal they cancel out, and

$$\Delta E = -1.09\ \text{V} - \frac{2.3}{2} \times \frac{8.3 \times 10^{-3}\ \text{kJ}}{\text{K mole}} \times 310\ \text{K} \times \frac{\text{V mole}}{96\ \text{kJ}} \log \frac{10}{1}$$

$$= -1.09\ \text{V} - 0.03\ \text{V}$$

$$\Delta E = -1.12\ \text{V}$$

At these concentrations

$$\Delta G = -2 \times \frac{96\ \text{kJ}}{\text{V mole}} \times -1.12\ \text{V}$$

$$\Delta G = 215\ \text{kJ/mole (or 108 kJ/mole for each electron)}$$

These calculations make it very clear that reduction of $NADP^+$ by Fe^{2+}

is extremely unfavorable. Yet *T. ferrooxidans* manages to do exactly that! Problem 14–69 shows how.

Reference: Ingledew JW (1982) *Thiobacillus ferrooxidans*: the bioenergetics of an acidophilic chemolithotroph. *Biochim. Biophys. Acta* 683, 89–117.

14–33 Reversing the $NAD^+/NADH$ half reaction and summing E_0' values gives a $\Delta E_0'$ value of 1.14 V (0.82 V + 0.32 V). Noting that the balanced equation is a four-electron reaction, the standard free-energy change is

$$\Delta G^\circ = -nF\Delta E_0'$$

$$= -4 \times \frac{96\ \text{kJ}}{\text{V mole}} \times -1.14\ \text{V}$$

$$= -438\ \text{kJ/mole}$$

The balanced equation that uses $\frac{1}{2}O_2$ has the same $\Delta E_0'$ value of 1.14 V, but because it is a two-electron reaction, the standard free-energy change is only –219 kJ/mole, just half the value calculated above. When the values from these calculations are expressed per mole of electrons, they agree: –109 kJ/mole of electrons.

DATA HANDLING

14–34

A. The reduced (electron-rich) forms of the cytochromes give rise to the bands. Oxygen accepts electrons from the electron-transport chain and is reduced to H_2O. Therefore, in the presence of oxygen, the cytochromes would be drained of their electrons; that is, oxidized. Since the absorption bands do not show up in the presence of oxygen, the oxidized forms must not absorb light. The reduced forms of the cytochromes absorb light and thus are responsible for the characteristic absorption patterns that Keilin observed. In the absence of oxygen, the cytochromes pick up electrons from substrates (become reduced) but cannot get rid of them by transfer to oxygen. In the presence of oxygen, the electrons are transferred efficiently, leaving the cytochromes in their electron-deficient or oxidized state.

B. Keilin's observations indicate that the order of electron flow through the cytochromes is

$$\begin{matrix}\text{reduced}\\ \text{substrates}\end{matrix} \rightarrow \begin{matrix}\text{cytochrome}\\ b\end{matrix} \rightarrow \begin{matrix}\text{cytochrome}\\ c\end{matrix} \rightarrow \begin{matrix}\text{cytochrome}\\ a\end{matrix} \rightarrow O_2$$

This order can be deduced from Keilin's results. Since the bands become visible in the absence of oxygen, they represent the reduced (electron-rich) forms of the cytochromes. When oxygen is added, they are all converted to the oxidized (electron-poor) form. When cyanide is added, all the cytochromes are reduced, indicating that cyanide blocks the flow of electrons from the cytochromes to oxygen; that is, all the cytochromes are "upstream" of oxygen (in the sense of electron flow).

When urethane is added, cytochrome *b* remains reduced but cytochromes *a* and *c* become oxidized. Thus, urethane interrupts the flow of electrons from cytochrome *b* to cytochromes *a* and *c*, indicating that cytochrome *b* is "upstream" of cytochromes *a* and *c*.

These results indicate that either cytochrome *a* or *c* transfers electrons to oxygen. The inability of oxygen to oxidize a preparation of cytochrome *c* suggests, by elimination, that cytochrome *a* is responsible for the transfer of electrons to oxygen. This ordering of cytochromes *a* and *c* is weak since it is based on a negative result (which could have other interpretations). Keilin himself confirmed this order by observing subtle spectral shifts in the cytochrome *a* band in the presence of cyanide under reducing conditions; he named the active component cytochrome a_3. We now

know that cytochrome *a* is a large complex with several redox centers, one of which reacts with molecular oxygen.

C. The rapid oxidation of glucose to CO_2 prevents the disappearance of the absorption bands by providing a source of reduced substrates (ultimately NADH and $FADH_2$) that transfer electrons into the electron-transport chain faster than oxygen can remove them. Under these conditions, the cytochromes remain reduced (electron-rich) and therefore continue to absorb light.

Reference: Keilin D (1966) The History of Cell Respiration and Cytochrome. Cambridge, UK: Cambridge University Press.

14–35 The rates of oxidation of the electron carriers, if measured rapidly enough, reveal their order in the respiratory chain. The carriers closest to oxygen will be oxidized first, and those farthest from oxygen will be oxidized last. This rationale allows you to deduce the order of electron flow through the carriers. Cytochromes *b* and c_1 are part of cytochrome *c* reductase and cytochromes *a* and a_3 are part of cytochrome oxidase.

$$\underset{b}{\text{cytochrome}} \rightarrow \underset{c_1}{\text{cytochrome}} \rightarrow \underset{c}{\text{cytochrome}} \rightarrow \underset{(a+a_3)}{\text{cytochrome}} \rightarrow O_2$$

14–36

A. Malonate, cyanide, and butylmalonate all give the oxygen trace shown in Figure 14–5A. Each of these inhibitors blocks the flow of electrons to oxygen, thereby stopping oxygen consumption. Butylmalonate and malonate block the uptake and oxidation of succinate, respectively, thereby eliminating the flow of electrons at the source. Cyanide stops the flow of electrons at the cytochrome *c* oxidase complex, which normally transfers the electrons to oxygen.

Atractyloside and oligomycin give the oxygen trace shown in Figure 14–5B. By blocking exchange of ADP for ATP, atractyloside prevents entry of ADP into mitochondria and subsequent ATP synthesis. This "removal" of ADP returns the rate of respiration to the rate before ADP was added. By inhibiting ATP synthase, oligomycin prevents synthesis of ATP, which has the same effect as eliminating ADP, and returns the rate of respiration to the rate before ADP was added.

FCCP gives the oxygen trace shown in Figure 14–5C. By making the inner membrane permeable to protons, FCCP uncouples electron transport from oxidative phosphorylation, thereby allowing the maximum rate of electron flow through the respiratory chain to oxygen (since it is not opposed by the electrochemical gradient).

B. The oxygen traces expected for the three combinations of inhibitors are shown in **Figure 14–20**.

1. FCCP uncouples the flow of electrons from oxidative phosphorylation, allowing a maximum rate of electron flow and oxygen consumption. The subsequent addition of cyanide stops the flow of electrons directly and, thereby, stops oxygen consumption (Figure 14–20A).
2. Since FCCP uncouples electron flow from oxidative phosphorylation, oligomycin (which inhibits ATP synthase) has no effect on the rate of oxygen consumption (Figure 14–20B).
3. Oligomycin slows the rate of oxygen consumption by blocking ATP synthase. This block is bypassed by FCCP because it uncouples electron transport from oxidative phosphorylation (Figure 14–20C).

Reference: Nicholls DG & Ferguson SJ (1992) Bioenergetics 2, pp. 82–87. London: Academic Press.

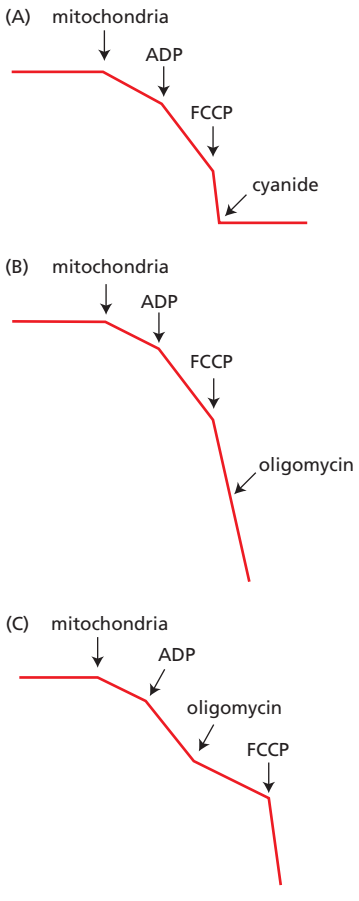


Figure 14–20 Oxygen traces expected for three combinations of inhibitors (Answer 14–36). (A) FCCP followed by cyanide. (B) FCCP followed by oligomycin. (C) Oligomycin followed by FCCP.

MEDICAL LINKS

14–37 In just the right amounts, an uncoupler such as dinitrophenol will promote weight loss by partially uncoupling electron flow from ATP

synthesis, thereby decreasing the efficiency of oxidative phosphorylation. For example, if sufficient uncoupler were ingested to reduce the efficiency of oxidative phosphorylation to 50%, twice as many calories (from food or internal stores, mainly fat) would have to be burned to generate the same amount of ATP. Dinitrophenol is no longer prescribed because its use led to several deaths; if oxidative phosphorylation is too efficiently compromised, not enough ATP will be generated to support essential cell functions and death is the result.

ATP PRODUCTION IN MITOCHONDRIA

DEFINITIONS

14–38 ATP synthase

TRUE/FALSE

14–39 False. An average person contains a much smaller amount of ATP, which they reuse by cycling it between ATP and ADP. An average person turns over roughly 50 kg of ATP per day in order to support their daily energy needs.

14–40 True. The *c* subunits act like cogs in a gear wheel. When the supply of protons is limited, as in mitochondria, there are fewer subunits than when the proton gradient is high, as in chloroplasts.

THOUGHT PROBLEMS

14–41 In the intact mitochondrion, NADH donates its electrons to the respiratory chain from the matrix side of the inner membrane. Likewise, ATP is made from ADP and phosphate on the matrix side of the inner membrane. With intact mitochondria it is difficult for a scientist to manipulate the concentrations of these small molecules in the matrix because the inner membrane encloses it. By contrast, in submitochondrial particles with the matrix side exposed to the surroundings, it is easy to add different concentrations of NADH, ATP, ADP, and phosphate (as well as inhibitors and artificial electron donors and acceptors) and follow the consequences for electron transport and ATP synthesis.

14–42 As soon as protons enter the intermembrane space, they move rapidly throughout the cytosol. They are not confined to the intermembrane space because of the large aqueous channels that are present in the outer mitochondrial membrane. The infolding of the inner membrane into cristae is thought to provide a proton trap of sorts to prolong the local pH difference across the membrane.

14–43

A. You would expect ATP to be produced, as shown in **Figure 14–21**. When the vesicles are exposed to light, protons will be pumped inside by bacteriorhodopsin, creating a pH difference across the vesicle membranes. The proton-motive force represented by the pH difference would drive the protons back out of the vesicles through the ATP synthase, causing ATP to be generated from ADP and phosphate in the external medium.

B. If the vesicles were leaky to protons, no pH difference would be generated, hence no ATP could be synthesized. The protons pumped into the vesicles by bacteriorhodopsin would immediately leak back out without generating a pH difference.

C. If the ATP synthase molecules were randomly oriented, you would still expect ATP to be synthesized, although at about half the rate. The

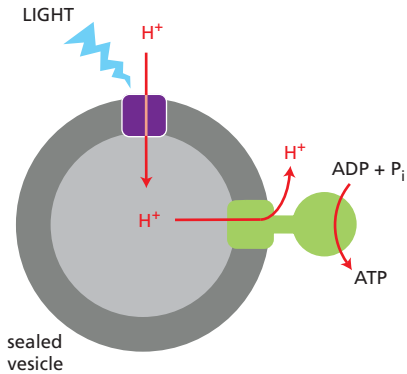


Figure 14–21 ATP synthesis in reconstituted lipid vesicles containing bacteriorhodopsin and ATP synthase (Answer 14–43).

molecules that were oriented correctly would make ATP; the oppositely oriented ATP synthase molecules would be inert.

If bacteriorhodopsin were randomly oriented, you would expect much less ATP to be synthesized. In vesicles with equal numbers of oppositely oriented bacteriorhodopsin molecules, no pH difference would be generated upon exposure to light because the proton pumping in both directions would be equal. In vesicles with an excess of outwardly directed proton pumps, the pH difference would be in the wrong direction to be utilized by ATP synthase and, thus, no ATP would be made. In vesicles with an excess of inwardly directed proton pumps, a pH difference of the right orientation would be generated; thus, those vesicles would be capable of synthesizing some ATP.

D. Using components from widely divergent organisms can be a very powerful experimental tool. Because the two proteins come from such different sources, it is very unlikely that they form a direct functional interaction. The experiment therefore strongly suggests that the pumping of protons (normally carried out by the respiratory chain) and the synthesis of ATP are separate events. The notion that electron transport and ATP synthesis are separate is now clearly established (this experiment was one of the earliest demonstrations); thus, this approach is a valid one.

Reference: Racker E & Stoeckenius W (1974) Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. *J. Biol. Chem.* 249, 662–663.

14–44 The stator plays the crucial role of anchoring the $\alpha_3\beta_3$ complex to the rotor component of the ATP synthase. Fixing the $\alpha_3\beta_3$ complex allows the γ subunit to turn inside it and drive the set of conformational changes that are responsible for ATP synthesis. In the absence of the stator, the $\alpha_3\beta_3$ complex would rotate along with the γ subunit, and there would be no conformational changes. Thus, ATP would not be synthesized in the absence of the stator.

CALCULATIONS

14–45 The number of protons in the matrix of an actively respiring liver mitochondrion at pH 7.5 (3.16×10^{-8} M H^+) is about 10.

$$\frac{H^+}{\text{mitochondrion}} = \frac{3.16 \times 10^{-8} \text{ mole } H^+}{\text{L}} \times \frac{6 \times 10^{23} \text{ } H^+}{\text{mole } H^+} \times \frac{(4/3)(3.14)(0.5\ \mu\text{m})^3}{\text{mitochondrion}} \times \frac{\text{L}}{10^{15}\ \mu\text{m}^3}$$

$$= 9.9$$

If the matrix of the mitochondrion started at pH 7 (10^{-7} M H^+), it originally held about 31 protons (31.4). Thus, to reach pH 7.5, about 21 protons would need to be pumped out. These are remarkable results. Regardless of the particulars of mitochondrial size and exact pH values, it is clear that only a few tens of protons are normally involved in establishing the proton-motive force. More than anything, these results emphasize the dynamic nature of proton pumping and ATP synthesis.

14–46

A. When the concentrations of the reactants and products are all 1 M, the reaction is at standard conditions and ΔG equals $\Delta G°$, which is –30.5 kJ/mole.

$$\Delta G = \Delta G° + 2.3RT \log \frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]}$$

$$= -30.5 \text{ kJ/mole} + 2.3 \times \frac{8.3 \times 10^{-3} \text{ kJ}}{\text{K mole}} \times 310 \text{ K} \times \log \frac{1 \times 1}{1}$$

Since the log of 1 is 0,

$$\Delta G = -30.5 \text{ kJ/mole}$$

When the concentrations of the reactants and products are all 1 mM, ΔG equals –48.2 kJ/mole. (At 37°C, 2.3RT is 5.9 kJ/mole.)

$$\Delta G = -30.5 \text{ kJ/mole} + (5.9 \text{ kJ/mole}) \log \frac{10^{-3} \times 10^{-3}}{10^{-3}}$$

$$= -30.5 \text{ kJ/mole} + (5.9 \text{ kJ/mole})(-3)$$

$$= -30.5 \text{ kJ/mole} - 17.7 \text{ kJ/mole}$$

$$\Delta G = -48.2 \text{ kJ/mole}$$

B. At the given concentrations of ATP, ADP, and P_i, ΔG for ATP hydrolysis is –46.4 kJ/mole.

$$\Delta G = -30.5 \text{ kJ/mole} + (5.9 \text{ kJ/mole}) \log \frac{10^{-3} \times 10^{-2}}{5 \times 10^{-3}}$$

$$= -30.5 \text{ kJ/mole} + (5.9 \text{ kJ/mole})(-2.7)$$

$$= -30.5 \text{ kJ/mole} - 15.9 \text{ kJ/mole}$$

$$\Delta G = -46.4 \text{ kJ/mole}$$

C. At equilibrium, ΔG is 0. At equilibrium, there is no longer any tendency for a reaction to proceed. If $[P_i]$ is 10 mM at equilibrium, then the ratio of [ATP] to [ADP] will be 6.3×10^{-8}.

$$0 = -30.5 \text{ kJ/mole} + (5.9 \text{ kJ/mole}) \log \frac{[\text{ADP}] \times 10^{-2}}{[\text{ATP}]}$$

$$-30.5 \text{ kJ/mole} = (5.9 \text{ kJ/mole})(\log 10^{-2} + \log [\text{ADP}]/[\text{ATP}])$$

$$= (-2)(5.9 \text{ kJ/mole}) + (5.9 \text{ kJ/mole}) \log [\text{ADP}]/[\text{ATP}]$$

$$\log [\text{ADP}]/[\text{ATP}] = \frac{42.3 \text{ kJ/mole}}{5.9 \text{ kJ/mole}}$$

$$= 7.2$$

$$\log [\text{ATP}]/[\text{ADP}] = -7.2$$

$$[\text{ATP}]/[\text{ADP}] = 6.3 \times 10^{-8}$$

D. At a constant $[P_i]$, every 10-fold change in the ratio of [ATP] to [ADP] will alter ΔG by 5.9 kJ/mole. As shown below, a 10-fold increase in [ATP]/[ADP] will decrease ΔG by 5.9 kJ/mole.

$$\Delta G = \Delta G^\circ + 5.9 \text{ kJ/mole } \log \frac{[\text{ADP}][P_i]}{[\text{ATP}]}$$

$$= \Delta G^\circ + 5.9 \text{ kJ/mole } \log [P_i] + 5.9 \text{ kJ/mole} \log \frac{[\text{ADP}]}{[\text{ATP}]}$$

A 10-fold increase in [ATP]/[ADP], which is equal to a 10-fold decrease in [ADP]/[ATP], causes the log of the ratio in the expression above to decrease by –1. Thus each 10-fold increase in the ratio causes 5.9 kJ/mole to be subtracted from the right-hand side, thereby decreasing ΔG by 5.9 kJ/mole. A 100-fold increase in the ratio of [ATP]/[ADP] decreases ΔG by 11.8 kJ/mole; a 1000-fold increase in the ratio decreases ΔG by 17.7 kJ/mole.

DATA HANDLING

14–47

A. Presumably, the hydrolysis of an individual ATP molecule provides the driving force for the 120° rotation of the γ subunit, hence the

corresponding revolution of the actin filament. Since a low concentration of ATP was used in these experiments, the pauses represent the variable times it takes for the next molecule of ATP to bind. Rotation through 120° corresponds to one αβ dimer, the unit of ATP hydrolysis (or of synthesis in ATP synthase's normal direction).

B. If three ATP molecules must be hydrolyzed to drive one complete rotation of the γ subunit, then in its normal operation, ATP synthase must synthesize three ATP molecules per rotation of the γ subunit.

Reference: Masaike T, Mitome N, Noji H, Muneyuki E, Yasuda R, Kinosita K & Yoshida M (2000) Rotation of F_1-ATPase and the hinge residues of the β subunit. *J. Exp. Biol.* 203, 1–8.

14–48 The crystal structure of the $\alpha_3\beta_3$ complex, with ADP and an analog of ATP bound, shows clearly that the correct arrangement is the one in Figure 14–8B. This arrangement can be deduced from the *counterclockwise* revolution of the actin filament when ATP synthase *hydrolyzes ATP*. When ATP synthase is operating to hydrolyze ATP—the reverse of its normal mode of action—the sequence of conformational changes is also reversed, as shown for arrangement B in **Figure 14–22**. An actin filament is shown arbitrarily between the ATP-binding conformation and the empty conformation in step I in Figure 14–22. After ATP hydrolysis to step II, a 120° counterclockwise revolution of the actin filament leaves it in the same relative position: between an ATP-binding conformation and an empty conformation. Likewise, after hydrolysis of another ATP to step III, a 120° counterclockwise revolution of the actin filament once again places it between an ATP-binding and an empty conformation. (If you try the same analysis for the arrangement in Figure 14–8A, you'll find that the neighbors of the actin filament change at each step, indicating that its revolution does not match the sequence of conformation changes.) Another way you might recognize that the arrangement in Figure 14–8B is the correct one is to notice that a particular αβ conformation, for example the ATP-binding conformation, moves counterclockwise—the same direction as filament rotation—in going from step I to II to III during ATP hydrolysis (Figure 14–22).

References: Noji H, Yasuda R, Yoshida M & Kinosita K (1997) Direct observation of the rotation of F_1-ATPase. *Nature* 386, 299–302.

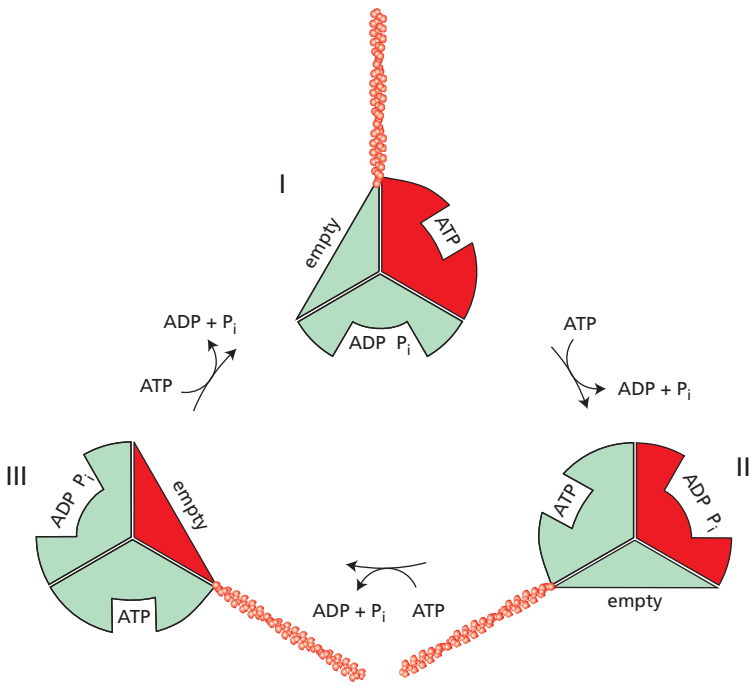


Figure 14–22 The correct arrangement of αβ conformations (Answer 14–48). *Roman numerals* indicate successive steps in the cycle of conformational changes driven by hydrolysis of ATP.

Abrahams JP, Leslie AG, Lutter R & Walker JE (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. *Nature* 370, 621–628.

14–49 Such a result is entirely reasonable; mechanical force has just been substituted for the proton-motive force to turn the axle-like γ subunit. This experiment would suggest a two-step model for ATP synthase: (1) proton flow causes rotation of the γ subunit and (2) rotation of the γ subunit inside the $\alpha_3\beta_3$ complex causes synthesis of ATP. In this experiment, the authors have succeeded in uncoupling these two steps: mechanically rotating the γ subunit inside the $\alpha_3\beta_3$ complex is sufficient to cause synthesis of ATP. This would be a very exciting experiment, indeed, because it would directly demonstrate the relationship between mechanical movement and enzymatic activity. There is no doubt that it should be published and that it would become a "classic."

14–50 The key to understanding these results is to recognize that an exchange of ADP for ATP is not electrically neutral. ATP carries four negative charges; ADP carries only three (see Figure 14–9). As a result, each exchange of ADP for ATP increases the negative charge on the side of the membrane that receives the ATP. In a respiring mitochondrion, there is an electrochemical gradient across the mitochondrial inner membrane such that the outside of the membrane is positive. The resulting proton-motive force drives the exchange of an external ADP for an internal ATP, thereby reducing the positive charge on the outside of the membrane. In your results, the exchange of external ADP was favored over that of external ATP under conditions in which the membrane was charged: when substrate was present without an inhibitor and when substrate was present with an inhibitor of ATP synthase (see Table 14–3, experiments 2 and 4). When the electrochemical gradient was absent—that is, when there was no substrate or when the membrane was made permeable to protons—ADP and ATP were exchanged at equal rates (see Table 14–3, experiments 1 and 3).

The electrogenic nature of the ADP/ATP transporter has important physiological consequences. In essence, the exchange harnesses the energy of the electrochemical gradient to drive transport so that the cytosolic ratio of ATP/ADP remains high (up to 50). This concentration difference provides up to one-third of the free-energy change (ΔG) for ATP hydrolysis in the cytoplasm.

References: Nicholls DG & Ferguson SJ (1992) Bioenergetics 2, pp. 215–218. London: Academic Press.

Tzagoloff A (1982) Mitochondria, pp. 212–213. New York: Plenum Press.

CHLOROPLASTS AND PHOTOSYNTHESIS

DEFINITIONS

14–51 Photosynthetic electron transfer

14–52 Antenna complex

14–53 Carbon fixation

14–54 Photochemical reaction center

14–55 Chloroplast

14–56 Chlorophyll

14–57 Stroma

TRUE/FALSE

14–58 True. The thylakoids and cristae have the same arrangement of membrane proteins—ATP synthase, for example, with its lollipop-like head protruding into the matrix or stroma.

14–59 False. When an electron in a chlorophyll molecule in the antenna complex is excited, it transfers its energy—not the electron—from one chlorophyll molecule to another by resonance energy transfer.

THOUGHT PROBLEMS

14–60 Protons are pumped across the crista membrane into the crista space in mitochondria. By contrast, they are pumped across the thylakoid membrane into the thylakoid space in chloroplasts. The thylakoid and cristae membranes and spaces are not precisely analogous, but cristae would be converted to something resembling thylakoids if they were pinched off from the inner membrane; similarly, if thylakoids fused to the inner membrane, they would resemble cristae. ATP is synthesized in the corresponding compartments in the two organelles: in the matrix in mitochondria and in the stroma in chloroplasts.

14–61 In the absence of light, but in the presence of CO_2, the amount of ribulose 1,5-bisphosphate will decrease and 3-phosphoglycerate will increase. The presence of CO_2 allows ribulose 1,5-bisphosphate to be converted to 3-phosphoglycerate but, in the absence of light (and therefore with decreased amounts of NADPH and ATP), 3-phosphoglycerate will accumulate because subsequent reactions require NADPH and ATP.

In the absence of CO_2, ribulose 1,5-bisphosphate will accumulate (and 3-phosphoglycerate will decrease) because its conversion to 3-phosphoglycerate is dependent on CO_2.

14–62 Plants are green because they absorb light efficiently at the blue and red ends of the spectrum but poorly at the green and yellow wavelengths. Thus the light that reaches our eyes after striking a plant is enriched for green and yellow wavelengths; hence, plants appear green.

14–63 If a chemical reagent that accepts electrons from plastoquinone can restore O_2 evolution in DCMU-treated chloroplasts, then the components from photosystem II to plastoquinone cannot be affected by DCMU. Because DCMU blocks photophosphorylation, it must act before the sites at which protons are pumped within the cytochrome b_6-f complex. These two arguments restrict the site of action of DCMU to a component after plastoquinone but before the first site of proton pumping in the cytochrome b_6-f complex (see Figure 14–10).

DCMU kills plants because it blocks their photosynthetic production of ATP and NADPH. Deprived of their ability to fix CO_2 and generate energy, plants die.

14–64 Protons pumped across the crista membrane into the crista space can exit to the intermembrane space, which equilibrates with the cytosol, a huge H^+ sink. Both the mitochondrial matrix (pH 8) and the cytosol (pH 7.4) house many metabolic reactions that require a pH around neutrality. The largest H^+ concentration difference between the mitochondrial matrix and cytosol that is compatible with function is therefore relatively small (less than 1 pH unit). Much of the energy stored in the mitochondrial electrochemical gradient is instead due to the membrane potential (about 140 mV of the 200 mV potential difference is due to the membrane potential).

By contrast, chloroplasts have a smaller, dedicated compartment—the thylakoid space—into which H^+ ions are pumped. Much higher

concentration differences can be achieved (more than 3 pH units), and nearly all of the energy stored in the thylakoid electrochemical gradient is due to the pH difference between the stroma and the thylakoid space.

14–65 Even during daylight hours in chloroplast-containing cells, it is the mitochondria that supply the cell with ATP, which is produced by oxidative phosphorylation. Glyceraldehyde 3-phosphate made by photosynthesis in chloroplasts moves to the cytosol and is eventually used as a source of energy to drive ATP production in mitochondria.

CALCULATIONS

14–66

A. The energy of a mole of photons at any particular wavelength is the energy of one photon times Avogadro's number (N). Therefore, the energy of a mole of photons at a wavelength of 400 nm is

$$E = Nhc/\lambda$$

$$= \frac{6 \times 10^{23} \text{ photons}}{\text{mole}} \times \frac{6.6 \times 10^{-37} \text{ kJ/sec}}{\text{photon}} \times \frac{3 \times 10^{17} \text{ nm}}{\text{sec}} \times \frac{1}{400 \text{ nm}}$$

$E = 297$ kJ/mole for 400-nm light

This calculation for 680-nm and 800-nm light gives

$E = 175$ kJ/mole for 680-nm light

$E = 149$ kJ/mole for 800-nm light

B. If a square meter receives 1.3 kJ/sec of 680-nm light, which is worth 175 kJ/mole of photons, then the time it will take for a square meter to receive one mole of photons is

$$\text{time} = \frac{\text{sec}}{1.3 \text{ kJ}} \times \frac{175 \text{ kJ}}{\text{mole}}$$

time = 135 sec/mole

C. If it takes 135 seconds for a square meter of tomato leaf to receive a mole of photons and eight photons are required to fix a molecule of CO_2, it will take just under 2 hours to synthesize a mole of glucose:

$$\text{time} = \frac{135 \text{ sec}}{\text{mole photons}} \times \frac{8 \text{ mole photons}}{\text{mole } CO_2} \times \frac{6 \text{ mole } CO_2}{\text{mole glucose}}$$

time = 6480 seconds or 108 minutes

The actual efficiency of photon capture is considerably less than 100%. Under optimal conditions for some rapidly growing plants, the efficiency of utilization of photons that strike a leaf is about 5%. However, even this value greatly exaggerates the true efficiency of utilization of the energy in sunlight. For example, a field of sugar beets converts only about 0.02% of the energy that falls on it during the growing season. Several factors limit the overall efficiency, including saturation of the photosystems far below maximum sunlight, availability of water, and low temperatures.

D. In contrast to the very low overall efficiency of light utilization, the efficiency of conversion of light energy to chemical energy *after photon capture* is 33%.

$$\text{efficiency} = \frac{\text{mole } CO_2}{8 \text{ mole photons}} \times \frac{\text{mole photons}}{175 \text{ kJ}} \times \frac{468 \text{ kJ}}{\text{mole } CO_2}$$

$$= 0.33 \text{ or } 33\%$$

14–67 When a photon is absorbed by P700, 91% of the energy of 700-nm light is captured. The energy in a photon of 700-nm light is

$$E = hc/\lambda$$

$$= \frac{6.6 \times 10^{-37}\ \text{kJ/sec}}{\text{photon}} \times \frac{3 \times 10^{17}\ \text{nm}}{\text{sec}} \times \frac{1}{700\ \text{nm}}$$

$$= 2.83 \times 10^{-22}\ \text{kJ/photon}$$

Reversing the $P700^+/P700^*$ half-cell and summing gives a standard redox potential ($\Delta E_0'$) of 1.6 V. Under standard conditions, ΔG is

$$\Delta G = -nF\Delta E_0'$$

$$= -1 \times \frac{96\ \text{kJ}}{\text{V mole}} \times 1.6\ \text{V}$$

$$= -154\ \text{kJ/mole}$$

Dividing by Avogadro's number (6×10^{23} molecules/mole) gives -2.57×10^{-22} kJ/molecule. Thus, 90% ($2.57 \times 10^{-22}/2.83 \times 10^{-22}$) of the energy of 700-nm light is captured when a photon is absorbed by P700.

14–68 Eight photons are required: four by photosystem II and four by photosystem I. Four electrons (ultimately from water, generating O_2) are excited by absorption of four photons in photosystem II and then re-excited by absorption of four photons in photosystem I. The four electrons from photosystem I are used to reduce two molecules of $NADP^+$ to NADPH.

14–69 A minimum of eight protons (8×-26.8 kJ/mole = –214 kJ/mole) would be required to drive electrons from Fe^{2+} to $NADP^+$, a process with an unfavorable free-energy change of 211 kJ/mole. The free-energy change for transfer of two electrons from Fe^{2+} to $NADP^+$ ($\Delta E = -1.1$ V) is

$$\Delta G = -nF\Delta E$$

$$= -2 \times \frac{96\ \text{kJ}}{\text{V mole}} \times -1.1\ \text{V}$$

$$= 211\ \text{kJ/mole}$$

These thermodynamic considerations say nothing about the mechanism of coupling. Reasoning from what is known in other organisms, it is likely that an electron-transport chain links the Fe^{2+}/Fe^{3+} redox pair to the $NADP^+$/NADPH redox pair. In other organisms, the flow of electrons in the favorable direction is coupled to the pumping of protons across a membrane. This is likely to be the arrangement in *T. ferrooxidans* as well; however, the naturally imposed pH difference across the membrane drives an inward proton flow that reverses the proton pumps to cause an upward (otherwise unfavorable) flow of electrons from Fe^{2+} to $NADP^+$ (see Problem 14–29).

DATA HANDLING

14–70 One measure of photosynthesis is the evolution of O_2. In 1882, none of the sensitive devices now used for measuring O_2 were available. Instead, Engelmann made use of bacteria that grow best in the presence of O_2 and actively seek it. When the alga was illuminated with a spectrum of light, only those portions that received light at the blue or red ends of the spectrum were able to carry out photosynthesis and evolve O_2. Bacteria that use O_2 tend to collect around those portions of the alga that give off O_2. Thus the density of bacteria is a crude measure of the rate of O_2 evolution. The action spectrum (the rate of O_2 evolution at different wavelengths)

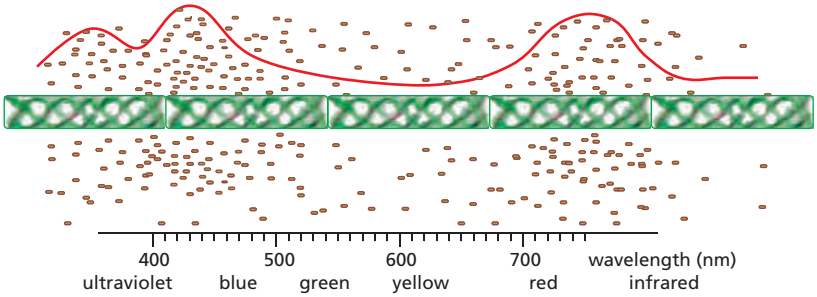


Figure 14–23 Action spectrum of a filamentous green alga (Answer 14–70).

can be approximated by the density of the bacteria at different places in the spectrum (**Figure 14–23**).

14–71

A. Since stimulation by 680-nm light removes electrons from the cytochromes, causing their oxidation, 680-nm light must preferentially stimulate photosystem I, which transports electrons from the cytochromes to $NADP^+$ (**Figure 14–24**). The subsequent stimulation by 562-nm light causes electrons to flow into the cytochromes at a faster rate than before, thereby causing them to become more reduced. Consequently, 562-nm light must stimulate photosystem II, which transfers electrons from water to the cytochromes (Figure 14–24). Thus, in these algae, as in most plants, the longer wavelength preferentially stimulates photosystem I, and the shorter wavelength preferentially stimulates photosystem II.

B. These results support the Z scheme of photosynthesis in several ways. First, the different effects at the two wavelengths suggest that there are at least two components that differ in their responses to these wavelengths of light. Second, the two wavelengths have opposite effects on the redox poise of the cytochromes—680-nm light causing oxidation and 562-nm light causing reduction. Finally, the effects at the two wavelengths could be separated by DCMU, which indicates that the two photosystems communicate through the cytochromes (Figure 14–24).

C. These results indicate that DCMU blocks electron transport through the cytochromes on the upstream side; that is, on the side nearer photosystem II (Figure 14–24). When photosystem I is stimulated by 680-nm light in the presence of DCMU, it transfers what electrons are available out of the cytochromes, causing their oxidation. In addition, in the presence of DCMU, electrons cannot be transferred into the cytochromes by stimulation of photosystem II by 562-nm light (see Figure 14–12B). These two effects indicate that DCMU blocks electron transport very near the beginning of the cytochrome chain.

Reference: Duysens LNM, Amesz J & Kamp BM (1961) Two photochemical systems in photosynthesis. *Nature* 190, 510–511.

14–72

A. These results support a gear-wheel connection between the abstraction of electrons from water and their activation in photosystem II reaction centers. The periodicity of O_2 production in response to light flashes rules out the possibility that four photons must be delivered simultaneously to the reaction center. If four photons were needed simultaneously, each flash should yield an equal burst of O_2.

The periodicity also argues against cooperation among four reaction centers to produce a molecule of O_2. At saturating light intensities, most of the reaction centers should be stimulated during each flash; if they could cooperate, they would generate O_2 at each flash. Furthermore, the results of the DCMU experiment definitely eliminate the possibility of cooperation. If four reaction centers were required to cooperate, one

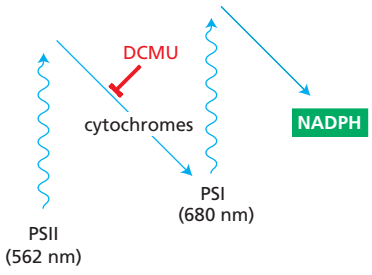


Figure 14–24 Simplified Z scheme of photosynthesis showing the relationship of the two photosystems (PS), the cytochromes, and the point of action of DCMU (Answer 14–71). Electron flow is shown in *blue* lines.

might expect a fourth-power dependence on the concentration of active centers. However, a 30-fold reduction in active centers (DCMU inhibited 97% of the active centers) gave only a 30-fold reduction in O_2 production (peaks of oxygen production were 3% of those in the absence of DCMU) instead of the enormous reduction (30^4) expected from a fourth-power dependence.

A periodicity in O_2 production is exactly what one would expect from a gear-wheel link between extraction of multiple electrons from water and photon excitation of single electrons in photosystem II reaction centers. Furthermore, each gear wheel must service a single reaction center. If one gear wheel could interact with four reaction centers, for example, then it could donate its four electrons from water during each flash, which would allow it to produce O_2 during each flash, eliminating the periodicity.

B. The four-flash periodicity in the production of O_2 argues strongly that the gear wheel picks up four electrons at a time from two water molecules and passes them on to the photosystem II reaction center one at a time. The timing of the appearance of the first burst of O_2 says something about the dark-adapted state of the gear wheel; namely, that it holds three electrons. The first three flashes transfer those electrons. The gear wheel can then pick up four new electrons from water (in a reaction that depends on light), generating a molecule of oxygen in the process. (Actually, about a quarter of the gear wheels carry four electrons in the dark-adapted state, which is why there is significant oxygen production on the fourth flash.)

C. The periodicity is gradually damped out with increasing flash number because the multiple photosystems fall out of phase with one another. During a single flash, most of the photosystem reaction centers capture one photon, but some capture two photons, and others capture no photons. Those reaction centers that capture zero or two photons are out of step with the majority. After several flashes, the number of out-of-step reaction centers increases sufficiently to obscure any periodicity. The period of dark adaptation at the beginning of the experiment is required to bring the majority of the reaction centers to the same state so that periodicity can be observed at all.

Reference: Forbush B, Kok B & McGloin M (1971) Cooperation of charges in photosynthetic oxygen evolution II. Damping of flash yield, oscillation and deactivation. *Photochem. Photobiol.* 14, 307–321.

14–73

A. The switch in solutions creates a pH gradient across the thylakoid membrane. The flow of protons down their electrochemical potential drives ATP synthase, which converts ADP to ATP.

B. No light is needed because the proton gradient is established artificially without a need for the light-driven electron-transport chain.

C. No ATP would be synthesized because the proton gradient would be in the wrong direction for ATP synthase to make ATP. In fact, one might expect that ATP would be hydrolyzed because the backward proton gradient would drive ATP synthase in reverse, causing hydrolysis of ATP.

D. These experiments provided early supporting evidence for the chemiosmotic model by showing that a pH difference alone is sufficient to drive ATP synthesis.

THE GENETIC SYSTEMS OF MITOCHONDRIA AND CHLOROPLASTS

DEFINITIONS

14–74 Maternal inheritance

TRUE/FALSE

14–75 False. The mitochondrial genetic code differs slightly from the nuclear code, and also varies slightly from species to species.

14–76 False. The presence of introns in organellar genes is surprising precisely because corresponding introns are so uncommon in related bacterial genomes.

14–77 True. Inheritance of organellar genomes is very different from the inheritance of nuclear genes, which is governed by Mendelian rules. A pattern of inheritance that does not obey Mendelian rules is unlikely to be due to a nuclear gene, which leaves the organellar genomes—the only other genomes in a cell.

THOUGHT PROBLEMS

14–78 It is likely that your organism is derived from an ancient eukaryote that once possessed an endosymbiont. Transfer of DNA from the endosymbiont to the nuclear genome occurred, giving rise to the precursors of the scattered bits of "bacterial" DNA you found in your organism's genome. At some later point, the endosymbiont-derived organelles (mitochondria) were lost, perhaps in adaptation to the anaerobic niche in which the organism now lives. An alternative hypothesis, which is difficult to rule out, is that the organism never possessed any mitochondria and that the bits of bacterial DNA were picked up by lateral gene transfer directly from other bacteria; that is, that there was no endosymbiont stage.

This hypothetical organism resembles *Giardia*, one of the rare eukaryotes without mitochondria. *Giardia* is thought to have been derived from a more typical eukaryotic cell that lost its mitochondria (see Problem 1–45).

14–79 What you have neglected in your scheme is a mechanism to get the ATP out of the proto-*Paracoccus*. An adenine nucleotide carrier is absent from all free-living bacteria, as expected, since they must retain ATP inside if it is to do them any good. All mitochondria have an ATP/ADP transporter in their membranes to allow free exchange of ATP and ADP between the cytoplasm and the mitochondrial matrix. Only after the acquisition of this carrier would ATP synthesized by the endosymbiont be available to the host.

14–80 Variegation occurs because the plants have a mixture of normal and defective chloroplasts. These sort out by mitotic segregation to give patches of green and yellow in leaves. Many of the green patches have cells that still retain defective chloroplasts in addition to the normal ones. As such patches grow, they can segregate additional cells that have only defective chloroplasts, giving rise upon cell division to an island of yellow cells in a sea of green ones. By contrast, yellow patches are due to cells that retain only defective chloroplasts. Thus, yellow cells cannot give rise to green cells by mitotic segregation; hence, there are no green islands surrounded by yellow.

14–81 Pedigree A is for an autosomal recessive mutation. Both the mother and the father must be heterozygotes who carry one copy of the mutant chromosome. One-quarter of their children, irrespective of gender, are expected to get a defective copy of the autosome from each parent, thereby becoming homozygous (and affected).

Pedigree B is for an X-linked recessive mutation. The mother carries the mutation on one copy of her two X chromosomes, and is thus unaffected. She passes the X chromosome with the mutation randomly to half her offspring. The males who get that X chromosome are affected because

it's their only X chromosome. Females who get the mutant X chromosome are unaffected because they have a normal X chromosome from their father. Note that without additional information (a larger pedigree) it would be difficult to be certain that this pedigree truly resulted from an X-linked recessive mutation and not from an autosomal recessive mutation. The distinguishing feature—a small number of affected individuals who are all male—could have resulted by chance in a small pedigree for an autosomal recessive mutation.

Pedigree C is for an autosomal dominant mutation. The mother is heterozygous, but affected, because one copy of a pair of autosomal chromosomes carries a dominant mutation. She will pass the affected chromosome to half her children, regardless of gender, all of whom will be affected because the mutation is dominant.

Pedigree D is for a mitochondrial mutation. The mother is affected and passes on her defective mitochondria to all the children because the fertilized egg contains only her mitochondria. In reality, mitochondrial inheritance is rarely as clear-cut as this example would indicate. The mother's mitochondria are rarely all of the defective type; thus, mitotic segregation can give rise to a range of mixtures of mutant and normal mitochondria in the children, who may display phenotypes that range from unaffected to severely affected. Real pedigrees for mitochondrial mutations are thus sometimes difficult to distinguish from pedigrees for autosomal dominant mutations.

DATA HANDLING

14–82

A. Chloroplast DNA, whether it is pure or a contaminant in the mitochondrial DNA preparation, will be cut into the same restriction fragments. Therefore any band that shows up at the same position in the chloroplast and mitochondrial lanes is likely to be due to contamination. Thus, the middle band in the zucchini digest and both bands in the pea digest are likely to have resulted from contamination. Only the fragments that are uniquely represented in the mitochondrial DNA lanes are candidates for chloroplast-to-mitochondrion DNA transfer.

B. Only zucchini and corn show evidence of bands that are likely to have arisen by transfer of DNA from chloroplasts to mitochondria, since they alone have bands that are uniquely represented in the mitochondrial DNA lanes.

Reference: Stern DB & Palmer JD (1984) Extensive and widespread homologies between mitochondrial DNA and chloroplast DNA in plants. *Proc. Natl Acad. Sci. USA* 81, 1946–1950.

14–83

A. Initiation of protein synthesis in mitochondria differs from that in the cytoplasm in two distinct ways. The first is straightforward: the codon AUA in mitochondria can serve as an initiation codon and encodes methionine (see Figure 14–19, mRNA 13). In cytoplasmic protein synthesis, AUA encodes isoleucine and does not serve as an initiator of protein synthesis. The second difference is more subtle: the encoded protein can begin immediately at the 5′ end of the mRNA (see Figure 14–19, mRNAs 7 and 16). Cytoplasmic (and bacterial) mRNAs typically have a short stretch of untranslated nucleotides at their 5′ ends that are thought to help guide ribosomes onto the mRNA. In bacteria, there is a short sequence in front of the start codon to which a ribosomal RNA hybridizes. In the cytoplasm, ribosomes bind to a 5′ cap (a modified G attached post-transcriptionally to the end of the mRNA) and thread onto the mRNA for some distance before reaching the first start codon. Neither of these features is present at the 5′ ends of mitochondrial mRNA.

B. The termination codons for protein synthesis in mitochondria are unusual in two ways. First, the termination codon in mRNA 16 is AGA (see Figure 14–19), which in the nucleus encodes arginine. Second, the termination codons in mRNAs 7 and 13 are not completely encoded in the DNA; instead, they are generated by addition of the poly-A tail (see Figure 14–19). In mRNA 7, only the initial U of the UAA stop codon is encoded; in mRNA 13, only the initial UA of the stop codon is encoded.

C. The presence of tRNA genes at the exact boundaries of the mRNA genes suggests that they might be involved in processing the mRNAs out of the single, long primary transcript. The tRNAs are thought to serve as structural signposts for the processing of the primary transcript. The folding of the tRNAs into cloverleaf structures would place distinctive structures at the ends of the mRNAs. It is thought that the tRNA structures are recognized and cleaved at their ends to remove them from the primary transcript. The mRNAs are then the remains of tRNA processing. This scheme is referred to as the tRNA punctuation model of RNA processing.

References: Montoya J, Ojala D & Attardi G (1981) Distinctive features of the 5′-terminal sequences of the human mitochondrial mRNAs. *Nature* 290, 465–470.

Ojala D, Montoya J & Attardi G (1981) tRNA punctuation model of RNA processing in human mitochondria. *Nature* 290, 470–474.

14–84 The abnormal patterns of cytochrome absorption suggest that both *poky* and *puny* affect mitochondrial function. The genetic analysis is consistent with a cytoplasmic mode of inheritance for *poky*, but a nuclear mode of inheritance for *puny*.

Crosses 7, 8, and 9 in Table 14–4 are control crosses, which show that wild type always yields fast-growing progeny and that mutants always yield slow-growing progeny.

Crosses 1 and 2 show the cytoplasmic mode of inheritance of *poky*. When *poky* was present in the protoperithecial parent (cytoplasmic donor), all the spores grew slowly (cross 1); when it was in the fertilizing parent, the spores grew rapidly (cross 2). This result is expected if the cytoplasmic donor determines the type of mitochondria present in the spores. In cross 1, *poky* was the cytoplasmic donor and the spores grew slowly. In cross 2, wild type was the cytoplasmic donor and the spores grew rapidly.

Crosses 3 and 4 show the nuclear mode of inheritance of *puny*. In both crosses, *puny* contributes a mutant gene to the fusion and the wild type contributes a normal gene to the fusion. These genes are divided up equally (in a Mendelian fashion) among the spores so that half the progeny grow rapidly and half the progeny grow slowly.

Crosses 5 and 6 are slightly more complicated because they involve the interplay of the two mutations. In cross 5, *poky* is the cytoplasmic donor, and since all spores receive "*poky*" mitochondria, all spores grow slowly. Some spores (about half) will also carry the *puny* mutation in their nuclei (the other half will have wild-type nuclei—from *poky*), but it makes no difference whether the nuclei are normal or mutant because the mitochondria are already compromised by the *poky* mutation. In cross 6, *poky* is the nuclear donor (*puny* is the cytoplasmic donor); therefore, the *poky* mutation is present in *none* of the spores. Once again, half the spores will carry the *puny* mutation and half will be wild type; however, in the absence of "*poky*" mitochondria, the nuclear phenotypes are expressed. Thus, half the spores will grow rapidly and half will grow slowly.

It was this sort of distortion from the expected Mendelian behavior of genes that led ultimately to the realization that mitochondria (and later chloroplasts) carried genetic material.

References: Mitchell MB & Mitchell HK (1952) A case of "maternal" inheritance in *Neurospora crassa*. *Proc. Natl Acad. Sci. USA* 38, 442–449.

Mitchell MB, Mitchell HK & Tissieres A (1953) Mendelian and non-Mendelian factors affecting the cytochrome system in *Neurospora crassa*. *Proc. Natl Acad. Sci. USA* 39, 606–613.

MCAT STYLE

14–85

B. Finding that electron-transport complexes span the inner mitochondrial membrane indicated that, in principle, they could pump protons across the membrane. Choice A is not correct because detecting electron transport in purified membranes does not provide any evidence that electron transport and ATP production are separable events. Choice C is not correct because a requirement to be embedded in the membrane does not distinguish between a direct mechanism of ATP production and chemiosmotic coupling. Choice D is incorrect because if all transport complexes were exposed only to the matrix, they would not be able to pump protons across the membrane.

14–86

C. The observation that the fragmented membranes could transport electrons, but not generate ATP, indicates that electron transport and ATP generation are separable events. The fragmented membranes were not able to generate ATP because they had lost the ability to generate and maintain a proton gradient. Choice A does not distinguish between chemiosmotic coupling and direct linkage between electron transport and ATP production. Choice B only shows that the electron-transport chains are embedded in the membrane. Choice D is not correct because production of protons does not necessarily mean that they are used to make ATP.

14–87

C. Replacement of the electron-transport chain with an alternative mechanism for generating a proton gradient provided direct proof that ATP production was completely independent of electron transport. Choice A is not correct because protons must flow into the matrix space down a concentration gradient to generate ATP. A decrease in pH inside the matrix (an increase in proton concentration) would generate a backward gradient that would prevent proton flow and ATP production. Choice B is incorrect because it does not rule out the possibility that ATP synthase is directly linked to electron transport. Choice D is not correct because it does not show that electron transport and ATP production are separable events; it shows only that electron transport results in transport of protons across the membrane.

CHAPTER 15

Cell Signaling

IN THIS CHAPTER

PRINCIPLES OF CELL SIGNALING

DEFINITIONS

15–1 Guanine nucleotide exchange factor (GEF)

15–2 Receptor

15–3 Adaptation or desensitization

15–4 Interaction domain

15–5 Paracrine signaling

15–6 Kinase cascade

15–7 Second messenger

15–8 Endocrine cell

15–9 Extracellular signal molecule

15–10 Protein kinase

15–11 Neurotransmitter

15–12 GTPase-activating protein (GAP)

15–13 Scaffold protein

15–14 Contact-dependent signaling

TRUE/FALSE

15–15 False. Signaling molecules that bind to cell-surface receptors do not have to cross the plasma membrane; thus, they can be large or small, hydrophilic or hydrophobic. By contrast, signaling molecules that bind to intracellular receptors must be sufficiently small and hydrophobic to diffuse across the plasma membrane.

15–16 False. Most second messengers, including cyclic AMP, Ca^{2+}, and IP_3, are water-soluble and diffuse freely through the cytosol; however, second messengers such as diacylglycerol are lipid-soluble and diffuse in the plane of the membrane.

THOUGHT PROBLEMS

15–17 Both types of signaling can occur over long distances: neurons can send action potentials along very long axons (from the spinal cord to the fingers, for example), and hormones are passed through the bloodstream throughout the organism. Neurons secrete large amounts of neurotransmitters into a small, well-defined space at the synapse, yielding a high

local concentration. Neurotransmitter receptors, therefore, need to bind to neurotransmitters with only low affinity (high K_d). By contrast, hormones are diluted extensively in the bloodstream, where they circulate at minuscule concentrations; hormone receptors, therefore, generally bind their hormones with extremely high affinity (low K_d).

Neuronal signaling is very fast, limited only by the speed of propagation of the action potential and the workings of the synapse. In addition to speed, nerves communicate directly with one or a few cells. Hormonal signaling is slower, limited by blood flow and diffusion over relatively large distances, but it communicates at the same time with all the diverse and widely dispersed target cells in the body.

15–18

A. A telephone conversation is analogous to synaptic signaling in the sense that it is a private communication from one person to another, usually some distance away and sometimes very far away. It differs from synaptic signaling because it is (usually) a two-way exchange, whereas synaptic signaling is a one-way communication.
B. Talking to people at a cocktail party is analogous to paracrine signaling, which occurs between different cells (individuals) and is locally confined.
C. A radio announcement is analogous to an endocrine signal, which is sent out to the whole body (the audience) with only target cells (individuals tuned to the specific radio station) affected by it.
D. Talking to yourself is analogous to an autocrine signal, which is a signal that is sent and received by the same cell.

15–19 Cells with identical receptors can respond differently to the same signal molecule because of differences in the internal machinery to which the receptors are coupled. Even when the entire signaling pathway is the same, cells can respond differently if they express different effector proteins at the ends of the pathways.

15–20 In the normal kinase cascade, PK2 activates PK1. If PK1 is permanently activated, a response is observed independent of the status of PK2. If the order were reversed—that is, PK1 activates PK2—signaling would not occur when PK2 carried an inactivating mutation.

If the experimental set-up were changed so that PK1 was mutationally inactive and PK2 carried an activating mutation, no response would be observed since PK2 would not be able to activate the mutationally inactive PK1.

15–21 Phosphorylation/dephosphorylation offers a simple, universal solution to the problem of controlling protein activity. In a signaling pathway, the activities of several proteins must be rapidly switched from the off state to the on state, or vice versa. Attaching a negatively charged phosphate to a protein is an effective way to alter its conformation and activity. And it is an easy modification to reverse. It is a universal solution in the sense that one activity—that of a protein kinase—can be used to attach a phosphate, and a second activity—a protein phosphatase—can be used to remove it. About 2% of the protein-coding genes in the human genome encode protein kinases, which presumably arose by gene duplication and modification to create appropriate specificity. Because serines, threonines, and tyrosines are common amino acids on the surfaces of proteins, target proteins can evolve to have appropriate phosphorylation sites at places that will alter their conformations. Finally, phosphorylation/dephosphorylation provides a flexible response that can be adjusted to give rapid on/off switches or more long-lasting changes.

All of these attributes of phosphorylation/dephosphorylation are missing with allosteric regulators. While it is possible, in principle, for

small molecules to turn proteins on or off, it is not a universal solution. Specific molecules would have to be "designed" for each target protein, which would require the evolution of a metabolic pathway for the synthesis and degradation of each regulatory molecule. Even if such a system evolved for one target protein, that specific solution would not help with the evolution of a system for other target proteins. In addition, regulation by binding of small molecules is very sensitive to the concentration of the regulator. For a monomeric target protein, the concentration of a small molecule would have to change by 100-fold to go from 9% bound to 91% bound—a minimal molecular switch (see Problem 3–86). Few metabolites in cells vary by such large amounts.

15–22 GTP-binding proteins are uniformly on when GTP is bound and off when GDP is bound; thus, GEFs turn GTP-binding proteins on and GAPs turn them off. The same is not true for protein kinases and phosphatases. Attachment of a phosphate will turn some target proteins on and others off. Indeed, attachment of a phosphate at one location in a protein can turn it on, while phosphorylation at a different location can turn the same protein off. Thus, while protein kinases throw the molecular switch, it is not always in the same direction.

15–23 The use of a scaffolding protein to hold the three kinases in a signaling complex increases the speed of signal transmission and eliminates crosstalk between pathways; however, there is relatively little opportunity for amplification of the signal from the receptor to the third kinase. Freely diffusing kinases offer the possibility for greater signal amplification since the first kinase can phosphorylate many molecules of the second kinase, which in turn can phosphorylate many molecules of the third kinase. The speed of signal transmission is likely to be slower, unless the concentration of kinases (and the potential for amplification) is high enough to compensate for their separateness. Finally, free kinases offer the potential for spreading the signal to other signaling pathways and to other parts of the cell. The organization that a cell uses for a particular signaling pathway depends on what the pathway is intended to accomplish.

15–24

A. 3. PH (pleckstrin homology) domains bind phosphorylated inositol phospholipids in the plasma membrane.
B. 1. PTB (phosphotyrosine-binding) domains bind phosphotyrosines in target proteins.
C. 1. SH2 (Src homology 2) domains bind phosphotyrosines in target proteins.
D. 2. SH3 (Src homology 3) domains bind proline-rich sequences in target proteins.

15–25

1. If more than one effector molecule must bind to activate the target molecule, the response will be sharpened in a way that depends on the number of required effector molecules. At low concentrations of the effector, most target proteins will have a single effector bound (and therefore be inactive). At increasing concentrations of effector, the target proteins with the requisite number of bound effectors will rise sharply, giving a correspondingly sharp increase in the cellular response.
2. If the effector activates one enzyme and inhibits another enzyme that catalyzes the reverse reaction, the forward reaction will respond sharply to a gradual increase in effector concentration. This is a common strategy employed in metabolic pathways involved in energy production and consumption.
3. The above mechanisms give sharp responses, but a true all-or-none

response can be generated if the effector molecule triggers a positive feedback loop so that an activated target molecule contributes to its own further activation. If the product of an activated enzyme, for example, binds to the enzyme to activate it, a self-accelerating, all-or-none response will be produced.

CALCULATIONS

15–26 At a circulating concentration of hormone equal to 10^{-10} M, about 1% of the receptors will have a bound hormone molecule {$[R–H]/[R]_{TOT} = 10^{-10}$ M/(10^{-10} M + 10^{-8} M) = 0.0099}. Half of the receptors will have a bound hormone molecule when the concentration of hormone equals the K_d; that is, at 10^{-8} M {$[R–H]/[R]_{TOT} = 10^{-8}$ M/(10^{-8} M + 10^{-8} M) = 0.5}. Thus, the hormone concentration will have to rise 100-fold to elicit a response. The relationships between concentration of ligand (hormone, in this case), K_d, and fraction bound are developed in Problem 3–86.

15–27

A. The concentrations of insulin in each of the unknown samples can be read directly from the standard curve by finding where the measured bound/free ratio intersects the calibration curve and reading the value for unlabeled insulin from the *x* axis.
   Sample 1 1.1 pg/mL
   Sample 2 6.1 pg/mL
   Sample 3 2.7 pg/mL
B. The most accurate part of the curve lies between bound/free ratios of about 0.4 to 0.7. Below 0.4 the curve flattens out so that large differences in insulin concentration give small differences in bound/free ratios.
C. The assay for human insulin will work even if the antibodies are directed against pig insulin, provided that the antibodies were raised in animals other than humans. As long as there are structural features (antigenic determinants) that are shared between the pig and human insulins—so that the antibodies raised in the pig react with human insulin—the assay will still be valid for measuring human insulin concentrations. The validity of RIA depends on the identical behavior of the antigen in the unknown samples with the antigen in known standards. Since the unknown sample and the standard are human insulins, the assay is valid.

   You may have wondered why the assay would not work if the antibodies were raised in humans. If antibodies against pig insulin were raised in humans, the antibodies would recognize those portions of pig insulin that are different from human insulin. That is because humans (like other animals) do not normally produce antibodies against their own proteins. As a result, the antibodies generated in humans would not bind to human insulin and the assay would not work.

Reference: Yalow RS (1978) Radioimmunoassay: a probe for the fine structure of biologic systems. *Science* 200, 1236–1245.

15–28

A. A cell will contain 100,000 molecules of A and 10,000 molecules of B at these rates of synthesis and average lifetimes. The number of molecules equals the rate of synthesis times the average lifetime. For A, the number of molecules = (1000 molecules/sec)(100 sec).
B. After 1 second, the number of A molecules will have increased by 10,000 to a total of about 110,000 molecules per cell—a 10% increase over the number present before the boost in synthesis. The number of B molecules will also increase by 10,000 to a total of about 20,000, which represents a doubling of its concentration. (For simplicity, the breakdown in A and B over a one-second interval can be neglected.)
C. Because of its larger proportional increase in the short term, molecule B would be the preferred signal molecule. Note that after a sufficiently

long time both molecules would increase by a factor of 10 in response to a 10-fold increase in rate of synthesis. For signaling, it is the rapidity of the change that is most critical. This calculation illustrates the surprising principle that the time it takes to switch a signal on is determined by the lifetime of the signal molecule.

DATA HANDLING

15–29 The results shown in Figure 15–3 are consistent with chemical signaling: in particular, the limited range of the signal (experiments A and B); the ability to bend around corners (experiment B); the ability to penetrate a semipermeable membrane but not a glass coverslip (experiments A and C); and the profound influence of a gentle stream of liquid (experiment D). These experiments go a long way toward demonstrating a secreted chemical signal, but the final proof is the isolation of the signal molecule, which was accomplished many years afterward and shown to be cyclic AMP.

Reference: Bonner JT & Savage LJ (1947) Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum. J. Exp. Zool.* 106, 1–26.

15–30

A. If phosphorylation of the two subunits occurs independently and at equal rates, four different types of receptor will exist: nonphosphorylated receptor, receptor phosphorylated on the γ subunit, receptor phosphorylated on the δ subunit, and receptor phosphorylated on both subunits. At 0.8 mole phosphate/mole receptor, each subunit would be 40% phosphorylated and 60% nonphosphorylated. Thus, the ratio of the various receptor forms would be 36% with no phosphate (0.6 × 0.6), 24% with only the γ subunit phosphorylated (0.6 × 0.4), 24% with only the δ subunit phosphorylated (0.6 × 0.4), and 16% with both subunits phosphorylated (0.4 × 0.4). At 1.2 mole phosphate/mole receptor, the ratios would be: 16% with no phosphate, 24% with the γ subunit phosphorylated, 24% with the δ subunit phosphorylated, and 36% with both subunits phosphorylated.

B. These experiments suggest that desensitization requires only one phosphate per receptor and that phosphorylation of either the γ or the δ subunit is sufficient for desensitization. For both preparations, the fraction that behaved like the untreated receptor matched best the fraction calculated to carry no phosphate: 36% versus 36% at 0.8 mole phosphate/mole receptor and 18% versus 16% at 1.2 mole phosphate/mole receptor. This result suggests that phosphorylation of either subunit is sufficient to promote desensitization. If a specific subunit were required to be phosphorylated, then the expected fractions behaving like the untreated receptor would have been 60% (24% + 36%) at 0.8 mole phosphate/mole receptor and 40% (24% + 16%) at 1.2 mole phosphate/mole receptor.

Reference: Huganir RL, Delcour AH, Greengard P & Hess GP (1986) Phosphorylation of the nicotine acetycholine receptor regulates its rate of desensitization. *Nature* 321, 774–776.

MEDICAL LINKS

15–31 Although succinylcholine binds to the acetylcholine receptor very tightly, it does not trigger the conformational change necessary to open the ion channel and initiate muscle contraction. Succinylcholine prevents normal muscle contraction by competing with acetylcholine for binding to the receptor, thereby blocking its action. For this reason, succinylcholine is referred to as an acetylcholine *antagonist.*

SIGNALING THROUGH G-PROTEIN-COUPLED RECEPTORS

DEFINITIONS

15–32 Stimulatory G protein (G_s)

15–33 Trimeric GTP-binding protein (G protein)

15–34 Calmodulin

15–35 Cyclic AMP phosphodiesterase

15–36 G-protein-coupled receptor (GPCR)

15–37 GPCR kinase (GRK)

15–38 Ryanodine receptor

15–39 Phospholipase C-β (PLCβ)

15–40 Regulator of G protein signaling (RGS)

15–41 Inositol 1,4,5-trisphosphate (IP_3)

15–42 Cyclic AMP-dependent protein kinase (PKA)

15–43 Protein kinase C (PKC)

15–44 Rhodopsin

15–45 Ca^{2+}/calmodulin-dependent kinase (CaM-kinase)

15–46 CRE-binding (CREB) protein

TRUE/FALSE

15–47 False. It is the substrates for PKA, not PKA itself, that differ in different cell types.

15–48 True. The activity of a population of protein molecules whose activity is regulated by phosphorylation depends on the percentage of the molecules that are phosphorylated, which in turn depends on the relative rates of phosphate addition and removal.

15–49 True. Intracellular signaling pathways that involve enzymes or ion channels can significantly amplify a signal. Once activated, a protein kinase, for example, can phosphorylate hundreds of its target proteins. Similarly, activation of an ion channel can raise the cytosolic concentration of a critical ion by many fold.

THOUGHT PROBLEMS

15–50 The mutant G protein would be constantly active. Each time the α subunit hydrolyzed GTP to GDP, the GDP would spontaneously dissociate, allowing GTP to bind and reactivate the α subunit. Normally, GDP is tightly bound by the α subunit, which keeps the G protein in its inactive state until release of GDP is stimulated by interaction with an appropriate GPCR.

15–51 Each time a G protein picked up a nonhydrolyzable analog of GTP it would be locked into its active form, from which it could not escape by the usual route of GTP hydrolysis. In the absence of adrenaline, most of the G protein would be in the GDP-bound form, and GDP is released only slowly in the absence of stimulation by an activated receptor. Thus, you might

expect a slow activation of G protein after injection of a nonhydrolyzable GTP analog and a correspondingly slow increase in glycogen breakdown. In the presence of adrenaline, GDP would be rapidly released and nonhydrolyzable GTP would be bound. A brief exposure to adrenaline normally would stimulate glycogen breakdown for a short time, until adrenaline was removed and the signaling pathway was turned off. However, in the presence of a nonhydrolyzable analog of GTP, the pathway would remain on even after adrenaline was removed. Thus, the nonhydrolyzable analog would cause a prolonged response to a pulse of adrenaline.

15–52 RGS proteins are GAPs that have a critical role in shutting off G protein responses in animals and yeasts. They stimulate the GTPase activity of G proteins, converting them to their inactive (GDP-bound) form, and thereby limit the duration of a response.

15–53 The "cyclic" in cyclic AMP refers to the ring of atoms formed by the phosphorus atom, its two oxygen atoms, and the carbons at the 3′, 4′, and 5′ positions of the ribose sugar (**Figure 15–29**). The ball-and-stick representations above and below the chemical formula give a more realistic representation of the chemical structure. The six-member phosphodiester ring is fused to the five-member ribose ring, forming a fairly planar structure that resembles the adenine ring in size and shape. In the more common representation (center), the phosphodiester ring looks very strained, but in reality it's not.

15–54 Rapid breakdown keeps cyclic AMP levels low, typically about 10^{-7} M. An extracellular signal can increase this concentration more than twentyfold in seconds. The lower the cyclic AMP level, the larger the proportional increase achieved upon activation of adenylyl cyclase, which makes new cyclic AMP. By analogy, if you have $100 in the bank, and you deposit another $100, you have doubled your wealth. If you have only $10 to start with and you deposit $100, you have increased your wealth tenfold, a much larger proportional increase resulting from the same deposit.

15–55 Since β-adrenergic receptors are coupled to adenylyl cyclase through a G protein, a reasonable guess would be GTP. Activation of the receptor by isoproterenol would stimulate the G protein to exchange bound GDP for free GTP. In the absence of free GTP, the G protein would not be activated and thus could not activate adenylyl cyclase. The requirement for GTP

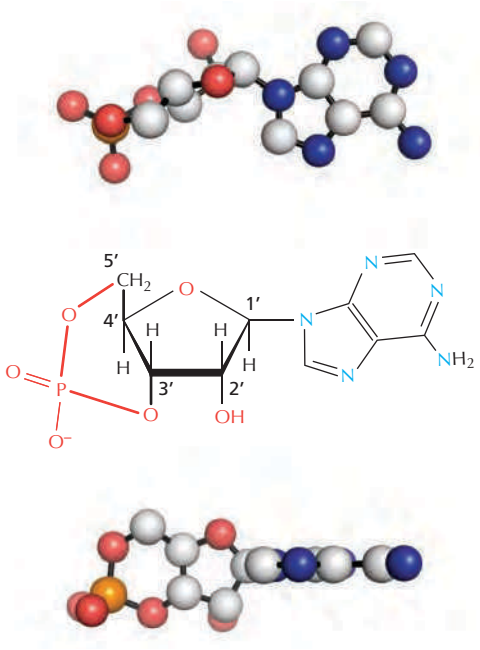


Figure 15–29 Chemical formula and ball-and-stick representations of cyclic AMP (Answer 15–53). The ball-and-stick models are rotated 90° relative to each other to illustrate the similarities between the adenine ring and the fused ribose and phosphodiester rings.

in the receptor-mediated activation of adenylyl cyclase was one of the original clues that led to the discovery of G proteins.

15–56 Any mutation that generated a regulatory subunit incapable of binding to the catalytic subunit would produce a permanently active PKA. When the catalytic subunit is not bound to the regulatory subunit, it is active.

Two general types of mutation in the regulatory subunit could produce a permanently inactive PKA. A regulatory subunit that was altered so that it could bind the catalytic subunit, but not bind cyclic AMP, would not release the catalytic subunit, rendering PKA permanently inactive. Similarly, a mutant regulatory subunit that could bind cyclic AMP, but not undergo the conformational change needed to release the catalytic subunit, would permanently inactivate PKA.

15–57 Because the intracellular concentration of Ca^{2+} is so low, an influx of relatively few Ca^{2+} ions leads to large changes in its cytosolic concentration. Thus, a tenfold increase in Ca^{2+} can be achieved by raising the concentration of Ca^{2+} into the micromolar range, which would require entry of relatively few ions into the cytosol. By contrast, many more Na^+ ions (10^4 more) would need to enter the cytosol to change its concentration by the same amount. In muscle, more than a tenfold change in Ca^{2+} can be achieved in microseconds by releasing Ca^{2+} from the intracellular stores of the sarcoplasmic reticulum, a task that would be difficult to accomplish if changes in the millimolar range were required.

15–58 EGTA, by chelating Ca^{2+}, would be expected to interfere with signaling pathways that use Ca^{2+} as a second messenger. Glucagon triggers glycogen breakdown in liver via a cyclic AMP pathway and thus would not be affected by EGTA. By contrast, vasopressin signals glycogen breakdown via a Ca^{2+} pathway and would be blocked by injection of EGTA.

15–59 The time that the catalytic kinase subunit spends in its active conformation depends on the extent to which its regulatory subunits are modified. Each modification by phosphorylation or by Ca^{2+} binding nudges the equilibrium toward the active conformation of the kinase subunit; that is, each modification increases the time spent in the active state. By summing the inputs from multiple pathways in this way, phosphorylase kinase integrates the signals that control glycogen breakdown.

15–60 When CaM-kinase II is exposed to Ca^{2+}/calmodulin, it becomes an active protein kinase and phosphorylates adjacent copies of itself in its multicopy complex. In its phosphorylated state, CaM-kinase II remains active even in the absence of Ca^{2+}, thereby prolonging the duration of the kinase activity beyond that of the initial activating Ca^{2+} signal. Its self-phosphorylation allows it to "remember" its exposure to Ca^{2+}/calmodulin. It finally "forgets" when a protein phosphatase removes the phosphate, shutting off its activity.

15–61

A. An inhibitor of cyclic GMP phosphodiesterase would prevent the reduction in cyclic GMP that normally occurs in response to light activation of rhodopsin. High levels of cyclic GMP would keep the cation channels open, preventing the membrane hyperpolarization that is essential for the visual response.
B. A nonhydrolyzable analog of GTP would lead to prolonged activation of transducin in response to activated rhodopsin. Continued activation of transducin would keep cyclic GMP phosphodiesterase high, which would lead in turn to a protracted decrease in cyclic GMP, a prolonged hyperpolarization of the membrane, and an extended visual response.
C. An inhibitor of rhodopsin-specific kinase would prolong the visual response by increasing the signaling lifetime of the activated form of

rhodopsin. Normally, rhodopsin-specific kinase adds a phosphate to the cytoplasmic tail of rhodopsin, inhibiting the interaction of activated rhodopsin with transducin.

15–62 The concentration of cyclic GMP in the smooth muscle cells lining the blood vessels of the penis is controlled by its rate of synthesis by guanylyl cyclase and its rate of degradation by cyclic GMP phosphodiesterase. The natural signal molecule, NO, binds to guanylyl cyclase and stimulates its activity, thereby increasing the concentration of cyclic GMP by increasing its rate of synthesis. The drug Viagra® binds to cyclic GMP phosphodiesterase and inhibits its activity, thereby increasing the concentration of cyclic GMP by decreasing its rate of degradation.

15–63 The signal is amplified at four points in the pathway. (1) A single, activated β-adrenergic receptor can activate many copies of the G protein, acting as a GEF to promote GDP release and GTP binding. Adenylyl cyclase, by contrast, is activated stoichiometrically; that is, adenylyl cyclase is active only so long as the activated Gα subunit is bound. (2) While active, a single molecule of adenylyl cyclase can convert many molecules of ATP to cyclic AMP. The next step—activation of PKA—is stoichiometric: four cyclic AMP molecules activate two PKA molecules. (3) A single molecule of PKA can add phosphates to many molecules of phosphorylase kinase. (4) A single molecule of phosphorylase kinase can add phosphates to many molecules of glycogen phosphorylase. Glycogen phosphorylase represents the end of the signaling pathway and the beginning of the biochemical pathway for utilization of the energy stored in glycogen (thus, its ability to cleave many molecules of glucose from glycogen is not considered an amplification step in the signaling pathway).

15–64 The β-adrenergic receptor is turned off directly by the conformational change that occurs when adrenaline is no longer bound. The Gα subunit becomes inactive when it hydrolyzes the attached GTP to GDP, which allows it to reassociate with the βγ subunits. Adenylyl cyclase becomes inactive as soon as Gα dissociates. Cyclic AMP is constantly being converted to AMP by cyclic AMP phosphodiesterase. In the absence of its continued synthesis, cyclic AMP quickly returns to its pre-stimulated level. At low concentrations, cyclic AMP dissociates from the regulatory subunits of PKA, which rebind the catalytic subunits to turn off PKA. In the absence of ongoing phosphorylation of phosphorylase kinase, a protein phosphatase removes the phosphates, turning off phosphorylase kinase. Similarly, a protein phosphatase quickly removes phosphates from glycogen phosphorylase, thereby turning it off.

CALCULATIONS

15–65

A. The specific binding curve is obtained by subtracting the nonspecific curve from the total. As illustrated in **Figure 15–30**, the specific binding curve reaches a plateau above 4 nM alprenolol. Thus, the β-adrenergic receptors are saturated with alprenolol above this concentration.

B. There are 1500 β-adrenergic receptors per frog erythrocyte. Since one alprenolol molecule binds per receptor, the number of bound alprenolol molecules is equal to the number of receptors. At saturation, 20,000 cpm of alprenolol binds per mg of erythrocyte membrane (Figure 15–30). Thus, the amount of bound alprenolol is

$$\text{bound alprenolol} = \frac{20 \times 10^3 \text{ cpm}}{\text{mg}} \times \frac{\text{mmol}}{10^{13} \text{ cpm}} \times \frac{6 \times 10^{20} \text{ molecules}}{\text{mmol}} \times \frac{\text{mg}}{8 \times 10^8 \text{ erythrocyte}}$$

$$= 1500 \text{ molecules per erythrocyte}$$

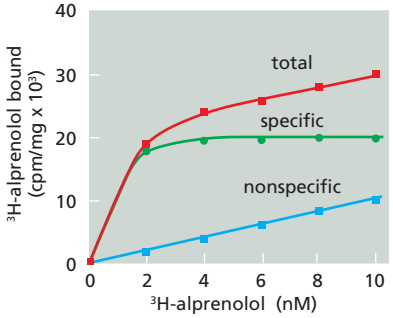


Figure 15–30 Specific binding of alprenolol to frog erythrocyte membranes (Answer 15–65).

Since one molecule of alprenolol binds per β-adrenergic receptor, there are 1500 β-adrenergic receptors per erythrocyte.

Reference: Lefkowitz RJ, Limbird LE, Mukherjee C & Caron MG (1976) The β-adrenergic receptor and adenylate cyclase. *Biochim. Biophys. Acta* 457, 1–39.

15–66

A. Since 5.5 mmol of GppNp were bound per mole of total rhodopsin and 0.0011% of the rhodopsin was activated, 5.5 mmol GppNp were bound per 0.011 mmol of activated rhodopsin. Thus, 500 molecules (5.5 mmol/0.011 mmol) of transducin were activated per activated rhodopsin. This experiment indicates that the first stage of amplification during visual excitation is achieved through the effect of activated rhodopsin on transducin. The calculation implies that there is very little additional amplification in the interaction of transducin with cyclic GMP phosphodiesterase.

B. These relative binding affinities indicate that transducin-GDP binds to activated rhodopsin and transducin-GTP is released, permitting activated rhodopsin to interact with many transducin molecules, as expected by the deduced amplification mechanism. Furthermore, the tight binding of transducin-GTP to cyclic GMP phosphodiesterase suggests that activation of phosphodiesterase is stoichiometric; that is, one molecule of activated transducin activates one molecule of cyclic GMP phosphodiesterase.

References: Fung BK-K & Stryer L (1980) Photolyzed rhodopsin catalyzes the exchange of GTP for bound GDP in retinal rod outer segments. *Proc. Natl Acad. Sci. USA* 77, 2500–2504.

Fung BK-K, Hurley JB & Stryer L (1981) Flow of information in the light-triggered cyclic nucleotide cascade of vision. *Proc. Natl Acad. Sci. USA* 78, 152–156.

DATA HANDLING

15–67

A. While all the mutations cause a sterile phenotype, loss of the G-protein α-subunit gene causes proliferation to arrest even in the absence of the α-factor pheromone. Since arrested proliferation is one of the normal responses to the binding of the α- factor pheromone to its receptor, it must be that the βγ subunits, rendered free by the absence of the α subunit, normally transmit the mating signal to the downstream effector molecules. This interpretation is reinforced by the phenotypes of the double mutants, which no longer show the arrested-proliferation phenotype characteristic of the simple α-subunit deletion. (Note that the simple deletion mutants of the α subunit described here cannot be isolated in reality because they do not proliferate under normal conditions. The phenotype of cells missing α-subunit activity was determined in other ways. Can you guess how?)

B.

1. An α subunit that can bind GTP but not hydrolyze it would be expected to exhibit an arrested-proliferation, sterile phenotype in the presence or absence of the α-factor pheromone. An α subunit with bound GTP releases the βγ subunit, which would trigger the downstream pathway leading to proliferation arrest.
2. An α subunit that cannot be myristoylated would not be properly localized to the membrane. Since the βγ subunit is localized to the membrane by a lipid group attached to the γ subunit, the cytosolic α subunit would not interact properly with the membrane-bound βγ subunit. As a result, the βγ subunit would trigger the downstream pathway, leading to

an arrested-proliferation, sterile phenotype in the presence or absence of the α-factor pheromone.

3. An α subunit that could not bind to the pheromone receptor could not "read" the signal from an α-factor-stimulated receptor and the mutant strain would therefore be sterile. The α subunit would remain in its GDP-bound form, and thus remain complexed with the βγ subunits. Such a mutant would be expected to display a normal-proliferation phenotype in the absence and presence of the α-factor pheromone.

Reference: Kurjan I (1992) Pheromone response in yeast. *Annu. Rev. Biochem.* 61, 1097–1129.

15–68

A. The concentration of cyclic AMP is a balance between its synthesis (by adenylyl cyclase) and its breakdown (by cyclic AMP phosphodiesterase). Since *Dunce* flies are missing one form of cyclic AMP phosphodiesterase, they hydrolyze cyclic AMP more slowly than usual, which results in an elevated level.

B. Homozygous duplications would be expected to produce twice the enzyme activity of normal flies, and homozygous deletions would be expected to give no activity. The results with the duplications and deletions of the *Dunce* gene are contrary to this expectation because there are two different genes coding for two distinct cyclic AMP phosphodiesterases in flies, as revealed by the sucrose-gradient analysis in Figure 15–10. Since the activities are about equal in normal flies, a doubling of one activity increases the total activity to about 1.5 times normal and removal of one activity lowers the total to half normal.

C. Since caffeine is an inhibitor of cyclic AMP phosphodiesterase, it would be expected to raise the level of cyclic AMP in flies. If the increased level of cyclic AMP were responsible for the learning defect associated with the *Dunce* mutation, caffeine would be expected to impair learning ability. Experiments show that caffeine does indeed interfere with learning in flies. This result strongly supports the idea that the learning defect in *Dunce* mutants is caused by the loss of phosphodiesterase activity and not, for example, by a regulatory defect that shuts off phosphodiesterase and independently interferes with a learning pathway.

It is worth pointing out the element of luck in this story. It would have been very difficult for the scientists working on *Dunce* to deduce the biochemical nature of the gene product (though its sequence would have revealed its function ultimately); similarly, the scientists interested in cyclic nucleotide deficiencies had not noticed a learning defect. The story emerged much more rapidly than normal because the two groups happened to work at the same institution.

References: Byers D, Davis RL & Kiger JA (1981) Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. *Nature* 289, 79–81.

Chen C-N, Denome S & Davis RL (1986) Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila dunce*$^+$ gene, the structural gene for cyclic AMP phosphodiesterase. *Proc. Natl Acad. Sci. USA* 83, 9313–9317.

15–69

A. The complete G protein does not activate the K^+ channels in the absence of acetylcholine presumably because, like other trimeric G proteins, the active portion is inhibited by one of the subunits. The ability of the Gβγ subunit to open the K^+ channel in the absence of acetylcholine and GTP suggests that it is the active portion of the G protein. This is different from the active component (Gα) of G proteins triggered by GPCRs that activate adenylyl cyclase.

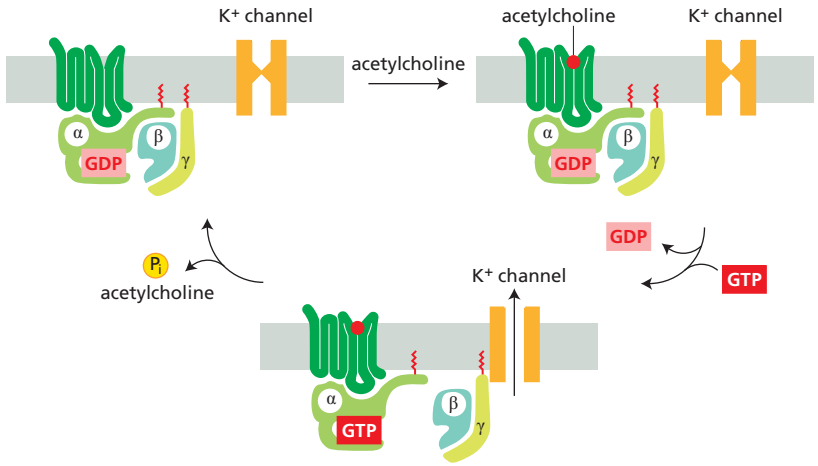


Figure 15–31 Diagram illustrating the activation of K^+ channels in heart by acetylcholine (Answer 15–69).

B. The opening of the K^+ channel in the presence of GppNp and absence of acetylcholine may seem somewhat surprising, since the release of GDP and the binding of GTP by G proteins are normally stimulated by an activated receptor. Even in the absence of an activated receptor, G proteins exchange their bound nucleotides with nucleotides in the cytoplasm. Exchange is slow, and any bound GTP is quickly hydrolyzed in the absence of an activated receptor, thereby keeping the channel closed. The K^+ channels open slowly when GppNp is present because each time a GDP is released and a GppNp is bound, the G protein is locked into an active form. Over the course of a minute, enough G protein is activated in this way to open the K^+ channels in the absence of acetylcholine.

C. A scheme for the G-protein-mediated activation of K^+ channels by acetylcholine is shown in **Figure 15–31**.

References: Logothetis DE, Kurachi Y, Galper J, Neer EJ & Clapham DE (1987) The βγ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature* 325, 321–326.

Reuveny E, Slesinger PA, Inglese J, Morales JM, Iñiguez-Lluhi JA, Lefkowitz RJ, Bourne HR, Jan YN & Jan LY (1994) Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature* 370, 143–146.

MEDICAL LINKS

15–70 Both glycogen and triglyceride breakdown depend on cyclic-AMP-dependent signaling pathways. By inhibiting cyclic AMP phosphodiesterase, caffeine increases cyclic AMP levels to promote glycogen breakdown in muscle and liver and to promote triglyceride breakdown in fat cells. The effects on glycogen are not so important as the effects on triglycerides. By breaking down triglycerides to release fatty acids earlier in the race, runners decrease their dependence on carbohydrate oxidation at that stage, thereby preserving carbohydrates for use throughout the race.

15–71 Because these patients recover abnormally slowly from a flash of bright light, it is likely that they are defective in the return of activated rhodopsin to its inactive state. This recovery process begins with the phosphorylation of the cytosolic tail of rhodopsin by rhodopsin-specific kinase. Phosphorylated rhodopsin is then bound by arrestin. Additional reactions remove the phosphate and replace the all-*trans*-retinal with 11-*cis*-retinal, finally regenerating a rhodopsin molecule that is ready for another cycle of phototransduction. Thus far, patients with Oguchi's disease have been found to have defects in the gene for rhodopsin-specific kinase or in the gene for arrestin.

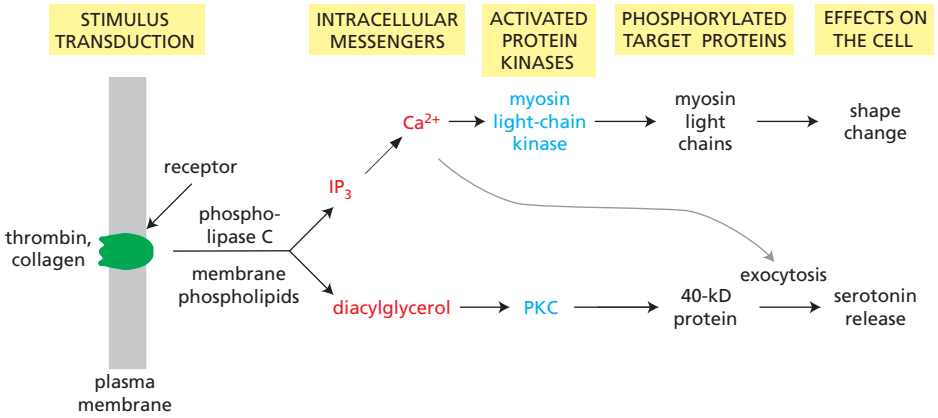


Figure 15–32 Overall pathway for platelet activation (Answer 15–72).

15–72

A. The activities of Ca^{2+} and diacylglycerol suggest that the normal sequence of events involves phospholipase C. Collagen fibers and thrombin stimulate a receptor on the surface of the platelet, which in turn activates phospholipase C, presumably through a G protein. Phospholipase C cleaves phosphatidylinositol bisphosphate to produce IP_3 and diacylglycerol. IP_3 mobilizes internal Ca^{2+} stores, activating myosin light-chain kinase, which phosphorylates the myosin light chain. This branch of the pathway can be stimulated by the calcium ionophore. Diacylglycerol activates PKC, which phosphorylates the 40-kD protein. This branch of the pathway can be stimulated directly by diacylglycerol. These two individual pathways interact to stimulate serotonin release. The overall pathway for platelet activation is diagrammed in **Figure 15–32**.

B. Secretion of serotonin evidently requires both Ca^{2+} and diacylglycerol, since neither alone causes efficient secretion (Figure 15–12B). These experiments imply that the 40-kD protein is involved, although direct proof is lacking. Ca^{2+} is thought to be more directly involved in secretion, enabling the fusion of membranes that is required for exocytosis.

Reference: Nishizuka Y (1983) Calcium, phospholipid turnover and transmembrane signalling. *Phil. Trans. R. Soc. Lond. B* 302, 101–112.

SIGNALING THROUGH ENZYME-COUPLED RECEPTORS

DEFINITIONS

15–73 Ephrins

15–74 Transforming growth factor-β (TGFβ) superfamily

15–75 Receptor tyrosine kinase (RTK)

15–76 Ras

15–77 Rho family

15–78 Focal adhesion kinase (FAK)

15–79 Phosphoinositide 3-kinase (PI 3-kinase)

15–80 Enzyme-coupled receptor

15–81 Tyrosine-kinase-associated receptor

15–82 MAP kinase module

15–83 JAK–STAT signaling pathway

15–84 Pleckstrin homology (PH) domain

15–85 SH2 domain

15–86 TOR or mTOR

TRUE/FALSE

15–87 False. Ligand binding usually causes a receptor tyrosine kinase to assemble into dimers, which, because of their proximity activates the kinase domains. The receptors then phosphorylate themselves to initiate the intracellular signaling cascade. In some cases, the insulin receptor for example, the receptor exists as a dimer and ligand binding is thought to rearrange their receptor chains, causing the kinase domains to come together.

15–88 False. PI 3-kinase phosphorylates inositol head groups at a position (number 3 on the inositol ring) that is not phosphorylated in IP_3. (IP_3 carries phosphates at the 1, 4, and 5 positions on the inositol ring.) Phosphorylation at position 3 serves an entirely different function; it creates inositol head groups that can serve as docking sites for intracellular signaling proteins.

15–89 True. Protein tyrosine phosphatases, unlike serine/threonine protein phosphatases, remove phosphate groups only from selected phosphotyrosines on a subset of tyrosine-phosphorylated proteins.

THOUGHT PROBLEMS

15–90 The added antibody is likely to activate the receptor tyrosine kinase. Because antibodies carry two identical binding sites, they bind to two receptor tyrosine kinase molecules, allowing them to phosphorylate each other and activate the signaling pathway. Experiments of this type were the first to demonstrate that receptor dimerization is the critical step in activation of most receptor tyrosine kinases. It should be noted that not all antibodies that cross-link receptors activate them. Presumably these antibodies cross-link the receptors in a way that does not properly juxtapose the kinase domains and target sites.

15–91

A. The mutant receptor tyrosine kinase will be inactive for signaling because it cannot bind its ligand in the absence of an extracellular domain. The mutant form of the kinase will have no effect on normal signaling mediated by the cell's own receptor tyrosine kinases; ligands will bind to them normally, causing their dimerization and activation (**Figure 15–33A**).

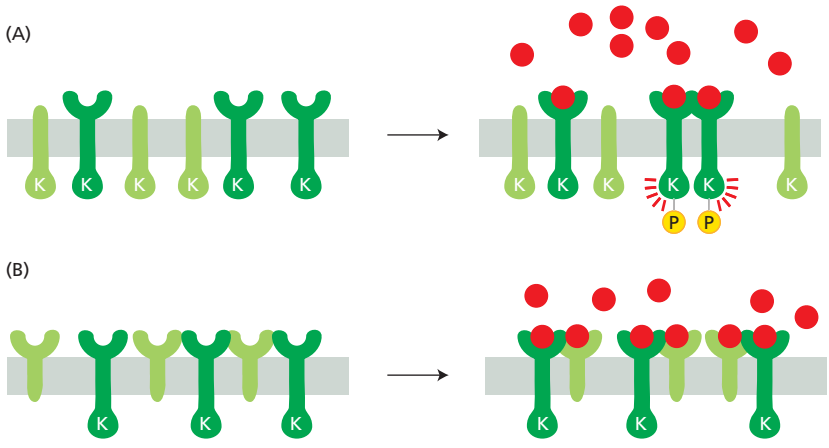


Figure 15–33 Effects of mutant receptors on receptor tyrosine kinase signaling (Answer 15–91). (A) Mutant receptors lacking their extracellular domain. (B) Mutant receptors lacking their intracellular domain.

B. The mutant receptor tyrosine kinase lacking an intracellular domain will also be inactive for signaling because it is missing its kinase domain and phosphorylation sites. Because it retains its ability to bind to the ligand, it will interfere with signaling by the cell's normal receptor tyrosine kinase. Because the mutant kinase is expressed at considerably higher levels than the cell's kinase, the mutant receptor will account for most of the cell's binding of ligand. Even when a normal receptor manages to bind a ligand, it will usually be dimerized with an inactive mutant receptor (**Figure 15–33B**). Under these conditions, the normal receptor will remain inactive because it cannot cross-phosphorylate the mutant partner, nor can its mutant partner phosphorylate it. This is called a "dominant-negative" effect.

15–92

A. Biotin-tagged GST–SH2 proteins could not be used for detecting proteins that bind to SH2 domains in the same way that GST–SH3 proteins can. That is because SH2 domains bind only to target proteins that carry a phosphotyrosine—a modification that doesn't occur normally in bacteria. One way to make the screen work would be to incubate the filters first with a protein tyrosine kinase and ATP. Alternatively, a protein tyrosine might be engineered into *E. coli* so that it could be turned on at the same time the cDNA library was expressed.

B. The main difference between protein interactions with short sequences and the subunit–subunit interactions in multisubunit enzymes lies in their stability and reversibility. Both types of interaction depend largely on the total number and aggregate strength of the weak bonds involved in their formation. Large contact surfaces such as those found among subunits in multisubunit enzymes make for very stable structures, whereas most of the examples of short-sequence recognition are more transient, and in some cases conditional, as in the interaction of SH2 domains with phosphotyrosine-containing proteins.

Reference: Ren R, Mayer BJ, Cicchetti P & Baltimore D (1993) Identification of a ten-amino-acid proline-rich SH3 binding site. *Science* 259, 1157–1161.

15–93 You would expect to see several differences. (1) You would expect a high background of Ras activity in the absence of an extracellular signal because Ras cannot be turned off efficiently. Since Ras activity depends on the balance between its binding to GTP and its GAP-enhanced hydrolysis of GTP, the balance would be somewhat more in favor of the GTP-bound (active) form than normal. (2) As some Ras molecules will already be in their GTP-bound form, Ras activity in response to an extracellular signal would be greater than normal, but would saturate when all Ras molecules were converted to the GTP-bound form. (3) The response to a signal would be less rapid because the signal-dependent increase in GTP-bound Ras would occur over an elevated background of preexisting GTP-bound Ras. (4) The response would be expected to be more prolonged than normal and to persist for a while even after the extracellular signal was removed because of the slower rate of conversion of GTP-bound Ras to its inactive GDP-bound form.

15–94 Activation in both cases depends on proteins that catalyze GDP/GTP exchange on the G protein or Ras protein. Whereas the GPCRs perform this function directly for G proteins, enzyme-coupled receptors assemble multiple adaptor proteins into a signaling complex when the receptors are activated by phosphorylation and one of these recruits a Ras-activating protein that fulfills this function for Ras. Inactivation of G proteins and Ras proteins is also similar. Ras is turned off by a GAP that promotes hydrolysis of GTP. Similarly, the ability of Gα subunits to hydrolyze GTP,

which is intrinsically higher than that of Ras, is also stimulated by their interactions with downstream targets such as adenylyl cyclase.

15–95 In order for activation of Ras to depend on inactivation of a GAP, both the GAP and the GEF would need to be active in the absence of the signal. In this way, the GEF would constantly load GTP onto Ras and the GAP would keep the concentration of Ras-GTP low by constantly inducing GTP hydrolysis to return Ras to its GDP-bound state. Under these conditions, inactivation of the GAP would result in a rapid increase in the Ras-GTP level, allowing rapid signaling. Although this would be a perfectly effective way to regulate the level of active Ras, it would be wasteful of energy. In order to keep Ras in its inactive state, GTP would be constantly hydrolyzed to GDP, which would then need to be reconverted to GTP (by ATP)—a drain on cellular energy metabolism. Regulation by activation of a GEF avoids this problem.

Although avoiding constant GTP hydrolysis is a rational explanation, eukaryotic cells are notoriously profligate in their energy expenditures. At several points in energy metabolism, for example, they operate so-called "futile" cycles that hydrolyze ATP as a means for rapid regulation of the flux through metabolic pathways. Thus, it could be that constant hydrolysis of GTP by a Ras GEF and GAP would not unduly tax the cell's energy budget. Perhaps the cell's method of regulating Ras by controlling the activity of a GEF is simply an evolutionary happenstance.

15–96 The small molecule would not be effective in treating cancers with mutationally activated Ras proteins. Activated Ras causes problems because it signals independently of any upstream influences. Thus, preventing receptors from dimerizing would have no effect on activity of the mutant Ras.

DATA HANDLING

15–97

A. If individual receptor tyrosine kinases phosphorylated themselves, only the band corresponding to the normal receptor (with a functional kinase domain and phosphorylation sites) would appear on the autoradiograph in Figure 15–13C.
B. If receptor tyrosine kinases can phosphorylate each other, then not only would normal receptors pair with each other and be labeled, but mutant receptor 2 (with a dead kinase domain but functional phosphorylation sites) should be labeled whenever it is paired with a receptor that has an active kinase domain (the normal receptor and mutant receptor 3).
C. The results in Figure 15–13C support the cross-phosphorylation model for autophosphorylation. Receptor 2 is labeled when paired with either receptor 1 or receptor 3 (see Figure 15–13C). You might wonder why receptor 1 was labeled when expressed along with receptor 2. In such a mixture, receptor 1 would be expected to pair with itself roughly half the time, allowing cross-phosphorylation.

References: Honegger AM, Kris RM, Ullrich A & Schlessinger J (1989) Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation. *Proc. Natl Acad. Sci. USA* 86, 925–929.

Honegger AM, Schmidt A, Ullrich A & Schlessinger J (1990) Evidence for epidermal growth factor (EGF)-induced intermolecular autophosphorylation of the EGF receptors in living cells. *Mol. Cell. Biol.* 10, 4035–4044.

15–98 PDGF receptors that bind only PI 3-kinase (PI3K) or PLCγ stimulate DNA synthesis to about 70% of the normal value (see Figure 15–15, lanes 2 and 5); thus, both PI3K and PLCγ mediate signaling pathways that increase

DNA synthesis. These two pathways must be somewhat redundant because receptors that bind both PI3K and PLCγ (but nothing else) give the same 70% response as either alone (see lane 6). GAP-mediated signaling seems to inhibit the mitogenic response, since receptors with intact PLCγ and GAP-binding sites stimulate DNA synthesis to a significantly lesser extent (see lane 7) than receptors with just the PLCγ-binding site (see lane 5). PTP appears to play no role in PDGF stimulation of DNA synthesis, since it does not stimulate DNA synthesis alone (see lane 4) or alter the response when paired with a PLCγ-binding site (see lane 8).

References: Valius M & Kazlauskas A (1993) Phospholipase C-γ1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell* 73, 321–334.

Valius M, Secrist JP & Kazlauskas A (1995) The GTPase-activating protein of Ras suppresses platelet-derived growth factor β receptor signaling by silencing phospholipase C-γ1. *Mol. Cell. Biol.* 15, 3058–3071.

15–99 The very steep response curve for activation of MAPK converts it into a molecular switch. Thus, MAPK goes from inactive to active over a very narrow range of input stimulus. This kind of behavior keeps the cascade turned off below a threshold concentration of the input signal, yet delivers a maximum response once that threshold is exceeded.

Reference: Huang C-YF & Ferrell JE (1996) Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA* 93, 10078–10083.

15–100 If scaffold proteins linked the kinases, the activation curves for MAPKK and MAPK would resemble more closely that of MAPKKK; that is, MAPK would behave less like a molecular switch. The gain in speed and precision of signal transmission and the avoidance of cross-talk between pathways evidently compensate for the loss of signal amplification and switchlike behavior, since cells use scaffold proteins to organize many different MAP kinase modules. The (evolutionary) choice of scaffold or independent components for a particular MAP kinase module presumably reflects the functional consequences of the signaling pathway.

15–101 The analysis of individual frog oocytes shows clearly that the response to progesterone is all-or-none, with no oocytes having a partially activated MAP kinase. Thus, the graded response in the population results from an all-or-none response in individual oocytes, with different mixtures of fully mature or immature oocytes giving rise to intermediate levels of MAP kinase activation (**Figure15–34**). It is not so clear why individual oocytes respond differently to different concentrations of progesterone, although there is significant variability among oocytes in terms of age and size (and presumably in the number of progesterone receptors, the concentrations of components of the MAP kinase signaling module, and downstream targets).

Whether a graded response in a population of cells indicates a graded response in each cell or a mixture of all-or-none responses is a question that arises in many contexts in biology.

Reference: Ferrell JE & Machleder EM (1998) The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes. *Science* 280, 895–898.

15–102 The data in Figure 15–19 suggest that Akt phosphorylates itself at serine 473. The kinase-dead mutant, Akt-K179M, is phosphorylated correctly at threonine 308 but not at serine 473. In addition, phosphorylation at serine 473 depends on phosphorylation of threonine 308, as shown by the results with Akt-T308A; thus, it is unlikely that PDK1 carries out this second phosphorylation.

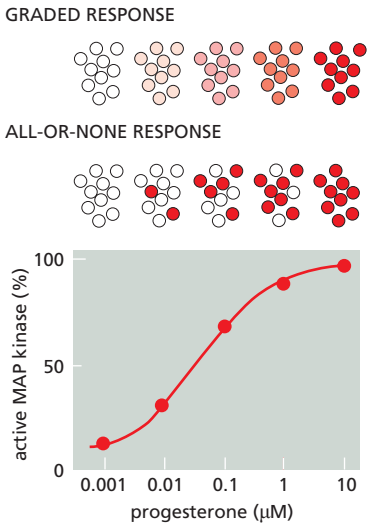


Figure 15–34 Graded or all-or-none responses in individual oocytes that give rise to a graded response in the population (Answer 15–101).

Reference: Toker A & Newton AC (2000) Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J. Biol. Chem.* 275, 8271–8274.

15–103

A. Many transcription factors are phosphorylated in response to cell stimulation: in some instances, this results in tighter binding to DNA, whereas in others it creates an acidic activation domain that promotes transcription. In this case, phosphorylation seems to be necessary for DNA binding because anti-phosphotyrosine antibodies and phosphatase treatment inhibit DNA binding.

B. Free phosphotyrosine will bind to the SH2 domain of the transcription factor. If simple occupancy of the SH2 domain by a phosphotyrosine were all that was required, free phosphotyrosine would be expected to activate the transcription factor instead of inhibiting it. This implies that the SH2 domain must bind to the phosphotyrosine on the transcription factor. By interfering with this interaction, free phosphotyrosine interferes with the factor's ability to bind to DNA.

C. Tyrosine phosphorylation of the transcription factor could promote its dimerization in two general ways. It could be that in the absence of phosphorylation the heptad repeats are masked by the tertiary structure of the protein and that phosphorylation and intramolecular binding to the SH2 domain causes a conformational change that exposes the heptad repeats and allows dimerization (**Figure 15–35A**). Alternatively, it could be that the heptad repeats do not promote a strong enough interaction for formation of a stable dimer and that phosphorylation and intermolecular binding to the SH2 domain is required (**Figure 15–35B**).

The dyad symmetry of the DNA sequence element suggests that it is composed of two half-sites for binding. The formation of the dimer allows the transcription factor to interact with both half-sites simultaneously, which greatly increases the strength of binding.

Reference: Sadowski HB, Shuai K, Darnell JE & Gilman MZ (1993) A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* 261, 1739–1744.

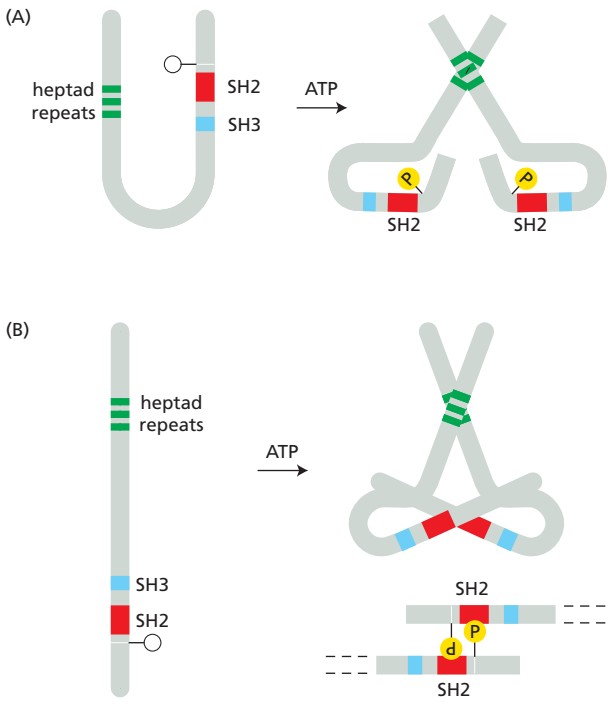


Figure 15–35 Two general ways for tyrosine phosphorylation to promote dimerization of the transcription factor (Answer 15–103).

ALTERNATIVE SIGNALING ROUTES IN GENE REGULATION

DEFINITIONS

15–104 Notch

15–105 Wnt proteins

15–106 Wnt/β-catenin pathway

15–107 Hedgehog proteins

15–108 Cubitus interruptus (Ci)

15–109 NFκB proteins

15–110 Steroid hormone

TRUE/FALSE

15–111 False. Although some signaling pathways activate latent gene regulatory proteins by regulated proteolysis, others control their activity by phosphorylation.

15–112 True. Notch carries both its functions—cell-surface receptor and latent gene regulator—in one polypeptide chain. When activated by a ligand such as Delta, its cytoplasmic tail is cleaved off, enters the nucleus, and activates gene expression.

15–113 True. Activated NFκB increases expression of the *IκBα* gene, and IκBα then binds to NFκB and inactivates it, thereby shutting off the response. If the initial activating signal persists, then additional cycles of NFκB activation and inactivation may follow.

THOUGHT PROBLEMS

15–114 In both cases the signaling pathways themselves are rapid. When the pathway modifies a protein that is already present in the cell, its activity is changed immediately, leading to a rapid response. When the pathway modifies gene expression, however, there will be a delay corresponding to the time it takes for the mRNA and protein to be made and for the cellular levels of the protein to be altered sufficiently to invoke a response, which would usually take an hour or more.

15–115 The extracellular fragments of APP aggregate to form amyloid plaques outside the cells. The amyloid plaques are thought to interfere with nerve function, leading to the characteristic loss of mental acuity that is typical of Alzheimer's disease.

15–116 Cells of flies with the heterozygous $Dsh^{\Delta}/+$ genotype probably make just half the normal amount of Dishevelled. Thus, underexpression of Dishevelled corrects the multi-hair phenotype generated by the overexpression of Frizzled. This relationship suggests that Frizzled acts upstream of Dishevelled; it is easy to imagine how underexpression of a downstream component could correct the overexpression of an upstream component. All this makes sense, as Frizzled is a Wnt receptor and Dishevelled is an intracellular signaling protein. However, if you knew nothing of the functions of Dishevelled and Frizzled, with only the genetic interactions as a guide, it would be possible to imagine more complex relationships (involving other unknown components) with Dishevelled acting upstream of Frizzled that could account for the phenotypes given in this problem. See if you can design such a pathway.

Reference: Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD & Luo L (2001) *Drosophila* Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. *Cell* 105, 81–91.

15–117 The *Apc* gene is a tumor suppressor gene. Its normal function is to inhibit β-catenin by helping to hold it in the cytosol until a proper signal has been received. When both copies of the *Apc* gene are inactivated, β-catenin is free to enter the nucleus in the absence of any signal, leading to uncontrolled stimulation of its target genes.

15–118

1. The latent gene regulatory protein is attached to the membrane as part of the covalent structure of a transmembrane protein. When a valid signal is received, the regulatory protein is cleaved and enters the nucleus. Notch is an example.
2. The latent gene regulatory protein is actively degraded in the cytosol. When a valid signal is received, the protein is stabilized against degradation, allowing it to enter the nucleus. β-Catenin is an example.
3. The latent gene regulatory protein is anchored to a cytosolic structure and released in response to an appropriate signal. Cubitus interruptus is an example.
4. The latent gene regulatory protein is bound to a protein that holds it in an inactive form. Upon receipt of an appropriate signal, the inhibitory protein is modified so that the gene regulatory protein is released in an active form and transported into the nucleus. NFκB is an example.

15–119 The modifications of cholesterol to make steroid hormones increase the hydrophilicity of the molecules by removing the hydrocarbon tail and by introducing polar groups. These modifications make the molecules sufficiently hydrophilic to diffuse from their carrier molecules in the bloodstream to cells, but not so hydrophilic as to prevent their crossing the plasma membrane to enter cells. By contrast, cholesterol is so hydrophobic that it normally spends all its time in the membrane. A lipid that is virtually insoluble in water could not serve as a hormone because it could not move readily from one cell to another via the extracellular fluid.

15–120 A specialized group of cells in the hypothalamus—the cells of the suprachiasmatic nucleus (SCN)—regulates our circadian rhythm. These cells receive neural cues from the retina, although not from rods and cones—the light receptors for vision—but from a subset of retinal ganglion cells that responds to light. These retinal signals entrain the cells of the SCN to the daily cycle of light and dark. In totally blind people, information about the light and dark cycle does not reach the SCN cells. As a consequence, they operate on their own inherent rhythm, which is slightly longer than 24 hours. Blind people typically report recurrent periods of insomnia and daytime sleepiness, as their circadian rhythms drift in and out of phase with the normal 24-hour cycle.

Reference: Sack RL, Brandes RW, Kendall AR & Lewy AJ (2000) Entrainment of free-running circadian rhythms by melatonin in blind people. *N. Engl. J. Med.* 343, 1114–1116.

DATA HANDLING

15–121 These results indicate that phosphorylation of β-catenin sensitizes it for degradation in proteasomes. If phosphorylation were irrelevant to degradation or if it protected against degradation, slower migrating, ubiquitylated forms of β-catenin should have been present in cell lines that were unable to phosphorylate β-catenin.

15–122

A. Although the Hedgehog precursor protein was purified from the bacteria in which it was expressed, it is unlikely to be 100% pure (no purified

protein ever is). Incubation over a wide range of concentrations of the protein argues against a contaminating protease. If the cleavage were a bimolecular reaction between the precursor protein and the contaminating protease, the rate of reaction should be exquisitely sensitive to concentration. Each fourfold dilution of the contaminating protease would slow the rate of reaction fourfold. But the dilution also lowers the concentration of substrate, which would also lower the rate. The absence of any effect of dilution on the rate of reaction makes it extremely likely that the precursor protein is cleaving itself.

B. The lack of effect of dilution also indicates that the reaction must be intramolecular. If it were not—if precursor molecules only cleaved other precursor molecules—then the rate of reaction would slow with increasing dilution. Thus, the precursor cleaves itself in an autoproteolytic reaction.

Reference: Porter JA, von Kessler DP, Ekker SC, Young KE, Lee JJ, Moses K & Beachy PA (1995) The product of *hedgehog* autoproteolytic cleavage active in local and long-range signaling. *Nature* 374, 363–366.

15–123 The N-terminus of the Hedgehog precursor protein remains associated with the cells when cleaved naturally, but it is secreted when it is synthesized from the truncated construct. These data do not define the nature of the cell association. As a part of its cleavage mechanism, the N-terminal fragment could, for example, become associated with a component of the cell, either inside the cell or on the membrane; alternatively, it could be trapped in an intracellular compartment. The actual explanation is very surprising; the cleavage mechanism uses a membrane cholesterol molecule to complete the cleavage, leaving the N-terminal fragment attached to the membrane via a covalent linkage between glycine 257 and cholesterol.

Reference: Porter JA, von Kessler DP, Ekker SC, Young KE, Lee JJ, Moses K & Beachy PA (1995) The product of *hedgehog* autoproteolytic cleavage active in local and long-range signaling. *Nature* 374, 363–366.

15–124

A. Overexpression of constructs encoding the full-length Hedgehog and the N-terminal segment both caused a dramatic increase in the level of Wnt expression (see Figure 15–25, embryos 2 and 4). Thus, the N-terminal segment must contain the portion of Hedgehog that is important in signaling Wnt expression.

B. Although all the cells of the embryo overexpress Hedgehog, the target receptors through which it acts are localized to cells in the stripes. In the absence of the appropriate receptor, as in the cells outside the stripes, Hedgehog cannot elicit a cellular response.

C. The stripes of Wnt expression in the absence of Hedgehog overexpression arise as a result of expression from the flies' normal *Hedgehog* gene.

Reference: Porter JA, von Kessler DP, Ekker SC, Young KE, Lee JJ, Moses K & Beachy PA (1995) The product of *hedgehog* autoproteolytic cleavage active in local and long-range signaling. *Nature* 374, 363–366.

15–125

A. The cycloheximide-induced alteration of the puffing pattern is due to its effect on protein synthesis. The result indicates that newly synthesized proteins are required to turn off the early puffs and to turn on the late puffs. Presumably, the proteins are synthesized from the early puffs.

The shut-off of transcription from the intermolt puffs is insensitive to cycloheximide treatment. This suggests that the receptor–ecdysone complex turns off these puffs directly.

B. The immediate regression of the early puffs upon ecdysone removal indicates that the ecdysone–receptor complex is required continuously to keep the genes turned on. The premature activation of the late puffs

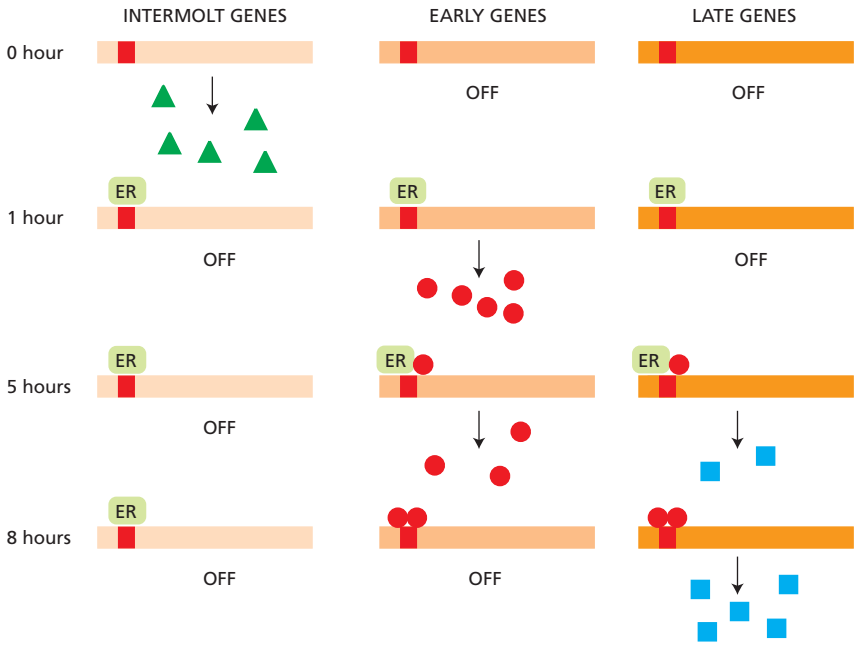


Figure 15–36 Diagram relating ecdysone–receptor (ER) binding to the pattern of gene activity (Answer 15–125).

under these conditions was unexpected. If activation of the late puffs depended only on products of the early puffs, then they should be turned on at the same time (or even delayed due to a lower level of early product). The premature activation suggests that the receptor–ecdysone complex actually functions as an inhibitor of late puff formation, delaying activation until the concentrations of the presumptive early-puff products reach some critical level. Removal of ecdysone allows the puffs to be induced at lower concentrations of early products.

C. These experimental observations are summarized in the diagram shown in **Figure 15–36**. The ecdysone–receptor complex binds to regulatory regions of intermolt, early, and late puffs. Binding at intermolt puffs turns them off, binding at early puffs turns them on, and binding at late puffs keeps them off. Products from one or more early puffs bind at the regulatory regions of early and late puffs, ultimately turning off the early puffs and turning on the late puffs.

Reference: Ashburner M, Chihara C, Meltzer P & Richards G (1974) Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harb. Symp. Quant. Biol.* 38, 655–662.

SIGNALING IN PLANTS

DEFINITIONS

15–126 Phytochrome

15–127 Ethylene

15–128 Growth regulator (plant hormone)

15–129 Cryptochrome

15–130 Auxin

15–131 Leucine-rich repeat (LRR) receptor kinase

TRUE/FALSE

15–132 False. Although there is some overlap in the cell–cell communication molecules used in plants and animals, there are many significant

differences. For example, plants do not use the nuclear receptor family, Ras, JAK, STAT, TGFβ, Notch, Wnt, or Hedgehog proteins.

15–133 True. When the gravity vector is changed, vesicles filled with the auxin efflux transporter fuse with the plasma membrane so that the transporters are correctly positioned to pump auxin toward the side of the root that points downward.

THOUGHT PROBLEMS

15–134 The similarities in signaling mechanisms between animals and fungi support the phylogenetic tree in which fungi branched from the animal lineage after plants and animals separated (see Figure 15–27B). This branching order is supported by a wide variety of other data, including genomic sequence comparisons.

15–135 If the basic mechanisms of cell communication arose in response to multicellularity, then fungi must have separated from the animal lineage after multicellularity evolved. This reasoning would suggest that unicellular fungi may have been derived from multicellular precursors. Not long ago—and to great surprise—it was shown that *Saccharomyces cerevisiae* will form multicellular filamentous forms. Many members of the fungal kingdom have this ability, termed dimorphism, to switch between two morphological forms: a cellular form and a multicellular invasive form.

Reference: Madhani HD & Fink GR (1998) The control of filamentous differentiation and virulence in fungi. *Trends Cell Biol.* 8, 348–353.

15–136 Systemic growth regulators have specific effects in those cells that express their receptors. This situation is no different from that of hormone action in animals: steroid hormones, for example, circulate throughout the body but have specific effects in cells that express appropriate receptors.

DATA HANDLING

15–137

A. The accepted explanation for the ability of an antisense RNA to block expression of the normal gene is that the two RNAs—the antisense RNA and the normal RNA—hybridize to make a double-stranded RNA that cannot be translated. This would effectively block synthesis of the ACC synthase enzyme and prevent the formation of ethylene. But this may not be the true mechanism. In some plants, a phenomenon called RIPing pairs duplicated sequences in meiosis and introduces mutations into both. Thus, it may be that the normal ACC synthase gene is inactivated in your transgenic tomatoes.

B. In all likelihood! Unless the GM uproar puts paid to your plans.

Reference: Oeller PW, Lu M-W, Taylor LP, Pike DA & Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* 254, 437–439.

MCAT STYLE

15–138

B. Ras GTPases are in their active forms when they are bound to GTP. Thus, a mutation that blocks GTPase activity of Ras would lock Ras into its active form. Choices A, C, and D are incorrect because stimulation of binding of Ras-GAP, which stimulates Ras GTPase activity, or blocking the binding of Ras-GEF, which would prevent activation of Ras, would both decrease the activity of Ras.

15–139

B. Many signaling pathways are regulated by negative feedback, which limits the strength and duration of the signal. In the Raf pathway, Raf activation leads to activation of Erk, which phosphorylates and inhibits the RTK. Thus, inhibition of Raf leads to decreased Erk activity, decreased phosphorylation of the RTK, and a rebound in RTK signaling. Choice A is not correct because Ras is upstream of Raf; thus, inhibition of Raf would have no effect on Ras activity. Choice C is incorrect because inhibition of Erk would decrease the stimulation of the RTK, which would lead to less Ras activity. Choice D is not correct because inhibition of Raf-V600E would prevent its activation of RTK, which would decrease Ras activity.

15–140

B. One hypothesis for why the cancer does not respond to the Raf inhibitor is that it also has a mutation in a kinase downstream of Raf. Thus, an inhibitor of Erk, the last kinase in the MAP kinase module, might give a better outcome. Choices A, C, and D are not correct because each of these drugs would inhibit proteins that are upstream of Raf-V600E in the signaling pathway; thus, drugs that block Raf-V600E would also block signals from these upstream proteins.

Reference: Lito P, Pratilas CA, Joseph EW, Tadi M, Halilovic E et al. (2012) Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. *Cancer Cell* 22, 668–682.

15–141

B. The dissociation of Ste7 from Ste5 on a time scale of seconds is inconsistent with the scaffold sequestration model. If activated Ste7 could rapidly dissociate from Ste5, it could activate Kss1, potentially stimulating the starvation response. Since mating-pheromone signaling takes place on a time scale of 5–10 minutes, Ste7 would need to remain bound to the scaffold for at least that long to ensure that it does not activate other MAPKs. Choices A, C, and D are not correct because the indicated observations are all consistent with the scaffold sequestration model.

15–142

B. The Ste5 domain "unlocks" Fus3 so that its phosphorylation site is accessible by Ste7. This hypothesis is consistent with Ste5 increasing the K_{cat} without having an effect on K_m; that is, the conformational change does not affect the binding of Ste7 to Fus3, but rather permits access to the site of phosphorylation, increasing the rate of the reaction. Choices A and C are not consistent with the observation that Ste7 robustly phosphorylates Kiss1 in the absence of Ste5. Choice D is incorrect because stronger binding of Ste7 to Fus3 would change the K_m for the reaction, since K_m is a measure of the affinity of an enzyme for its substrate.

15–143

C. Hypothesis II is not correct because feedback activation of Ste7 by Fus3 in the presence of full-length Ste5 would predict that Fus3 would be more active in the presence of full-length Ste5, in contrast to the observed lower activity.

References: Good M, Tang G, Singleton J, Reményi A & Lim WA (2009) The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation. *Cell* 136, 1085–1097.

Zalatan JG, Coyle SM, Rajan S, Sidhu SS & Lim WA (2012) Conformational control of the Ste5 scaffold protein insulates against MAP kinase misactivation. *Science* 337, 1218–1222.

CHAPTER 16

The Cytoskeleton

IN THIS CHAPTER

FUNCTION AND ORIGIN OF THE CYTOSKELETON

DEFINITIONS

16–1 Protofilament

16–2 Cytoskeleton

TRUE/FALSE

16–3 False. Actin filaments accomplish this list of functions. Microtubules determine the positions of membrane-enclosed organelles, direct intracellular transport, and form the mitotic spindle that segregates chromosomes during cell division.

16–4 True. The actin filaments that make up the actin bundle in stereocilia display the dynamic properties typical of all actin filaments.

16–5 False. Bacteria contain homologs of all three types of cytoskeletal filament, with bacterial actins and tubulins being more diverse than their eukaryotic versions, both in the types of assemblies they form and the functions they serve.

THOUGHT PROBLEMS

16–6 Intermediate filaments provide mechanical stability and resistance to shear stress. Microtubules determine the positions of membranous organelles and direct intracellular transport. Actin filaments determine the shape of the cell's surface and are necessary for whole-cell locomotion.

16–7 Although the subunits are indeed held together by noncovalent bonds that are individually weak, there are a very large number of them, distributed among a very large number of filaments. As a result, the stress a human being exerts by lifting a heavy object is dispersed over so many subunits that their interaction strength is not exceeded. By analogy, a single thread of silk is not nearly strong enough to hold a human, but a rope woven of such fibers is.

16–8 A few examples of the differences between bacteria and animal cells are listed below. This is by no means a complete list.

1. Animal cells are much larger, diversely shaped, and do not have a cell wall. Cytoskeletal elements are required to provide mechanical strength and shape in the absence of a cell wall.
2. Animal cells, and all other eukaryotic cells, have a nucleus that is shaped and held in place by intermediate filaments; the nuclear lamins attached to the inner nuclear membrane support and shape the nuclear membrane, and a meshwork of intermediate filaments surrounds the nucleus and spans the cytosol.

3. Animal cells can move by a process that requires a change in cell shape. Actin filaments (and myosin motor proteins) are required for these activities.
4. Animal cells have a much larger genome than bacteria; this genome is fragmented into many chromosomes. For cell division, chromosomes need to be accurately distributed to the daughter cells, which requires the microtubules that form the mitotic spindle.
5. Animal cells have internal organelles. Their localization in the cell depends on motor proteins that move them along microtubules. The long-distance travel of membrane-enclosed vesicles along microtubules in an axon, which can be up to a meter long in the case of the nerve cells that extend from your spinal cord to your feet, provides a remarkable example.

16–9 The evolution of actins and tubulins is constrained not only by the requirement that they bind to one another, but also by the necessity that they interact with a large number of other proteins that bind to the same or overlapping sites on their surfaces. A mutation in actin that results in a desirable change in its interaction with one protein might cause undesirable changes in its interactions with a half-dozen other proteins that bind at or near the same site. These multiple interactions constrain the evolution of most of the surfaces of actins and tubulins. By contrast, the proteins that bind to actin filaments and microtubules need only preserve their filament-binding sites—which are, in fact, the portions of their structures that are most conserved—and the binding sites for the limited number of other proteins they interact with. This reduced constraint allows them considerably more evolutionary freedom.

CALCULATIONS

16–10

A. The average time for a small molecule such as ATP to diffuse across a cell 10 μm (10^{-3} cm) in diameter is

$$t = x^2/2D$$
$$= (10^{-3}\ \text{cm})^2/2\ (5 \times 10^{-6}\ \text{cm}^2/\text{sec})$$
$$= 0.1\ \text{sec}$$

Similarly, a protein molecule takes 1 second and a vesicle 10 seconds, on average, to travel 10 μm.

B. The diffusion of long cytoskeletal filaments is even slower than that of membrane vesicles; hence, it would take much longer to rearrange the cytoskeleton by diffusion. In addition to time, there is also the problem of length: polymerization allows filaments to be constructed to fit. Finally, if the long cytoskeletal elements were to rearrange by diffusion, they would become hopelessly entangled with one another.

DATA HANDLING

16–11

A. The ascending portions of the plots in Figure 16–1 are more consistent with growth at the tip of an acrosomal process than with growth at the base. All six points on the ascending portion of the plot of length versus square root of time fall on a straight line; however, no more than three or four points lie on a straight line on the ascending portion of the plot of length versus time. These results indicate that the rate of growth of the process slows down in the manner expected for a diffusion-controlled reaction, and they suggest that the addition of new subunits occurs at the tip. Independent experiments, using myosin decoration, indicate that the tip of the process is the plus end, where growth is expected to occur.

B. The slower rates of growth at the beginning and end of the acrosomal reaction are not surprising. Growth is slow at the beginning presumably

because it takes a short time for the subunits to diffuse from their site of storage to the site of assembly. Growth slows down (and essentially stops) at the end because the supply of subunits is exhausted.

Reference: Tilney LG & Inoué S (1982) Acrosomal reaction of *Thyone* sperm. II. The kinetics and possible mechanism of acrosomal process elongation. *J. Cell Biol.* 93, 820–827.

ACTIN AND ACTIN-BINDING PROTEINS

DEFINITIONS

16–12 Treadmilling

16–13 Cell cortex

16–14 Arp2/3 complex

TRUE/FALSE

16–15 True. Arp2/3 complexes cap the minus ends of actin filaments and attach them to the sides of other actin filaments, generating the highly branched network of actin filaments that makes up the cell cortex.

16–16 False. While it is true that end-binding proteins such as the Arp2/3 complex proteins cap the ends and prevent further polymerization, other proteins such as the formins can bind to the end and promote growth.

THOUGHT PROBLEMS

16–17

A. Phase A corresponds to a lag phase (**Figure 16–43A**), during which actin monomers must assemble to form a nucleus for polymerization (thought to be a trimer of subunits). Formation of a nucleus (nucleation) is followed by rapid growth (phase B), as actin monomers are added to the ends of the growing filaments. At phase C, equilibrium is reached between the rate of addition of actin at the ends and its rate of release. Once equilibrium is reached, the concentration of free actin remains constant.

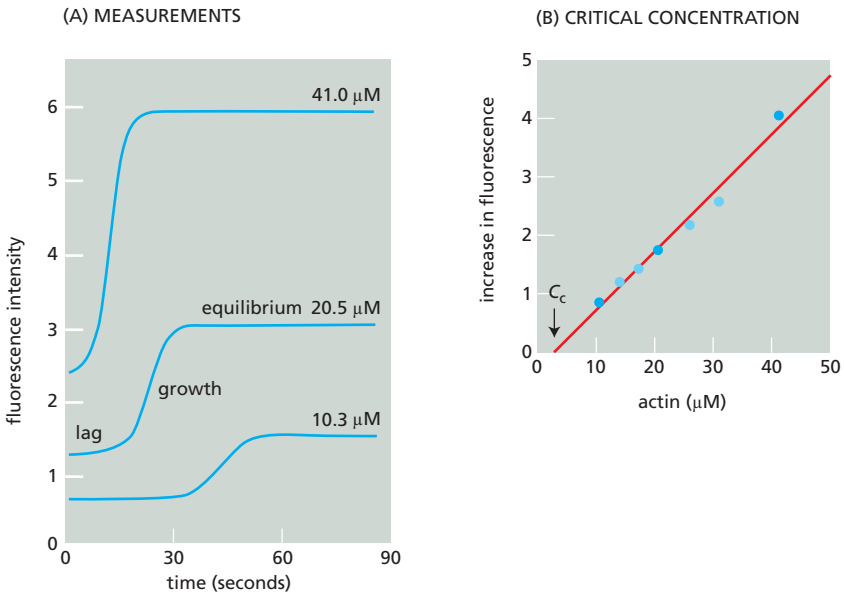


Figure 16–43 Analysis of actin polymerization (Answer 16–17). (A) Actin polymerization at three different actin concentrations, as indicated on the individual curves. (B) The critical concentration determined from many such experiments. The plateau values for increase in intensity of fluorescence (corrected for fluorescence of the monomers) are plotted against actin concentration. The *dark blue circles* indicate the data shown in (A); *light blue circles* are additional data points from similar experiments. The critical concentration (C_c) is estimated by extrapolating to a value of zero increase in intensity.

B. If the starting concentration of actin were doubled, the lag phase would be shorter, the growth phase would be more rapid (steeper), and the mass of polymer at equilibrium would be twice as great. The experimental curves generated at twice and half the initial actin concentrations illustrate these relationships (Figure 16–43A). The concentration of free actin monomers at equilibrium—the critical concentration (C_c)—would be the same regardless of the initial actin concentration. It can be derived from the data in Figure 16–43A, as shown in **Figure 16–43B**.

Reference: Carlier M-F, Pantaloni D & Korn ED (1985) Polymerization of ADP-actin and ATP-actin under sonication and characteristics of the ATP-actin equilibrium polymer. *J. Biol. Chem.* 260, 6565–6571.

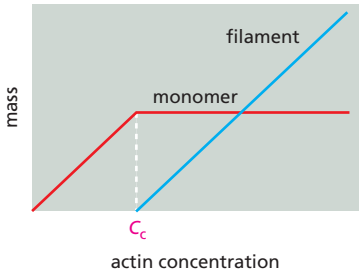


Figure 16–44 Critical concentration (C_c) of actin (Answer 16–18).

16–18 The critical concentration is the concentration of actin at which filaments initially form (**Figure 16–44**). Above the critical concentration, the mass of free actin remains constant.

16–19 Subunit 1 will add faster to the right end of the polymer than to the left end, subunit 2 will add to both ends at equal rates, and subunit 3 will add faster to the left end of the polymer. A difference in growth rates at the two ends reflects a change in conformation of the free subunit as it adds to the polymer. For example, subunit 1 can add to the right end of the polymer through an existing binding site (its pointed end) and change conformation later. To add to the left end, however, it must make the conformational change before or during addition.

For the simple polymerization described here, both ends must grow or shrink; there is no concentration of subunit that can allow one end to grow while the other shrinks (or stays the same length). This is because the conformations of the subunits at the two ends of the polymer are identical and they involve identical contacts. You could not tell from which end a free subunit derived. Thus, the ΔG for subunit loss, which determines the equilibrium constant for subunit association at an end, must be the same for both ends.

16–20 Any actin-binding protein that stabilizes complexes of two or more actin monomers will facilitate the initiation (nucleation) of a new filament. The actin-binding proteins must not block the ends required for filament growth.

16–21 In cells, most of the actin subunits are bound to thymosin, which locks actin into a form that cannot hydrolyze its bound ATP and cannot be added to either end of a filament. Thymosin reduces the concentration of free actin subunits to around the critical concentration. Actin subunits are recruited from this inactive pool by profilin, whose activity is regulated so that actin polymerization occurs when and where it is needed. The advantage of such an arrangement is that the cell can maintain a large pool of subunits for explosive growth at the sites and times of its choosing.

16–22 Cofilin binds preferentially to actin with bound ADP. When cofilin binds to ADP-containing actin filaments, it introduces strain into the filament by twisting it, which makes it easier for the filament to be severed and for ADP-actin subunits to dissociate. Because polymerization is faster than ATP hydrolysis, the newly added subunits are resistant to depolymerization by cofilin. Thus, cofilin efficiently dismantles older filaments in the cell, which contain more ADP-actin.

DATA HANDLING

16–23

A. The plus end of the myosin-decorated filament is defined as the one that

grows more rapidly and, thus, has the longer newly synthesized actin segment, which is the end on the left in Figure 16–5. The minus end grows more slowly; hence, it is the end on the right in Figure 16–5. The myosin heads form arrowheadlike structures on an actin filament, with the point corresponding to the minus end and the barb corresponding to the plus end. If you examine the myosin-decorated actin filament in Figure 16–5, you should see a chain of about 15 of these arrowheadlike structures. This easy way of visualizing the two ends is why the ends of an actin filament are commonly referred to as "pointed" or "barbed."

B. If the mixture were diluted below the critical concentration of actin monomers, the actin filaments would depolymerize. The plus end, the one with the longer tail, would depolymerize more rapidly. The plus end is the more dynamic end, always polymerizing and depolymerizing more rapidly than the minus end.

C. During depolymerization, actin monomers dissociate exclusively from the ends because fewer noncovalent bonds hold them in place. A terminal actin monomer is held in place by two sets of interactions: those between it and the next monomer in the chain and those between it and the actin monomers in the adjacent chain. In addition to these two sets of interactions, an internal actin monomer is held in place by noncovalent bonds to a second actin monomer in the same chain (**Figure 16–45**).

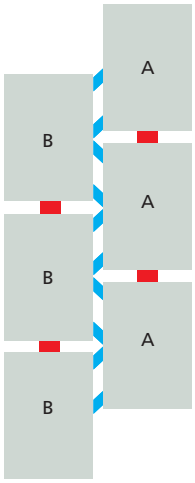


Figure 16–45 Interactions between actin subunits in an actin filament (Answer 16–23). *Red* and *blue* indicate defined interactions between the subunits, which are designated A and B to distinguish between the two actin protofilaments.

References: Pollard TD, Blanchoin L & Mullins RD (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* 29, 545–576.

Oosawa F (2001) A historical perspective of actin assembly and its interactions. *Results Probl. Cell Differ.* 32, 9–21.

16–24

A. An enzyme-catalyzed reaction reaches a plateau when the enzyme becomes saturated with substrate. Beyond that point an increase in substrate concentration cannot increase the rate of the reaction, because the enzyme is already working at maximum capacity. In contrast, growth of an actin filament does not saturate. Each time a monomer is added to the filament, a new site for addition of the next monomer is created. Addition of new monomers occurs through productive collisions with the end of the filament. The number of productive collisions increases linearly with the concentration of actin monomers.

B. At concentration A, both ends would shrink. At concentration B, the minus end would shrink and the plus end would be unchanged. At concentration C, the plus end would grow and the minus end would shrink. At concentration D, the plus end would grow and the minus end would remain unchanged. At concentration E, both ends would grow, with the plus end growing faster than the minus end. The critical concentration is the concentration of actin at which an end neither shrinks nor grows. For plus ends the critical concentration is concentration B (0.12 μM); for the minus ends the critical concentration is concentration D (0.62 μM). At any concentration between these two critical concentrations, the filament would exhibit treadmilling. At concentration C, the plus end would grow at exactly the same rate as the minus end would shrink, giving treadmilling with no change in length of the filament.

References: Pollard TD, Blanchoin L & Mullins RD (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* 29, 545–576.

Pollard TD (1986) Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J. Cell Biol.* 103, 2747–2754.

16–25

A. Although the end points for polymerization and ATP hydrolysis were the same, the initial rate of ATP hydrolysis was less than the initial rate of polymerization. (Compare the slopes of the two curves in Figure 16–7 at short times.) At the time when all the actin was polymerized (about 30 seconds), less than half the ATP was hydrolyzed. It is the difference in initial rates that your advisor noticed, and, as he said, it proves that actin polymerization can occur in the absence of ATP hydrolysis.

B. Since the rate of polymerization is faster than the rate of ATP hydrolysis, newly added actin subunits must still retain bound ATP. Since the bound ATP is not hydrolyzed until some time after assembly, growing actin filaments have ATP caps. Once an ATP-actin monomer has bound to a filament, the ATP can be hydrolyzed, giving rise to the bound ADP found interior to the ATP caps.

Reference: Carlier M-F, Pantaloni D & Korn ED (1984) Evidence for an ATP cap at the ends of actin filaments and its regulation of the F-actin steady state. *J. Biol. Chem.* 259, 9983–9986.

16–26

A. Figure 16–9 shows that cytochalasin B interferes with filament assembly by stopping actin polymerization at the plus end, the preferred end for the addition of monomers. One plausible mechanism to explain this inhibition is that cytochalasin B binds to the plus end of the actin filament and physically blocks the addition of new actin monomers.

This mechanism can also account for the viscosity measurements. Since growth at the minus end is unaffected, the filaments continue to grow, but much more slowly. The slower growth rate explains the slower increase in viscosity in the presence of cytochalasin B. The lower viscosity at the plateau indicates that the actin filaments are shorter in the presence of cytochalasin B. The filaments are shorter when they are growing only from the minus ends because the critical concentration for assembly at the minus end is higher than the critical concentration for assembly at the plus end.

B. An actin filament normally grows at different rates at the plus and minus ends. This observation indicates that the monomer probably undergoes a conformational change upon addition to an actin filament. If all subunits, assembled and free, were identical in conformation, the rates of growth at the two ends should be the same (see Problem 16–19).

Reference: MacLean-Fletcher S & Pollard TD (1980) Mechanism of action of cytochalasin B on actin. *Cell* 20, 329–341.

16–27 The gold particles in **Figure 16–46A** follow parallel helical paths that are staggered relative to one another, just like the two protofilaments in an actin filament. The spacing of gold particles (5.5 nm per particle) also matches the spacing of actin subunits in the actin filament. In **Figure 16–46B**, the positions of the gold particles have been mapped onto the surface of the actin filament.

Reference: Steinmetz MO, Stoffler D, Hoenger A, Bremer A & Aebi U (1997) Actin: from cell biology to atomic detail. *J. Struct. Biol.* 119, 295–320.

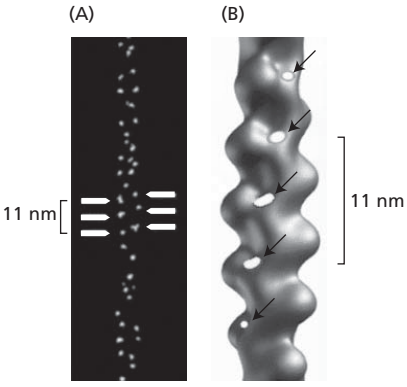


Figure 16–46 Relationship of phalloidin to actin monomers in an actin filament (Answer 16–27). (A) An actin filament with gold-tagged phalloidin at high contrast. (B) The mapped positions of the gold particles on the surface of an actin filament. The positions of the gold particles are indicated by *arrows*.

16–28

A. Phalloidin increases the growth rate of actin filaments by eliminating the off rate. Because the slopes of the lines in Figure 16–11A are identical, k_{on} is the same in the presence and absence of phalloidin. The y intercept ($-k_{off}$) is dramatically altered, from about –12 molecules/sec in the absence of phalloidin to 0 molecules/sec in its presence. These results suggest that the off rate is zero in the presence of phalloidin.

B. The results in Figure 16–11B confirm the interpretation in part A. Actin filaments made in the absence of phalloidin disassemble as expected when diluted in the absence of actin monomers. Filaments made in the presence of phalloidin, however, are rock-solid stable, as expected if the off rate were zero.
C. The critical concentration for actin assembly is the concentration of actin at which no growth occurs. In the absence of phalloidin, this point occurs at about 1 μM. In the presence of phalloidin, it occurs at an actin concentration of 0 μM. This result is also consistent with phalloidin reducing the off rate to zero: when phalloidin is present, the filament will grow at any concentration of actin.
D. Phalloidin interferes with actin assembly by binding to the filament to prevent dissociation of actin subunits. The requirement for a 1:1 molar mixture suggests that phalloidin is required stoichiometrically with actin but does not tell you whether it binds to free monomers or to each actin subunit in the filament. The stability of phalloidin-treated filaments upon dilution indicates that phalloidin binds to the filaments. It is thought that phalloidin binds to actin subunits and locks them in place.

Reference: Coluccio LM & Tilney LG (1984) Phalloidin enhances actin assembly by preventing monomer dissociation. *J. Cell Biol.* 99, 529–535.

16–29

A. The fluorescence in Figure 16–12B reaches a plateau when all the actin has been converted to monomers (or a swinholide A bound pair of monomers). The plateau is not at zero because the monomers themselves exhibit a low level of fluorescence.
B. The humps in the depolymerization curves (see Figure 16–12B) show that the rates of depolymerization *increase* with time in the presence of swinholide A. Since the rate of depolymerization depends on the number of ends, an increase in rate is consistent with an increase in the number of ends. This feature of the depolymerization curves supports the idea that swinholide A severs actin filaments, thereby increasing the number of ends. It is not consistent with the idea that swinholide A brings about depolymerization through mass-action effects by binding to actin subunits, which predicts a linear loss of fluorescence with time.
C. Multiple molecules of swinholide A are required to sever an actin filament. This conclusion is based on the data in Figure 16–12C, which shows that the increments in swinholide A concentration have progressively larger effects on depolymerization. Were a single molecule of swinholide A required, the relationship would have been linear, with each increment in swinholide A concentration producing the same increment in depolymerization rate.

Reference: Bubb MR, Spector I, Bershadsky AD & Korn ED (1995) Swinholide A is a microfilament disrupting marine toxin that stabilizes actin dimers and severs actin filaments. *J. Biol. Chem.* 270, 3463–3466.

16–30 The two alternatives make different predications about the kinds of structures that should be generated. As shown in **Figure 16–47A**, if the Arp2/3 complex were to bind to plus ends, the capped actin filament might not be a substrate for Arp2/3 binding, in which case no branches would be seen. Alternatively, the Arp2/3 complex might bind to the capped structure, in which case a kinked filament would be generated (Figure 16–47A). If the Arp2/3 complex were to bind instead to the sides of the filament, then the plus-end cap would be irrelevant and a typical branched structure would be generated (**Figure 16–47B**). The results of such experiments revealed highly branched structures, supporting the idea that the Arp2/3 complex binds to the sides of actin filaments.

References: Amann KJ & Pollard TD (2001) The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. *Nat. Cell Biol.* 3, 306–310.

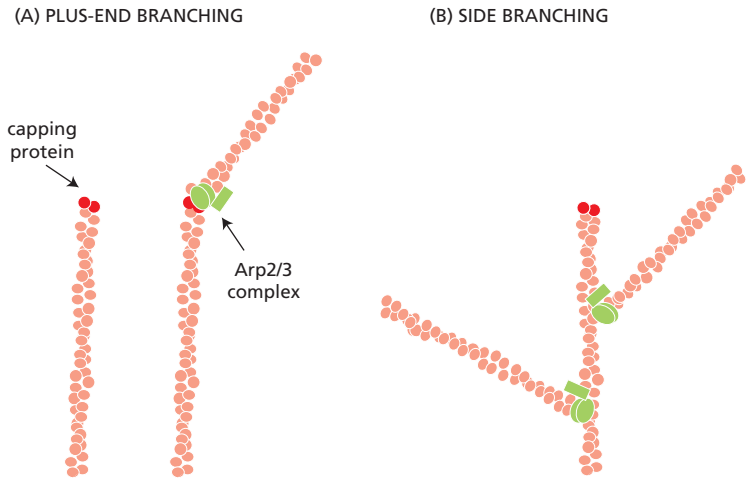


Figure 16–47 Expectations of two models for the branching of actin filaments induced by the binding of the Arp2/3 complex (Answer 16–30). (A) Arp2/3-complex binding at the plus end. (B) Arp2/3-complex binding to the side.

Higgs HN & Pollard TD (2001) Regulation of actin filament network formation through ARP2/3 complex: Activation by a diverse array of proteins. *Annu. Rev. Biochem.* 70, 649–676.

16–31 Protein 1 lowers the critical concentration, allowing actin filaments to polymerize at a low concentration of actin, as is typical of plus ends. Thus, protein 1 must cap the minus end, like the Arp2/3 complex does. Protein 2, which raises the critical concentration, must cap the plus end, like CapZ, a protein involved in muscle contraction. Note that in the absence of either protein, the critical concentration is a balance of those for the plus and minus ends.

MEDICAL LINKS

16–32

A. ActA alone has no effect on actin polymerization. The Arp2/3 complex (ARP) stimulates the rate of actin polymerization but does not substantially decrease the delay (lag) before polymerization begins (see Figure 16–15B), which is a measure of the rate of nucleation. Thus, the absence of an effect on the lag indicates that the Arp2/3 complex does not efficiently nucleate actin polymerization under these conditions. The combination of ActA and the Arp2/3 complex dramatically stimulates nucleation (decreases the lag time) and enhances the rate of polymerization. The increase in rate may be a consequence of accelerated nucleation, which would generate many more ends, and hence faster polymerization rates.

B. The ActA protein stimulates nucleation of new actin filaments by the Arp2/3 complex, so that actin polymerization occurs in the immediate vicinity of the bacterium (since ActA is attached to the bacterial surface). The actin polymerization is oriented so that the growing ends—the plus ends—are pointed toward the bacterium. In this orientation, the growing ends can "push" on the bacterium and move it forward (much as the meshwork of actin filaments pushes on the plasma membrane at the leading edge of a lamellipodium).

How the actin filaments actually push the bacterium is not certain. A thermal ratchet provides one plausible way to think about it. Thermal motion allows enough separation between the bacterium and the ends of the nucleated filaments to permit actin to polymerize at the end, and the end then acts like a ratchet to prevent backward motion of the bacterium. The actin meshwork is likely anchored in some way to the cell's cytoskeleton, which prevents its own backward movement. Thus, the bacterium is continually moved forward by random thermal motion and

by the unidirectional polymerization of actin. As it moves forward, the bacterium triggers more nucleation sites in its wake, thereby perpetuating its movement.

References: Dramsi S & Cossart P (1998) Intracellular pathogens and the actin cytoskeleton. *Annu. Rev. Cell Dev. Biol.* 14, 137–166.

Welch MD, Rosenblatt J, Skoble J, Portnoy DA & Mitchison TJ (1998) Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. *Science* 281, 105–108.

MYOSIN AND ACTIN

DEFINITIONS

16–33 Myosin

16–34 Myofibril

TRUE/FALSE

16–35 True. An individual myosin II molecule, with its two motor domains and tail, would be insufficient to slide actin filaments past each other efficiently. By polymerizing into bipolar filaments, however, the motor domains at each end of the filament are properly arranged to slide oppositely oriented actin filaments past each other.

16–36 False. The entry of Ca^{2+} through the voltage-sensitive Ca^{2+} channels in T tubules is not sufficient, by itself, to trigger rapid muscle contraction. Instead, this initial burst of Ca^{2+} opens Ca^{2+}-release channels in the sarcoplasmic reticulum, which flood the cytoplasm with Ca^{2+}, initiating rapid muscle contraction by binding to troponin C.

16–37 True. In resting muscle, the troponin I–T complex pulls tropomyosin out of its normal binding groove in actin, so that it interferes with the binding of myosin heads. Binding of Ca^{2+} to troponin C alters its conformation, which forces troponin I to release its hold on actin; this allows tropomyosin to slip back into its preferred position, thereby exposing binding sites for the myosin heads.

THOUGHT PROBLEMS

16–38

A. In each cycle, the chemical free energy that drives the cycle is provided by hydrolysis of ATP. Although ATP hydrolysis is a common source of chemical free energy, it is not the only one. The free energy in a Na^+ ion gradient, for example, drives active transport of sugars in animal cells, and GTP hydrolysis powers the movements of ribosomes during protein synthesis.

   The mechanical work accomplished during muscle contraction is the motion of actin thin filaments relative to myosin thick filaments. The mechanical work done during active transport of Ca^{2+} is the pumping of ions to outside the cell, against their concentration gradient.

B. Actin is bound tightly and then released in each cross-bridge cycle during muscle contraction; Ca^{2+} is bound tightly and then released during its active transport.

   In the diagram in Figure 16–16A, actin is tightly bound to myosin at each point where the two are in contact. The binding of ATP to the myosin head converts it to a weakly binding form, allowing it to detach from actin. (Although each of these steps is shown separately in the diagram,

the binding of ATP is thought to initiate a conformational change, which in turn reduces the affinity of myosin for actin, thereby promoting the detachment of actin and the completion of the conformational change.)

In the diagram in Figure 16–16B, Ca^{2+} is tightly bound to the transport protein when it is on the inside of the cell (upper drawing) but only weakly bound when it faces the outside of the cell (lower drawing). Although the tightness of binding is not immediately apparent in the diagrammatic representation, it follows from the concept of active transport. Since the pump transports Ca^{2+} against its concentration gradient, the pump must have a high affinity for Ca^{2+} on the inside of the cell (so that Ca^{2+} can be bound effectively at its low intracellular concentration) and a low affinity for Ca^{2+} on the outside of the cell (so that Ca^{2+} can be released effectively at its high external concentration).

C. In both cycles, the "power stroke" is the conformational change indicated on the right side of the cycles as drawn in Figure 16–16. The "return stroke" in each case is the conformational change indicated on the left side of the drawings.

Reference: Eisenberg E & Hill TL (1985) Muscle contraction and free energy transduction in biological systems. *Science* 227, 999–1006.

16–39

D. The sarcomeres become shorter. Upon contraction, the Z discs move closer together. Neither actin nor myosin filaments contract: they slide past one another.

16–40

A. The locations of the striated muscle components in the electron micrograph are illustrated schematically in **Figure 16–48A**. α-Actinin is a component of the Z disc, titin links the myosin II filaments to the Z disc, and nebulin binds along the length of each actin filament.

B. The micrograph in Figure 16–17B shows a hypercontracted muscle. The light band has entirely disappeared, and a new band, caused by the overlap of actin filaments, has appeared in the middle of the sarcomere. The relationship between the two electron micrographs in Figure 16–17 is shown schematically in Figure 16–48.

16–41 Successive actin molecules in an actin filament are identical. After the first troponin molecule had bound to the actin filament, there would be no way a second troponin could recognize every seventh actin subunit

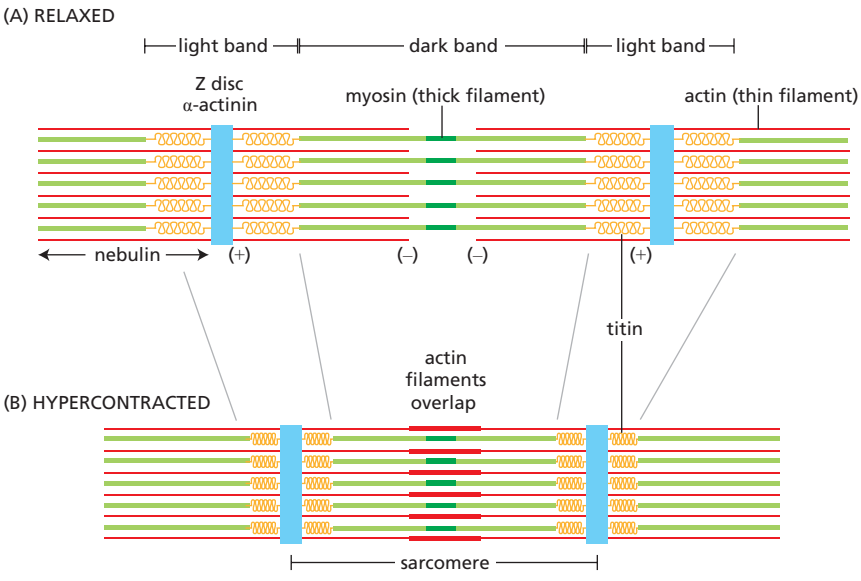


Figure 16–48 Schematic diagrams of electron micrographs in Figure 16–17 (Answer 16–40). (A) Relaxed muscle. (B) Hypercontracted muscle.

in a naked actin filament. Tropomyosin, however, binds along the length of an actin filament, spanning precisely seven subunits and providing a molecular ruler that measures the length of seven actin monomers. Troponin becomes localized by binding to actin at the end of each tropomyosin molecule, and thus is present every seventh actin subunit.

16–42 ATP hydrolysis by the myosin motor domain is required for filament sliding in muscle contraction, and hydrolysis by the ATP-dependent Ca^{2+}-pump is required to pump Ca^{2+} out of the cytosol, to allow the myofibrils to relax.

DATA HANDLING

16–43

A. The presence of ATP in the suspension buffer did not cause contraction because the Ca^{2+} was absent. In the absence of Ca^{2+}, troponin and tropomyosin block the myosin-binding sites on actin, thereby preventing contraction.

B. Contraction upon removal of ATP is perhaps the most difficult result to understand. After all, when Ca^{2+} is absent, how can myosin bind to actin? One clue is the slow rate of contraction. The open and closed configurations of the myosin-binding sites on actin are in equilibrium with one another. In the absence of Ca^{2+}, the equilibrium is far in the direction of the closed configuration. Nevertheless, the equilibrium nature of the switch guarantees that new sites will continually be exposed. As they become available, the sites are bound by myosin-ADP, and when the myosin head loses its bound ADP, it undergoes a conformational change, which generates tension. In the absence of ATP to promote dissociation and relaxation, the resulting myosin–actin complex is trapped. (This complex is known as the "rigor" complex because it is the principal cause of rigor mortis in a corpse.) The tension accumulates as more myosin heads become involved. Presumably, myosin also binds at some background level in normal resting muscle (in the absence of Ca^{2+}), but ATP continually dissociates the myosin heads, thereby keeping the muscle relaxed.

C. The muscle fiber relaxes suddenly upon illumination by laser light because the ATP released from its "cage" binds to the myosin heads, causing their dissociation from actin. The release of all the myosin cross-bridges allows the actin filaments to return to their original resting position.

Reference: Goldman YE, Hibberd MG, McCray JA & Trentham DR (1982) Relaxation of muscle fibres by photolysis of caged ATP. *Nature* 300, 701–705.

16–44 Sketches representing sarcomeres at each of the arrows in Figure 16–20 are shown in **Figure 16–49**. As illustrated in these pictures, the increase in tension with decreasing sarcomere length in segment I is due to increasing numbers of interactions between myosin heads and actin. In segment II, actin begins to overlap with the bare zone of myosin, yielding a plateau at which the number of interacting myosin heads remains constant. In segment III, the actin filaments begin to overlap with each other, interfering with the optimal interaction of actin and myosin and producing a decrease in tension. In segment IV, the spacing between the Z discs is less than the length of the myosin thick filaments, causing their deformation and a precipitous drop in muscle tension.

Reference: Gordon AM, Huxley AF & Julian FJ (1966) The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J. Physiol.* 184, 170–192.

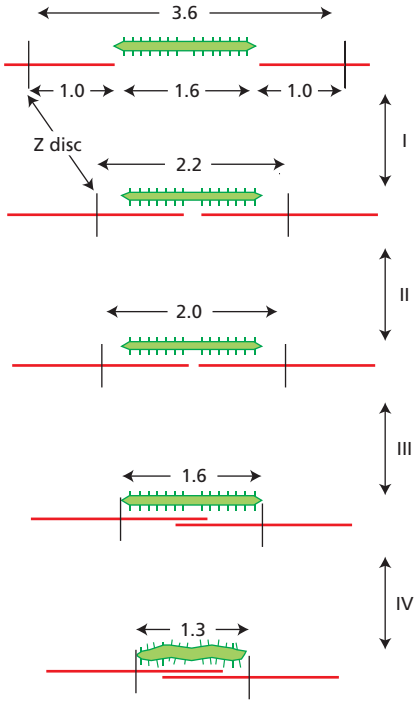


Figure 16–49 Schematic diagrams of sarcomeres at the points indicated by *arrows* in Figure 16–20 (Answer 16–44). Numbers refer to lengths in micrometers.

MICROTUBULES

DEFINITIONS

16–45 Dynamic instability

16–46 Centrosome

16–47 γ-Tubulin ring complex (γ-TuRC)

16–48 Centriole

16–49 Dynein

16–50 Kinesin

16–51 Axoneme

16–52 Flagellum

TRUE/FALSE

16–53 True. Each protofilament in a microtubule is assembled from subunits that all point in the same direction; thus, each protofilament has α-tubulin at one end and β-tubulin at the other. Since the protofilaments in a microtubule are aligned in parallel, α-tubulin is always at one end and β-tubulin is always at the other.

16–54 True. When ATP in actin filaments (or GTP in microtubules) is hydrolyzed, much of the free energy released by cleavage of the high-energy bond is stored in the polymer lattice, making the free energy of the ADP-containing polymer higher than that of the ATP-containing polymer. This shifts the equilibrium toward depolymerization so that ADP-containing actin filaments disassemble more readily than ATP-containing actin filaments.

16–55 False. Although centrosomes, the major microtubule-organizing centers in almost all animal cells, do contain centrioles, a number of microtubule-organizing centers in plants, animals, and fungi do not. The common feature of all microtubule-organizing centers is an electron-dense matrix that usually contains γ-tubulin, which is used to nucleate microtubules.

16–56 False. The centrosome, which establishes the principal array of microtubules in most animal cells, nucleates microtubule growth at the minus end. Thus, the plus ends of the microtubules are near the plasma membrane, and the minus ends are buried in the centrosome at the center of the cell. This orientation of the array requires that plus-end directed motors be used to transport cargo to the cell periphery and that minus-end directed motors be used for cargo delivery to the center of the cell.

THOUGHT PROBLEMS

16–57 Two tubulin dimers have a lower affinity for each other (because of a more limited number of interaction sites) than a tubulin dimer has for the end of a microtubule. At the end of an existing microtubule there are multiple possible interaction sites, both end-to-end as the tubulin dimers add to a protofilament, and side-to-side as they bind to adjacent protofilaments in the microtubule lattice. Thus, to initiate a microtubule from scratch, enough tubulin dimers have to come together and remain bound to one another for long enough for other tubulin molecules to add to them. Only when several tubulin dimers have already assembled will the binding of the next subunit be favored.

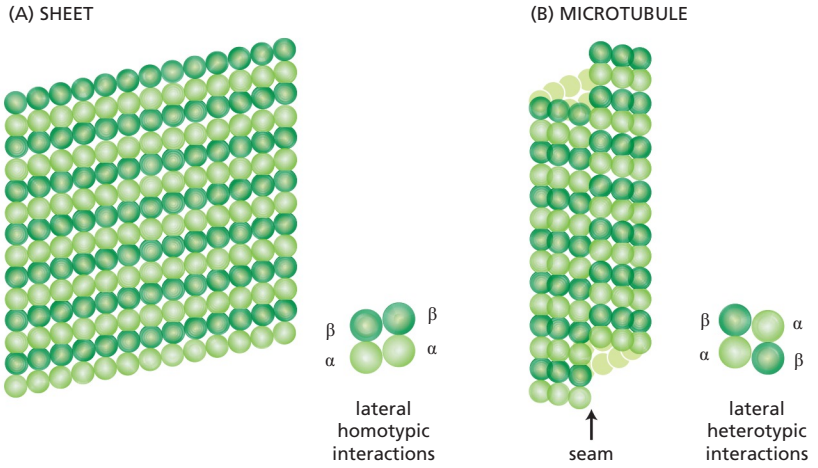


Figure 16–50 Interactions between protofilaments composed of αβ-tubulin dimers (Answer 16–58). (A) A sheet of protofilaments. An example of lateral homotypic interactions between αβ-tubulin dimers in the protofilaments is shown on the *right*. (B) A microtubule. An example of lateral heterotypic interactions between αβ-dimers at the seam is shown on the *right*.

16–58 The heterotypic interactions between the protofilaments are likely to be weaker than the homotypic interactions between them. If the interactions between α-tubulin and β-tubulin were stronger than the homotypic interactions, the protofilaments would preferentially align so that heterotypic interactions, rather than homotypic ones, were maximized. If the two sets of interactions were the same strength, the arrangement of protofilaments might be mixed within the same microtubule, or two different types of microtubule—with protofilaments aligned either by homotypic or heterotypic lateral interactions—might be observed. You can imagine the formation of the microtubule as building a sheet of protofilaments that curl into a microtubule by forming the seam (**Figure 16–50**).

Reference: Desai A & Mitchison TJ (1997) Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* 13, 83–117.

16–59 The ends of the shrinking microtubule are visibly frayed, and the individual protofilaments appear to come apart and curl as the end depolymerizes. This micrograph therefore suggests that the GTP cap (which is lost from shrinking microtubules) holds the protofilaments properly aligned with each other, perhaps by strengthening the side-to-side interactions between αβ-tubulin dimers when they are in their GTP-bound form.

The rapidly growing microtubules, by contrast, have nonfrayed ends. The one on the left in Figure 16–22A has an end that appears cylindrical. The one at the right, however, has a different kind of end, one that may reflect the pattern of addition of αβ-tubluin to a growing end.

Reference: Chrétien D, Fuller SD & Karsenti E (1995) Structure of growing microtubule ends: two-dimensional sheets close into tubes at variable rates. *J. Cell Biol.* 129, 1311–1328.

16–60

A. The microtubule is shrinking because it has lost its GTP cap; that is, the tubulin subunits at its end are all in their GDP-bound form. GTP-loaded tubulin subunits from solution will still add to this end, but they will be short-lived—either because they hydrolyze their GTP or because they fall off as the microtubule rim around them disassembles. If enough GTP-loaded subunits are added quickly enough to cover up the GDP-containing tubulin subunits at the microtubule end, then a new GTP cap can form and regrowth will be favored.

B. The rate of addition of GTP-tubulin will be greater at higher tubulin concentrations. The frequency with which shrinking microtubules switch to the growing mode will therefore increase with increasing tubulin

concentration. The consequence of this regulation is that the system is self-balancing. The more microtubules shrink (resulting in a higher concentration of the free tubulin), the more frequently microtubules will start to grow. As microtubules grow, the concentration of free tubulin will fall and the rate of GTP-tubulin addition will slow down. At some point GTP hydrolysis will catch up with new GTP-tubulin addition, the GTP cap will be destroyed, and the microtubule will switch to the shrinking mode.

C. If only GDP were present, microtubules would continue to shrink and eventually disappear, because tubulin dimers with bound GDP have very low affinity for each other and will not add stably to microtubules.

D. If a GTP analog that cannot be hydrolyzed were present, microtubules would continue to grow until all free tubulin subunits had been used up.

16–61 Severing a microtubule in the middle would generate new plus and minus ends, both of which would lack a GTP cap. The β-tubulin subunits in the middle of a microtubule will have already hydrolyzed their GTP caps and thus will have bound GDP. Thus, the simple notion that ends with GTP caps grow and ends without caps shrink leads to the expectation that the newly exposed plus and minus ends will both shrink. In reality, in this sort of experiment, the plus end shrinks, as expected, but the minus ends are stable and immediately resume a slow rate of polymerization. These results may indicate that a GTP cap is important for growth at a plus end but is not important for stability and growth at a minus end.

Reference: Walker RA, Inoué S & Salmon ED (1989) Asymmetric behavior of severed microtubule ends after ultraviolet-microbeam irradiation of individual microtubules *in vitro*. *J. Cell Biol.* 108, 931–937.

16–62 Cell division depends on the ability of microtubules to polymerize and to depolymerize. During mitosis, cells first depolymerize most of their microtubules and then repolymerize them to form the mitotic spindle. Taxol-treated cells are prevented from depolymerizing their existing microtubules, and thus cannot form a mitotic spindle. Colchicine-treated cells cannot polymerize new microtubules, and thus are also prevented from forming a mitotic spindle. On a more subtle level, both drugs would block the dynamic instability of microtubules and thus would interfere with the workings of the mitotic spindle, even if one could be formed.

16–63 Once the first lateral association has occurred, the next αβ-dimer can bind much more readily because it is stabilized by both lateral and longitudinal contacts (**Figure 16–51**). The formation of a second protofilament stabilizes both protofilaments, allowing the rapid addition of new αβ-tubulin dimers to form adjacent protofilaments and to extend

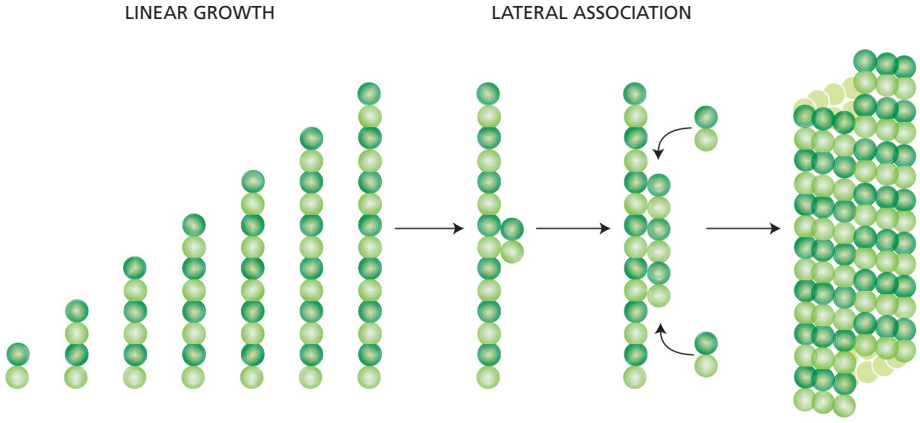


Figure 16–51 Rapid addition of αβ-tubulin dimers to nucleation structure (Answer 16–63).

existing ones. At some point the sheet of tubulin curls into a tube to form the microtubule.

Reference: Leguy R, Melki R, Pantaloni D & Carlier M-F (2000) Monomeric γ-tubulin nucleates microtubules. *J. Biol. Chem.* 275, 21975–21980.

16–64 The centrosome nucleates a three-dimensional, starburst array of microtubules that grow until they encounter an obstacle, ultimately the plasma membrane. Dynamic instability of the microtubules, coupled to the requirement for equal pushing of oppositely directed microtubules, eventually positions the centrosome in the middle of the cell. One way to think about the notion of equal and opposite forces is to realize that the microtubules are not absolutely rigid structures. Imagine pushing an object with a short steel rod versus a very long one; the short rod transmits force effectively, but the long rod will bend, delivering less force. The same principle may operate inside the cell, with microtubules of equal length delivering the same force. When all the oppositely directed microtubules emanating from a centrosome are the same length, the centrosome will be in the center of the cell.

16–65 Both γ-TuRC and the Arp2/3 complex nucleate growth by binding at the minus end of microtubules and actin filaments, respectively, allowing rapid growth at the plus end. Both contain subunits that are evolutionarily related to the subunits of the filaments they nucleate: γ-tubulin for γ-TuRC, and Arp2 and Arp3, which are related to actin, for the Arp2/3 complex.

γ-TuRC most often nucleates microtubule growth deep within the cell cytoplasm, whereas the Arp2/3 complex most frequently nucleates actin filaments near the plasma membrane. The Arp2/3 complex can bind to the side of an actin filament and thereby build a weblike network, whereas γ-TuRC does not promote branching.

16–66 Katanin cleaves microtubules all along their length. The fragments that form therefore contain GDP-tubulin at their exposed ends and rapidly depolymerize. Katanin thus provides a very quick means for destroying existing microtubules.

16–67 Kinesin-1 molecules need to be highly processive in order to accomplish their biological function of transporting organelles over long distances. For example, a kinesin molecule can transport a mitochondrion all the way down a nerve axon. Their high processivity translates into high efficiency of transport. By contrast, it is essential for muscle function that myosin II molecules not be processive. Because myosin II motors in skeletal muscle always function as part of a large array, it doesn't matter that individual motors let go; others will always be bound. In fact, if myosin II bound to actin tightly enough to be highly processive, it would inhibit muscle contraction, whose speed depends on the low processivity of its motors.

16–68

A. The components of the flagellum and their locations in Figure 16–24 are listed below.
   1. Inner sheath
   2. Radial spoke
   3. A microtubule
   4. Inner dynein arm
   5. B microtubule
   6. Singlet microtubule
   7. Nexin
   8. Outer dynein arm

B. The A and B microtubules of the outer doublets and the central pair of singlet microtubules are composed of α- and β-tubulin.

16–69 One pattern of dynein activity that could account for the planar bending of an axoneme is depicted in **Figure 16–52**. The axonemes shown in this figure are oriented with their tips *below* the plane of the page. If the dynein arms on just the left half of the axoneme were active (arrows in Figure 16–52A), the cilium would bend upward toward the top of the page. This is difficult to imagine in three dimensions, but consider it step by step. First, the dynein arms push their neighbor doublets toward the *tip* of the axoneme, so the doublets are being pushed *below* the plane of the page. Second, the doublet at the *top* of the diagram in Figure 16–52A will be pushed the farthest below the page because its total displacement is the sum of incremental displacements produced by all four active dynein arms. Third, the doublet that moves the farthest defines the "inside" of the bend (see Figure 16–28). Therefore, since the top doublet moves the farthest, the axoneme will bend upward (toward the top of the page) when the dynein arms on the left half of the axoneme are active.

The same reasoning argues that the axoneme will bend downward (toward the bottom of the page) if the dynein arms on the right half of the axoneme are active and the ones on the left half are passive (Figure 16–52B).

The actual pattern of dynein activity that gives rise to planar bending is not known. The two central singlet microtubules are natural candidates for regulatory elements: they are surrounded by nonidentical proteins; they contact different subsets of outer doublets; and they are linked (indirectly) to the two sets of dynein arms used in the model proposed above.

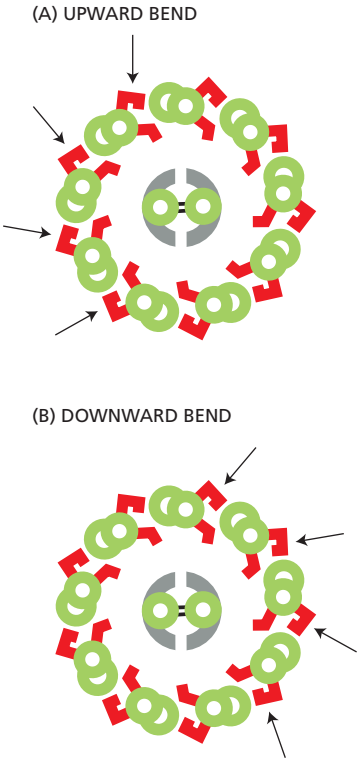


Figure 16–52 One possible pattern of dynein activity that could produce planar bending of an axoneme (Answer 16–69). (A) Upward bend. (B) Downward bend. *Arrows* indicate active dynein arms.

Reference: Satir P & Matsuoka T (1989) Splitting the ciliary axoneme: implications for a "switch-point" model of dynein arm activity in ciliary motion. *Cell Motil. Cytoskel.* 14, 345–358.

16–70 Since all hooks curve in the same (clockwise) direction, all the microtubules have the same orientation. If there were a mixture of microtubule orientations—as there would be in a cross section of a dendrite, for example—some of the microtubules would have hooks that curved in the opposite (counterclockwise) direction. (Note that you would never expect to see a single microtubule with some hooks curved in one direction and other hooks curved in the opposite direction.)

CALCULATIONS

16–71 A growth rate of 2 μm/min (2000 nm/60 sec = 33 nm/sec) corresponds to the addition of 4.2 αβ-tubulin dimers [(33 nm/sec) × (αβ-tubulin/8 nm) = 4.17 dimers/sec] to each of 13 protofilaments, or about 54 αβ-tubulin dimers/sec to the ends of a microtubule.

Reference: Detrich HW, Parker SK, Williams RC, Nogales E & Downing KH (2000) Cold adaptation of microtubule assembly and dynamics. *J. Biol. Chem.* 275, 37038–37047.

16–72

A. Centrosomes lower the critical concentration by providing nucleation sites for microtubule growth. Nucleation sites make it easier to start new microtubules; moreover, they protect the bound end from disassembly. Thus, once started, a microtubule is more likely to persist. In the absence of such a nucleation site, it is much more difficult to start a microtubule, and both ends serve as sites for disassembly.

B. The shapes of the curves in the presence and absence of centrosomes differ because of the nature of the assays used to detect polymerization. In the absence of centrosomes (see Figure 16–26A), the assay was for total polymer formed, which depends only on the concentration of

added αβ-tubulin. Thus, it increases indefinitely in a linear fashion with increasing concentration of tubulin. In the presence of centrosomes (see Figure 16–26B), the assay was the number of microtubules per centrosome. Since each centrosome has a limited number of nucleation sites (about 60 for the centrosomes used in this experiment), the measurement must reach a plateau at high tubulin concentrations.

C. A concentration of αβ-tubulin dimers of 1 mg/mL corresponds to 9.1 μM.

$$[\text{tubulin}] = \frac{1 \text{ mg tubulin}}{\text{mL}} \times \frac{\text{mmol tubulin}}{1.1 \times 10^5 \text{ mg tubulin}} \times \frac{1000 \text{ mL}}{\text{L}}$$

$$= 9.1 \times 10^{-3} \text{ mmol/L}$$

$$= 9.1 \times 10^{-3} \text{ mM, or } 9.1 \text{ μM}$$

This value is below the critical concentration for microtubule assembly in the absence of centrosomes. Thus, without a nucleation site for growth, commonly provided by the centrosome, a cell would have no microtubules. This simple consideration probably explains why the majority of microtubules originate from centrosomes in animal cells.

Reference: Mitchison T & Kirschner M (1984) Microtubule assembly nucleated by isolated centrosomes. *Nature* 312, 232–237.

16–73

A. The decrease in the lag time for assembly of microtubules in the presence of γ-tubulin indicates that γ-tubulin monomers accelerate the nucleation event in microtubule polymerization. Assembly in the presence of γ-tubulin occurs more rapidly because there are more sites of polymerization, as a result of the more efficient nucleation by γ-tubulin.

B. The critical concentration in the absence of γ-tubulin is a combination of the critical concentrations for the plus and minus ends. γ-Tubulin lowers the critical concentration by capping the minus end, preventing polymerization and depolymerization from that end. Thus, the critical concentrations in the presence of γ-tubulin represents the critical concentration for the plus end, which is lower than that for the minus end (discussed for actin assembly in Problem 16–24) and lower than the combination of critical concentrations measured in the absence of γ-tubulin (see Figure 16–27B). The greater extent of polymerization in the presence of γ-tubulin (see Figure 16–27A) happens because growth occurs at the plus ends, which have a lower critical concentration. As a result, they reach a greater extent of polymerization before they come into equilibrium with the concentration of free αβ-tubulin.

Reference: Leguy R, Melki R, Pantaloni D & Carlier M-F (2000) Monomeric γ-tubulin nucleates microtubules. *J. Biol. Chem.* 275, 21975–21980.

16–74 It would take a vesicle an average of 10^9 seconds—nearly 32 years—to diffuse the length of a 10 cm axon:

$$t = x^2/2D$$

$$= \frac{(10 \text{ cm})^2}{2 \times (5 \times 10^{-8} \text{ cm}^2/\text{sec})}$$

$$= 10^9 \text{ second, or } 31.7 \text{ years}$$

16–75 The mitochondrion is about 12 times faster. It moves at 10^6 body lengths per day. The swimmer moves at 100 body lengths/1.75 min, which is 8.2×10^4 body lengths per day.

16–76

A. If the flagellum bends into a semicircle, then the "inside" doublet will protrude beyond the "outside" doublet by a length equal to the difference in the perimeters of the semicircles they form (see Figure 16–28).

Since the perimeter of a semicircle is πr, the difference in length between the "outside" and "inside" perimeters will be $\pi(r + 180\text{ nm}) - \pi r$, which equals $\pi \times 180$ nm, or about 565 nm. Thus, the "inside" doublet protrudes beyond the "outside" doublet by 565 nm. This distance corresponds to about 70 tubulin dimers.

It may come as a mild surprise that this calculation is independent of the radius of the semicircle. Since radius is irrelevant, the flagellum could be wrapped half way around the world and the result would be the same: the "inside" doublet would still protrude 565 nm beyond the "outside" doublet.

B. The length of the stretched nexin molecule can also be calculated from simple geometric principles. The stretched nexin molecule is the hypotenuse of a right triangle with a base equal to 30 nm (the distance between the adjacent doublets) and a side whose length is the difference between the perimeters of the semicircles formed by the adjacent doublets (which can be calculated as described in part A). The length of the side is $\pi(r + 30\text{ nm}) - \pi r$, which equals $\pi \times 30$ nm, or about 94 nm. The hypotenuse of the right triangle is the square root of the sum of the squares of the two sides. Thus, the stretched nexin molecule is $[(30\text{ nm})^2 + (94\text{ nm})^2]^{0.5}$, which equals about 99 nm. This calculation suggests that nexin molecules can stretch to more than three times their normal length. Independent experimental measurements verify this striking elasticity.

References: Brokaw CJ, Luck DJL & Huang B (1982) Analysis of the movement of *Chlamydomonas* flagella: the function of the radial-spoke system is revealed by comparison of wild-type and mutant flagella. *J. Cell Biol.* 92, 722–732.

Gibbons IR (1981) Cilia and flagella of eukaryotes. *J. Cell Biol.* 91, 107s–124s.

DATA HANDLING

16–77 These observations show that αβ-tubulin dimers are oriented in microtubules with β-tubulin exposed at the plus end and α-tubulin facing the minus end. Because the GTP that is bound to the α-tubulin monomer is physically trapped at the dimer interface, it is never hydrolyzed or exchanged. By contrast, the GTP in β-tubulin is hydrolyzed and can be exchanged. Thus, when a microtubule is exposed to GTP-coated fluorescent beads, the GTP can bind to the β-tubulin subunits exposed at the end of the microtubule. Finding the fluorescent beads at the plus ends indicates that the αβ-tubulin dimer must be oriented with the β-tubulin monomer at the plus end. The presence of the beads only at one end, and not all along the microtubule, indicates that GTP can be exchanged only at the exposed ends.

Similarly, the presence of gold beads coated with antibodies specific for α-tubulin at the minus ends indicates that the αβ-tubulin dimer must be oriented with the α-tubulin monomer exposed at the minus end. The presence of beads only at one end indicates that the portion of α-tubulin with which the antibody reacts is buried at the interface between adjacent αβ-tubulin dimers, and thus is available only at the end.

References: Mitchison TJ (1993) Localization of an exchangeable GTP binding site at the plus end of microtubules. *Science* 261, 1044–1047.

Fan J, Griffiths AD, Lockhart A, Cross RA & Amos LA (1996) Microtubule minus ends can be labelled with a phage display antibody specific to α-tubulin. *J. Mol. Biol.* 259, 325–330.

Nogales E, Whittaker M, Milligan RA & Downing KH (1999) High-resolution model of the microtubule. *Cell* 96, 79–88.

16–78

A. The two ends of an individual microtubule appear to behave independently of one another. One end can grow while the other shrinks, and both ends can grow or shrink at the same time. Furthermore, the transitions between growth states at the two ends do not correlate with one another in any obvious way.

B. The GTP-cap hypothesis predicts that the faster-growing end, which has the longer GTP cap, should be more stable than the slower-growing end, which has a shorter GTP cap. Thus, a fast-growing end should persist in a growth state longer than a slow-growing end; that is, a fast-growing end should switch from a growth state to a shrinking state less frequently than a slow-growing end. (The hypothesis says nothing about how frequently a shrinking end, which does not have a cap, will be converted into a growing end.)

   The experimental results appear, if anything, to run counter to the predictions of the GTP-cap hypothesis. The growth periods at the plus ends do not seem to be significantly longer (they actually appear somewhat shorter) than the growth periods at the minus ends. Thus, these results do not support this version of the GTP-cap hypothesis. In cells, proteins other than tubulin may bind to GTP caps and help to stabilize fast-growing ends.

C. Since centrosomes nucleate growth of microtubules by binding to the minus end, all the free ends would be plus ends. As a consequence, the ends would behave uniformly—like plus ends (see Figure 16–29A). The minus ends would be stably associated with the centrosome.

   Since MAPs tend to stabilize microtubules against disassembly, they would be expected to reduce the frequency of switching between the two growth states and extend the length of time microtubules remain in the growing state. (This is the result that is observed in the actual experiment. The switches in the growth state are abolished and growth is smooth and continuous until the steady-state length is reached, after which the length remains constant.)

Reference: Horio T & Hotani H (1986) Visualization of the dynamic instability of individual microtubules by dark-field microscopy. *Nature* 321, 605–607.

16–79

A. You should expect the amino acid changes to strengthen primarily the lateral interactions in the microtubule lattice; that is, interactions between tubulin subunits in adjacent protofilaments. The surest way to strengthen the lattice is to strengthen the weakest interactions. The interactions between the α- and β-tubulin subunits in the αβ-tubulin dimer are the strongest interactions in the lattice; the dimer is so stable that it is the predominant form of tubulin in a cell. Increasing the affinity between adjacent dimers in a protofilament would tend to increase the stability of the microtubule; however, the appearance of microtubule ends undergoing catastrophic shrinkage—frayed ends with curled protofilaments (see Figure 16–22)—indicates that interactions within protofilaments are stronger than interactions between protofilaments. Thus, interactions between the protofilaments are the weakest interactions in the lattice. Therefore, it is not surprising that the amino acid changes in the tubulins in notothenioid fish strengthen interactions between adjacent protofilaments, thereby increasing the overall stability of the microtubules.

B. It seems highly unlikely that notothenioid fish cells could exist if they had a stable microtubule cytoskeleton. In normal cells, microtubules are constantly shifting, and they undergo a dramatic rearrangement during mitosis when the cytoskeletal microtubules disassemble to form the mitotic spindle. It seems inconceivable that fish cells could divide with a

fixed microtubule architecture. It is much more likely that notothenioid microtubules are just as dynamic in cells as normal microtubules, but that accessory proteins control the instability.

Reference: Detrich WH, Parker SK, Williams RC, Nogales E & Downing KH (2000) Cold adaptation of microtubule assembly and dynamics. *J. Biol. Chem.* 275, 37038–37047.

16–80 This simple purification procedure takes advantage of the properties of tubulin and microtubules. At 0°C, microtubules dissociate into αβ-tubulin dimers, which remain in the supernatant when subjected to high centrifugal force. In the presence of GTP at 37°C, the tubulin dimers polymerize into microtubules, which are large enough to form a pellet when centrifuged. This repetitive, two-step procedure purifies tubulin away from all other cell components. Large cell components are discarded each time the supernatant is saved; small cell components, including other proteins, are discarded each time the pellet is saved.

Reference: Sloboda RD, Dentler WL & Rosenbaum JL (1976) Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*. *Biochemistry* 15, 4497–4505.

16–81

A. Microtubules assembled on flagellar axonemes are extensions of the microtubules already present in the axonemes. Therefore, the polarity of growth is fixed: the plus end of the axoneme will nucleate a microtubule that has its plus end free for the addition of new subunits. The newly assembled microtubule therefore has its plus end pointing away from the axoneme and its minus end attached to the axoneme (**Figure 16–53A**).

B. The plus end of the microtubule must grow faster since microtubules with free plus ends (attached to the plus end of the axoneme) are longer than those with free minus ends (attached to the minus end of the axoneme).

C. For axonemes, where the plus and minus ends can be distinguished, it is clear that the growth rate at the plus end is faster than at the minus end, since the microtubules attached to the plus end are longer than those attached to the minus end. It is this difference in growth rates that allows one to decide the polarity of growth nucleated by centrosomes. Microtubules nucleated on centrosomes have lengths that indicate their plus ends are free. Thus, centrosomes nucleate microtubule growth by binding to the minus end of the microtubule (**Figure 16–53B**).

Reference: Mitchison T & Kirschner MW (1985) Properties of the kinetochore *in vitro*. I. Microtubule nucleation and tubulin binding. *J. Cell Biol.* 101, 755–765.

16–82 Whether γ-TuRC is present or not makes no difference to the lengths of the bright segments at the plus ends of the microtubules (and, as expected, they tend to be much longer that those at the minus ends). However, γ-TuRC shifts the distribution of bright segments at the minus ends, suggesting that it blocks (or at least retards) growth at that end. γ-TuRC appears to cap the minus end, suggesting that it nucleates growth from the minus end. You might reasonably ask why there are any bright segments at all at the minus ends. Although 50% have very short or nonexistent bright segments, the remainder have a distribution of lengths that is not much different from that observed in the absence of γ-TuRC. Some of these microtubules may have nucleated spontaneously, γ-TuRC may have dissociated from some, or some microtubules may have been broken.

Reference: Zheng Y, Wong ML, Alberts B & Mitchison T (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* 378, 578–583.

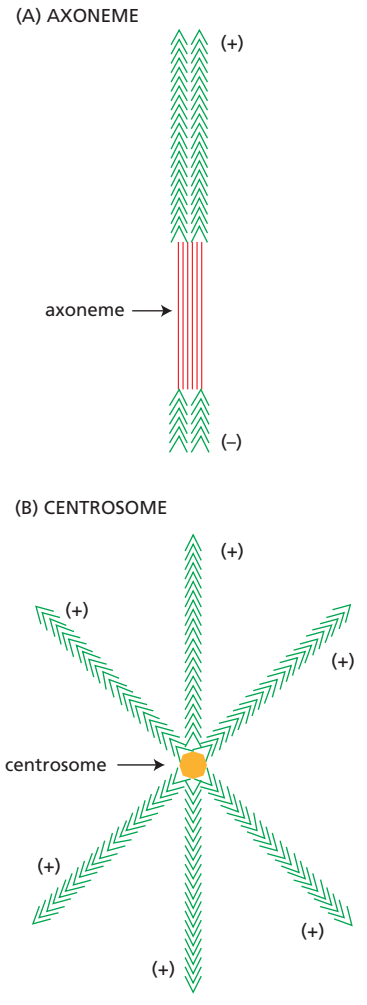


Figure 16–53 Polarities of microtubules (Answer 16–81). (A) Nucleation on a flagellar axoneme. (B) Nucleation on a centrosome.

16–83

A. Motor proteins are unidirectional in their action; nearly all kinesins move toward the plus end of a microtubule and dyneins always move toward the minus end. Thus, if dynein molecules, for example, were attached to the coverslip, only those individual molecules that were correctly oriented relative to the microtubule that settles on them could attach to it, exert force, and propel it forward.

B. On a bed of dynein motors, microtubules will always move plus-end first over the coverslip. The dynein motors "walk" toward the minus end; thus, since the motors are fixed, the microtubule moves plus-end first.

C. The protein on the coverslip is a plus-end directed motor. Since the bead, which marks the minus end of the microtubule, is moving forward, the motor must be walking toward the opposite end—the plus end.

Reference: Fan J, Griffiths AD, Lockhart A, Cross RA & Amos LA (1996) Microtubule minus ends can be labelled with a phage display antibody specific to α-tubulin. *J. Mol. Biol.* 259, 325–330.

16–84 The investigators knew which end of the microtubule was attached to the gold beads because they determined the direction of motion on a bed of plus-end directed kinesin motors. The microtubules were observed to move bead-end first (see Figure 16–32, which are video frames from these experiments). Since kinesin motors propel the microtubules with their minus ends forward (by walking toward the plus end), the gold beads must be at the minus ends of the microtubules.

If you designed your experiment using a minus-end directed motor such as dynein, you would have observed the gold bead at the trailing end of the microtubule.

Reference: Fan J, Griffiths AD, Lockhart A, Cross RA & Amos LA (1996) Microtubule minus ends can be labelled with a phage display antibody specific to α-tubulin. *J. Mol. Biol.* 259, 325–330.

16–85

A. The differences in landing rates at low densities of two-headed and one-headed kinesins indicate that individual two-headed kinesin motors can move a microtubule, but that multiple one-headed kinesin motors are required. At high motor protein density, the landing rates for both motors are about the same. The landing rate for two-headed kinesin declines linearly with density, whereas the landing rate for one-headed kinesin drops abruptly at lower densities. This behavior indicates that a single, two-headed kinesin is sufficient to move a microtubule, but that several one-headed kinesins (four to six according to the authors) are required. A one-headed kinesin can bind a microtubule, but when it lets go to take the next step the microtubule floats away. Thus, several one-headed kinesins are required so that some can hold onto the microtubule while others release and rebind.

B. Two heads are better than one. In principle, a single kinesin motor with two heads could move a vesicle for long distances along a microtubule track because it holds on with one "hand," while it releases and rebinds with the other. A one-headed motor would lose its way each time it released the microtubule to take a step.

Reference: Hancock WO & Howard J (1998) Processivity of the motor protein kinesin requires two heads. *J. Cell Biol.* 140, 1395–1405.

16–86

A. The unidirectional movement of kinesin along a microtubule is driven by the free energy of ATP hydrolysis. ATP binding and hydrolysis are coupled to a series of conformational changes in the kinesin head that bring about the unidirectional stepping of the kinesin motor domains along the microtubule.

B. In the first trace, the kinesin moves 80 nm in 9 sec at an average rate of about 9 nm/sec. In the second trace, the kinesin moves 80 nm in 5 sec at an average rate of about 16 nm/sec. These rates are about 100-fold slower than the *in vivo* rate because the experimental conditions (ATP concentration and force exerted by the interference pattern) were adjusted to slow the movements of kinesin so individual steps could be observed.
C. As can be seen in Figure 16–35B, the two kinesin molecules each took 10 steps to move 80 nm, indicating that the length of an individual step is about 8 nm.
D. Since the step length and the interval between β-tubulin subunits along a microtubule protofilament are both 8 nm, a kinesin appears to move by stepping from one β-tubulin to the next along a protofilament. Because kinesin has two domains that can bind to β-tubulin, it presumably keeps one domain anchored as it swings the other domain to the next β-tubulin binding site—much like a person walking along a path of stepping-stones.
E. The data in Figure 16–35B contain no information about the number of ATP molecules hydrolyzed per step. Other experiments by these same investigators suggest that hydrolysis of one ATP does not cause multiple steps. By lowering ATP concentrations to slow movement along the microtubule, the investigators showed the same sort of stepping patterns as in Figure 16–35B, although on a longer time scale. If hydrolysis of a single ATP could cause multiple steps, a clustering of steps might have been expected under these experimental conditions. None of these experiments rule out the possibility that more than one ATP molecule might need to be hydrolyzed for each step.

Reference: Svoboda K, Schmidt CF, Schnapp BJ & Block SM (1993) Direct observation of kinesin stepping by optical trapping interferometry. *Nature* 365, 721–727.

MEDICAL LINKS

16–87 Kinesin motors use microtubules as tracks to deliver organelles and materials to nerve endings. The similar neuropathies that develop in mice and humans with only one functional copy of the gene for the kinesin motor KIF1B suggest that half the normal number of these motors is not sufficient to keep up with the needs of the nerves.

Reference: Zhao C, Takita J, Tanaka Y, Setou M, Nakagawa T, Takeda S, Yang HW, Terada S, Nakata T, Takei Y, Saito M, Tsuji S, Hayashi Y & Hirokawa N (2001) Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβ. *Cell* 105, 587–597.

INTERMEDIATE FILAMENTS AND SEPTINS

DEFINITIONS

16–88 Neurofilament

16–89 Keratin

TRUE/FALSE

16–90 False. In contrast to actin filaments and microtubules, which are present in all eukaryotic organisms, intermediate filaments are found only in some metazoans, including vertebrates, nematodes, and snails. Even in these organisms, intermediate filaments are not required in every cell

type. The nuclear lamins, which are the ancestors of the intermediate filaments, form a meshwork of protein that lines the nuclear membrane; they are much more widely distributed among eukaryotes.

16–91 False. The cytoplasmic cytoskeleton and the nuclear lamina are connected by proteins that are embedded in the nuclear membrane. The KASH protein in the outer nuclear membrane and SUN proteins in the inner nuclear membrane bind to one another in the lumen of the nuclear envelope. The KASH proteins bind to elements of the cytoplasmic cytoskeleton and the SUN proteins bind to the nuclear lamina.

THOUGHT PROBLEMS

16–92 The building blocks—soluble subunits—of the three types of filaments are the basis for their polarity differences. The building blocks for actin filaments (an actin monomer) and microtubules (αβ-tubulin) have polarity—distinct ends—and thus form a polymer with distinct ends when they are linked together. By contrast, the building block of intermediate filaments is a symmetrical tetramer with identical ends. Thus, when these subunits are linked together, the ends of the resulting filament are also identical.

16–93 Cells that migrate rapidly from one place to another, like amoebae (A) and sperm cells (E), do not, in general, need intermediate filaments in their cytoplasm, since they do not develop or sustain large tensile forces. Plant cells (F) are pushed and pulled by the forces of wind and water, but they resist these forces by means of their rigid cell walls, rather than by their cytoskeleton. Epithelial cells (B), smooth muscle cells (C), and the long axons of nerve cells (D) are all rich in cytoplasmic intermediate filaments, which prevent them from rupturing as they are stretched and compressed by the movements of surrounding tissues.

16–94 The disulfide bonds that cross-link keratin filaments in skin cells form after the cells have died. In the absence of cellular metabolism to maintain the reducing environment characteristic of the interior of a living cell, a dead cell's contents quickly become oxidized. It is in this postmortem environment that the keratin filaments become cross-linked by disulfide bonds.

16–95 It is surprising that so many knockouts of genes for intermediate filaments have little effect in mice. The amino acid sequence of vimentin, for example, is 98% identical in hamster, chicken, mouse, and human, implying an important function, yet mouse knockouts appear entirely normal. The absence of an effect of knockout of such a conserved gene is usually interpreted in terms of a "backup" system that compensates for the loss. In the case of intermediate filament genes, the backup system may be other intermediate filaments. That the combined knockout of vimentin and GFAP does have a phenotype—defective astrocytes—suggests that these two intermediate filaments back each other up in astrocytes.

Astrocytes express genes for three intermediate filament proteins—vimentin, GFAP, and nestin, whose properties have been studied using the pure proteins. Nestin cannot form intermediate filaments on its own, which presumably explains its inability to compensate for the loss of vimentin and GFAP. Vimentin can form intermediate filaments, but only with nestin or GFAP as a partner, and GFAP can form somewhat abnormal intermediate filaments on its own. Thus, these three proteins appear to cooperate in the formation of correct intermediate filaments in astrocytes: disruption of one can be tolerated, but not the ablation of two.

References: Herrmann H & Aebi U (2000) Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. *Curr. Opin. Cell Biol.* 12, 79–90.

Eliasson C, Sahlgren C, Berthold CH, Stakeberg J, Celis JE, Betsholtz C, Eriksson JE & Pekny M (1999) Intermediate filament protein partnership in astrocytes. *J. Biol. Chem.* 274, 23996–24006.

16–96 Intermediate filaments have no polarity; their ends are indistinguishable. It would therefore be difficult for a hypothetical motor protein bound to the middle of the filament to sense a defined direction. Such a motor protein would be equally likely to attach to the filament facing in one direction as the other. The known molecular motors all move in one direction along a filament of defined polarity, allowing them to move toward their intended destinations.

16–97 Both filaments are composed of subunits of protein dimers that are held together by coiled-coil interactions. Moreover, in both cases, the dimers polymerize through their coiled-coil domains into filaments. Intermediate filament dimers assemble head-to-head to generate symmetric building blocks that are joined end-to-end to create a filament that has no polarity. By contrast, asymmetrical myosin molecules are assembled into a polar chain, two of which join tail-to-tail to form the bipolar myosin filament. As a result, all myosin molecules in the same half of the myosin filament are oriented with their heads pointing in the same direction. This polarity is necessary for them to be able to develop a contractile force in muscle.

DATA HANDLING

16–98 In BHK-21 cells, the entire vimentin network depolymerizes in preparation for mitosis and then reassembles afterward (see Figure 16–36A). Note the absence of obvious filaments during the two phases of mitosis and their clear presence in the daughter cells. By contrast, in PtK2 cells, the vimentin network remains largely intact until late cytokinesis, when the portion of the network in the connecting cytoplasmic bridge is finally "dissolved." Thus, in these cells, only a small portion of the vimentin network is disassembled during mitosis. It is unclear how these two quite different strategies are accomplished.

Reference: Yoon M, Moir RD, Prahlad V & Goldman RD (1998) Motile properties of vimentin intermediate filament networks in living cells. *J. Cell Biol.* 143, 147–157.

16–99

A. Treatment with alkaline phosphatase reduces the number of spots to three—one for each lamin—because it removes all phosphates from the individual lamins. Lamin molecules of the same type migrate to different positions because they carry different numbers of attached phosphate groups. The charges on the phosphates alter the total charge on the lamins, thereby changing their migration under conditions that are sensitive to charge.

B. The untreated mixture of lamins from interphase and mitotic cells shows most clearly the total number of different types of lamin molecules present overall. For example, lamin C shows up as four equally spaced spots in the untreated mixture. The spot farthest to the right in Figure 16–37A (most positively charged) corresponds to the one lamin C spot that is present when all phosphates have been removed by alkaline phosphatase. Thus, that spot is derived from lamin C molecules that carry zero phosphate groups. The equal spacing between adjacent spots of lamin C suggests that they correspond to molecules that carry one, two, or three

phosphates (although in principle they could carry two, four, or six phosphates, or any such multiple). A comparison of the interphase pattern with the mixture indicates that lamin C molecules in interphase cells carry zero or one phosphate. Similarly, lamin C molecules from mitotic cells carry two or three phosphates.

The same analysis indicates that lamin A molecules carry zero or one phosphate in interphase cells and three phosphates in mitotic cells. (The gap in the pattern in the mixture indicates that lamin A molecules rarely, if ever, carry two phosphates.) Analysis of lamin B molecules indicates that they carry zero phosphates in interphase cells and one (or perhaps two) phosphates in mitotic cells.

C. ^{35}S-methionine was used instead of ^{32}P-phosphate in order to see the positions of all the lamins, even those that do not carry phosphates. If ^{32}P-phosphate had been used to label the cells, the three spots visible after alkaline phosphatase treatment would not have been present.

D. Although these results are highly suggestive that phosphorylation plays an important role in the disassembly of the lamin network, they stop short of proving the point. There are many examples of cellular proteins whose state of phosphorylation can change without any apparent alteration in function. Site-directed mutagenesis of phosphorylation sites in lamins has now demonstrated conclusively that phosphorylation is essential for disassembly of the nuclear lamina during mitosis.

References: Ottaviano Y & Gerace L (1985) Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* 260, 624–632.

Ward GE & Kirschner MW (1990) Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell* 61, 561–577.

Heald R & McKeon F (1990) Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* 61, 579–589.

Peter M, Nakagawa J, Dorée M, Labbé JC & Nigg EA (1990) *In vitro* disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61, 591–602.

CELL POLARIZATION AND MIGRATION

DEFINITIONS

16–100 Rho protein family

16–101 Lamellipodium

16–102 Filopodium

16–103 Blebbing

TRUE/FALSE

16–104 True. Such fragments continue to crawl normally, even though they lack microtubules and membrane-enclosed organelles.

16–105 True. A site of bacterial infection is a source of bacterial proteins, some of which have retained the *N*-formylmethionine used for initiation of bacterial protein synthesis. As these proteins are degraded, *N*-formylated peptides are released and diffuse outward from the source, generating a gradient that can be sensed by neutrophils via membrane receptors. The binding of *N*-formylated peptides triggers changes in the cytoskeleton that allow the neutrophil to travel up the gradient to the site of infection.

THOUGHT PROBLEMS

16–106 During protrusion, cells extend actin-rich structures—filopodia, lamellipodia, or pseudopodia—in front of them. During attachment, the actin cytoskeleton in the extended structures makes connections with the substratum. During traction, contraction of the anchored actin cytoskeleton pulls the bulk of the cytoplasm forward.

16–107 The minus ends of the growing actin filaments are anchored to the rest of the actin cytoskeleton, which supports the growing actin filaments and allows them to push on the membrane without simply sliding back into the cell's interior. The solution to the problem at the plus end is not so straightforward. Once the filament contacts the membrane, there would be no room for a new subunit to fit onto the end of the growing chain. It is thought that random thermal motions briefly expose the plus end of the filament, allowing a new subunit to be added. By taking advantage of these small windows of opportunity, actin polymerization acts as a ratchet to capture random thermal motions. It is unclear what motions the actin ratchet is capturing. It could be that membranes "breathe" thermally, allowing polymerization. Alternatively, the actin filament may bend elastically, moving the plus end sufficiently to allow subunit addition.

16–108 Injection of activated Rac triggers actin polymerization over the entire membrane periphery, forming essentially one giant lamellipodium (see Figure 16–38C). Injection of activated Rho promotes the bundling of actin filaments with myosin II filaments to form stress fibers (see Figure 16–38B), which associate with other proteins at focal contacts. Injection of activated Cdc42 triggers actin polymerization and bundling to form filopodia (see Figure 16–38D).

Reference: Hall A (1998) Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514.

16–109 The unidirectional motion of a lamellipodium results from the nucleation and growth of actin filaments at the leading edge of the cell and depolymerization of the older actin meshwork more distally. Cofilin plays a key role in differentiating the new actin filaments from the older ones. Because cofilin binds cooperatively and preferentially to actin filaments containing ADP-actin, the newer filaments at the leading edge, which contain ATP-actin, are resistant to depolymerization by cofilin. As the filaments age and ATP hydrolysis proceeds, cofilin can efficiently disassemble the older filaments. Thus, the delayed ATP hydrolysis by filamentous actin is thought to provide the basis for a mechanism that maintains an efficient, unidirectional treadmilling process in the lamellipodium.

DATA HANDLING

16–110

A. If the oligomers were subjected to cycles of phosphorylation and dephosphorylation as they moved down the axon, they would move back and forth between the motor and the neurofilaments, depending on their phosphorylation state. An oligomer that spends a high fraction of its time in the dephosphorylated form would mostly be associated with kinesin and would therefore keep moving. It would be at the leading edge of the transport wave. By contrast, an oligomer that is phosphorylated most of the time would spend more of its time unattached to kinesin, and thus would move down the axon more slowly. The farther the oligomers travel down the axon, the greater would be the difference between the fastest and the slowest ones; hence, the broader the transport wave would be.

B. Oligomers at both the leading edge and the trailing edge of the transport wave would be expected to move at the same rate when they were attached to kinesin. When they were not attached to the motor, they would be stationary. Thus, both oligomers would be expected to move and stop as they traveled along the axon. The difference is that an oligomer at the trailing edge would have spent more of its time stationary because it was phosphorylated and attached to the neurofilament for more of the time. Note that the position of an oligomer—at the leading or trailing edge of the transport wave—indicates its past history of stopping and starting, but does not predict its future behavior.

References: Yabe JT, Pimenta A & Shea TB (1999) Kinesin-mediated transport of neurofilament protein oligomers in growing axons. *J. Cell Sci.* 112, 3799–3814.

Shea TB & Yabe J (2000) Occam's razor slices through the mysteries of neurofilament axonal transport: Can it really be so simple? *Traffic* 1, 522–523.

16–111 Extension and contraction of this filopodium were regulated by the rate of actin polymerization. The rate of retrograde flow was constant at about –1 μm/min, whereas the rate of actin polymerization varied from 0 to 2 μm/min. The movements of the tip correlated with the rate of actin polymerization, extending when the polymerization rate was high and retracting when the polymerization rate was low. Observations on many individual filopodia support this general conclusion.

Reference: Mallavarapu A & Mitchison T (1999) Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. *J. Cell Biol.* 146, 1097–1106.

16–112 These experimental results clearly support a role for N-WASp in the Cdc42-initiated polymerization of actin and, by implication, in the natural rearrangements of the actin cytoskeleton in cells as a result of Cdc42 activation. The inability of the mutant H208D to restore actin polymerization indicates that a direct interaction between Cdc42 and N-WASp is a critical feature of the activation pathway. The inability of the mutant Δcof to restore actin polymerization indicates that the cofilin domain of N-WASp is required for actin polymerization. The cofilin domain would allow N-WASp to bind to actin filaments, or perhaps to the actin-like subunits of the Arp2/3 complex, suggesting that such binding is essential for N-WASp-mediated actin polymerization.

Reference: Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, Takenawa T & Kirschner MW (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97, 221–231.

16–113

A. From Figure 16–41B, it is clear that both Cdc42-GTPγS and vesicles with PIP_2, in addition to ARP, are required to activate N-WASp fully for actin polymerization. Cdc42-GTPγS alone activates N-WASp partially, but vesicles with PIP_2 do not stimulate at all. Only when both components are present is the activity of N-WASp equal to that of its C-terminal VCA segment.

B. Because the C-terminal VCA segment of N-WASp has full activity, it must be the only portion of N-WASp actually involved in actin polymerization. The rest of N-WASp appears to function as an inhibitor of the C-terminal segment. The function of Cdc42 and PIP_2 is to relieve that inhibition, freeing the C-terminal segment to stimulate actin polymerization. A speculative model that includes these ideas and incorporates the known functions of the domains is shown in **Figure 16–54**.

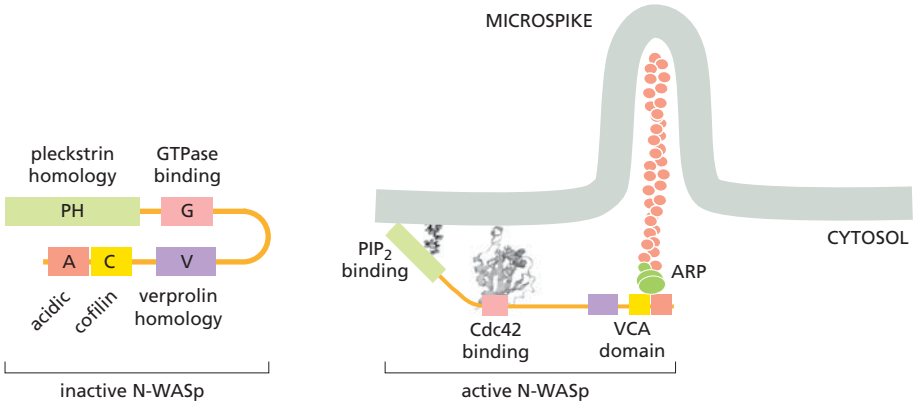


Figure 16–54 A model for Cdc42-activated polymerization of actin (Answer 16–113).

Reference: Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, Takenawa T & Kirschner MW (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97, 221–231.

MCAT STYLE

16–114

C. Observation II shows that expression of ActA in nonmotile bacteria is *sufficient* to allow bacterial motility. Observation III shows that ActA is *necessary* for motility in *Listeria*. Observation I may be critical for the mechanism of polymerization—indeed, it is—but the observation itself does not indicate that ActA is either necessary or sufficient.

16–115

C. Since inhibition of myosins did not alter bacterial motility, actin-based motor proteins (myosins) are unlikely to be involved in bacterial motility. If myosins are not involved, then actin polymerization—the only other known force-producing mechanism involving actin filaments—must be responsible for bacterial motility. Choices A and D are not correct because they do not distinguish between the two mechanisms. Both ActA and the Arp2/3 complex are required for actin polymerization, but actin polymers are critical to both mechanisms. Choice B is not correct for the same reason; it indicates that actin polymers are critical, but that observation does not distinguish between the proposed mechanisms, both of which require actin filaments.

16–116

C. Cofilin binds preferentially to ADP-actin filaments and causes them to depolymerize, releasing actin monomers from the comet tail so that they can be reused at the growing ends of filaments to propel bacteria forward. The other answers do not describe functions of cofilin.

16–117

D. Plus ends of microtubules have a lower critical concentration than minus ends. Thus, microtubules capped by centrosomes at their minus ends will grow at their plus ends, which polymerize at lower concentrations of tubulin because they have a lower critical concentration. Because the minus ends are capped by centrosomes, they are stable, even though uncapped minus ends would normally depolymerize at the lower tubulin concentration. This lack of depolymerization also contributes to the apparent increase in growth rates of the centrosome-capped microtubules. Choice A is not correct because centrosomes, which bind only to the minus ends, cannot influence events at the plus ends of microtubules. Choice B is incorrect because centrosomes cap microtubule minus ends,

eliminating growth, not increasing it. Because centrosomes are confined to the minus end, they do not affect the rate of microtubule growth at the plus end. Choice C is not correct because centrosomes completely block growth at the minus end; they do not lower the critical concentration at the minus end, they raise it infinitely.

16–118

C. After dilution to 7.5 μM, the tubulin concentration is still above the critical concentration for the plus end, so some microtubules will continue to grow. At this lower concentration, however, the rate of growth will be slower, increasing the chance that GTP hydrolysis will eliminate the GTP cap, which will expose GDP-bound subunits and cause rapid depolymerization. As a result, many microtubules will be eliminated, even as the remaining microtubules continue to grow. Choice A is not correct because GTP hydrolysis does not promote microtubule growth. Choice B is incorrect because if the centrosomes were diluted to a tubulin concentration below the critical concentration of the plus end, all microtubules would shrink. Choice D is inconsistent with the data because if dilution caused release of microtubules, the effect would be expected to occur equally at all tubulin concentrations. However, the decrease in microtubule number is proportional to the tubulin concentration.

16–119

D. Since GTP hydrolysis occurs after tubulin polymerization, the subunits found in the middle of microtubules will be bound to GDP. Thus, breakage of microtubules by shear forces would expose GDP-bound tubulin subunits at the broken ends, leading to rapid depolymerization from the newly exposed GDP-capped ends of the microtubule. The loss of turbidity is transient because the tubulin concentration is above the critical concentration, allowing rapid new polymerization, which would quickly increase the turbidity of the solution. Shear forces would not be expected to cause any of the effects described in the other answers.

CHAPTER 17

The Cell Cycle

IN THIS CHAPTER

OVERVIEW OF THE CELL CYCLE

DEFINITIONS

17–1 Interphase

17–2 Cell cycle

17–3 Start or restriction point

17–4 G_1 phase

TRUE/FALSE

17–5 False. Although a number of cells equivalent to an adult human is replaced about every three years, not all cells are replaced at the same rate. Blood cells and cells that line the gut are replaced at a high rate, whereas cells in most organs are replaced more slowly, and neurons are rarely replaced.

17–6 True. The G_1 phase is the most critical time for cell growth. Its length can vary greatly depending on external conditions and extracellular signals from other cells.

THOUGHT PROBLEMS

17–7 The gaps between S phase and M phase are required partly to permit cells the time they need to grow and double their mass of proteins and organelles. The two gaps also provide time for the cell to monitor the internal and external environment to ensure that conditions are suitable and preparations are complete before beginning S phase and mitosis.

17–8 Enzymes for most metabolic reactions function in isolation; that is, their enzymatic competence does not depend on critical interactions with other proteins. So long as the enzyme folds properly and its small-molecule substrate is present, the reaction will proceed. By contrast, cell-cycle proteins must interact with many other proteins to form the complexes that are critical for coordinated progression through the cell cycle. The ability of many human cell-cycle proteins to interact with yeast components implies that the binding surfaces responsible for these interactions have been preserved through more than a billion years of evolution. That's remarkable.

17–9 A yeast mutant that is defective in a cell-cycle gene can be isolated if its phenotype is conditionally defective—that is, it can be isolated if the product of the mutant gene fails to function under one set of conditions, but functions adequately under another. Most conditional cell-cycle mutants are temperature sensitive: the mutant protein fails to function at high temperature, but performs well enough at low temperature to allow the cell to divide.

17–10

A. During the second thymidine block, all the cells will accumulate at the beginning of S phase, since they cannot synthesize DNA. Thus, upon release of the second block, the synchronized population will begin S phase.

B. The first thymidine block halts all cells that are in S phase. Cells that are not in S phase traverse the cell cycle normally until they reach the beginning of S phase, where they stop. Since $G_2 + M + G_1$ is 15 hours long, the presence of thymidine for 18 hours should be sufficient for all the cells not originally in S phase to reach the beginning of S phase. Thus, at the end of the first thymidine block, the majority of the population will be at the beginning of S phase, but the rest of the population will be distributed throughout S phase. The release of the first thymidine block for 10 hours allows the entire population to move through S phase, but does not allow any of the population to re-enter S phase. When the second thymidine block is applied, none of the cells should be in S phase. Application of the second thymidine block for 16 hours allows the entire population to move through the cell cycle until they reach the beginning of S phase, where they accumulate.

(In reality, a thymidine block does not completely stop DNA synthesis: it slows it to a fraction of its normal rate. Thus, a double thymidine block does not result in the entire population accumulating exactly at the G_1–S boundary. The population is actually distributed within the first bit of S phase.)

References: Bootsma D, Budke L & Vos O (1964) Studies on synchronous division of tissue culture cells initiated by excess thymidine. *Exp. Cell Res.* 33, 301–309.

Bostock CJ, Prescott DM & Kirkpatrick JB (1971) An evaluation of the double thymidine block for synchronizing mammalian cells at the G_1-S border. *Exp. Cell Res.* 68, 163–168.

Rao PN & Johnson RT (1970) Mammalian cell fusion: I. Studies on the regulation of DNA synthesis and mitosis. *Nature* 225, 159–164.

Xeros N (1962) Deoxyriboside control and synchronization of mitosis. *Nature* 194, 682–683.

CALCULATIONS

17–11 Since you examined 25,000 cells and found 3 in mitosis, the mitotic index is 3/25,000, which equals 0.00012. If mitosis is 30 minutes (0.5 hours) long and the frequency of cells in mitosis is 0.00012, then 0.5 hours is 0.00012 of the length of the cell cycle. Thus, the cell cycle is 0.5/0.00012 = 4167 hours in length, on average, which is nearly half a year.

17–12 The overall length of the cell cycle is equivalent to the time it takes for the entire population of cells to double in number. To find the length of the cell cycle, select any two points on the graph in Figure 17–1, between which the number of cells has doubled. The time separating those two points is the length of the cell cycle. For example, the first two data points in Figure 17–1 are at 3×10^5 cells (10 hours) and 6×10^5 cells (30 hours). Since the population of mouse L cells doubled in 20 hours (30 hours – 10 hours), the length of the cell cycle is 20 hours.

DATA HANDLING

17–13

A. The execution point for your temperature-sensitive mutant is marked on **Figure 17–32**. Cells that had not reached the execution point in the cell

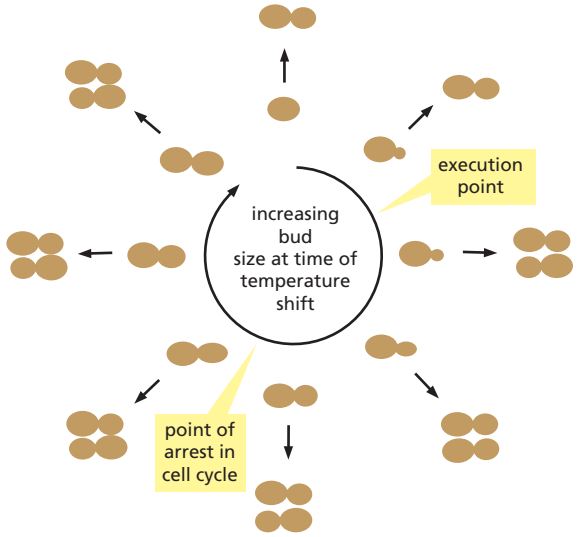


Figure 17–32 The execution point and the point of arrest for the gene product affected in your mutant (Answer 17–13).

cycle when the temperature was raised grew to the characteristic landmark morphology (large buds) but did not divide. Cells that were beyond the execution point when the temperature was raised divided and then stopped at the landmark morphology during the next cell cycle.

B. The characteristic landmark morphology defines the time at which the cell stops its progress through the cell cycle, as indicated for your mutant in Figure 17–32. The landmark morphology is clearly different from the morphology at the execution point. Therefore, the execution point and the point of cell-cycle arrest do not correspond in your mutant.

At first glance, it may seem odd that the execution point and the point of arrest do not coincide. An analogy may make the situation clearer. The addition of engine mounts to the chassis is an early step in the assembly of an automobile. Without engine mounts the engine cannot be added and a complete car cannot be built. In the absence of engine mounts, assembly of other parts of the automobile can continue until a point is reached at which all further assembly depends on the engine mounts. In this case, the normal execution point for engine-mount addition is early, but the arrest point for assembly is relatively late, with a characteristic landmark morphology, which resembles a complete automobile (until one looks under the hood).

Reference: Hartwell LH (1978) Cell division from a genetic perspective. *J. Cell Biol.* 77, 627–637.

17–14

A. Only the cells that were in the S phase of their cell cycle—those that were making DNA—during the 30-minute labeling period would contain any radioactive DNA.

B. The first mitotic cells to appear contained no radioactive DNA because they were not engaged in DNA synthesis during the labeling period; they were in G_2. It takes about 3 hours before the first labeled mitotic cells appear because it takes that long for cells to progress from the tail end of S phase to mitosis.

C. The initial rise of the curve corresponds to cells that were just finishing DNA replication when the radioactive thymidine was added. The curve rises to a peak that corresponds to those times when all of the mitotic cells were in S phase during the time of labeling. The curve then falls when the labeled cells exit mitosis, being replaced by unlabeled mitotic cells that were not yet in S phase during the labeling period. After 20 hours the curve starts rising again, because the labeled cells enter their second round of mitosis.

D. The ascending curve passes through 50% labeled mitoses at 3 hours, which corresponds to the length of the G_2 phase. The initial 3-hour lag before labeled mitotic cells appear corresponds to the time between the end of the S phase and the beginning of mitosis (**Figure 17–33**). The first labeled cells seen in mitosis were those that were just finishing S phase when the radioactive thymidine was added. The length of S phase can be estimated from the width of the first peak at 50% labeled mitoses, which is about 10.5 hours in this experiment (Figure 17–33). The overall length of the cell cycle is the time between the 50% points on the two ascending curves, which is about 27 hours (Figure 17–33). The total cell cycle minus G_2, S, and M is equal to G_1. Thus, G_1 is 13 hours long [27 – (3 + 0.5 + 10.5)].

Reference: Baserga R & Wiebel F (1969) The cell cycle of mammalian cells. *Int. Rev. Exp. Pathol.* 7, 1–30.

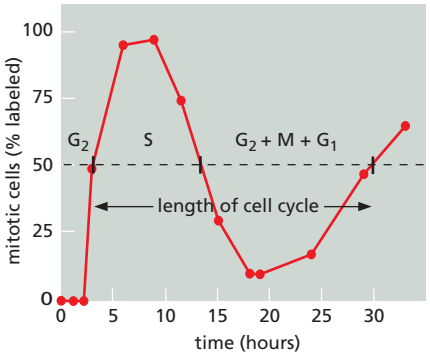


Figure 17–33 Lengths of phases of the cell cycle deduced from pulse labeling (Answer 17–14).

17–15

A. The relationships between cell fluorescence and position in the cell cycle are indicated in **Figure 17–34A**. Because Hoechst 33342 binds to DNA, cellular fluorescence is proportional to DNA content. The peak with the lowest fluorescence corresponds to cells in G_1, which are diploid. The peak with the highest fluorescence corresponds to cells in G_2 and M, which have finished replication and are tetraploid (and thus have twice the fluorescence of G_1 cells). Cells in S phase, which are replicating their DNA, are between diploid and tetraploid and thus have intermediate levels of fluorescence.

B. The distributions of fluorescence for cells treated with agents that block the cell cycle in G_1, S, and M phases are shown in **Figure 17–34B, C, and D**. Cells blocked in either G_1 or in M form sharp distributions because all the cells have the same amount of DNA (diploid for G_1 and tetraploid for M). Cells treated with an inhibitor that blocks in S phase give a biphasic distribution. Cells that were in S phase at the time the inhibitor was added give a broad distribution because the cells are distributed through all stages of replication. Cells that were in other phases of the cell cycle, however, pile up at the beginning of S phase, giving a sharp peak with a DNA content very close to that of G_1 cells.

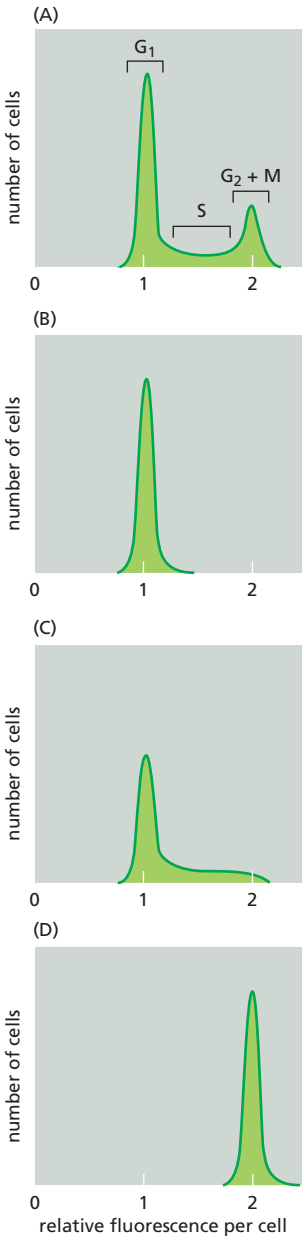


Figure 17–34 Relationships between fluorescence and the cell cycle (Answer 17–15). (A) Distribution of fluorescent cells among phases of the cell cycle for a normal population of dividing cells. (B) A cell population blocked in G_1. (C) A cell population blocked in S. (D) A cell population blocked in M.

THE CELL-CYCLE CONTROL SYSTEM

DEFINITIONS

17–16 Cyclin-dependent kinase (Cdk)

17–17 Anaphase-promoting complex or cyclosome (APC/C)

17–18 M-Cdk

17–19 Cyclin

17–20 Metaphase-to-anaphase transition

17–21 Cell-cycle control system

17–22 Cyclin–Cdk complex

TRUE/FALSE

17–23 False. Although cyclin–Cdk complexes are indeed regulated by phosphorylation and dephosphorylation, they can also be regulated by the binding of Cdk inhibitor (CKI) proteins. Moreover, the rates of synthesis and proteolysis of the cyclin subunits are extremely important for regulating Cdk activity.

17–24 True. If the length of the cell cycle were shorter than it takes for the cell to double in size, the cell would get progressively smaller with each division; if it were longer, the cells would get bigger and bigger.

THOUGHT PROBLEMS

17–25 At different transitions in the cell cycle in budding yeast, the single Cdk (Cdk1) binds to different cyclins. These cyclins not only activate Cdk1, but also influence its target specificity. As a result, each cyclin–Cdk1 complex phosphorylates a different set of substrate proteins. Even though vertebrates use multiple Cdks, they use a variety of cyclins to target the cyclin–Cdk complexes to different substrates at different stages of the cell cycle.

DATA HANDLING

17–26

A. Since each transfer accomplishes a 20-fold dilution (50 nL/1000 nL), 10 transfers yield a dilution factor of 20^{10}, which is equal to 10^{13}. It is unreasonable for a molecule to have an undiminished biological effect over this range of dilution.

B. The appearance of MPF activity in the absence of protein synthesis suggests that an inactive precursor of MPF is being activated. In principle, activation could involve one of several kinds of post-translational modifications such as protease cleavage or changes in protein phosphorylation. It is now known that MPF is held in an inactive state by phosphorylation of two key residues on its Cdc2 subunit. MPF is activated when those inhibitory phosphates are removed by the protein phosphatase, Cdc25.

C. In order for MPF to propagate its activated state through serial transfers, it must be able to activate itself. If it were a protease, for example, active MPF might activate its inactive precursor by cleavage, in the same way that trypsin-mediated cleavage of trypsinogen produces more trypsin. Since MPF is a protein kinase, it is reasonable to suggest that it operates via phosphorylation to trigger the activation of the inactive pre-MPF. In principle, MPF could activate pre-MPF directly by adding one or more phosphates. Or it could act indirectly through other kinases or phosphatases to alter the phosphorylation status of pre-MPF and thereby activate it. As it turns out, MPF triggers the addition of activating phosphates to Cdc25, which then removes the inhibitory phosphates from pre-MPF, activating it. This sets up an auto-amplification cycle that rapidly activates all the MPF, thereby stimulating egg maturation in each serial transfer.

   Progesterone induces egg maturation by setting up the same auto-amplification cycle, but initiates it in a different way. Progesterone stimulates translation of the mRNA for Mos, which is the frog's mitogen-activated protein (MAP) kinase kinase kinase. Expression of Mos activates the MAP kinase cascade, which inhibits the Wee1-like protein kinase that is responsible for adding inhibitory phosphates to pre-MPF. Inhibition of this kinase allows a small amount of pre-MPF to become active, thereby triggering auto-amplification of MPF activity and subsequent egg maturation.

References: Karaïskou A, Jessus C, Brassac T & Ozon R (1999) Phosphatase

2A and Polo kinase, two antagonistic regulators of Cdc25 activation and MPF auto-amplification. *J. Cell Sci.* 112, 3747–3756.

Wasserman WJ & Masui Y (1975) Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. *Exp. Cell Res.* 91, 381–388.

17–27 In the normal situation, Wee1 adds phosphates to Cdk1 to inactivate it, and Cdc25 removes those phosphates, activating Cdk1. In principle, different types of mutation in any one of these genes might produce the *giant* and *tiny* temperature-sensitive strains of yeast. For example, a mutation in the *Cdk1* gene that led to an inactive protein at high temperature would generate a yeast strain like *giant* that was unable to proceed through the cell cycle. A mutation in the same gene that prevented Cdk1 from being inactivated by Wee1 at the restrictive temperature would lead to a constitutively active Cdk1 that would continually push the cell through the cell cycle, as in the *tiny* strain.

You could imagine analogous mutations in the *Wee1* and *Cdc25* genes. An inactive form of Wee1 (or a hyperactive form of Cdc25) would leave Cdk1 permanently active, generating cells with a short cell cycle, like the *tiny* strain. A hyperactive version of Wee1 (or an inactive form of Cdc25) that generated an inactive Cdk1 would produce cells that were unable to progress through the cell cycle, as in the *giant* strain.

In various screens, inactivating mutations in *Wee1* and *Cdc25* have been found, and they have the expected phenotypes, but mutations that lead to hyperactive forms of these proteins have not been isolated. The mutations described in this problem correspond to two alleles of *Cdc2* (the original name for the *Cdk1* gene) discovered by Paul Nurse in 1974. Nurse had been very excited by his discovery of the *Wee1* gene, which, when mutated, gave rise to small yeast cells that entered mitosis at a smaller-than-usual size compared to wild-type cells. To see if any other genes could be identified with similar properties, he set up a genetic screen based on looking at the mutants in the microscope, hunting for small cells. After identifying 49 new temperature-sensitive, small-cell strains, all of which proved to be allelic to the original *Wee1* gene, he was on the point of giving up when the 50th mutant strain turned out not to map in the *Wee1* locus. Instead, it corresponded to a different, but already known gene, *Cdc2*. Existing mutations in Cdc2 interfered with cell-cycle progression, giving rise to large cells under restrictive conditions. Because this gene could be mutated to inactivity or hyperactivity, Nurse concluded that it must encode a really important cell cycle regulator. Many genes can be mutated so that they block cell-cycle progression, but this was the only one that could apparently be made more active. Time proved this conclusion to be absolutely correct.

References: Nurse P (1975) Genetic control of cell size at cell division in yeast. *Nature* 256, 547–551.

Nurse P & Thuriaux P (1980) Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 96, 627–637.

S PHASE

DEFINITIONS

17–28 Cohesin

17–29 Origin recognition complex (ORC)

17–30 Geminin

TRUE/FALSE

17–31 True. Origins of replication are licensed for replication by the binding of a large complex of initiator proteins, called the prereplicative complex.

17–32 True. The origin recognition complex serves as a scaffold at origins of replication in eukaryotic cells around which other proteins are assembled and activated to initiate DNA replication.

THOUGHT PROBLEMS

17–33 S-Cdk could initiate the firing of replication origins—directly or indirectly—by activating origin-binding proteins via phosphorylation events. Such binding proteins might bind to prereplicative complexes at different times in S phase as a result of their accessibility or surrounding DNA sequences, thereby accounting for the characteristic firing times for different origins. Alternatively, S-Cdk could modify all origins at the same time, preparing them for the subsequent binding of a key initiation factor, the timing of which determines when origins fire.

Preventing re-replication is not in conflict with the role of S-Cdk in promoting origin firing. When DNA replication is initiated, S-Cdk phosphorylates ORC and Cdc6, inhibiting them and preventing reassembly of the pre-RC until after the next mitosis.

Reference: Bell SP & Dutta A (2002) DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* 71, 333–374.

17–34 Cohesins must be present during S phase because it is only while DNA is being replicated that sister chromatids can be reliably identified by the cellular machinery that links them together. Once sister chromatids have separated, it is impossible for a nonspecific DNA-binding protein like cohesin to tell which chromosomes are sisters. And it would be virtually impossible for any protein to distinguish sister chromatids from homologous chromosomes. If sister chromatids are not kept together after their formation, they cannot be accurately segregated to the two daughter cells during mitosis.

Reference: Uhlmann F & Nasmyth K (1998) Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* 8, 1095–1101.

DATA HANDLING

17–35 When a G_1 cell is fused with an S-phase cell, DNA replication occurs in the G_1 nucleus, presumably triggered by S-Cdk activity in the S-phase cell. Fusion of a G_2 cell with an S-phase cell, however, does not cause DNA replication in the G_2 nucleus, indicating that G_2 nuclei are refractory to the effects of S-Cdk. Thus, cells that have completed S phase cannot replicate their DNA again, consistent with a block to re-replication. Fusion of a G_2 cell with a G_1 cell does not drive the G_1 nucleus into S phase, indicating that S-Cdk in G_2 cells is no longer capable of triggering entry of G_1 nuclei into S phase.

Reference: Johnson RT & Rao PN (1971) Nucleo-cytoplasmic interactions in the achievement of nuclear synchrony in DNA synthesis and mitosis in multinucleate cells. *Biol. Rev. Camb. Philos. Soc.* 46, 97–155.

17–36

A. Sister chromatids behave as expected for wild-type cells: they are stuck together in small-budded cells and separated into the mother and daughter cells at mitosis. The single spot of fluorescence in the small-budded

cell in Figure 17–7 likely results from two sites of binding that are close together on the paired sister chromatids. The spot is brighter than the individual spots in cells that have two separated spots, and the size of the bud indicates that the site should have been replicated (by comparison with the size of the small-budded *Scc1*^{ts} cell with two spots).

B. In one of the small-budded cells from the *Scc1*^{ts} strain, it is clear from the presence of two spots of fluorescence that sister chromatids have already separated. Although the sister chromatids are separated in the large-budded cells, they are abnormal because they have both remained in the mother cell.

C. Prematurely separated sister chromatids prevent the formation of a normal spindle apparatus, and thus prevent normal segregation of chromosomes into the mother and daughter cells. It is likely that they trigger the spindle assembly checkpoint; that is, that unpaired sister chromatids behave as if they are unattached.

Reference: Michaelis C, Ciosk R & Nasmyth K (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91, 35–45.

MITOSIS

DEFINITIONS

17–37 Microtubule flux

17–38 Anaphase B

17–39 Spindle assembly checkpoint

17–40 Centrosome

17–41 Metaphase plate

17–42 Interpolar microtubules

17–43 Telophase

17–44 Condensin

17–45 Separase

17–46 Astral microtubule

TRUE/FALSE

17–47 True. Microtubules nucleated by the centrosomes grow outward toward the chromosomes in a highly dynamic process, alternately growing and shrinking. When they eventually attach to the kinetochore of a chromosome, they become stabilized and are referred to as kinetochore microtubules.

17–48 False. Equal and opposite forces that tug the chromosomes toward the two spindle poles would tend to position them at random locations between the poles. The poleward force on each chromosome is opposed by a polar ejection force that pushes the chromosome away from the pole. The ejection force is mediated by plus-end directed kinesin motors on chromosome arms that interact with interpolar microtubules and transport the chromosomes away from the spindle poles. This balance of forces tends to position the chromosomes at the midpoint between the poles—the metaphase plate.

17–49 False. Kinetochore microtubules polymerize at their plus ends up to anaphase, at which point they begin to depolymerize. Prior to anaphase, kinetochore microtubules maintain a fairly constant length by treadmilling, with addition to the plus ends being balanced by removal at the minus ends. At anaphase, coincident with sister-chromatid separation, kinetochore microtubules begin to depolymerize at their plus ends as well, and therefore become shorter, moving the chromosomes toward the spindle poles.

17–50 False. The five stages of mitosis occur in strict sequential order, but cytokinesis begins during anaphase and continues through the end of M phase.

THOUGHT PROBLEMS

17–51 Prophase (see Figure 17–8E), prometaphase (see Figure 17–8D), metaphase (see Figure 17–8C), anaphase (see Figure 17–8A), telophase (see Figure 17–8F), and cytokinesis (see Figure 17–8B).

17–52 The sharp activation of M-Cdk at the end of G_2 is due to the events shown in **Figure 17–35**. M-Cyclin accumulates gradually by steady synthesis. As it accumulates, it will bind to Cdk1 molecules to form M-Cdk. M-Cdk is phosphorylated at two sites: an activating site by CAK and an inhibitory site by Wee1 kinase (Figure 17–35). The inhibitory phosphate keeps the M-Cdk largely inactive, but it is slowly removed by the phosphatase Cdc25. After a certain threshold level has been reached, active M-Cdk initiates two positive feedback loops that trigger its explosive activation. In one loop, active M-Cdk stimulates Cdc25 phosphatase, causing removal of the inhibitory phosphate from inactive molecules of M-Cdk. In the second loop, active M-Cdk inhibits phosphorylation by Wee1, thereby preventing further addition of the inhibitory phosphate.

17–53 Astral, kinetochore, and interpolar microtubules all radiate from the spindle poles, with their plus ends directed outward. Astral microtubules, which radiate in all directions, act as "handles" for orienting and positioning the spindle in the cell. In addition, those in contact with the cell cortex aid in separation of the spindle poles during anaphase. Kinetochore microtubules link chromosomes to the spindle and are responsible for movement of the sister chromatids to the poles. Interpolar microtubules interdigitate at the equator of the spindle and are responsible for the symmetrical, bipolar shape of the spindle. Their movement relative to one another pushes the poles apart at anaphase.

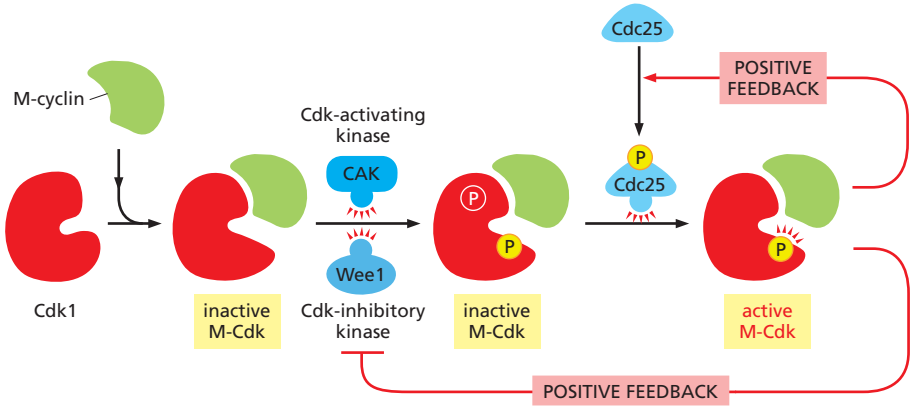


Figure 17–35 The activation of M-Cdk (Answer 17–52). Inhibitory phosphate is shown in *red*; activating phosphates are shown in *yellow*.

17–54 When the two motor domains of a kinesin-5 molecule attach to different microtubules, they will each move toward the plus end. When the kinesin-5 motors reach the plus ends, they can move no farther. At that point, they will link the plus ends tightly together. Multiple copies of kinesin-5 would tie multiple microtubules together by their plus ends, leaving the minus ends free. Thus, the astral array generated by the plus-end directed kinesin-5 motor proteins will have the plus ends of the microtubules at the center and the minus ends at the periphery.

Reference: Hyman AA & Karsenti E (1996) Morphogenetic properties of microtubules and mitotic spindle assembly. *Cell* 84, 401–410.

17–55 Centrosome duplication is semiconservative. The pair of centrioles in the centrosome separates, and each serves to nucleate synthesis of a new centriole. As a consequence, each new centrosome consists of one old and one new centriole. Thus, centrosome duplication is analogous to DNA replication: a new duplex consists of one old strand and one newly replicated strand.

Reference: Stearns T (2001) Centrosome duplication: a centriolar pas de deux. *Cell* 105, 417–420.

17–56 There are 46 human chromosomes, each with two kinetochores—one for each sister chromatid—thus, there are 92 kinetochores in a human cell at mitosis.

17–57 Both sister chromatids could end up in the same daughter cell for any of a number of reasons. If the microtubules or their connections with a kinetochore were to break during anaphase, both sister chromatids could be drawn to the same pole, hence the same daughter cell. If microtubules from the same spindle pole attached to both kinetochores, the chromosome would be pulled to the same pole. If the cohesins that link sister chromatids were not degraded, the pair of chromatids might be pulled to the same pole. If a chromosome never engaged microtubules, and was left out of the spindle, it would also end up in one daughter cell. Some of these errors in the mitotic process would be expected to engage a checkpoint mechanism—for example, the spindle assembly checkpoint—and allow most such errors to be corrected, which is one reason why such errors are so rare.

As a consequence of this error, one daughter cell would contain only a single copy of all the genes carried on that chromosome, and the other daughter cell would contain three copies. The altered gene dosage, leading to correspondingly changed amounts of the mRNAs and proteins produced, is often detrimental to the cell. In addition, there is the possibility that the cell with a single copy of the chromosome may be defective for a critical gene, a defect that was hidden by the presence of a second, good copy of the gene on the other chromosome.

17–58 The kinetochore microtubules and the interpolar microtubules treadmill by constantly adding tubulin subunits to their plus ends and removing an equivalent number from their minus ends. This balanced addition and removal results in a movement of subunits toward the poles (the minus ends of the microtubules), but leaves the overall appearance of the spindle unchanged.

17–59 Nocodazole arrests cells in M phase of the cell cycle. By preventing microtubule polymerization—hence spindle formation—nocodazole triggers the spindle assembly checkpoint, which inhibits the APC/C ubiquitin ligase so that the metaphase-to-anaphase transition cannot occur.

17–60 In the absence of benomyl, the majority of spindles form normally and the spindle assembly checkpoint plays no role. As a consequence, Mad2

is irrelevant. In the presence of benomyl, however, cells that are defective for Mad2 cannot stop cell-cycle progression, with the result that chromosomes are segregated incorrectly, causing the cells to die.

Reference: Li R & Murray AW (1991) Feedback control of mitosis in budding yeast. *Cell* 66, 519–531.

17–61 This experiment shows that for microtubules to remain attached to kinetochores, tension has to be exerted. Tension is normally achieved by the opposing pulling forces from the two spindle poles. The requirement for such tension ensures that if two sister kinetochores ever become attached to the same spindle pole, so that tension is not generated, one or both of the connections will break, and microtubules from the opposing spindle pole will have another chance to attach properly.

17–62 At a gross level this analogy appears valid. Chromosomes move to the spindle poles tethered on a microtubule line, much as fish move to a fishing pole on a fishing line. But in detail the analogy fails. A fishing line is shortened at the end opposite the fish, whereas a microtubule is shortened by disassembly at the end attached to the chromosome.

17–63 The events occur in the following order: duplication of the centrosome (F), separation of centrosomes (J), condensation of chromosomes (D), breakdown of nuclear envelope (C), attachment of microtubules to chromosomes (B), alignment of chromosomes at the spindle equator (A), separation of sister chromatids (K), elongation of the spindle (G), reformation of nuclear envelope (I), decondensation of chromosomes (E), and pinching of cell in two (H).

CALCULATIONS

17–64 The dose of caffeine required to interfere with the DNA replication checkpoint mechanism is much higher than the amount imbibed by even the most excessive drinkers of coffee and colas. The concentration of caffeine in a cup of coffee is about 3.4 mM.

$$[\text{caffeine}] = \frac{100\ \text{mg}}{150\ \text{mL}} \times \frac{\text{g}}{1000\ \text{mg}} \times \frac{\text{mole}}{196\ \text{g}} \times \frac{1000\ \text{mL}}{\text{L}}$$

$$= 3.4 \times 10^{-3}\ \text{M} = 3.4\ \text{mM}$$

Since the concentration in a cup is less than the 10 mM required to interfere with the DNA replication checkpoint mechanism, you cannot get a higher concentration by drinking it and diluting it in the water volume of the body. If you assume for the purposes of calculation that the caffeine is not metabolized or excreted (but that all the liquid is), then you can ask how many cups of coffee would you need to drink (at 100 mg of caffeine per cup) to reach a concentration of 10 mM in 40 L of body water. The answer is: you would need to drink 784 cups of coffee!

17–65 The average length of chromosomes in base pairs and millimeters, and the average number of base pairs carried per microtubule, are given in **Table 17–5**. The length of DNA carried by each microtubule varies about 50-fold from *S. cerevisiae* to *Haemanthus*. Although this represents a fair amount of variability, it is much more constant than the average length of chromosomal DNA, which spans three to four orders of magnitude over the same range of organisms.

Reference: Bloom K (1993) The centromere frontier: kinetochore components, microtubule-based motility, and the CEN-value paradox. *Cell* 73, 621–624.

TABLE 17–5 Average lengths of chromosomes and average numbers of base pairs per microtubule in a variety of organisms (Answer 17–65).

Type of organism	Species	Average length of chromosome (bp)	Average length of chromosome (mm)	Average DNA (bp)/ microtubule
Yeast	*S. cerevisiae*	0.9×10^6	0.3	0.9×10^6
Yeast	*S. pombe*	4.7×10^6	1.6	1.6×10^6
Protozoan	*Chlamydomonas*	5.8×10^6	2.0	5.8×10^6
Fly	*Drosophila*	4.3×10^7	15	4.3×10^6
Human	*Homo sapiens*	1.4×10^8	47	5.6×10^6
Plant	*Haemanthus*	6.1×10^9	2100	5.1×10^7

DATA HANDLING

17–66

A. The state of phosphorylation of Wee1 and Cdc25 is the result of the balance between the protein kinase and protein phosphatase activities that regulate them. By inhibiting the protein phosphatases, okadaic acid causes Wee1 and Cdc25 to accumulate in their phosphorylated forms (**Figure 17–36**). Since this change activates M-Cdk, Wee1 and Cdc25 must have originally been present in the extract in their nonphosphorylated forms. Thus, active Wee1 kinase is nonphosphorylated, as is inactive Cdc25 phosphatase (Figure 17–36). Knowing which forms are phosphorylated allows you to label the arrows that correspond to the kinases and phosphatases that control Wee1 and Cdc25 phosphorylation (Figure 17–36).

B. The protein kinases and phosphatases that control phosphorylation of Wee1 and Cdc25 must be specific for serine/threonine side chains because they are affected by okadaic acid, which inhibits only serine/threonine phosphatases.

C. Okadaic acid has no direct effect on Cdk1 phosphorylation because Cdk1 is phosphorylated on a tyrosine side chain. Tyrosine phosphatases are unaffected by okadaic acid. The decrease in Cdk1 phosphorylation is a consequence of the change in activities of Wee1 kinase, which becomes less active, and of Cdc25 phosphatase, which becomes more active.

D. As soon as some active M-Cdk appears, it would begin to phosphorylate Wee1 and Cdc25, inactivating the kinase and activating the phosphatase. The resultant decrease in Wee1 kinase activity and increase in Cdc25 phosphatase activity would lead to dephosphorylation (and activation) of more M-Cdk. This in turn would further decrease the activity of Wee1 kinase and further increase the activity of Cdc25 phosphatase, leading to still more M-Cdk activity. Thus, the initial appearance of a little M-Cdk activity would rapidly lead to its complete activation.

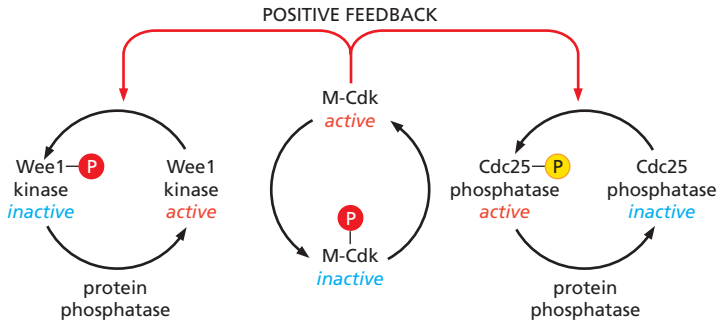


Figure 17–36 Control of M-Cdk activity by Wee1 kinase and Cdc25 phosphatase (Answer 17–66). Inhibitory phosphates are shown in *red*; activating phosphate is shown in *yellow*.

This sort of activation is referred to as a positive feedback loop (Figure 17–36). It is a common means of regulation when it is advantageous for a system to flip rapidly from one state to another without lingering in the intermediate states.

Reference: Kumagai A & Dunphy WG (1992) Regulation of the cdc25 protein during the cell cycle in *Xenopus* extracts. *Cell* 70, 139–151.

17–67

A. One fundamental difference in the proposed roles for cohesin and condensin is the number of duplexes that are involved. Cohesin is proposed to link sister chromatids together; that is, to link two duplexes. By contrast, condensin is proposed to operate on a single duplex, causing it to be coiled, and thus condensed. These experiments are in perfect accord with this fundamental difference. Incubation with cohesin generates catenanes, which must be produced by topoisomerase II action on juxtaposed, separate duplexes. Incubation with condensin generates knots, which arise by topoisomerase II action on juxtaposed parts of a single duplex.

B. **Figure 17–37A** shows a plausible mechanism by which cohesins might bring two duplexes close enough together to allow topoisomerase II to link them.

C. **Figure 17–37B** shows one way that condensins could organize a single circle of DNA so that topoisomerase could tie it into a knot with a single duplex-crossing event. Remarkably, the particular type of trefoil knot that is generated (a mirror image could have been produced in principle) places severe constraints on the type of coiling that condensin introduces. If you got this part of the problem correct, pat yourself on the back!

References: Kimura K, Rybenkov VV, Crisona NJ, Hirano T & Cozzarelli NR (1999) 13S condensin actively reconfigures DNA by introducing global positive writhe: implications for chromosome condensation. *Cell* 98, 239–248.

Losada A & Hirano T (2001) Intermolecular DNA interactions stimulated by the cohesin complex *in vitro*: implications for sister chromatid cohesion. *Curr. Biol.* 11, 268–272.

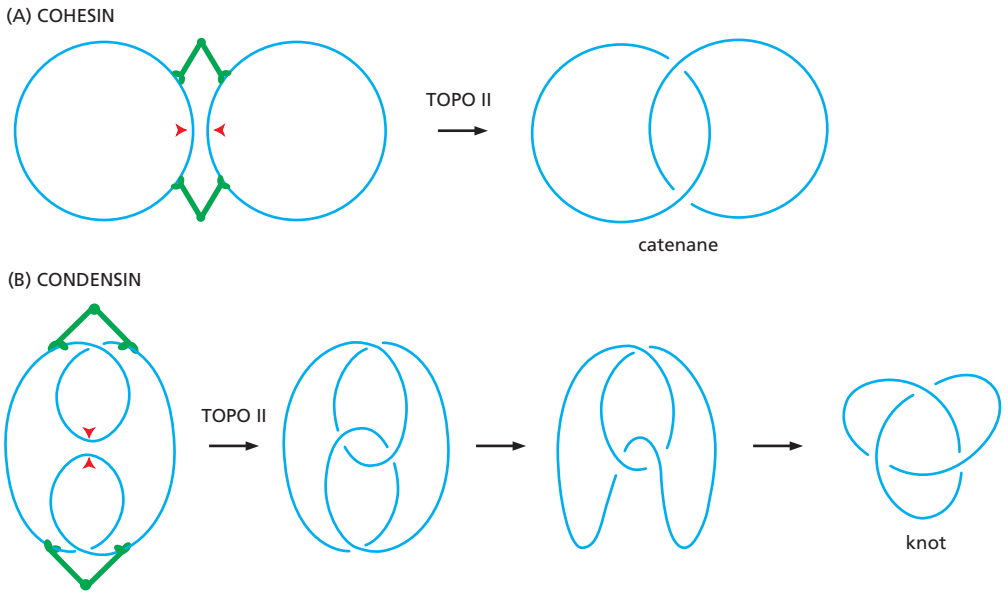


Figure 17–37 Cohesin- and condensin-mediated topological changes (Answer 17–67). (A) Cohesin-mediated catenane formation. *Arrowheads* indicate sites at which topoisomerase II (TOPO II) could catalyze duplex crossing to generate the indicated molecule. (B) Condensin-mediated knot formation. The relationship between the initial product, formed by duplex crossing at the *arrowheads*, and the trefoil knot is illustrated in discrete steps to try to make the topology clearer.

17–68

A. A kinetochore microtubule is relatively stable because both of its ends are protected from disassembly: one by attachment to the centrosome, and the other by attachment to the kinetochore. This stabilization suggests that kinetochores cap the plus ends of the microtubules, thereby altering the equilibrium for subunit dissociation.

B. Astral microtubules disassemble when tubulin is below the critical concentration for microtubule assembly. Under these conditions, the rate of addition does not balance the rate of dissociation, and microtubules get progressively shorter. The presence of kinetochore microtubules strengthens this interpretation relative to the other two possibilities mentioned. Neither detachment from the centrosome nor random breakage would explain the stability of kinetochore microtubules.

C. The possible mechanisms of disappearance would be readily distinguished by a time course. At intermediate time points, the number and length of microtubules will be sensitive indicators of which mechanism operates. If microtubules detached, the number of microtubules per centrosome would decrease, but the length would remain the same. If microtubules depolymerized from the end, the number would remain constant and their lengths would decrease relatively uniformly. If the microtubules broke at random, the number would remain relatively constant, but the distribution of lengths would be very broad.

Reference: Mitchison TJ & Kirschner MW (1985) Properties of the kinetochore *in vitro*. II. Microtubule capture and ATP-dependent translocation. *J. Cell Biol.* 101, 766–777.

17–69

A. Dicentric plasmids are stable in bacteria because bacteria use a completely different mechanism to segregate their chromosomes. A bacterial chromosome is polarized so that its single origin of replication (*OriC*) is located at one pole of the bacterium. As soon as *OriC* sequences are replicated, one copy is translocated to the opposite pole, ensuring that the daughter chromosomes lie on either side of the plane of fission that separates the bacterium into daughter cells. This mechanism of cell division makes bacteria indifferent to the presence of centromeres on the plasmid DNA.

B. Dicentric plasmids are unstable in yeasts for the same reason that dicentric chromosomes are unstable in higher eukaryotes. If the two centromeres attach to opposite poles, the spindle apparatus can exert enough force on the DNA molecule to break its phosphodiester backbone. Roughly half the time a plasmid would be expected to orient itself on the spindle so that its two centromeres are attached to opposite poles. Thus, there is a very high probability that a plasmid will be broken at each cell division, hence the instability.

C. Since monocentric plasmids are very stable, it seems most likely that the mechanism for deletion of centromeric sequences from dicentric plasmids relates to the breakage they suffer during mitosis. As illustrated in **Figure 17–38**, a circular plasmid must suffer two breaks to permit the centromeres to separate during mitosis. This breakage naturally separates the centromeres from one another onto linear fragments of the original plasmid. If the ends of a fragment join to make a circle, the resulting plasmid will contain a single centromeric sequence (Figure 17–38). Only those fragments that contain the yeast origin of replication (*Ars1*) and the selected marker (*Trp1*) can continue to grow in future generations in yeast.

This mechanism does not readily account for the loss of both centromeres; rather, it predicts that one centromere will be retained. Once the dicentric plasmid is reduced to a monocentric plasmid, it should be stable. The loss of both centromeres probably involves a process other

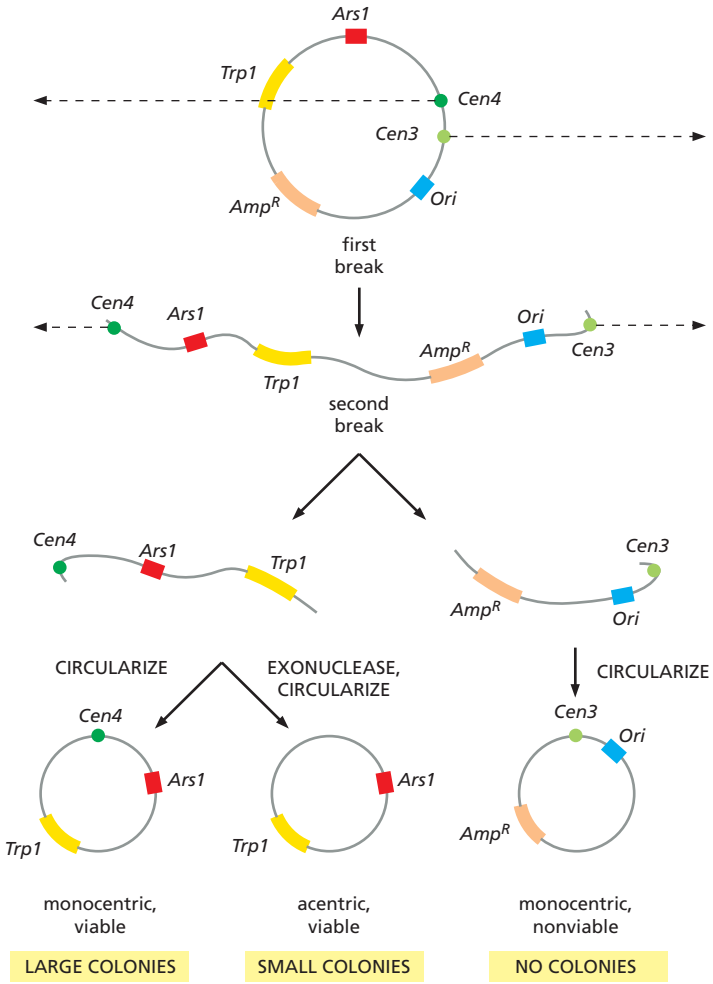


Figure 17–38 A mechanism for generating monocentric and acentric plasmids from a dicentric plasmid in yeast (Answer 17–69). *Dashed arrows* indicate the direction of pull toward the spindle poles. Viability refers to the ability of the plasmid to grow in yeast under selective conditions (which requires *Ars1* and *Trp1*).

than simple breakage. One likely possibility is that the broken ends are digested by exonucleases, which occasionally remove the remaining centromeric sequence before the fragment circularizes (Figure 17–38).

Reference: Mann C & Davis RW (1983) Instability of dicentric plasmids in yeast. *Proc. Natl Acad. Sci. USA* 80, 228–232.

17–70

A. Xkid helps align chromosomes on the metaphase plate by moving the chromosome arms toward the plus ends of the interpolar microtubules. Outside the central region of the spindle, where the interpolar microtubules overlap, Xkid will propel a chromosome unidirectionally toward the center of the spindle. Inside the region of overlap, Xkid motors will move a chromosome back and forth between the plus ends of the overlapping interpolar microtubules, confining the chromosome to the spindle equator.

B. Plus-end directed, microtubule-dependent motors that bind chromosome arms, as Xkid does, are plausible candidates for mediators of the polar ejection force, which pushes chromosomes away from the poles toward the spindle equator.

C. The separation of chromosomes is blocked in the presence of stable Xkid. The continued function of Xkid, which constantly pushes the chromosomes away from the poles toward the equator, is sufficient to oppose the normal force exerted by the shortening of the kinetochore

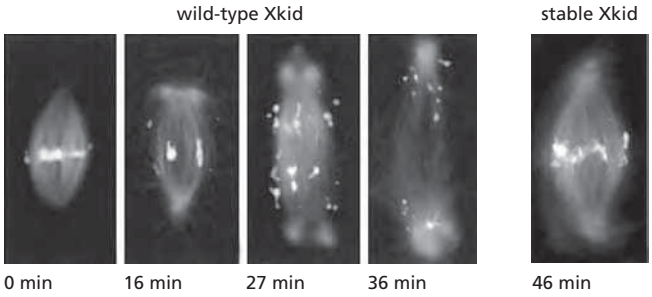


Figure 17–39 Anaphase in the presence of wild-type and stable Xkid (Answer 17–70). Metaphase spindles were assembled and then anaphase was initiated by addition of Ca^{2+} at time zero. *Faint fluorescence* marks the position of microtubules and *bright spots* mark the position of the chromosomes.

microtubules, as illustrated in **Figure 17–39**. Note that not all aspects of anaphase appear to be blocked by stable Xkid. The spindle, for example, has increased in size, suggesting that the poles have moved apart.

References: Antonio C, Ferby I, Wilhelm H, Jones MJ, Karsenti E, Nebreda AR & Vernos I (2000) Xkid, a chromokinesin required for chromosome alignment on the metaphase plate. *Cell* 102, 425–435.

Funabiki H & Murray AW (2000) The *Xenopus* chromokinesin Xkid is essential for metaphase chromosome alignment and must be degraded to allow anaphase chromosome movement. *Cell* 102, 411–424.

17–71 Spindles with astral microtubules will tend to repel one another, keeping themselves out of each other's way. When spindles with asters come close to one another, their astral microtubules will overlap, allowing them to be pushed apart by plus-end directed motors such as kinesin-5. As a consequence, the nuclei that are generated are well distributed in the common cytoplasm. By contrast, spindles without astral microtubules will not be able to repel one another and their nuclei will be clustered.

Reference: de Saint Phalle B & Sullivan W (1998) Spindle assembly and mitosis without centrosomes in parthenogenetic *Sciara* embryos. *J. Cell Biol.* 141, 1383–1391.

17–72

A. M-Cdk remains active in the presence of cyclin BΔ90 because cyclin BΔ90 is missing the destruction box and cannot be destroyed by APC/C. This so-called indestructible cyclin remains bound to Cdk1 and keeps it active.

B. M-Cdk remains active in the presence of cyclin B13-110 for a different reason. Excess cyclin B13-110 overwhelms APC/C, which would otherwise degrade the normal cyclin B that is complexed with Cdk1 in M-Cdk. By keeping APC/C occupied, cyclin B13-110 prevents degradation of normal cyclin B and keeps M-Cdk activity high.

C. The mutant forms of cyclin B, unlike normal cyclin B, both maintain a high level of M-Cdk activity in the presence of Ca^{2+}. Cyclin BΔ90, however, allows normal separation of sister chromatids. Therefore, sister-chromatid separation cannot depend directly on loss of M-Cdk activity, which suggests that dephosphorylation of a linker protein is not the critical event for sister-chromatid separation.

Addition of cyclin B13-110 prevents sister-chromatid separation. Since cyclin B13-110 blocks APC/C, it seems likely that the linker protein that holds sister chromatids together may itself be a target of APC/C.

Reference: Holloway SL, Glotzer M, King RW & Murray AW (1993) Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* 73, 1393–1402.

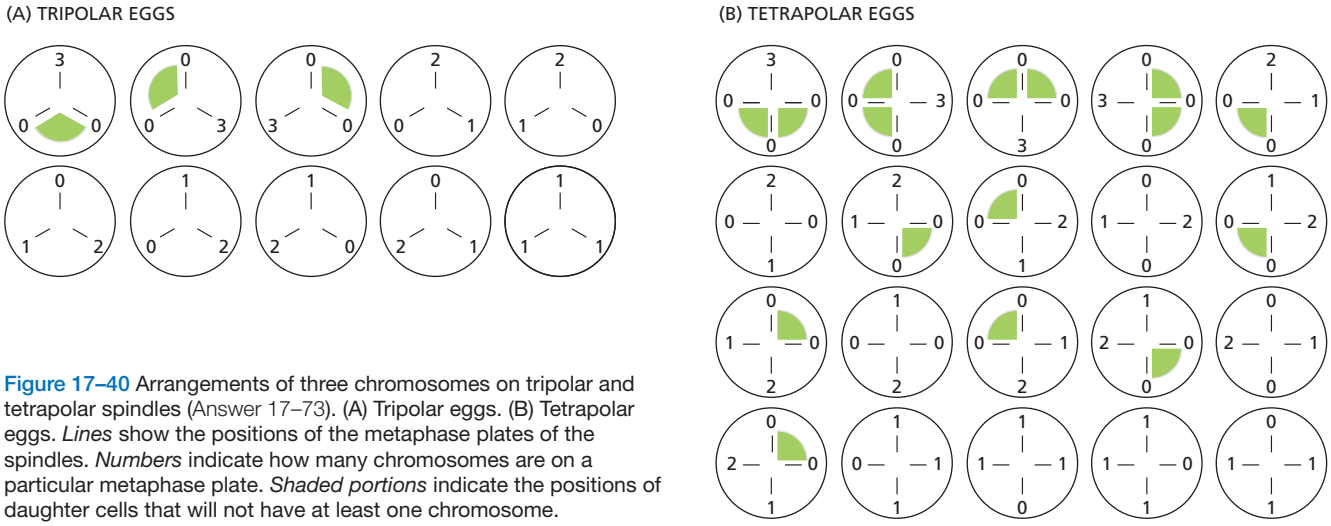


Figure 17–40 Arrangements of three chromosomes on tripolar and tetrapolar spindles (Answer 17–73). (A) Tripolar eggs. (B) Tetrapolar eggs. *Lines* show the positions of the metaphase plates of the spindles. *Numbers* indicate how many chromosomes are on a particular metaphase plate. *Shaded portions* indicate the positions of daughter cells that will not have at least one chromosome.

17–73

A. The 10 arrangements of chromosomes on tripolar spindles are illustrated schematically in **Figure 17–40A**. Upon separation of the sister chromatids and cell division, some of the arrangements yield cells that do not contain at least one chromosome, as indicated by shaded regions in Figure 17–40A. Of the 10 possible arrangements for tripolar eggs, 7 would give rise to three cells, each of which has at least one chromosome. Thus, 70% of tripolar eggs will produce three cells that each carry at least one chromosome.

(For tetrapolar eggs, there are 20 possible arrangements, 8 of which give rise to four cells that each have at least one chromosome, as illustrated in **Figure 17–40B**. Thus, 40% of tetrapolar eggs will give rise to four cells with at least one chromosome.)

B. If the total number of chromosomes were the critical factor, 70% of tripolar eggs would be expected to develop into normal plutei. If the distribution of chromosomes were the critical factor, then $(0.7)^9$, or 4%, of tripolar eggs would be expected to develop into normal plutei. Boveri found that 58 out of 695, or 8%, of tripolar eggs developed into normal plutei. These results agree remarkably well with expectations based on the idea that individual chromosomes carry only a portion of the total genetic information.

[For tetrapolar eggs, the expectations are 40% for total number of chromosomes and $(0.4)^9$, or 0.03%, for distribution of chromosomes. His observation that zero of 1170, or less than 0.09%, of tetrapolar eggs developed into normal plutei again matches the expectations based on the idea that individual chromosomes carry a fraction of the total genetic information.]

References: Baltzer F (1967) Theodor Boveri: Life and Work of a Great Biologist. Berkeley, CA: University of California Press.

Boveri T (1902) Über mehrpolige Mitosen als Mittel zur Analyse des Zellkerns. *Verh. d. Phys.-med. Ges. Würzburg, N.F.* 35, 67–90. [Available in English translation, B. Willier and J. Oppenheimer (eds) (1964) Foundations of Experimental Embryology. Englewood Cliffs, NJ: Prentice-Hall.]

Boveri T (1907) Zellenstudien VI: Die Entwicklung dispermer Seeigeleier. Ein Beitrag zur Befruchtungslehre und zur Theorie des Kernes. *Jenaische Zeitschr. Naturwissen.* 43, 1–292.

CYTOKINESIS

DEFINITIONS

17–74 Syncytium

17–75 Midbody

17–76 Cytokinesis

17–77 Phragmoplast

17–78 Contractile ring

TRUE/FALSE

17–79 False. For most cells—typical cells—this statement is true. There are exceptions, however. Osteoclasts, for example, undergo mitosis without cytokinesis and become multinucleate.

17–80 False. The position of the mitotic spindle anticipates the position of the cleavage furrow: it is positioned centrally for symmetric cleavage and asymmetrically for asymmetric cleavage.

THOUGHT PROBLEMS

17–81 The two cytoskeletal machines are the mitotic spindle and the contractile ring. The segregation of chromosomes and their distribution to daughter cells are accomplished by the bipolar mitotic spindle, which is composed of microtubules and a variety of microtubule-dependent motors and other proteins. The division of an animal cell into daughter cells by cytokinesis is accomplished by the contractile ring, which is composed of actin and myosin filaments and is located just under the plasma membrane. As the ring constricts, it pulls the membrane inward, ultimately dividing the cell in two.

17–82 The movement of chromosomes at anaphase depends on microtubules, not on actin or myosin. Injection of an antibody against myosin would therefore have no effect on chromosome movement during mitosis. Cytokinesis, on the other hand, depends on the assembly and contraction of a ring of actin and myosin II filaments, which forms the cleavage furrow that splits the cell into two. If the injected antibody interferes with myosin II, the contractile ring will not be able to initiate cleavage.

17–83 Nocodazole treatment disassembles microtubules, preventing formation of a spindle. Because nuclei break down and chromosomes condense, these events must be independent of aster formation by centrosomes, for example, and of any other microtubule-dependent process. Mitosis finally arrests because the unattached chromosomes trigger a signal that engages the spindle assembly checkpoint, which halts the cycle.

Treatment with cytochalasin D does not affect mitosis because actin filaments are not involved in the process. Moreover, the disassembly of actin filaments does not trigger a cell-cycle arrest; the cell completes mitosis and forms a binucleate cell. Thus, there does not seem to be a cytokinesis checkpoint.

CALCULATIONS

17–84

A. If the volume of the parental cell is 1, then the volume of each progeny cell is 0.5. (Note that it is not necessary to specify the units for the volume.

Since we are calculating a *fractional* increase in surface area, the units drop out at the end.) The radius of the parent cell can be calculated as follows

$$V = \frac{4\pi r^3}{3}$$

Substituting for $V = 1$ and rearranging

$$r^3 = \left(\frac{3}{4\pi}\right)$$

$$r = \left(\frac{3}{4\pi}\right)^{1/3} = 0.62$$

The surface area of the parental cell is

$$A = 4\pi r^2$$

Substituting for $r = 0.62$,

$$A = 4.83$$

The same calculation for a progeny cell, using 0.5 as the volume, gives $r = 0.49$. The surface area of a single progeny cell is 3.02, and the surface area of both progeny cells is 6.04. The fractional increase in surface area due to division is

$$\begin{aligned}\text{Increase} &= (6.04 - 4.83)/4.83 \\ &= 25\%\end{aligned}$$

Thus, at cell division there is a 25% increase in the amount of plasma membrane. This increase is unlikely to cause the cell any problem. The internal pools of membrane in most cells are much greater than the plasma membrane and serve as a ready source of new plasma membrane.

B. If there is a 25% increase in membrane at each cell division, then the total membrane area after a division is 1.25 times the membrane area before division. Thus, for 12 successive divisions with no increase in total cell volume, the total membrane area increases $(1.25)^{12} = 15$-fold. This increase is much more substantial than in a single cell division and suggests that eggs must have an extra large internal supply of membrane or that membrane synthesis keeps pace with cell division.

DATA HANDLING

17–85 Megakaryocyte precursors enter their first mitosis in exactly the same way as normal cells and proceed normally up to anaphase. Megakaryocytes can complete anaphase A, allowing sister chromatids to separate and move toward the spindle poles. They do not, however, begin the movements characteristic of anaphase B; that is, separation of the spindle poles. Because the spindle poles remain close together and cytokinesis is avoided, the chromosomes become enclosed within one nuclear envelope. Most importantly, the cell now contains two centrosomes. At the next division, the two centrosomes duplicate and divide, ultimately forming the four poles of the tetrapolar spindle that organizes the condensed, replicated chromosomes. Once again, the cells proceed to anaphase A, and then exit mitosis and enclose all the chromosomes in a single nuclear membrane.

Megakaryocytes must lack the motor activities that are responsible for the movements of anaphase B; namely, plus-end directed motors that push the poles apart and the minus-end directed motors that interact with the cell cortex and pull the spindle poles apart. It is unclear whether

the motors are missing or simply inactive. The megakaryocytes must also be missing the pathways that trigger cytokinesis.

References: Nagata Y, Muro Y & Todokoro K (1997) Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis. *J. Cell Biol.* 139, 449–457.

Zimmet J & Ravid K (2000) Polyploidy: occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. *Exp. Hematol.* 28, 3–16.

MEDICAL LINKS

17–86 In the absence of the psychosine receptor, the population of cells is distributed between 2N (unreplicated) cells and 4N (replicated) cells. In the presence of the receptor, but in the absence of psychosine, there is the same distribution. When treated with psychosine, however, prominent peaks corresponding to 8N and 16N cells appear. FACS analysis cannot determine whether the increase in DNA is due to multiple nuclei, or to a single nucleus with several copies of the genome. When the cells in these experiments were examined microscopically, they were shown to contain multiple nuclei.

References: Im D-S, Heise CE, Nguyen T, O'Dowd BF & Lynch KR (2001) Identification of a molecular target of psychosine and its role in globoid cell formation. *J. Cell Biol.* 153, 429–434.

Mitchison T (2001) Psychosine, cytokinesis, and orphan receptors. Unexpected connections. *J. Cell Biol.* 153, F1–F3.

MEIOSIS

DEFINITIONS

17–87 Nondisjunction

17–88 Meiosis I

17–89 Chiasma

17–90 Bivalent

17–91 Synaptonemal complex

TRUE/FALSE

17–92 False. At the start of meiosis, each diploid cell contains two sets of homologs: one from the mother and one from the father. During meiosis, these two sets of homologs are randomly assorted so that sperm and eggs will get one set of homologs, but each set will be a mixture of paternal and maternal homologs.

17–93 False. During meiosis I, each pair of sister chromatids remains together. At the beginning of meiosis I, paternal and maternal homologs pair and are separated into different cells. The sister chromatids are then separated during meiosis II.

17–94 True. A DNA double-strand break introduced into the paired homologs stimulates homologous recombination between the homologs, leading to a few crossover events that make one chromatid from one homolog continuous with a chromatid from the other homolog. These crossovers tie the two homologs together until they are pulled apart at the end of meiosis I.

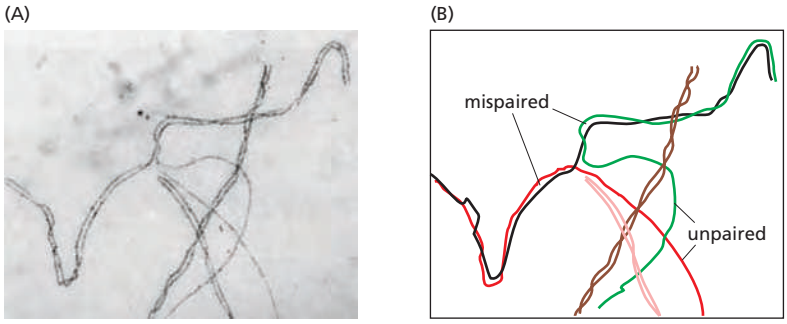


Figure 17–41 Chromosomes in *PamI* cells undergoing meiosis (Answer 17–95). (A) Electron micrograph of abnormal chromosome pairing. (B) An interpretive drawing of the chromosomes in the electron micrograph. Unpaired segments of chromosomes and mispaired regions are indicated.

THOUGHT PROBLEMS

17–95 As shown in the electron micrograph of the *PamI* cells, the pairing of homologs has partially failed. Indeed, it seems that two different chromosomes are paired with a third chromosome, suggesting that pairing is not homologous. In **Figure 17–41**, the mispaired and unpaired chromosomes are indicated.

Reference: Golubovskaya IN, Harper LC, Pawlowski WP, Schichnes D & Cande WZ (2002) The *pamI* gene is required for meiotic bouquet formation and efficient homologous synapsis in maize (*Zea mays L.*). *Genetics* 162, 1979–1993.

17–96 A high frequency of trisomy does not mean that the chromosomes are difficult to segregate. For a trisomy to be present in a human infant requires that two conditions be met. First, the chromosomes must suffer nondisjunction during meiosis. Second, the chromosome complement of the fertilized egg has to be sufficient to support embryonic development. Down syndrome, which occurs at a frequency of one affected individual per 700 live births, and Edwards syndrome, which occurs at a frequency of one per 3000 live births, are the most common autosomal trisomies that meet both conditions. The most common trisomy involves chromosome 16, which occurs in more than 1% of pregnancies, but it is not compatible with normal development.

CALCULATIONS

17–97 Since the assortment of homologs is a binary choice for each chromosome, the number of possible combinations is 2^{23}, which is 8.4×10^6. If recombination were allowed at any possible position between homologs, as it is in reality, the number of possible combinations would increase immeasurably.

DATA HANDLING

17–98

A. Coexpression of Rec8, the meiosis-specific cohesin, and Sgo1 in mitotic cells is lethal because Sgo1 prevents cleavage of Rec8, which continues to hold the sister chromatids together. The inability to separate sisters during mitosis is lethal.

B. During meiosis I, Sgo1 protects Rec8 from cleavage. As a result, Rec8 continues to hold the sister chromatids together through the completion of meiosis I.

C. If Sgo1 were not expressed during meiosis I, Rec8 would be cleaved and sister chromatids would be separated, which would prevent the orderly reduction division that meiosis I is meant to accomplish.

Reference: Kitajima TS, Kawashima SA & Watanabe Y (2004) The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. *Nature* 427, 510–517.

CONTROL OF CELL DIVISION AND CELL GROWTH

DEFINITIONS

17–99 G_0

17–100 Growth factor

17–101 Telomere

17–102 Replicative cell senescence

17–103 Mitogen

17–104 E2F protein

17–105 ATM

TRUE/FALSE

17–106 False. Serum-deprived cells continue through the current cell cycle until they reach the G_1 checkpoint, which diverts them into G_0. Cells can only enter G_0 from G_1.

17–107 False. Budding yeast resume their cell cycle, even if they are unable to repair the damage; evidently life with mutation is better than no life at all. By contrast, mammalian cells do not resume the cycle, but instead commit suicide by undergoing apoptosis. In multicellular organisms, the health of the organism takes precedence over the life of an individual cell. Cells with severe damage threaten the life of the organism, since genetic damage can lead to cancer and other lethal defects.

17–108 False. Organism senescence (aging) is distinct from replicative cell senescence, which occurs in the absence of telomerase. Aging is thought to depend largely on progressive oxidative damage to macromolecules. Strategies that reduce metabolism—for example, restricted caloric intake—decrease the production of reactive oxygen species, and can extend the life-span of experimental animals.

THOUGHT PROBLEMS

17–109 Mitogens stimulate cell division, primarily by relieving intracellular negative controls that otherwise block progress through the cell cycle. Growth factors stimulate cell growth (an increase in cell mass) by promoting the synthesis of proteins and other macromolecules and by inhibiting their degradation. Survival factors promote cell survival by suppressing apoptosis.

17–110

A. Most of the cells in an adult human are in this class, having withdrawn from the cell cycle into G_0. Liver cells, for example, remain quiescent for long periods, although they can grow and divide when the need arises.
B. Nerve cells grow as they extend axons over long distances, but do not divide. Fat cells can accumulate large quantities of triglyceride, which causes them to increase in size (although this is not properly growth, per se). Oocytes grow to become very large cells prior to fertilization.
C. This is the most rare category of cell, but the production of red blood cells is a good example. During production of red blood cells, precursor

reticulocytes undergo five cell divisions with little increase in overall volume, ultimately generating very small red blood cells from a much larger precursor cell.

D. Most cells in the human body grow and divide actively at some point during development, until we become adults. Even in adults some cells continue to grow and divide; most notably, intestinal cells and hematopoietic cells, which must constantly renew the lining of the gut and the cells in the blood, respectively. Red blood cells, for example, are produced at a rate of 2 million per second in an adult human. Most other cells grow and divide often enough to balance cell death.

Reference: Dolznig H, Bartunek P, Nasmyth K, Müllner EW & Beug H (1995) Terminal differentiation of normal chicken erythroid progenitors: shortening of G_1 correlates with loss of D-cyclin/cdk4 expression and altered cell size control. *Cell Growth Differ.* 6, 1341–1352.

17–111 For multicellular organisms, the control of cell division is extremely important. Individual cells must not proliferate unless it is to the benefit of the whole organism. The G_0 state offers protection from aberrant activation of cell division, because the cell-cycle control system is partly or completely dismantled. If a cell just paused in G_1, it would still contain all the cell-cycle machinery and might still be induced to divide. It would also have to remake the "decision" not to divide almost continuously. To re-enter the cell cycle from G_0, a cell has to resynthesize the components that have disappeared, which is unlikely to occur by accident.

17–112 The on-demand, limited release of PDGF at a wound site triggers cell division of neighboring cells for a limited time, until PDGF is degraded. This is different from the continuous release of PDGF from mutant cells, where PDGF is made in an uncontrolled way at high levels. Moreover, the mutant cells that make PDGF often inappropriately express their own PDGF receptor, so that they can stimulate their own proliferation, thereby promoting the development of cancer.

17–113

A. Radiation leads to DNA damage, which activates ATM and ATR kinases, which phosphorylate and activate Chk1 and Chk2 kinases, which phosphorylate and stabilize p53, which induces expression of p21, which binds to and inactivates G_1/S-Cdk and S-Cdk, which stops progression through the cell cycle.

B. In the absence of a functional DNA damage checkpoint, the cell will replicate the damaged DNA, introducing mutations into the genomes inherited by the daughter cells.

C. A checkpoint-deficient cell will be able to divide normally, but it will be prone to mutations, because some DNA damage always occurs as the result of natural processes (for example, by cosmic rays). The checkpoint mediated by p53 is mainly required as a safeguard against the devastating effects of DNA damage, but not for the natural progression of the cell cycle in undamaged cells.

D. Cell division is an ongoing process that does not cease when we reach maturity. Blood cells, epithelial cells in the skin or lining the gut, and the cells of the immune system, for example, are being constantly produced by cell division to meet the body's needs. Our bodies produce about 10^{11} new blood cells each day.

17–114

A. Cells that cannot degrade M-phase cyclins would be unable to divide. The cells would enter mitosis, but would not be able to exit.

B. Cells that always expressed high levels of p21 would be unable to divide. The cells would arrest permanently in G_1 because their G_1/S-Cdk and their S-Cdk would be inactivated.

C. Cells that cannot phosphorylate Rb would be unable to divide. The cells would not be able to activate the transcription of genes required for entry into S phase because the required regulatory protein, E2F, would be sequestered by unphosphorylated Rb.

17–115 The gene for telomerase is turned off early in development and remains off in most cells in humans. Thereafter, each time a cell replicates its chromosomes, it fails to copy a short segment of the telomeric DNA at the very end of the chromosome (see Problem 5–55). As a result, the telomere becomes progressively shorter with each cell division; thus, the length of the telomere is a rough gage of the number of times a cell has divided. When the telomere gets too short to function properly, it triggers a p53-dependent cell-cycle arrest.

17–116 In alcoholism, liver cells proliferate because the organ is overburdened and becomes damaged by the large amounts of alcohol that have to be metabolized. This need for more liver cells activates the control mechanisms that normally regulate proliferation. Unless badly damaged, the liver will usually shrink back to a normal size after the patient stops drinking. In a liver tumor, in contrast, mutations abolish normal cell-proliferation control, and as a result, cells divide and keep on dividing in an uncontrolled manner.

DATA HANDLING

17–117 The results in Table 17–2 indicate that 3T3 cells require these growth factors in an ordered sequence. If the growth factors were required simultaneously, none of the pretreatments should have advanced entry into S phase. If the growth factors were required independently (that is, regardless of order), all of the pretreatments should have advanced the entry into S phase equally. Since the order of addition clearly makes a difference, the cells must respond sequentially to the growth factors. Since the pretreatment in experiment 3 (PDGF, EGF, and then IGF1) advances entry into S phase most markedly, the cells must respond to the growth factors in this order.

From more extensive experiments, it appears that PDGF induces a state of competence in quiescent 3T3 cells that permits them to respond to EGF and IGF1. Exposure of competent 3T3 cells to EGF causes them to progress about 6 hours toward S phase; exposure of EGF-treated competent cells to IGF1 causes them to progress another 5 hours toward S phase.

Reference: O'Keefe EJ & Pledger WJ (1983) A model of cell cycle control: sequential events regulated by growth factors. *Mol. Cell. Endocrinol.* 31, 167–186.

17–118 Cyclin D antibodies bind to cyclin D and make it unavailable for binding to its Cdk partners, thereby preventing formation of G_1-Cdk, which is required for cells to progress through Start, the G_1 checkpoint. Once Start has been passed—about 14 hours after addition of mitogenic growth factors—the cell is committed to enter S phase independent of any further requirement for G_1-Cdk. Thus, after 14 hours, antibodies to cyclin D do not affect the eventual entry of cells into S phase.

Reference: Baldin V, Lukas J, Marcote MJ, Pagano M & Draetta G (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. *Genes Dev.* 7, 812–821.

17–119

A. Careful examination of the time-lapse pictures in Figure 17–27 shows that all the cells without buds arrested at the dumbbell stage, whereas

all of the cells with buds formed viable colonies. The appearance of a bud corresponds with the beginning of S phase. Haploid cells that have partially or fully replicated their genomes are more resistant to x-ray-induced breaks because a break in one chromosome can be repaired by recombination with the intact sister chromatid. Haploid cells in G_1 are especially sensitive to breaks because they contain no second intact copy of the chromosome with which to recombine. After replication, such a cell will contain two copies of the chromosome, but both will be broken at the same position. Thus, even in G_2, a haploid cell that suffers a break in G_1 will not have an intact chromosome with which to repair itself by homologous recombination.

B. The observation that dumbbell-stage cells have a single nucleus and no spindle indicates that the cells are arrested in G_2 prior to mitosis. The presence of a bud indicates that the cells have already passed the G_1 checkpoint and such cells will complete S phase.

C. Half the wild-type cells temporarily arrest in the dumbbell stage while they wait for damage to be repaired. A cell-cycle checkpoint senses damaged DNA and halts the cell cycle until the damage is repaired. When repair is complete, the cells enter mitosis and divide to produce viable colonies. The nonviable cells died, either because they suffered damage too late to stop and divided with damaged chromosomes, or because they suffered so much damage that it could not be repaired.

D. *Rad52* cells remain arrested at the dumbbell stage because they are incapable of repairing their damaged chromosomes. The continued signal from the damaged DNA prevents the cells from passing the mitotic entry checkpoint.

E. Very few *Rad9* mutant cells arrest at the dumbbell stage because they are defective in their ability to sense DNA damage. In these cells, the mitotic entry checkpoint does not function. Division in the absence of repair leads to haploid cells that have broken chromosomes and cells that are missing pieces of chromosomes. Both situations lead to nonviable cells. Only a small fraction of cells (30%) manages to repair their chromosomes in the absence of a checkpoint delay.

F. If *Rad9* cells were artificially delayed in mitosis, the number of viable cells would increase. The artificial delay would allow the cells time to repair their damaged chromosomes, so that they then could complete mitosis with an intact genome. The important point is that *Rad9* cells contain all the necessary enzymes required for DNA repair; they are defective only in sensing DNA damage.

References: Hartwell LH & Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* 246, 629–634.

Weinert TA & Hartwell LH (1988) The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae. Science* 241, 317–322.

17–120 Your results with mutant strains indicate that signals from damaged DNA and unreplicated DNA interact with the mitotic entry checkpoint via a branched pathway. If the two signals shared the same pathway, all the mutants would show mitotic delay in response to either damaged or unreplicated DNA. If they used independent pathways, each mutant would respond to only one or the other kind of DNA. As indicated in **Figure 17–42**, *Rad24* affects a part of the pathway that is specific for damaged DNA, *Cdc2-3w* and *Cdc2*-F15 affect a part of the pathway that is specific for unreplicated DNA, and *Rad1*, *Hus1*, and *Hus2* affect a common part of the pathway.

Reference: Murray A & Hunt T (1993) The Cell Cycle: An Introduction, pp. 143–144. New York: WH Freeman.

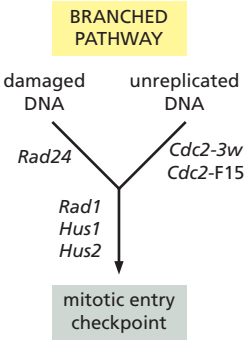


Figure 17–42 Branched signaling pathway from damaged and unreplicated DNA to the mitotic entry checkpoint (Answer 17–120).

17–121 If the G_1 phase of the cell cycle did not increase in length, ts *Wee1* cells would become smaller and smaller with each cell division until they could no longer carry out DNA synthesis and mitosis. It is likely that G_1 becomes longer because of a size requirement to enter S phase. In fission yeast, normal cells are born at a size that is adequate to pass Start and begin DNA synthesis (S phase) almost as soon as they finish mitosis. Although the mechanism is poorly understood, cells of most species measure their own size to make sure they have reached the proper threshold for entering S. If this size-determination mechanism were intact in the ts *Wee1* cells, then the small cells generated by division at 37°C would be held in G_1 until they grew to the proper size. Thus, the lengthening of G_1 would be a consequence of the size requirement for entering S.

Reference: Nurse P (1975) Genetic control of cell size at cell division in yeast. *Nature* 256, 547–551.

17–122 Enlargement of cells that are no longer cycling indicates that the primary effect of cyclin D and Cdk4 is on cell growth. If the primary effect were on the cell cycle, the differentiated cells should have been the same size as normal cells. The surprising conclusion of this work is that the cyclin D–Cdk4 complex is a growth regulator in flies and may not be involved in cell-cycle progression.

(As an aside, when the authors of this work overexpressed cyclin D and Cdk4 in the whole eye, they noted "...we found that all ommatidiae were enlarged, as was the entire eye, which bulged out of the head in an ominous fashion.")

References: Datar SA, Jacobs HW, de la Cruz AF, Lehner CF & Edgar BA (2000) The *Drosophila* cyclin D-Cdk4 complex promotes cellular growth. *EMBO J.* 19, 4543–4554.

Meyer CA, Jacobs HW, Datar SA, Du W, Edgar BA & Lehner CF (2000) *Drosophila* Cdk4 is required for normal growth and is dispensable for cell cycle progression. *EMBO J.* 19, 4533–4542.

MCAT STYLE

Passage 1 (Answers 17–123 to 17–126)

17–123

B. Choice I is correct because chromosome condensation occurs in early mitosis, and is therefore a good marker for entry into mitosis. Choice II is correct because M-Cdk is specifically activated during mitosis. Thus, cells with M-Cdk activity have entered mitosis. Choice III is incorrect because DNA replication occurs during S phase.

17–124

C. Cdc25 is a critical regulator of entry into mitosis; it promotes mitotic entry by removing the inhibitory phosphate from M-Cdk. Thus, inhibiting Cdc25 could block entry into mitosis. The other choices are incorrect because they would all promote entry into mitosis.

17–125

C. In the spindle assembly checkpoint, free kinetochores that are not stably attached to microtubules send a repressive signal that blocks entry into anaphase. Thus, the spindle assembly checkpoint appears to operate by a mechanism that is superficially analogous to your proposed checkpoint mechanism. The checkpoint mechanisms in the other answers do not exist.

17–126

B. Transcription of G_1 cyclin, which leads to its increased levels in G_1, is a critical event that drives cells to enter the cell cycle and proceed into S phase. Thus, inhibiting G_1 cyclin transcription would block entry into the cell cycle. Choice A is incorrect because inhibiting DNA polymerase would arrest cells in S phase, not before S phase. Choices C and D are incorrect because they would promote entry into the cell cycle. Rb inhibits transcription of G_1 cyclins, so inhibiting Rb would increase production of G_1 cyclin, accelerating entry into the cell cycle. Similarly, E2F promotes G_1 cyclin transcription, so overproduction of E2F would also promote entry into the cell cycle.

CHAPTER 18

Cell Death

DEFINITIONS

18–1 Caspase

18–2 Apoptosome

18–3 Apoptosis

18–4 Death-inducing signaling complex (DISC)

18–5 Extrinsic pathway

18–6 Survival factor

18–7 Intrinsic pathway

18–8 Death receptor

18–9 Executioner caspase

TRUE/FALSE

18–10 True. Adult tissues are maintained at a constant size, so that there must be a balance between cell death and cell division. If this were not so, the tissue would grow or shrink.

18–11 True. Cytochrome *c* mediates apoptosis from signals within a mammalian cell—the intrinsic pathway of apoptosis. This has been confirmed directly by generating cytochrome *c*-deficient mouse embryo fibroblasts (MEFs) by reverse genetics. Although mice with knockouts of their cytochrome *c* genes die about midway through gestation because of problems with mitochondrial function, fibroblasts from such embryos can be cultured under special conditions and tested for sensitivity to various apoptotic signals. They are resistant to a variety of agents that induce the intrinsic pathway of apoptosis.

Reference: Li K, Li Y, Shelton JM, Richardson JA, Spencer E, Chen ZJ, Wang X & Williams RS (2000) Cytochrome *c* deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* 101, 389–399.

THOUGHT PROBLEMS

18–12 Because apoptosis occurs on a large scale in both developing and adult tissues, it is important that it does not trigger the alarm reactions normally associated with cell injury. In tissue injury, for example, signals are released that can cause a destructive inflammatory reaction. Moreover, the release of intracellular contents could elicit an immune response against molecules that are normally not encountered by the immune system. In normal development, such reactions would be self-defeating, even dangerous, if they occurred in response to apoptosis.

18–13 Cells in an animal must behave for the good of the organism as a whole—to a much greater extent than people generally act for the good of society as a whole. In the context of an organism, unsocial behavior would lead to loss of organization and to cancer. Many of the rules that cells have to obey would be unacceptable in a human society. Most people, for example, would be reluctant to kill themselves for the good of society, yet our cells do it all the time.

18–14 The plasma membrane of the cell that died by necrosis (see Figure 18–1A) is ruptured; several clear breaks are visible, for example, at 8, 9, and 12 o'clock. The cell's contents, mostly membranous and cytoskeletal debris, are seen spilling into the surroundings. The cytosol stains lightly, as most soluble components had been lost before the cell was fixed. By contrast, an intact membrane surrounds the cell that underwent apoptosis (see Figure 18–1B), and its cytosol is densely stained, indicating a normal concentration of cellular components. The cell's interior is remarkably different from a normal cell, however. Particularly characteristic are the large blobs that extrude from the nucleus, probably as the result of the breakdown of the nuclear lamina. The cytosol also contains many large, round, membrane-enclosed vesicles of unknown origin that are not normally seen in healthy cells. The pictures visually confirm the notion that necrosis involves cell lysis, whereas cells undergoing apoptosis remain relatively intact until they are engulfed and digested inside a normal cell.

18–15 Somewhat surprisingly, cytochrome *c* seems not to be required for apoptosis in *C. elegans*. However, even if it were required, *C. elegans* mutants that were defective for cytochrome *c* would not have been isolated because they would not have been viable. Cytochrome *c* is an essential component of the electron-transport chain in mitochondria. Without it, no production of ATP by oxidative phosphorylation would be possible, and such a mutant organism could not survive.

Reference: Ellis HM & Horvitz HR (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44, 817–829.

18–16 Upon microinjection of cytochrome *c*, both cell types undergo apoptosis. The presence of cytochrome *c* in the cytosol is a signal for the assembly of apoptosomes and the downstream events that lead to apoptosis. Cells that are defective for both Bax and Bak cannot release cytochrome *c* from mitochondria in response to upstream signals, but there is no defect in the downstream part of the pathway that is triggered by cytosolic cytochrome *c*. Thus, microinjection bypasses the defects in the doubly defective cells, triggering apoptosis.

Reference: Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB & Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292, 727–730.

18–17 Mice that are deficient for Apaf1 or caspase-9 are defective for cytochrome *c*-dependent apoptosis. Apoptosis is a critical event in development, allowing excess brain cells to be weeded out. The extent of brain overgrowth and the size of the cranial protrusions indicate that the pruning process in the developing brain must be massive. The dramatic effects of the deficiencies of Apaf1 and caspase-9 suggest that the cytochrome *c*-dependent apoptotic pathway—the intrinsic pathway—must be critically important in brain development.

References: Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA & Gruss P (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* 94, 727–737.

Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, de la Pompa JL, Kagi D, Khoo W, Potter J, Yoshida R, Kaufman SA, Lowe SW, Penninger JM & Mak TW (1998) Differential requirement for caspase 9 in apoptotic pathways *in vivo*. *Cell* 94, 339–352.

Kuida K, Haydar TF, Kuan C-Y, Gu Y, Taya C, Karasuyama H, Su MS-S, Rakic P & Flavell RA (1998) Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. *Cell* 94, 325–337.

Yoshida H, Kong Y-Y, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM & Mak TW (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* 94, 739–750.

18–18 The retention of the web cells in *Apaf1*$^{-/-}$ mice indicates that Apaf1 is essential for web-cell apoptosis, presumably in conjunction with cytochrome *c*. The absence of web cells in *Casp9*$^{-/-}$ mice indicates that caspase-9 is not required for web-cell apoptosis. These observations suggest that Apaf1 may activate a different caspase in web cells, in addition to or instead of caspase-9.

Reference: Earnshaw WC, Martins LM & Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* 68, 383–424.

CALCULATIONS

18–19

A. One-eighth of Fas–Fas ligand complexes on the lymphocytes from an individual with ALPS would be expected to be composed entirely of normal Fas subunits. Since half the Fas protein in the lymphocytes is normal and there are three Fas subunits per complex, the probability of three normal Fas subunits coming together in a complex is $(\frac{1}{2})^3$.
B. In an individual heterozygous for a mutation that eliminates Fas expression, all the expressed Fas protein would be normal; thus, 100% of the Fas–Fas ligand complexes would be composed entirely of normal Fas subunits. The total number of Fas molecules, however, would be half the number present in an individual with two normal genes for Fas.
C. Fas mutations associated with ALPS are dominant because they reduce the number of normal Fas–Fas ligand complexes by a factor of eight in heterozygotes. Mutations that eliminate Fas expression are recessive because they reduce the number of Fas–Fas ligand complexes by only a factor of two.

Reference: Siegel RM, Chan FK-M, Chun HJ & Lenardo MJ (2000) The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat. Immunol.* 1, 469–474.

DATA HANDLING

18–20 The two cells in Figure 18–4 have released cytochrome *c*–GFP from all their mitochondria within a few minutes: within 6 minutes for the cell in Figure 18–4A and within 8 minutes for the cell in Figure 18–4B. The time after exposure to UV light at which the release occurred varied dramatically for the two cells: after 10 hours for the cell in Figure 18–4A and after 17 hours for the cell in Figure 18–4B. These observations indicate that individual cells release cytochrome *c* from all their mitochondria rapidly, but that release is triggered in different cells at widely varying times after exposure to apoptosis-inducing levels of UV light.

Reference: Goldstein JC, Waterhouse NJ, Juin P, Evan GI & Green DR (2000) The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2, 156–162.

18–21 If the activated downstream caspases were required to accelerate cytochrome *c* release in order to yield a rapid response, the presence of the caspase inhibitor should slow the release of cytochrome *c*. Since the caspase inhibitor did not increase the time required for cytochrome *c* release, it is unlikely that a caspase-mediated positive feedback loop is involved for apoptosis induced by actinomycin D, staurosporine, or UV light.

Reference: Goldstein JC, Waterhouse NJ, Juin P, Evan GI & Green DR (2000) The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2, 156–162.

18–22 Apoptosis induced by the expression of tBid is prevented only in MEFs in which both Bax and Bak have been eliminated. Thus, tBid-induced oligomerization of either Bax or Bak is sufficient to release cytochrome *c* and trigger cell death.

Reference: Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB & Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292, 727–730.

18–23

A. All the treatments, except for Fas ligand, induce signals that trigger apoptosis at a point after Bid but before Bax and Bak. These results indicate that there is at least one other way to activate Bax and Bak in addition to activation via Bid. Activation of apoptosis through Bax and Bak occurs via the intrinsic pathway of apoptosis. In apparent contradiction to the pathway shown in Figure 18–6, the results in Table 18–1 show that Fas ligand must also signal apoptosis through a pathway that does not involve Bid, Bax, or Bak.

B. It may seem surprising that Fas ligand, which binds to Fas to activate caspase-8-mediated cleavage of Bid (see Figure 18–6), still causes apoptosis in Bid-deficient cells. Caspase-8, however, is the initiator caspase for a variety of executioner caspases in the extrinsic pathway of apoptosis, which is independent of Bid, Bax, and Bak. In MEFs and some other cells, activation of caspase-8 normally triggers both the extrinsic and intrinsic pathways of apoptosis as a way of ensuring rapid cell death.

Reference: Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB & Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292, 727–730.

MEDICAL LINKS

18–24 Overexpression of a secreted protein that binds to Fas ligand would protect tumor cells from attack by killer lymphocytes. By binding to the Fas ligand on the surface of killer lymphocytes, the secreted protein would prevent the Fas ligand from binding to the Fas death receptor on the surface of tumor cells, thereby insulating them from death-inducing interactions with killer lymphocytes. Secreted proteins that bind to Fas ligand are commonly known as decoy receptors. They play a normal role in modulating the killing induced by interactions between Fas ligand and Fas. When tumor cells overproduce such decoy receptors, they subvert this normal mechanism into a cellular defense against Fas-mediated killing.

Reference: Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, Huang A, Donahue CJ, Sherwood SW, Baldwin DT, Godowski PJ, Wood WI, Gurney AL, Hillan KJ, Cohen RL, Goddard AD, Botstein D

& Ashkenazi A (1998) Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396, 699–703.

18–25 These results show that alkylation of DNA is responsible for the apoptotic signal. MGMT-deficient and MGMT-overexpressing cells are equally sensitive to γ-irradiation, indicating that the apoptotic apparatus is intact in both cell lines. MGMT-overexpressing cells are much more resistant to MNNG treatment than are MGMT-deficient cells, suggesting that efficient removal of O^6-methylguanine lesions prevents apoptosis.

Reference: Meikrantz W, Bergom MA, Memisoglu A & Samson L (1998) O^6-alkylguanine DNA lesions trigger apoptosis. *Carcinogenesis* 19, 369–372.

MCAT STYLE

18–26

B. The protein in the unbound fraction turned out to be Apaf1, which binds cytochrome *c* to form a complex that initiates apoptosis. Only choice B explains the requirement for both proteins to initiate apoptotic events. Choice A is not correct because activation of executioner caspases would be sufficient to induce apoptosis; there would be no need for cytochrome *c*. Choice C is not correct because addition of an apoptosis inhibitory protein would not be required for initiation of apoptosis; cytochrome *c* alone would be sufficient. Choice D is not correct because cytochrome *c* alone should initiate apoptosis, if the sole function of the unbound protein was to release cytochrome *c*.

18–27

B. Because Bcl2 blocks cytochrome *c* release from mitochondria and also inhibits apoptosis, this observation provides the best evidence that it is mitochondrial cytochrome *c* that triggers apoptosis. Choice A would not distinguish whether release of cytochrome *c* from mitochondria is the cause or a consequence of apoptosis. Since caspases drive apoptotic events, choice C would suggest that release of cytochrome *c* is a consequence of apoptosis, rather than the cause. Choice D is irrelevant to the source of cytochrome *c*, it just confirms that cytochrome *c* is not present in the cytosol in the absence of apoptosis.

18–28

C. Bcl2 blocks release of cytochrome *c* from mitochondria. Survival factors block apoptosis, and therefore would block release of cytochrome *c*. The Fas ligand promotes apoptosis and would be expected to promote release of cytochrome *c*.

Cell Junctions and the Extracellular Matrix

CHAPTER 19

IN THIS CHAPTER

CELL–CELL JUNCTIONS

DEFINITIONS

19–1 Selectin

19–2 Homophilic

19–3 Desmosome

19–4 Polarized

19–5 Gap junction

19–6 Adherens junction

19–7 Cadherin

19–8 Plasmodesmata

19–9 Adhesion belt

19–10 Tight junction

19–11 Apical

19–12 Connexon

TRUE/FALSE

19–13 False. Although cells can be readily dissociated by removing Ca^{2+} from the external medium, it is unlikely that Ca^{2+}-dependent cell–cell adhesions are regulated by changes in Ca^{2+} concentration. Cells have no way to control the Ca^{2+} concentration in their environment.

19–14 True. This like-to-like binding is the reason that cadherin–cadherin interactions between cells are referred to as homophilic.

19–15 True. Selectins are transmembrane proteins that bind to cell-surface carbohydrates on other cells. These proteins (lectins) mediate a variety of transient cell–cell interactions in the bloodstream.

19–16 False. The adhesions mediated by cadherins are much stronger than those mediated by Ig family members. Thus, cadherins are mainly responsible for holding cells together, segregating cell collectives into discrete tissues, and maintaining tissue integrity.

19–17 True. The polarity of essentially all epithelia is the same: the basal surface is anchored in the basal lamina, which abuts other tissues, while the apical surface is exposed.

19–18 True. The barriers formed by tight-junction proteins restrict the flow of

molecules between cells and the diffusion of proteins (and lipids) from the apical to the basolateral domain and vice versa.

19–19 False. All paracellular transport is passive; it results from the movement of material down an electrochemical gradient.

19–20 False. Individual gap-junction channels continuously flip between open and closed states.

19–21 True. Because the cells of a plant are all connected by plasmodesmata, all their nuclei share a common cytoplasm. Because the plasmodesmata restrict the flow of larger components, however, the cells do not share most of their macromolecules.

THOUGHT PROBLEMS

19–22 This quote is correct in spirit, though incorrect in detail. Warren Lewis was trying to draw attention to the importance of the adhesive properties of cells in tissues at a time when the problem had been largely ignored by the biologists of the day. The quote is incorrect because a large fraction of our bodies is made up of connective tissue such as bone and tendon, whose integrity depends on the matrix itself rather than on the cells that inhabit it. It is not at all easy to dissociate cells from tissues, as anyone who has eaten a tough piece of steak can testify.

19–23 IgG antibodies contain two identical binding sites; thus, they are able to cross-link the molecules they recognize (this is the basis for immune precipitation). If whole antibodies were used to block aggregation, they might cross-link the cells rather than inhibit their aggregation. By contrast, monovalent Fab fragments cannot cross-link cells. They bind to the cell adhesion molecules and prevent them from binding to their partners, thus preventing cell aggregation (**Figure 19–19**).

Reference: Beug H, Katz FE & Gerisch G (1973) Dynamics of antigenic membrane sites relating to cell aggregation in *Dictyostelium discoideum*. *J. Cell Biol.* 56, 647–658.

19–24

A. The final architecture can be understood most readily from the standpoint of competition for cadherin binding sites. If high-expressing cells surround a low-expressing cell, some of their cadherin binding capacity will be unutilized. If the low-expressing cell is replaced by a high-expressing cell, the full binding capacity will be satisfied. If sorting occurs to maximize cadherin binding, the high-expressing cells will ultimately sort themselves into a ball. The underutilized binding on the surface of such a ball promotes interaction with low-expressing cells, which can satisfy more of their total binding capacity in a single interaction with a high-expressing cell than they can with other low-expressing cells; hence, the low-expressing cells will form a layer around a central ball of high-expressing cells.

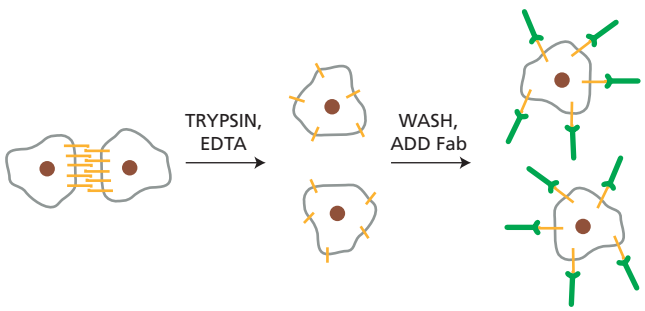


Figure 19–19 Fab antibody fragments block cell adhesion (Answer 19–23).

B. The same sort of logic applies to a mixture of cells that express P-cadherin in common, but with some cells expressing E-cadherin and others expressing N-cadherin. Each population will segregate in a way that maximizes its cadherin interactions. Thus, the population that expresses P-cadherin and E-cadherin will segregate together, as will those that express P-cadherin and N-cadherin. The two populations will remain in contact, however, because they share expression of P-cadherin. It is likely that they will form some sort of layered architecture like that in Figure 19–2B, but the specific arrangement will depend on the relative strengths of E-cadherin versus N-cadherin interactions and on their expression levels.

19–25

A. As the size of the particle approaches the size of the pore, a marked decline in permeability is expected. Imagine throwing larger and larger balls at a meshwork of fixed-size holes. When the balls are much smaller than the holes, many will go through. As the size of the balls approaches the size of the holes, many more will bounce off. Finally, when the ball just fits through the hole, nearly all will bounce off. The random diffusion of molecules through a pore is conceptually very similar.

B. The identity of the nonrestrictive paracellular passageway is not known. There are several possibilities that cannot be distinguished at present. It may be that the nonrestrictive pathway results from heterogeneity within the junction itself; that is, that within an epithelial sheet, a certain number of tight junctions are imperfectly formed. Another possibility is that the nonrestrictive passageways reflect a dynamic aspect of tight-junction behavior. If claudins are continually making and breaking their lateral associations on some time scale, then large, transient passageways may appear and disappear over time.

Reference: Watson CJ, Rowland M & Warhurst G (2001) Functional modeling of tight junctions in intestinal cell monolayers using polyethylene glycol oligomers. *Am. J. Physiol. Cell Physiol.* 281, C388–C397.

19–26 The many negative charges in the claudin-16 sequence suggest that it might function as a cation pore. Claudin-16 in humans is restricted to tight junctions in the ascending limb of the loop of Henle in the kidney, which is specialized for reabsorption of cations such as Na^+, K^+, and Mg^{2+}. Loss of claudin-16 in humans causes a rare disease in which Mg^{2+} is not retained in the body.

Reference: Van Itallie CM & Anderson JM (2006) Claudins and epithelial paracellular transport. *Annu. Rev. Physiol.* 68, 403–429.

19–27 When the intracellular concentration of Ca^{2+} rises beyond normal levels, it causes the gap-junction channels to close immediately. A high intracellular concentration of Ca^{2+} is an indicator of cell damage. The extracellular concentration of Ca^{2+} is about 1 mM, whereas the intracellular concentration normally does not rise above a few micromolar. In a damaged cell, Ca^{2+} quickly rises to extracellular levels. If gap junctions did not close under such circumstances, healthy cells in contact with a damaged cell would themselves suffer a dangerous disturbance in their internal chemistry. By closing its gap junctions, a damaged cell effectively isolates itself from its neighbors.

19–28 Because the plasmodesmata have such small diameters, it would take a long time for the cytoplasm to leak out, even if there were no other protection. In reality, the plasmodesmata that connect to a damaged cell are rapidly sealed off. Although the mechanisms are entirely different, plasmodesmata and gap junctions both respond to damage in the same way: by severing the connection.

19–29 The additional force required to remove P-selectin from the plasma membrane presumably reflects its attachments to other proteins on the cytoplasmic side of the membrane. Thus, to remove a P-selectin from a bilayer requires only that the hydrophobic interactions with the bilayer lipids be overcome; to remove it from the plasma membrane requires, in addition, that all bonds on the cytoplasmic side also be broken. (In case you were wondering, it takes a force of 10,000 pN to break a carbon–carbon bond.)

CALCULATIONS

19–30 The weight of an individual cell in seawater is 0.42 pN:

$$W = 4.2 \times 10^{-13} \text{ N, which is } 0.42 \text{ pN}$$

Thus, an individual bond with a strength of 125 pN could support nearly 300 cells (125 pN/0.42 pN = 297) against the force of gravity in seawater. Given that there are multiple interactions between cells, the cells of a sponge are firmly tied together.

Reference: Dammer U, Popescu O, Wagner P, Anselmetti D, Güntherodt H-J & Misevic GN (1995) Binding strength between cell adhesion proteoglycans measured by atomic force microscopy. *Science* 267, 1173–1175.

19–31

A. The fraction of the phage population that will be attached by at least one tail fiber at any one instant is equal to one minus the fraction not attached by any tail fibers, which is $(0.5)^{12} = 0.00024$ for wild-type bacteria and $(0.5)^6 = 0.0156$ for *ompC*$^-$ bacteria. Thus, at any instant, 99.98% of the phage population will be attached to wild-type bacteria and 98.44% will be attached to *ompC*$^-$ bacteria.

B. The tiny difference in the fraction of the phage population attached to wild-type and *ompC*$^-$ bacteria at first seems too small to account for the 1000-fold difference in infectivity. However, since T4 must wander around the surface of a bacterium to find an appropriate place to attach its baseplate, the instantaneous calculation is misleading. If, for example, T4 must stay bound to the bacterial surface for 500 "instants" during its wandering, then $(0.9998)^{500} = 90\%$ will remain attached to wild-type bacteria, but only $(0.984)^{500} = 0.03\%$ will remain attached to *ompC*$^-$ bacteria. This difference would be more than enough to account for the 1000-fold difference in infectivity.

By associating with the bacterial surface through multiple weak interactions, bacteriophage T4 can wander around the surface without falling off. This allows a search for relatively rare injection sites, which are at points of connection between the inner and outer membranes.

Reference: Goldberg E (1983) Recognition, attachment, injection. In Bacteriophage T4 (CK Mathews, EM Kutter, G Mosig, PB Berget eds.), pp. 32–39. Washington, DC: American Society for Microbiology.

19–32 Of the alternatives given, your measurements support the two-state model of resistance of tight junctions because the resistance of an epithelium is logarithmically related to the number of sealing strands in the junction, as shown by the straight line in **Figure 19–20**.

Analysis of these data suggests that an individual sealing strand has a relatively high probability of being in the open state. If there are enough strands, the probability that all will be open at the same time is very low, so the overall junction is very tight. What it means to be "open" or "closed" is still not clear, although it could relate to the rare, nonrestrictive paracellular passageway described in Problem 19–25.

The relationship described here between number of sealing strands and the electrical resistance of the junction was worked out before

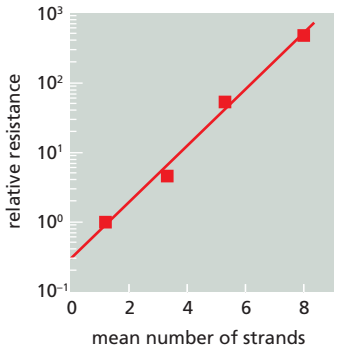


Figure 19–20 Plot of relative electrical resistance versus the number of strands in the tight junction (Answer 19–32).

claudins had been identified as the principal component of tight junctions. We now know that in addition to the number of sealing strands, some properties of tight junctions depend critically on the specific claudins that are expressed. Thus, the relative resistance of the tight junctions could reflect the specific claudins used in their construction, rather than the number of strands. This is an area of ongoing research.

References: Claude P (1978) Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. *J. Membr. Biol.* 39, 219–232.

Van Itallie CM & Anderson JM (2006) Claudins and epithelial paracellular transport. *Annu. Rev. Physiol.* 68, 403–429.

19–33 The volume of each plasmodesma is $\pi r^2 h$ or 7.1×10^4 nm^3 [$3.14 \times (15\text{ nm})^2 \times 100$ nm]. Since there are 10^9 nm^3 per μm^3, the volume of a single plasmodesma is 7.1×10^{-5} μm^3 and the volume of all 100 plasmodesmata is 7.1×10^{-3} μm^3. Given that the volume of the cytoplasm of the cell is 1000 μm^3, the volume of plasmodesmata is 7.1×10^{-6} the volume of the cytosol, or 1 part in 140,000. It is no surprise that the protein components of plasmodesmata have proven so difficult to characterize.

DATA HANDLING

19–34

A. Immunoprecipitation of proteins from cells transfected with the full-length construct gave three radiolabeled bands (lane 1, Figure 19–7A). One of these must be the cadherin itself, and the other two are proteins with which the cadherin interacts. The band corresponding to the cadherin can be identified because it is present in every lane, and it changes position, as expected since each construct expresses a protein of different length; it is the slowest migrating band (top band) in lanes 1, 3, 4, and 5 and the only band in lanes 2 and 6. On SDS polyacrylamide gels, smaller proteins migrate faster than larger ones; thus cadherins with larger deletions are found at lower positions on the gel. The two bands of constant size in lanes 1, 3, 4, and 5 must be the proteins that the cadherin binds. Lane 7 is blank, confirming the specificity of the antibody, and proving that the labeled bands truly represent cadherin binding partners. Incidentally, one of the proteins bound by this cadherin is β-catenin, a central player in the two-way interaction between adhesion and signaling.

B. These experiments define the C-terminal 72 amino acid residues of cadherin as the maximum stretch of amino acids required to bind the other proteins. Lane 2 in Figure 19–7A shows that the terminal 37 residues are required since no proteins were bound when that construct was expressed in cells. As shown in lane 5, an internal deletion of 70 amino acid residues did not alter binding, whereas deletion of a further 35 amino acid residues toward the C-terminus (lane 6) eliminated binding. Because the end points of the deletions in ΔC5 and ΔC10 coincide, the 35- and 37-residue segments form a contiguous 72 amino acid stretch that contains the domain (or domains) that is critical for binding of the other proteins.

C. To check whether this domain is sufficient for binding, you might fuse it by cloning to the C-terminus of a heterologous membrane-spanning protein. If this segment of protein is all that is needed for binding, immunoprecipitation of the fusion protein should bring down the same two proteins. The researchers who did this study attached the cadherin domain to a class I major histocompatibility antigen, H2-K, and found that antibodies against the extracellular domain of the MHC protein–cadherin fusion also precipitated the same proteins.

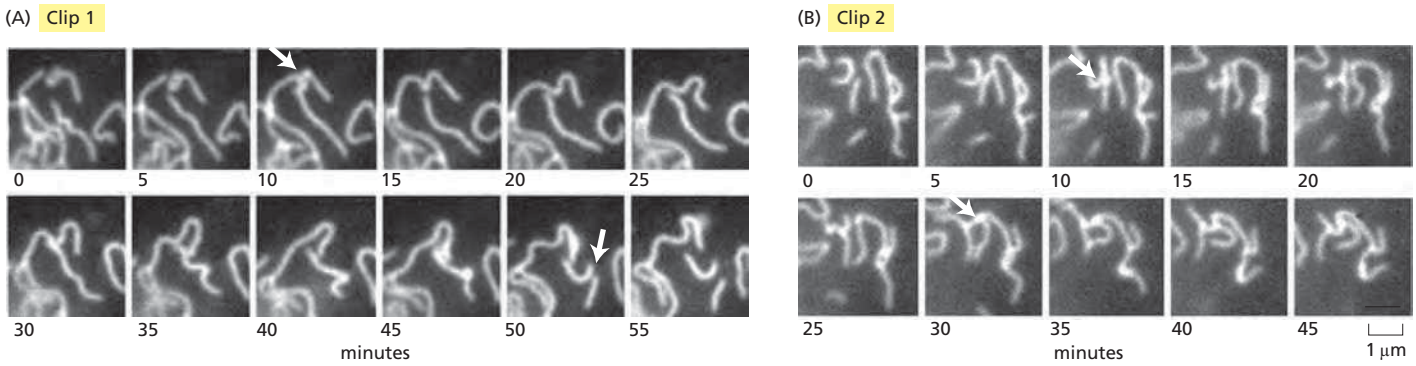


Figure 19–21 Changes in connections between paired claudin strands in apposing plasma membranes (Answer 19–35). (A) Frames from movie clip 1. End-to-end joining of strands and strand breakage are indicated by *white arrows*. (B) Frames from movie clip 2. Formations of T-junctions are indicated by *white arrows*.

Reference: Ozawa M, Ringwald M & Kemler R (1990) Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc. Natl Acad. Sci. USA* 87, 4246–4250.

19–35

A. In contrast to what is observed for intracellular polymers such as microtubules and actin filaments, tight-junction strands do not elongate or shorten appreciably on the time scale of observation in these studies.
B. Individual strands can join end-to-end and they can break apart, as indicated by the arrows in **Figure 19–21A**.
C. Formation of T-junctions is indicated by the arrows in **Figure 19–21B**.
D. The reviewer makes a good point. In cells that form tight junctions, the plasticity of the strands may well be affected by the binding of claudin monomers to scaffolding proteins that contain PDZ domains. Nevertheless, these movies show that claudins can organize themselves into strands that pair with similar strands in apposed cells. And they can form T-junctions with other strands, which resemble those in natural tight junctions.

Reference: Sasaki H, Matsui C, Furuse K, Mimori-Kiyosue Y, Furuse M & Tsukita S (2003) Dynamic behavior of paired claudin strands within apposing plasma membranes. *Proc. Natl Acad. Sci. USA* 100, 3971–3976.

19–36

A. HRP and fluorescein enter both cells at the early two-cell stage (but not at the late two-cell stage) because the cells are still connected by cytoplasmic bridges, which allow the passage of large molecules.
B. Gap junctions form at the compaction stage of embryo development. As a result, fluorescein can enter all cells in the compacted eight-cell embryo. Since gap junctions permit the passage only of molecules less than 1000 daltons or so, HRP is confined to the cell into which it was initially injected. The different results before and after compaction indicate that the formation of gap junctions is associated with compaction.
C. If you were to inject current from the HRP electrode, you would detect it in the fluorescein electrode only in the early two-cell embryo and in the compacted eight-cell embryo. Only at these two stages are the cells electrically coupled. At the two-cell stage, the cytoplasmic bridge remaining from cell division mediates the coupling; at the eight-cell stage, gap junctions mediate the coupling.

Reference: Lo CW & Gilula NB (1979) Gap junctional communication in the preimplantation mouse embryo. *Cell* 18, 399–409.

19–37 The lack of a requirement for the TMV coat protein suggests that the infection is spread by the RNA itself. The data in Table 19–3 do not restrict the possibilities sufficiently to define the mechanism. The increase in the size-exclusion limit to particles with a radius between 2.4 and 3.1 nm in MP^+ cells suggests that the larger size permits the TMV RNA to move through the plasmodesmata. Although you were not given this information, TMV RNA on its own folds into a structure with a radius of about 10 nm, which is too large to fit through the plasmodesmata. An unfolded RNA molecule could fit through a plasmodesma if it were pulled like a string. You might reasonably have suggested that the MP enlarges the plasmodesmata and facilitates the snakelike movement of the RNA through it. Additional experiments have shown that the MP binds to the RNA to form a filament that is 2–3 nm in diameter, which is small enough to fit. The MP apparently interacts with other components of the plasmodesmata to help move the RNA–MP filament.

References: Wolf S, Deom CM, Beachy RN & Lucas WJ (1989) Movement protein of tobacco mosaic virus modifies plamodesmatal size exclusion limit. *Science* 246, 377–379.

Lucas WJ (2006) Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. *Virology* 344, 169–184.

19–38

A. The flow experiments done in the presence of a P-selectin antibody, or with EDTA present, show that the cells bind to the bilayer surface in a way that is expected for P-selectin-mediated binding. Antibody to P-selectin blocks cell binding, as expected. And EDTA, which binds divalent metal ions such as Ca^{2+}, prevents interaction, as it should because P-selectin binding is Ca^{2+} dependent. These two controls encourage the idea that the interactions between the cells and the synthetic lipid bilayer are due to the specific binding of P-selectin to its ligand.

B. Two aspects of the data are consistent with the idea that transient tethering of cells to the bilayer surface is due to single P-selectin–neutrophil interactions. First, the number of tethering events increases in direct proportion to the density of P-selectin in the bilayer. Second, the off rate is unaffected by the density of P-selectin. If tethering depended on a pair of interactions, for example, the number of tethering events would be expected to increase as the square of the density of P-selectin in the bilayer. Similarly, the off rate would be expected to decrease substantially with increasing density of P-selectin.

C. Increasing the flow rate might be expected to increase the off rate by straining the noncovalent bonds holding P-selectin to its ligand. Imagine hanging onto a cliff by your fingertips. If someone else is hanging onto your foot, exerting an additional force by their weight, you will not be able to hold on as long: your off rate will increase.

Reference: Alon R, Hammer DA & Springer TA (1995) Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. *Nature* 374, 539–542.

MEDICAL LINKS

19–39

A. Even though all of the claudin-4 has disappeared, the cells still express claudin-1, which is not affected by the toxin. Using antibodies specific for claudin-1, the authors showed that it remained intact at the sites of the tight junctions in the presence of the toxin.

B. Because the tight junction prevents molecules from penetrating the junction, added toxin will have access to only one side of the junction.

Its inability to work from the apical side suggests that its binding sites on the claudin-4 molecules are accessible only from the basolateral side. If the toxin binds to monomers, as suggested above, then it could be that the monomers are delivered to the basolateral membrane domain, and therefore are accessible only from that side of the epithelial sheet. Alternatively, if the strands of claudin molecules are all oriented in the same way—that is, with their "top" surfaces all facing the apical side and their "bottom" surfaces all facing the basolateral side, as would be expected from symmetry principles—then a toxin-binding site on the bottom surface would only be accessible from the basolateral side.

Reference: Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y & Tsukita S (1999) *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J. Cell Biol.* 147, 195–204.

THE EXTRACELLULAR MATRIX OF ANIMALS

DEFINITIONS

19–40 Collagen

19–41 Matrix metalloprotease

19–42 Laminin

19–43 Glycosaminoglycan (GAG)

19–44 Fibronectin

19–45 Elastin

19–46 Fibroblast

19–47 Basal lamina (basement membrane)

TRUE/FALSE

19–48 False. The extracellular matrix plays an active role influencing the development, migration, proliferation, shape, and metabolism of cells that contact it.

19–49 False. The elasticity of elastin fibers derives from their lack of secondary structure: elastin forms random coils that are easily stretched. The set of hydrogen bonds that stabilizes an α helix is too strong, in aggregate, to be disrupted by the kinds of forces that deform elastin.

19–50 True. There is a single fibronectin gene in the human genome. This gene, which contains about 50 exons, can be spliced in many different ways to produce many different fibronectin isoforms.

19–51 True. The thin, tough, flexible sheet of matrix is an essential underpinning of all epithelia.

19–52 True. The basal lamina that separates the endothelial cells of the blood vessels from the epithelial cells of the kidney serves as a key component of the filter for molecules passing from the blood into the urine. The proteoglycan responsible for this filtering function is perlecan, a heparan sulfate proteoglycan. Gaps in both sheets of cells allow communication across the basal lamina, but also play roles in the filtering process. The glycocalyx of the endothelial cells of the blood vessels provides a crude, first-stage filter, whereas the wondrously designed slit diaphragms

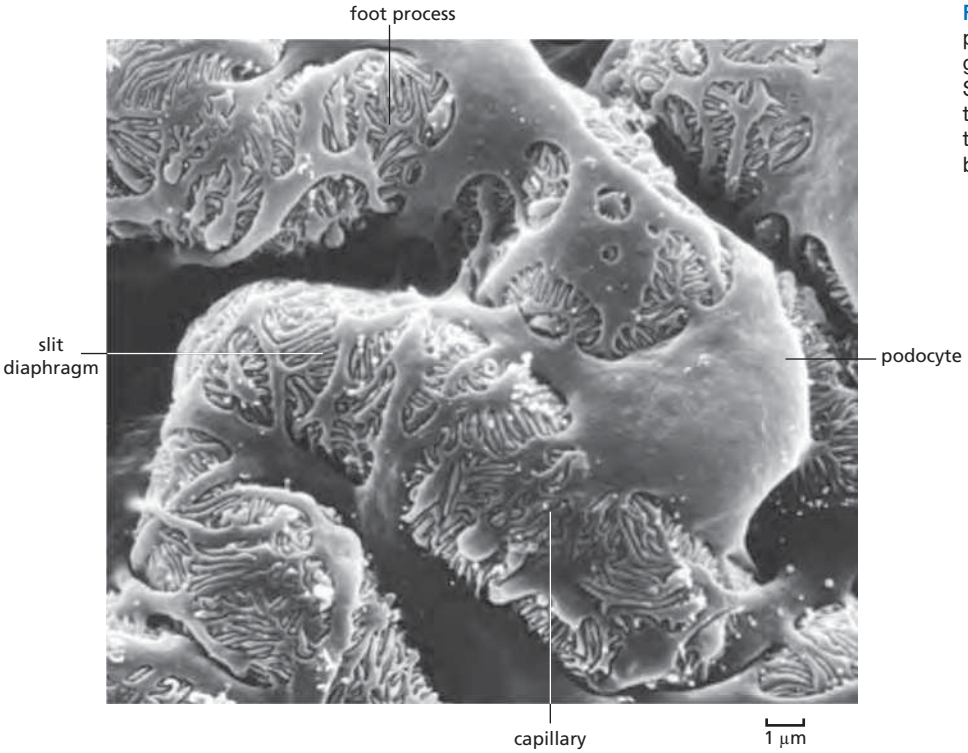


Figure 19–22 Podocytes and their foot processes enveloping a capillary in the glomerulus of the kidney (Answer 19–52). Slit diaphragms are constructed between the interdigitating foot processes of the podocytes. The capillary is hidden beneath the podocyte foot processes.

between the kidney epithelial cells (podocytes) prevent passage of virtually all proteins (**Figure 19–22**).

19–53 True. There are many examples. Congenital muscular dystrophy is caused by a mutation in laminin α-2. Duchenne muscular dystrophy is caused by a defect in dystrophin, an intracellular protein that links dystroglycan to the cytoskeleton. In addition, mutations in dystroglycan, α_7 integrin, and the α chains of type IV collagen all cause muscle-wasting diseases, in which muscles develop normally, but cannot be maintained, perhaps because of gradual muscle damage in the absence of a well-formed basal lamina, or because a defective lamina cannot perform its full range of signaling and survival-promoting functions.

Reference: Sanes JR (2003) The basement membrane/basal lamina of skeletal muscle. *J. Biol. Chem.* 278, 12601–12604.

THOUGHT PROBLEMS

19–54 Individual beads of carboxymethyl Sephadex swell because of osmotic effects. The negative charges on the cross-linked polymer trap an equal number of cations (which are present in the dry gel) to maintain electrical neutrality. These charges—both the fixed negative charges on the polymer and the mobile cations—are confined by electrostatic forces to the volume occupied by the cross-linked polymer. In a solution of pure water, the concentration of particles in the volume of the gel is higher than in the water; hence, the water flows into the gel to try to equalize the concentrations inside and outside the gel bead. The beads swell as the water enters until they reach a maximum size dictated by their cross-linked structure. At that point, the pressure exerted by the cross-linked structure of the polymer opposes further entry of water molecules. It is more common to think of osmotic effects across a semipermeable membrane, but the effects are exactly analogous in a cross-linked gel.

When a salt solution is added to the swollen gel, the osmotic effects are reversed. The concentration of particles in 50 mM NaCl is much higher than in the swollen gel, so water flows out of the gel (and Na^+ and Cl^- ions flow in) to equalize the concentration of ions. In the absence of the osmotic water pressure, the gel shrinks.

19–55 Because the racemization of L-aspartate to D-aspartate occurs slowly, proteins that turn over rapidly will have very low levels of D-aspartate, if it can be detected at all. Proteins that are degraded and replaced more slowly will be expected to have a higher percentage of D-aspartate, with the absolute level depending on the rate of turnover. What makes the observations on elastin remarkable is the age dependence of the D-aspartate levels. These observations have been interpreted to mean that our lifetime supply of elastin is made early on and never degraded. In addition, studies in humans that made use of the inadvertent metabolic ^{14}C-labeling due to atmospheric testing of nuclear weapons, led to the conclusion that elastin synthesis occurs almost exclusively during the fetal and postnatal periods of development. Experiments in mice support this idea.

Reference: Shapiro SD, Endicott SK, Province MA, Pierce JA & Campbell EJ (1991) Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. *J. Clin. Invest.* 87, 1828–1834.

19–56 Marfan's syndrome is caused by defects in the glycoprotein fibrillin, the major component of microfibrils, which binds to elastin and is essential for the integrity of elastic fibers. Patients with mutations in the fibrillin gene are often tall and lanky in appearance.

19–57 The high density of negative charges on the polysaccharide components of proteoglycans causes the sugar chains to be extended, occupying a large volume. The negative charges on the proteoglycans trap an equal number of cations to maintain electrical neutrality. Electrostatic forces confine these charges—both the fixed negative charges on the polysaccharide and the mobile cations—to the volume occupied by the proteoglycan. The concentration of particles in the volume of the proteoglycan is higher than in the surrounding solution; hence, the water flows in to try to equalize the concentrations inside and outside. The proteoglycans thus trap water to form a hydrated gel by drawing in water molecules by osmosis. In the absence of the negative charges, the sugar chains would collapse into fibers or granules, dramatically altering the properties of the extracellular matrix.

19–58

A. The effective pore size is smaller for negatively charged solutes because the basal lamina, itself, is highly negatively charged. These charges come from the glycosaminoglycan polysaccharide chains on its proteoglycans. The negative charge on the polysaccharide components tends to repel negatively charged solutes, making it more difficult for them to penetrate the basal lamina, thereby shrinking the effective pore size.
B. The shape selectivity arises because the pores have fixed dimensions. Elongated molecules can fit through smaller pores than can spherical molecules because they are thinner. By way of analogy, a long string can be threaded through a much smaller hole than can the ball of twine it could be wound into.

19–59 This statement encapsulates our growing recognition of the diverse roles the basal lamina plays. Although it provides structural support for the cells that rest upon it, mechanical stability is only one of the several functions the basal lamina supplies. For example, during the regeneration of

muscles or motor neurons, the neuromuscular junction is reestablished based on information contained in the basal lamina. Special molecules stuck in the basal lamina—like messages on a bulletin board—mark the site of the junction and allow it to be reconstituted exactly. Growing evidence indicates that similar processes occur during the original development of muscle and neuromuscular junctions.

Reference: Sanes JR (2003) The basement membrane/basal lamina of skeletal muscle. *J. Biol. Chem.* 278, 12601–12604.

19–60 The bacteria that secrete such enzymes are typically pathogenic bacteria that digest a protecting layer of basal lamina in order to invade their hosts and infect them.

DATA HANDLING

19–61

A. Hyaluronan synthase can add one residue at a time, as suggested by the results in Figure 19–13B, lane 3, where it has added one GlcNAc unit in the absence of the other activated sugar.

B. The presence of odd-numbered chains implies that hyaluronan synthase adds GlcNAc much more slowly than it adds GlcUA. As a result, the intermediates that end in GlcUA accumulate; they constitute the odd-numbered bands that are visible after chromatographic separation (see Figure 19–13B, lane 2).

C. The tetrasaccharide primer has GlcNAc at the reducing end and GlcUA at the nonreducing end. The only sugar monomer that can be added to this primer is GlcNAc (Figure 19–13B, lanes 3 and 4). Since we know that GlcNAc must be added to GlcUA, which is at the nonreducing end of the primer, hyaluronan synthase must add monomers to the nonreducing end.

Much of the controversy over how hyaluronan synthase generates hyaluronan chains came about because different groups of investigators studied enzymes from different sources—either bacterial or mammalian. Direct comparisons have now shown that the mammalian and bacterial enzymes synthesize their hyaluronan in different ways. It is still not clear how any of these membrane-embedded enzymes manage to synthesize external hyaluronan chains from activated sugar monomers that are made inside the cell.

Reference: DeAngelis PL (1999) Molecular directionality of polysaccharide polymerization by the *Pasteurella multocida* hyaluronan synthase. *J. Biol. Chem.* 274, 26557–26562.

19–62

A. The sticking of cells to the dishes means opposite things in the two experiments. In the first experiment, cell sticking indicates that the peptide is active. In the second experiment, it means the peptide is inactive. In the first experiment, the peptides are stuck to the dish. Only when the peptides contain the active segment will the cells stick to the peptides and, hence, to the dish. In the second experiment, the cells will stick to the fibronectin on the dish *unless* the receptor sites on the cell surface are already occupied by the small peptide, in which case binding to the dish will be inhibited. The two experiments represent alternative ways of measuring the same thing, namely, the specific interaction between a receptor on the cell surface and a ligand in the cell's environment.

B. In the first experiment, the only segments that show activity are those that contain the tripeptide RGD (arginine-glycine-aspartic acid). Peptide 9 shows that changes in this short sequence abolish the binding activity: substituting the bulky valine (V) for the compact glycine (G) inactivates

the peptide. The results of the second experiment confirm those of the first and suggest that the RGD sequence is stringently required for activity. Even the conservative substitution of lysine (K) for arginine (R), or of glutamic acid (E) for aspartic acid (D), abolishes the binding.

C. The specific binding of small peptides suggests the possibility of using affinity chromatography to isolate the fibronectin receptor. This strategy was used successfully to isolate the receptor. A peptide containing the receptor-binding sequence RGD was first attached to a column matrix, in this case Sepharose. Next, detergent-solubilized plasma membrane proteins were passed over the column under conditions that promote binding between the receptor and fibronectin. The column was then washed extensively to remove unbound protein. Finally, the receptor was detached from the column by adding the peptide GRGDSP to the wash buffer. A single 140-kd protein was eluted. (No such band was eluted if GRGESP was used instead.)

References: Pierschbacher MD & Ruoslahti E (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309, 30–33.

Pytela R, Pierschbacher MD & Ruoslahti E (1985) Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* 40, 191–198.

Ruoslahti E & Pierschbacher MD (1986) Arg-Gly-Asp: a versatile cell recognition signal. *Cell* 44, 517–518.

19–63 Mice that are homozygous for knockout of the gene for either nidogen-1 or nidogen-2 presumably have no phenotype because the two forms of nidogen can substitute for one another. Mice that are homozygous for the mutant form of laminin γ-1, which does not bind nidogen, have a much more severe phenotype than either of the individual nidogen gene knockouts because the mutation eliminates the ability of both nidogens to bind to laminin. As a result, these mice do not form proper basal laminae and die at birth with severe defects in kidney and lung. If this is the correct explanation for the genetic observations, then you would predict that mice that are homozygous for knockouts of both nidogen genes would have a very severe phenotype, comparable to that of the laminin γ-1 mutant. Such mice have been made and they do have a severe phenotype.

Reference: Sasaki T, Fassler R & Hohenester E (2004) Laminin: the crux of basement membrane assembly. *J. Cell Biol.* 164, 959–963.

19–64 The answer to this puzzle is not yet known. The basal lamina contains a complicated mixture of molecules including growth factors and growth inhibitors. Failure to cleave collagen (and other components) may lead to failure to release the signaling molecules that are essential for adipocyte development. Alternatively, it may be that the collagen cleavage products, themselves, provide differentiation signals to the preadipocytes. It could also be that these cells fail to correctly process signaling molecules on their own surface; or, because they cannot penetrate the collagen tangle, they do not come into contact with the signaling molecules on the surfaces of other cells. These hypotheses are difficult to test in intact animals, but isolated cells from mutant mice can be used to explore some of these possibilities.

Reference: Chun T-H, Hotary KB, Sabeh F, Saltiel AR, Allen ED & Weiss SJ (2006) A pericellular collagenase directs the three-dimensional development of white adipose tissue. *Cell* 125, 577–591.

MEDICAL LINKS

19–65

A. Since an individual who is heterozygous for a deletion of the *α1(I)* gene has one normal gene, and is missing the other gene entirely, all type I collagen molecules will be normal. However, this individual will make only about half the usual number of collagen molecules.

If the α1(I) collagen chain with the point mutation can be incorporated into a collagen molecule, then only about 25% of the collagen molecules will be normal. The probability of incorporating a normal chain at each of two positions in a type I collagen molecule is (1/2)(1/2) = 1/4. Similarly, there is a probability of 1/4 of incorporating a mutated chain at each of two positions in a type I collagen molecule. Finally, there is a 1/2 chance of incorporating one normal and one mutated chain.

If the mutant α1(I) chain cannot be incorporated into a collagen molecule, then the result will be the same as that described for the gene deletion.

B. A heterozygous individual with one deleted *α1(III)* gene will make 100% normal type III collagen, but in half the usual amount.

If the *α1(III)* gene with the point mutation can be incorporated into a collagen molecule, then only (1/2)(1/2)(1/2) or 1/8 of the type III collagen molecules will be normal. Similarly, 1/8 of the type III collagen molecules will consist of three chains with point mutations, 3/8 will have two normal chains and one chain with a point mutation, and 3/8 will have two mutant chains and one normal chain.

C. The calculations in parts A and B indicate that point mutations are potentially much more severe than gene deletions. If point mutations yield products that are incorporated into collagen molecules, and if the collagen molecules with mutant chains can assemble into collagen fibrils, a heterozygous individual will have vanishingly few completely normal collagen fibrils. By contrast, an individual with one deleted gene will have 50% the usual number of normal collagen molecules and therefore 50% the usual number of normal collagen fibrils. Thus, point mutations are more likely than deletions to be dominant and to display the mutant phenotype.

CELL–MATRIX JUNCTIONS

DEFINITIONS

19–66 Focal adhesion kinase (FAK)

19–67 Anchorage dependence

TRUE/FALSE

19–68 True. Tension—a mechanical signal—applied to an integrin can cause it to tighten its grip on intracellular and extracellular structures, including not only cytoskeletal and matrix components, but also intracellular molecular signaling complexes. Similarly, loss of tension can loosen its hold, so that molecular signaling complexes fall apart on either side of the membrane. Thus, the tension on the integrin can trigger or inhibit molecular signaling.

19–69 False. Of the 24 or so different kinds of integrins in humans, all but one are linked to actin filaments. The remaining variety connects to the intermediate filament network formed by keratin.

19–70 False. Integrins are dynamic molecules that fold to hide their binding sites in the absence of strong intracellular or extracellular ligands.

THOUGHT PROBLEMS

19–71 The high level of activation when alanine was substituted for D723 in the β chain, or for R995 in the α chain, indicates that those residues are somehow important for holding the $\alpha_{IIb}\beta_3$ integrin in an inactive state. The "charge-swap" experiment, which showed that D723R paired with R995D was as inactive as the wild type, suggests strongly that these two residues form an electrostatic attraction—a salt bridge—that helps to hold $\alpha_{IIb}\beta_3$ integrin in its inactive configuration. It follows that inside-out signaling is probably triggered by breaking this salt bridge.

Reference: Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ & Ginsberg MH (1996) Breaking the integrin hinge: A defined structural constraint regulates integrin signaling. *J. Biol. Chem.* 271, 6571–6574.

CALCULATIONS

19–72 Assuming that the area of a platelet can be approximated as the areas of two circles, each 2 μm in diameter, the surface area of a platelet is $2 \times \pi r^2 = 6.3\ \mu m^2$, which is $6.3 \times 10^6\ nm^2$. At 80,000 integrins per platelet, each integrin is allotted $78.8\ nm^2$ ($6.3 \times 10^6\ nm^2/80{,}000$ integrins). Assuming that each integrin is 10 nm in diameter, the cross-sectional area of an integrin is $78.5\ nm^2$ ($\pi \times 5^2$). With the assumptions stated in this problem, then, integrins would be cheek by jowl in the membranes of platelets. Regardless of the specific assumptions, integrins clearly occupy a large fraction of the surface area of platelets, as befits their critical role in platelet function.

DATA HANDLING

19–73

A. The $\alpha_{IIb}\beta_3$ integrin can exist in two conformations: one with very low affinity for fibrinogen and the other with very high affinity. Evidently, the cytoplasmic domain of the α_{IIb} chain controls the affinity of this integrin. In CHO cells, the cytoplasmic domain holds the integrin in its low-affinity conformation. Truncation of the cytoplasmic domain allows the integrin to flip into its high-affinity conformation.

B. If the affinity of the $\alpha_{IIb}\beta_3$ integrin is regulated by the cytoplasmic domain of α_{IIb}, as suggested by the answer to part A, then the factors that stimulate clotting presumably initiate a cell-signaling pathway that alters the affinity of the integrin via effects on the cytoplasmic domain of α_{IIb}. Thrombin has no effect on CHO cells that display $\alpha_{IIb}\beta_3$ integrin on their surface because the thrombin receptor or a portion of the thrombin-induced signaling pathway is missing in CHO cells.

C. Individuals who carry one gene for α_{IIb} with a mutation like the one described in this problem would be expected to have blood-clotting problems. Although half the $\alpha_{IIb}\beta_3$ integrin in their platelets would be normal, half would be expected to have a high affinity for fibrinogen and cause platelet aggregation and blood clotting in the absence of thrombin stimulation. Thus, such a mutation would be expected to be dominant and cause serious problems, even in individuals who are heterozygous for the mutation.

Reference: O'Toole TE, Mandelman D, Forsyth J, Shattil SJ, Plow EF & Ginsberg MH (1991) Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of α_{IIb}. *Science* 254, 845–847.

THE PLANT CELL WALL

DEFINITIONS

19–74 Primary cell wall

19–75 Cellulose microfibril

19–76 Turgor pressure

19–77 Lignin

19–78 Secondary cell wall

TRUE/FALSE

19–79 False. A mature cell may simply retain the primary cell wall or, far more commonly, produce a rigid secondary cell wall either by thickening the primary wall or by depositing new layers with a different composition underneath the old ones, so that the secondary cell wall lies inside the primary cell wall.

19–80 True. Without turgor pressure, growing cells could not expand to their full size, and mature plants would wilt.

19–81 False. Although the major components of plant cell walls are polysaccharides, structural proteins make a small contribution (on the order of 5% of the dry mass of the cell wall). These proteins are thought to strengthen the wall. They are also produced in higher amounts as a local response to attack by pathogens.

19–82 False. In the absence of cortical microtubules, new cellulose microfibrils tend to be deposited in the same orientation as the preexisting microfibrils. The cortical array of microtubules seems to be critically important for the initial deposition of cellulose microfibrils, as well as for any developmentally programmed change in orientation.

THOUGHT PROBLEMS

19–83 If you soak the lettuce in tap water, it will take up water due to osmosis and become crisper. Soaking the lettuce in salt water or sugar water will have the opposite effect, sucking even more water out of the lettuce, making it even limper. Your day-old lettuce is long past the point where photosynthesis can do it any good, and the bright light will dry it out even more.

19–84 The orientation of cellulose microfibrils follows the orientation of the cortical array of microtubules. Thus, in gibberellic-acid-treated cells the cellulose microfibrils will be oriented perpendicular to the long axis of the cell, and in ethylene-treated cells the microfibrils will be oriented parallel to the long axis. Because cell elongation occurs perpendicular to the orientation of the cellulose microfibrils, gibberellic-acid-treated cells will elongate in the direction of the long axis of the cell, producing long, thin shoots. By contrast, ethylene-treated cells will elongate perpendicular to the long axis, producing short, fat shoots.

CALCULATIONS

19–85 At 0.1 MPa the hydraulic conductivity of a single aquaporin water channel is 4.4×10^{-23} m^3/s [$(4.4 \times 10^{-22}$ m^3/s MPa) $\times$ 0.1 MPa = 4.4×10^{-23} m^3/s]. Thus, the question becomes how many water molecules are in 4.4×10^{-23} m^3. There are 3.33×10^{28} water molecules/m^3

[(55.5 moles/L)(10^3 L/m^3)(6×10^{23} water molecules/mole)]. Therefore, 1.5×10^6 water molecules flow through a water channel each second at 1 atmosphere of pressure [(4.4×10^{-23} m^3/s)(3.33×10^{28} water molecules/m^3)].

Reference: Tyerman SD, Bohnert HJ, Maurel C, Steudle S & Smith JAC (1999) Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. *J. Exp. Bot.* 50, 1055–1071.

DATA HANDLING

19–86 The 83-kd band in Figure 19–17B is the cellulose synthase. Several experimental results point to this conclusion. First, it is the only band that is labeled in the purified fraction. A faint band at the same position is visible in the soluble fraction in the presence of cyclic di-GMP, suggesting that the 83-kd protein is also present at lower concentration (or activity) in the soluble fraction. Second, addition of cyclic di-GMP increases its activity by about 50%. Presumably, the effect of added cyclic di-GMP is not greater because there is already some present in the enzyme preparation. Third, the 83-kd band is labeled only when it is exposed to UV light. By contrast, the 57-kd band is labeled in the presence or absence of UV light. Finally, excess UDP-glucose, the normal substrate, inhibits labeling of the 83-kd band. Labeling of the 57-kd band is not affected by excess UDP-glucose. These results argue convincingly that the 83-kd band is cellulose synthase.

Additional studies revealed that the 57-kd band was phosphoglucomutase, which catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate. The enzyme catalyzes this reaction by forming a covalent intermediate with the substrate, which is the basis for the cross-linking that does not depend on UV light. It is thought that the substrate for this reaction arises from the breakdown of ^{32}P-azido-UDP-glucose, or as a contaminant in the preparation of the photoaffinity label.

Reference: Lin FC, Brown RM, Drake RR & Haley BE (1990) Identification of the uridine 5′-diphosphoglucose (UDP-Glc) binding subunit of cellulose synthase in *Acetobacter xylinum* using the photoaffinity probe 5-azido-UDP-Glc. *J. Biol. Chem.* 265, 4782–4784.

MCAT STYLE

19–87

A. The best hypothesis is that the loss of E-cadherin frees up proteins that were bound to it, and that those proteins, in their free state, promote metastasis. This hypothesis is consistent with the observation that cells that express only 10% of the normal amount of E-cadherin lack cell adhesion and metastasize. There is not a sufficient number of E-cadherin molecules to permit cell adhesion, and the proteins that would have been bound are free to promote metastasis. The highly expressed cytoplasmic domain of the mutant E-cadherin prevents metastasis by binding to proteins that normally bind to the cytoplasmic domain of full-length E-cadherin and, as a result, it blocks adhesion with other tumor cells. It also keeps these proteins in a bound state, preventing them from acting to promote metastasis. Choice B is not correct because the cytoplasmic domain must bind to proteins that are required for both cell adhesion and metastasis. Overexpression of the cytoplasmic domain prevents cell adhesion by binding to the proteins that would normally bind to E-cadherin. However, the overexpressed cytoplasmic domain also occupies those same proteins so that they cannot act as free entities to promote metastasis. Choice C is incorrect because the transmembrane domain alone is not sufficient to promote cell–cell adhesion, since the normal

expression of full-length E-cadherin was not sufficient to cause cell–cell adhesion in the presence of the overexpressed cytoplasmic domain. Choice D is not correct because both cell lines lost cell adhesion, but only one metastasized. Thus, loss of adhesion alone is not sufficient to explain the effects of the E-cadherin mutations.

Reference: Onder TT, Gupta PB, Mani SA, Yang J, Lander ES & Weinberg RA (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res.* 68, 3645–3654.

19–88

A. Catenins bind to the cytoplasmic domain of cadherins and link them to the cytoskeleton, thereby mediating signaling by cadherins. None of the other proteins are involved in cadherin signaling.

19–89

B. Collagen and laminin are components of the extracellular matrix that are secreted from cells. By contrast, integrin is an integral membrane protein that mediates attachment to the extracellular matrix; it is not a component of the matrix.

19–90

C. Cells from line C proliferate in 3D culture conditions, and they form more extensive contacts with the Matrigel matrix than the other cell types. These observations suggest that cell line C expresses adhesion molecules that can interact with components of the 3D matrix. Combined with the cells' ability to metastasize, it seems likely that these adhesion molecules send signals that drive the abnormal cell proliferation. Together, these observations suggest that the ability of cell line C to undergo metastasis is due, at least in part, to the cells' adhesive properties. Choice A is not correct because it would not explain the unique properties of cell line C. Choice B is unlikely since the Matrigel contains a rich mixture of matrix components, and there are no known matrix components that promote cell metastasis. Choice D is not correct because the data suggest that cells from line C have the ability to interact with the extracellular matrix.

Reference: Shibue T & Weinberg RA (2009) Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc. Natl Acad. Sci. USA* 106, 10290–10295.

19–91

C. Integrin is a transmembrane adhesion molecule with an intracellular domain that plays diverse roles in signaling to the inside of the cell. The other molecules are secreted proteins found in the extracellular matrix. Since all three cell lines were exposed to the same extracellular matrix in the Matrigel, the components of the extracellular matrix cannot be involved in defining the individual behaviors of the cell lines. Analysis of the C cell line confirmed that it alone expressed an integrin that could be responsible for their unique behavior.

CHAPTER 20

Cancer

IN THIS CHAPTER

CANCER AS A MICROEVOLUTIONARY PROCESS

DEFINITIONS

20–1 Carcinogenesis

20–2 Malignant

20–3 Sarcoma

20–4 Primary tumor

20–5 Tumor progression

20–6 Genetically unstable

20–7 Benign

20–8 Replicative cell senescence

20–9 Carcinoma

20–10 Somatic mutation

TRUE/FALSE

20–11 False. A carcinoma consists of a variety of normal cells, along with the cancer cells. The fibroblasts in the supporting connective tissue, the associated inflammatory cells, and the cells of the newly formed blood vessels are all normal cells that are present because they are in contact with the cancer cell mass or have been recruited into the tumor. These normal cells do not evolve from the cancer cell population.

20–12 False. There is an optimum level of genetic instability for the development of cancer. A cell must be mutable enough to evolve rapidly, but not so mutable that it accumulates too many harmful changes and dies.

20–13 False. Cancer cells consume glucose much more rapidly than normal cells to provide the raw materials they need for synthesis of proteins, nucleic acids, and lipids. The cancer cells could easily meet their energy needs by oxidative phosphorylation, which would require much smaller amounts of glucose.

THOUGHT PROBLEMS

20–14 The incidence of cancer increases dramatically with age because it takes mutations in several critical genes to disable a cell's normal mechanisms for controlling its growth. Since growing cells continually accumulate mutations, which they pass on to their progeny cells, the chance

that a particular cell lineage will accumulate a critical set of mutations increases with age. The steep rise in cancer incidence in older women seen in Figure 20–1 reveals that colon cancer increases as the sixth power of age, suggesting that it arises only after mutations have occurred in six or so genes that regulate cell growth in the colon.

20–15 The key difference in the incidences of colon cancer and osteosarcomas is the size of the population of cells at risk for the disease. Colon cancer arises from the population of proliferating cells in the colon, which are present in roughly the same number throughout life. This population can accumulate mutations over time, giving rise to an abnormal lineage of cells that progresses to full-blown cancer with the age-dependence seen in Figure 20–1. By contrast, the cells responsible for osteosarcomas are present in much greater numbers during adolescence, when their proliferation is required to increase the size of the skeleton, than they are in young children or adults. It is in this large, proliferating population that an abnormal lineage of cancer cells is most likely to arise. In this case, it is the number of cells at risk that is the most important determinant of the frequency of cancer.

Reference: Knudson AG (2001) Two genetic hits (more or less) to cancer. *Nat. Rev. Cancer* 1, 157–162.

20–16 The time at which the death rates due to breast and cervical cancer slow corresponds to menopause, at which time the production of estrogen declines. Estrogen normally promotes the proliferation of cells in the breast and uterus. Thus, the decline in estrogen would reduce the population of proliferating cells, thereby reducing the risk of cancer in these tissues.

Reference: Armitage P & Doll R (1954) The age distribution of cancer and a multi-stage theory of carcinogenesis. Reprinted in (2004) *Br. J. Cancer* 91, 1983–1989.

CALCULATIONS

20–17 A tumor that arose from 50 cell doublings would contain 2^{50} cells, which is 1.1×10^{15} cells. If 10^8 cells is 1 gram, then the tumor would weigh 1.1×10^7 g, which is 24,000 pounds. Thus, a limit of 50 cell divisions, by itself, does not provide much protection against cancer. Even in a 40-year-old, whose fibroblasts divide about 40 times in culture, a tumor arising from 40 cell divisions would weigh more than 20 pounds.

This calculation assumes that all cells survive. It is likely, however, that many cells die by apoptosis, especially at early stages in the evolution of cancer. In combination with extensive cell death, a limit of 50 cell divisions could protect against cancer.

20–18

A. The cell-cluster model for cancer formation predicts an exponential relationship between chemical carcinogen concentration and cancer formation. In the expression Nx^n, the concentration of carcinogen would influence the probability of mutation, x, so that 1, 2, and 4 times the chemical carcinogen concentration would give $1x$, $2x$, and $4x$ probabilities of mutation, respectively. The numbers of cancers should then be $N(1x)^n$, $N(2x)^n$, and $N(4x)^n$, respectively. To illustrate this with numbers, let $N = 10^9$, $x = 10^{-2}$, and $n = 5$, as they are in the body of the problem. Substituting these numbers gives 0.1, 3.2, and 102.4 cancer clusters for 1, 2, and 4 times the carcinogen concentration. This predicted exponential dependence on carcinogen concentration does not match the linear dependence observed experimentally.

B. The tumor-progression model for cancer formation predicts a linear relationship between chemical carcinogen concentration and cancer formation, so long as the frequency of mutation is low. A slowly progressing tumor presents a moving target for mutation. At an early time, say when it has one mutation, carcinogen treatment can stimulate the acquisition of a second mutation in one of its cells, allowing that cell to progress to the next stage. It is unlikely, however, to introduce two mutations into one cell in the small population of cells that constitutes the early tumor. At a later stage, when the tumor cells have, say, three changes, the same argument applies: the carcinogen can realistically induce an additional mutation in only one of its cells. Induction of one mutation at a time gives a linear dependence on carcinogen concentration.

References: Fisher JC & Hollomon JH (1951) A hypothesis for the origin of cancer foci. *Cancer* 4, 916–918.

Armitage P & Doll R (1954) The age distribution of cancer and a multistage theory of carcinogenesis. Reprinted in 2004 in *Br. J. Cancer* 91, 1983–1989.

DATA HANDLING

20–19 Development of most cancers requires a gradual accumulation of mutations in several different genes. In the ongoing presence of cigarette smoke, these mutations evidently accumulate at an increased rate (over their accumulation in the absence of cigarette smoke). By stopping smoking, an individual returns to the normal, slower rate of mutation accumulation. Thus, whatever mutations remain to be generated in a reformed smoker are generated at a slower rate than in a continuing smoker. The slower rate of accumulation of mutations translates into a lower cumulative risk.

Reference: Peto R, Darby S, Deo H, Silcocks P, Whitley E & Doll R (2000) Smoking, smoking cessation, and lung cancer in the UK since 1950: combination of national statistics with two case-control studies. *Br. Med. J.* 321, 323–329.

MEDICAL LINKS

20–20 Although the numbers are small, there seems to be a reasonable correlation between smoking and lung cancer. Statistical analysis shows that these differences are significant ($P = 0.04$). Similarly, the consistent upward trend in heart attacks with increased smoking also turns out to be statistically significant ($P = 0.03$). Follow-up studies on this cohort (the most recent after 50 years) have confirmed the initial impression conveyed by the original, preliminary study. Compared to lifelong nonsmokers, lung cancer is 8-fold, 14-fold, and 25-fold more prevalent among smokers of 1–14, 15–24, and more than 25 cigarettes per day, respectively. Moreover, in these longer studies it is clear that other cancers—for example, mouth, nose, throat, stomach, liver, kidney, and bladder—are also significantly elevated in smokers, although not so dramatically as the incidence of lung cancer.

References: Doll R & Hill AB (1954) The mortality of doctors in relation to their smoking habits: A preliminary report. Reprinted in 2004 in *Br. Med. J.* 328, 1529–1533.

Doll R, Peto R, Boreham J & Sutherland I (2004) Mortality in relation to smoking: 50 years' observations on male British doctors. *Br. Med. J.* 328, 1519–1528.

CANCER-CRITICAL GENES: HOW THEY ARE FOUND AND WHAT THEY DO

DEFINITIONS

20–21 Oncogene

20–22 Proto-oncogene

20–23 *p53*

20–24 Cancer stem cell

20–25 Retinoblastoma

20–26 Colorectal cancer

20–27 Cancer-critical gene

20–28 Tumor suppressor gene

20–29 Passenger

TRUE/FALSE

20–30 False. Oncogenes, which are mutated, overactive forms of proto-oncogenes, can be detected in this way. Their addition to the genome can transform a cell to a cancer cell. By contrast, tumor suppressor genes have their effects because they are inactive; it is their absence that causes cancer. One cannot use the same sort of transformation assay to detect something that is not there.

20–31 False. It is not that DMBA is a specific mutagen, but rather that the *Ras* gene is converted to its activated, cancer-causing form by a particular A-to-T alteration that leads to a very specific amino acid change. DMBA causes mutations throughout the genome, but only those at the specific site in the *Ras* gene give rise to cells that have cancerous properties and thus are identified in the assay.

20–32 True. An inherited predisposition to cancer often occurs because one copy of a tumor suppressor gene is mutated. Heterozygous cells, which retain one wild-type copy of the gene, are fine, but they are at increased risk for cancer because a single event (rather than the two in a normal individual) can inactivate the remaining good copy, causing loss of heterozygosity.

20–33 True. That is why oncogenes in their overactive, mutant form tend to drive cell growth and proliferation, and why the loss of tumor suppressor genes removes regulatory brakes on these pathways, which also promotes cell growth and proliferation.

20–34 False. Although cancer cells from individual patients commonly have a mutation in the Rb pathway, they rarely have mutations in more than one component of the same regulatory pathway. Mutation in any one component of a pathway is usually sufficient to inactivate the pathway and promote cancer. Inactivation of more than one component in a pathway would have no benefit for the cancer's evolution.

20–35 True. p53 protein normally acts to limit the harm done by DNA damage. Cells that are severely damaged are driven to commit suicide by apoptosis; mildly damaged cells are prevented from dividing until the damage is repaired. In the absence of p53, these two safeguards are eliminated, allowing some cancer cells to proliferate even when exposed to the damaging effects of irradiation and many anticancer drugs.

20–36 True. For example, in ovarian cancers, chromosome breaks, translocations, and deletions are very common, whereas in glioblastomas point mutations are scattered throughout the genome. The basis for the different modes of instability in different tissues is not understood.

20–37 False. Activation of a single oncogene is generally not sufficient to convert a normal cell into a cancer cell. Typically, in mice engineered to express an oncogene such as *Myc* or *Ras*, cells in some tissues that express the oncogene show enhanced proliferation, and, over time, occasional cells will undergo further changes to give rise to cancers.

20–38 True. Many cancers appear to be maintained by a small population of cancer stem cells. These cells usually divide more slowly than the cells in the bulk of the tumor, and they are less sensitive to treatments aimed at rapidly dividing cells. If the stem cells are not killed, the cancer is likely to return.

THOUGHT PROBLEMS

20–39 Oncogenes correspond to stuck accelerators. In their mutated, overactive form they drive a cell to proliferate in a way that is not responsive to normal controls. Defective tumor suppressor genes correspond to broken brakes. They normally function to inhibit steps in regulatory pathways; that is, to act as brakes. When tumor suppressor genes are defective, the pathways are unrestrained. Defective genome maintenance genes correspond to bad mechanics. These genes normally operate to maintain the genome during its propagation and in the face of DNA damage. Defective genome maintenance genes lead to genetic rearrangements or increased point mutations, either of which can convert a proto-oncogene to an oncogene or eliminate a tumor suppressor gene.

Reference: Vogelstein B & Kinzler KW (2004) Cancer genes and the pathways they control. *Nat. Med.* 10, 789–798.

20–40 Antiproliferative genes such as *Rb* encode proteins that stop the cell cycle. During normal cell division, these proteins must be turned off. If they were overexpressed in all cells, it is likely that the machinery that keeps these proteins turned off would be overwhelmed, and cell division would stop. Thus, this cure for cancer might be successful but the patient would be dead.

20–41 Cancer cells have additional changes that typically disable cell-cycle checkpoints and apoptotic mechanisms. In the absence of these regulatory controls, which are fully operational in normal cells, overexpression of Myc drives cell growth and proliferation of cancer cells.

20–42 Mutant B-Raf with glutamate at position 599 is thought to be active because it carries a negative charge ($-COO^-$) in the same region of the protein at which a negative charge ($-PO_4^-$) is normally introduced by Ras-induced phosphorylation. Evidently, the negative charge alters the conformational equilibrium of B-Raf, shifting it toward the active form. It is common practice these days to test whether a phosphate at a site activates (or inactivates) a protein by engineering a version of the protein with a glutamate at that position. Often (but not always) the glutamate change will mimic the effect of the phosphate.

Reference: Wang L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, Burgart LJ, McDonnell SK, Schaid DJ & Thibodeau SN (2003) *BRAF* mutations in colon cancer are not likely attributable to defective DNA mismatch repair. *Cancer Res.* 63, 5209–5212.

20–43 These observations argue strongly that MMTV generates an oncogene upon integration into the mouse genome. It is extremely unlikely that

MMTV would integrate so often in the same region of the genome by chance. Moreover, a unique transcript is generated in the region of the integrated virus, suggesting that a gene is turned on in response to the neighboring viral sequences.

These results summarize the initial characterization of the *Int1* locus in mice. It was later shown to be homologous to the *wingless* locus in *Drosophila*, both of which are now referred to as *Wnt* genes. These genes secrete a molecule that triggers a signaling pathway in other cells and activates expression of a set of Wnt-responsive genes, some of which promote cell proliferation.

Reference: Nusse R & Varmus HE (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31, 99–109.

CALCULATIONS

20–44

A. Four different haploid gametes can combine to give 16 different diploid products. Of the several different ways of representing these possibilities, the most concise is the Punnett square, a time-honored tradition with geneticists (**Table 20–3**). However the possibilities are represented, the 16 combinations of gametes form 9 distinct genotypes. Four genotypes are represented once (the ones along the diagonal from upper left to lower right in Table 20–3). One genotype is represented four times (the one along the diagonal from lower left to upper right). The remaining genotypes are each represented twice (symmetrically arrayed about the diagonal from upper left to lower right). The ratios of the genotypes, along with their expected frequencies among 36 progeny, are given in **Table 20–4**.

Progeny with the genotypes $p53^{+/+}$ $Mdm2^{-/-}$ and $p53^{+/-}$ $Mdm2^{-/-}$ are underrepresented in these experiments: none were found. More detailed experiments have shown that these mice do not survive because they die early in embryonic development.

B. It is striking that $p53^{+/+}$ $Mdm2^{-/-}$ and $p53^{+/-}$ $Mdm2^{-/-}$ do not survive, whereas $p53^{-/-}$ $Mdm2^{-/-}$ mice do. In the absence of Mdm2, it seems that even a haploid amount of p53 is lethal, but the complete absence of p53 renders the mice viable. Since Mdm2 is a ubiquitin ligase, one reasonable explanation for this result is that Mdm2 normally keeps the cellular concentration of p53 very low by targeting it for destruction. In the absence

TABLE 20–3 A Punnett square showing all possible genotypes resulting from random assortment of *p53 Mdm2* gametes (Answer 20–44).

		Female			
	Gametes	$p53^{+}Mdm2^{+}$	$p53^{+}Mdm2^{-}$	$p53^{-}Mdm2^{+}$	$p53^{-}Mdm2^{-}$
Male	$p53^{+}Mdm2^{+}$	$p53^{+}Mdm2^{+}$ $p53^{+}Mdm2^{+}$	$p53^{+}Mdm2^{+}$ $p53^{+}Mdm2^{-}$	$p53^{+}Mdm2^{+}$ $p53^{-}Mdm2^{+}$	$p53^{+}Mdm2^{+}$ $p53^{-}Mdm2^{-}$
	$p53^{+}Mdm2^{-}$	$p53^{+}Mdm2^{-}$ $p53^{+}Mdm2^{+}$	$p53^{+}Mdm2^{-}$ $p53^{+}Mdm2^{-}$	$p53^{+}Mdm2^{-}$ $p53^{-}Mdm2^{+}$	$p53^{+}Mdm2^{-}$ $p53^{-}Mdm2^{-}$
	$p53^{-}Mdm2^{+}$	$p53^{-}Mdm2^{+}$ $p53^{+}Mdm2^{+}$	$p53^{-}Mdm2^{+}$ $p53^{+}Mdm2^{-}$	$p53^{-}Mdm2^{+}$ $p53^{-}Mdm2^{+}$	$p53^{-}Mdm2^{+}$ $p53^{-}Mdm2^{-}$
	$p53^{-}Mdm2^{-}$	$p53^{-}Mdm2^{-}$ $p53^{+}Mdm2^{+}$	$p53^{-}Mdm2^{-}$ $p53^{+}Mdm2^{-}$	$p53^{-}Mdm2^{-}$ $p53^{-}Mdm2^{+}$	$p53^{-}Mdm2^{-}$ $p53^{-}Mdm2^{-}$

TABLE 20–4 Actual and expected genotypes of progeny mice from crosses between doubly heterozygous *p53*$^{+/-}$ *Mdm2*$^{+/-}$ mice (Answer 20–44).

Genotype	Progeny mice (number)	Progeny mice (expected ratios)	Progeny mice (expected numbers)
p53$^{+/+}$ *Mdm2*$^{+/+}$	3	1	2 ¼
p53$^{+/+}$ *Mdm2*$^{+/-}$	5	2	4 ½
p53$^{+/+}$ *Mdm2*$^{-/-}$	0	1	2 ¼
p53$^{+/-}$ *Mdm2*$^{+/+}$	7	2	4 ½
p53$^{+/-}$ *Mdm2*$^{+/-}$	11	4	9
p53$^{+/-}$ *Mdm2*$^{-/-}$	0	2	4 ½
p53$^{-/-}$ *Mdm2*$^{+/+}$	1	1	2 ¼
p53$^{-/-}$ *Mdm2*$^{+/-}$	7	2	4 ½
p53$^{-/-}$ *Mdm2*$^{-/-}$	2	1	2 ¼

of Mdm2, p53 is no longer destroyed at the proper rate and accumulates to levels that are toxic. The basis for toxicity in this instance is not entirely clear, but it is thought that the abnormally high amounts of p53 arrest the cell cycle and promote apoptosis.

References: Montes de Oca Luna R, Wagner DS & Lozano G (1995) Rescue of early embryonic lethality in *mdm2*-deficient mice by deletion of *p53*. *Nature* 378, 203–206.

Jones SN, Roe AE, Donehower LA & Bradley A (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 378, 206–208.

DATA HANDLING

20–45

A. Most of the 463,248 sequence changes that remained after the reading errors were removed have nothing to do with cancer. The authors of this study applied six additional filters to eliminate sequence changes that are unlikely to contribute to the functional differences between the normal cells and the tumor cells. See if your suggestions are included in this list.

1. Filter out changes that do not alter the encoded amino acid sequence; for example, mutations to synonymous codons. (259,957 changes were eliminated by this criterion.)
2. Filter out changes that are also present in the DNA from the two normal individuals that were included in the analysis. (163,006 changes were eliminated by this criterion.)
3. Filter out changes that correspond to known sequence polymorphisms in the human population. (11,004 changes were eliminated by this criterion.)
4. Filter out changes that cannot be confirmed upon reamplifying and resequencing the sample. (Of the 29,281 sequence differences that remained after applying the above filters, 9295 were not confirmed and therefore eliminated.)
5. Filter out changes that are also present in normal tissue from the same individual that had the tumor. (18,414 of the 19,986 sequence differences that remained after applying filter 4 were eliminated by this criterion.)

6. Filter out changes in sequences that have closely related sequences elsewhere in the genome. There can be problems deciding which genomic location is the true source of the sequence read. (265 of the remaining 1572 sequence differences were eliminated by this criterion, leaving 1307 potential cancer-relevant mutations.)

B. Deciding which of these 1307 mutations are likely to contribute to the cancers is not an easy task. One approach is to look for mutant genes that are found in multiple breast tumors or in multiple colorectal tumors. The underlying assumption is that similar cancers should have similar sets of causative mutations. The authors used this sort of analysis to identify roughly 12 cancer-related mutations in breast tumors, and about 9 in colorectal tumors. (The rest of the mutations are likely to be passenger mutations.) Because only a little more than half the protein-coding genes in the genome were analyzed (13,023/25,000), the real number of cancer-relevant mutations in these tumors may be closer to 20.

C. The sequencing strategy used here—amplifying and sequencing exons—was designed to detect small changes in sequence: point mutations and short deletions. Larger deletions and gene rearrangements would not be detected because they would not give an informative PCR product.

Reference: Sjöblom T, Jones S, Wood LD et al. (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* 314, 268–274.

20–46

A. An *ARF* knockout mouse would be expected to be more prone to cancer than a wild-type mouse. In the absence of ARF, Mdm2 would be more active than in a wild-type mouse. Overactive Mdm2 would, in turn, tend to repress p53 activity more than normal. Thus, the consequence of an *ARF* knockout would be reduced p53 activity. If this lowered activity impaired the ability of p53 to force abnormal cells to undergo cell-cycle arrest or apoptosis, more precancerous cells would escape detection and more tumors would form.

B. A $p53^{+/+}$ $Mdm2^{-/-}$ mouse will not be rescued by knockout of the *ARF* gene. In the absence of Mdm2, the activity of p53 will be maximized. Because Mdm2 is absent, the link between ARF and p53 is missing. Thus, no change in ARF levels can affect p53 activity, and $p53^{+/+}$ $Mdm2^{-/-}$ $ARF^{-/-}$ mice will die just like $p53^{+/+}$ $Mdm2^{-/-}$ mice do.

C. Mice that express the *Myc* oncogene will overstimulate ARF activity, which will decrease Mdm2 activity, which will cause an increase in p53 activity. Increased p53 activity (so long as it is not increased to the point where it is toxic—see Problem 20–44) will tend to increase the mouse's ability to force abnormal cells into cell-cycle arrest and apoptosis. This increased activity of p53 operates in opposition to the proliferation-promoting activity of the *Myc* oncogene. In an $ARF^{+/-}$ mouse there is less ARF, hence, less of a decrease in Mdm2 activity and less of an increase in p53 activity. Because the proliferation-promoting activity of the *Myc* oncogene is opposed to a lesser extent (by the lower p53 activity), $ARF^{+/-}$ mice generate tumors more quickly and die at a younger age.

References: Quelle DE, Zindy F, Ashmun RA & Sherr CJ (1995) Alternative reading frames of the *INK4a* tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 83, 993–1000.

Zindy F, Williams RT, Baudino TA, Rehg JE, Skapek SX, Cleveland JL, Roussel MF & Sherr CJ (2003) *Arf* tumor suppressor promoter monitors latent oncogenic signals *in vivo*. *Proc. Natl Acad. Sci. USA* 100, 15930–15935.

20–47

A. As also discussed in Problem 20–51, the absence of a shoulder on any of the three curves suggests that in all cases only a single event is needed to

trigger tumor production in mice that are already expressing one or both oncogenes.

B. Although the rate of tumor production is much higher in mice with both oncogenes, activation of the cellular *Ras* gene cannot be a required event in the production of tumors in mice that are already expressing the *Myc* oncogene. Nor can activation of the cellular *Myc* gene be a required event in triggering tumor formation in mice that are already expressing the *Ras* oncogene. As indicated in part A, even when mice express both *Myc* and *Ras*, some additional event is required to produce a tumor. If expression of *Myc* plus *Ras* were sufficient for tumor formation, then all mice containing both oncogenes would develop tumors as soon as they passed through puberty and turned on their expression.

C. The rate of tumor production in mice with both oncogenes is much higher than expected if the effects of the individual oncogenes were additive. Thus, the two oncogenes together have a synergistic effect on the rate of tumor production. As argued in part B, activation of both oncogenes is not sufficient to generate a tumor. Thus, the two oncogenes acting together must open up a pathway to tumor production that can be triggered by any one of several low-frequency events or that can be triggered by one very common event. The nature of the activating events is unclear for these transgenic mice, but analysis of transformed cells suggests that inactivation of the p53 tumor suppressor pathway may be responsible.

Reference: Sinn E, Muller W, Pattengale P, Tepler I, Wallace R & Leder P (1987) Coexpression of MMTV/v-Ha-*ras* and MMTV/c-*myc* genes in transgenic mice: synergistic action of oncogenes *in vivo*. *Cell* 49, 465–475.

20–48

A. The formation of the three types of patches observed in speckled kernels is shown in **Figure 20–19**. The formation of a colorless, nonwaxy (*c-Wx*) patch results from a breakage that eliminates the dominant color (*C*) allele (Figure 20–19A). In the absence of the dominant allele, the color of the patch is determined by the recessive colorless (*c*) allele on the normal chromosome (which is not shown in the figure).

The formation of a colorless, waxy (*c-wx*) spot in a colorless, nonwaxy (*c-Wx*) patch is due to a second breakage event that eliminates the dominant nonwaxy (*Wx*) allele (Figure 20–19B). In the absence of the dominant allele, the spot is waxy (*wx*) due to the recessive allele on the normal chromosome (not shown).

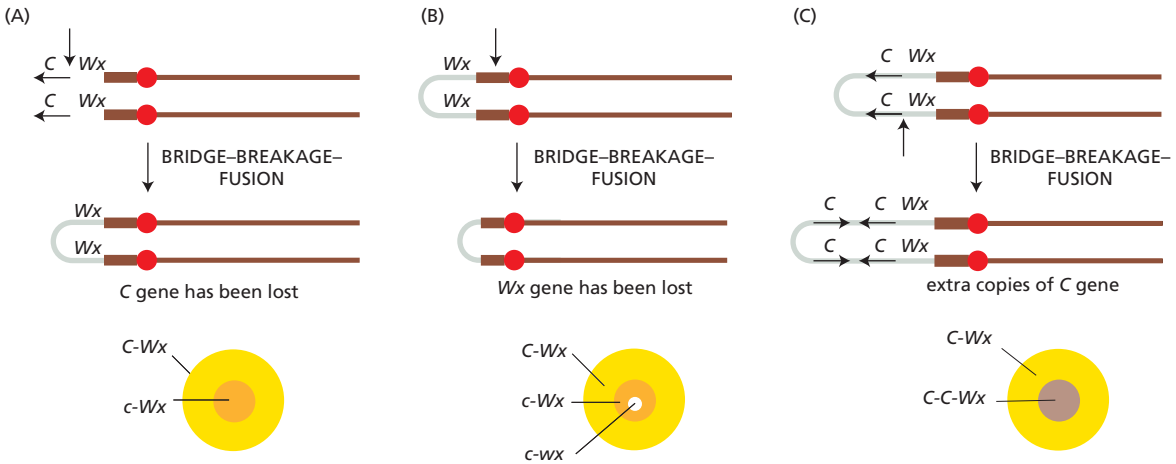


Figure 20–19 Formation of three different types of patches observed in speckled kernels (Answer 20–48). (A) Formation of a colorless, nonwaxy (*c-Wx*) spot. (B) Formation of a colorless, waxy (*c-wx*) spot inside a colorless, nonwaxy (*c-Wx*) spot formed as in (A). (C) Formation of an intensely colored, nonwaxy (*C-C-Wx*) spot. *Vertical arrows* pointing to the dicentric chromosomes show the positions of the breaks that lead to formation of the patches. In each case, the *upper half* of the starting dicentric chromosome gives rise to the new dicentric chromosome.

The formation of an intensely colored patch is due to a breakage event that leads to a dicentric chromosome with multiple copies of the dominant color allele (Figure 20–19C). Thus, the genetic constitution of the intensely colored patch is *C-C-Wx*.

B. You would never expect to see a colored spot within a colorless patch because, once eliminated, the dominant color (*C*) allele cannot be regained by further bridge–breakage–fusion cycles.

C. You would expect to see colorless spots within an intensely colored patch because the dominant color (*C*) allele could be lost by subsequent bridge–breakage–fusion cycles.

The demonstration by McClintock of bridge–breakage–fusion cycles in plants was one of the earliest indications that the broken ends of chromosomes are in some way "sticky"—entirely different from natural chromosome ends. It is clear now that cells have an active repair pathway for joining broken DNA ends together as a defense against potentially lethal double-strand breaks. So long as breaks are rare, the correct ends are joined. But when multiple breaks are present, the wrong partners can be joined, leading to translocations or other genetic rearrangements. Human cancer cells often carry similar kinds of chromosome rearrangements.

Reference: McClintock B (1939) The behavior in successive nuclear divisions of a chromosome broken at meiosis. *Proc. Natl Acad. Sci. USA* 25, 405–416.

20–49

A. The key observation about these multiple, localized rearrangements is that they give copy numbers that are either 0 or 1. As can be seen by examination of the rearranged chromosome in the progressive rearrangements model, different segments are present in 0 copies (I), 1 copy (A, E, G, H, J), 2 copies (B, D, F), and 3 copies (C) (**Figure 20–20**). By contrast, in the chromosome catastrophe model, segments are present in either 0 copies (A, E, G, J) or 1 copy (B, C, D, F, H, I) (Figure 20–20). Computer simulations indicate that it is virtually impossible for a sequence of rearrangements to produce a chromosome in which every segment is present either once or not at all.

B. The authors of the paper suggest two possible explanations for how such shattered chromosomes might arise. One possibility is ionizing

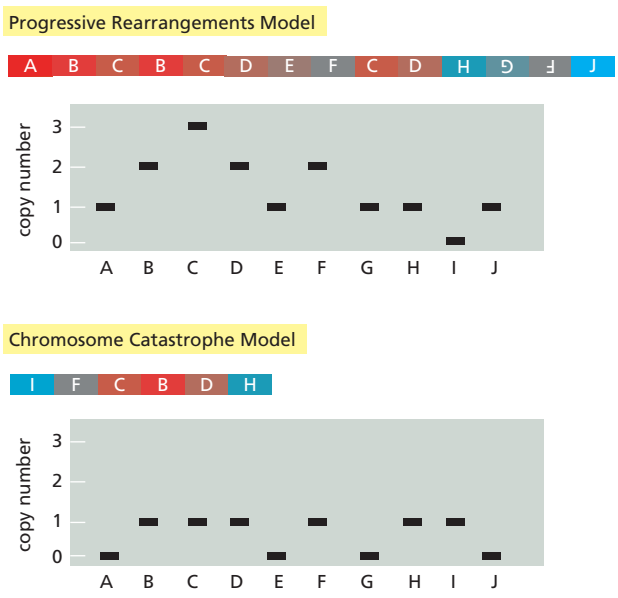


Figure 20–20 Copy number variation associated with the rearranged chromosomes generated by progressive rearrangements or chromosome catastrophe (Answer 20–49).

radiation, which commonly generates double-strand breaks. A pulse of ionizing radiation passing through a condensed mitotic chromosome could break the chromosome in multiple nearby places, giving rise to ends that could be rejoined in random order. A second possibility is that the damage is triggered by the fusion of two chromosomes that have lost their telomeres. When the two centromeres of such dicentric chromosomes are pulled to opposite daughter cells during anaphase, they form a so-called anaphase bridge. It is unclear how these bridges are resolved, but they appear to induce the formation of micronuclei containing fragmented DNA in the daughter cells, which could account for the localized chromosome fragmentation. Which, if either, of these explanations is correct must await further experimentation.

C. These rearrangements certainly have the capacity to be driver events. Rearrangements could inactivate one copy of a tumor suppressor gene by deleting it completely or partially, or by splitting it into two pieces. Similarly, rearrangements could activate an oncogene by placing it near a more active promoter, or by fusing it with another gene to give a hybrid protein with oncogenic properties. Examples of both these kinds of event were found among the set of cancers examined by the authors of the paper.

Reference: Stephens PJ, Greenman CD, Fu B et al. (2011) Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell* 144, 27–40.

MEDICAL LINKS

20–50

A. If the integration events were random, then 0.00005 [f_i = (100 kb/2 × 10^6 kb)] would be expected to occur in the target sequence. The fraction of integration events expected to occur outside the target would be 0.99995 ($f_o = 1 - f_i$).

B. The probability of not finding (P_N) a second integration at *Il2rg* in a survey of 600 tumors with a retroviral integration at *Lmo2* is the probability of not finding it in one, raised to the power of 600. Thus, $P_N = (0.99995)^{600} = 0.97$. This means that in 97 out of 100 times you survey a new set of 600 tumors, you would not expect to find a second integration at *Il2rg*.

C. The probability of finding (P_Y) a second integration at *Il2rg* in a survey of 600 tumors is 1 minus P_N, or 0.03. This means that 3 times out of 100, you would expect to find a second integration at *Il2rg*.

D. Given that only 2 out of 600 tumors actually had a retrovirus integrated at *Lmo2*, the probability of finding a tumor with dual integrations at *Lmo2* and *Il2rg* would be 0.0001 [0.03 × (2/600)], or 1 chance in 10,000.

E. The calculation in part D depends on the specific assumptions. If the target size were 10 kb (instead of 100 kb), the probability in part D would decrease to 1 chance in 100,000. If there were, on average, fewer than two retroviral integrations per tumor, the probability in part D would decrease; if there were more than two integrations per tumor, the probability would increase.

The assumption of randomness of retroviral integration is the most difficult to evaluate in terms of this calculation. Retroviruses are decidedly nonrandom in their integration pattern, with most varieties showing some degree of preference for actively transcribed genes. An entirely different approach to this question takes such preferences into account. If 2 out of 600 tumors had a retroviral integration at *Lmo2*, and 2 out of 600 had a retroviral integration at *Il2rg*, then the chance of having both is (2/600) × (2/600) = 0.000011, or about 1 in 100,000 tumors. The chance of not finding a dual integration in 600 tumors would be $P_N = (0.999989)^{600} = 0.993$. Thus, there would be a 7/1000 chance of finding a dual integration.

This differs by a factor of 100 from the result calculated in part D. Yet, both suggest that dual integration should be a relatively rare event.

References: Davé UP, Jenkins NA & Copeland NG (2004) Gene therapy insertional mutagenesis insights. *Science* 303, 333.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval J-L, Fraser CC, Cavazzana-Calvo M & Fischer A (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* 348, 255–256.

20–51

A. Fibroblasts and tumor cells from the same patient have different patterns of hybridization because the tumor cells have lost portions of the *Rb* gene. Loss of this gene is a very rare somatic event that affects less than one in a million cells. Only in the retina does its loss cause uncontrolled growth and tumor formation. No doubt the same proportion of fibroblasts also lose the *Rb* gene, but its loss from fibroblasts has no known biological consequence, so its absence cannot be readily detected.

B. The fibroblasts from the patient with unilateral retinoblastoma appear to be identical to those from normal cells, suggesting that the patient with unilateral retinoblastoma inherited two good *Rb* genes. Fibroblasts from the patient with bilateral retinoblastoma are not normal. Three of the four restriction fragments are present at half the normal intensity, suggesting that one of the *Rb* genes contains a deletion that encompasses those three restriction fragments. Note that the three affected fragments are adjacent on the map of the *Rb* gene (see Figure 20–12B).

   The tumor cells from both patients are abnormal. The patient with unilateral retinoblastoma is missing two fragments entirely and a third is present at half the normal intensity. This pattern indicates that each copy of the *Rb* gene has undergone deletion: one deletion encompasses the 9.8-kb and the 6.2-kb fragments; the other encompasses these two fragments and the 5.3-kb fragment. The patient with bilateral retinoblastoma is missing three fragments entirely and the remaining fragment is present at only half the normal intensity. This pattern indicates that the one good *Rb* gene has been entirely deleted, leaving only the 6.2-kb fragment from the original inherited deletion.

C. These results are exactly what is expected from the hypothesis that retinoblastoma is due to the loss of both copies of the *Rb* gene. Many cases of retinoblastoma have now been examined, and they all show loss, mutation, or epigenetic silencing of the *Rb* gene. Thus, retinoblastoma develops in the absence of functional Rb.

References: Fung Y-KT, Murphree AL, T'Ang A, Qian J, Hinrichs SH & Benedict WF (1987) Structural evidence for the authenticity of the human retinoblastoma gene. *Science* 236, 1657–1661.

Knudson AG (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl Acad. Sci. USA* 68, 820–823.

CANCER PREVENTION AND TREATMENT: PRESENT AND FUTURE

DEFINITIONS

20–52 Papillomavirus (HPV)

20–53 Multidrug resistance

TRUE/FALSE

20–54 True. The modifications introduced into dietary carcinogens by the action of the P-450 enzymes of the liver can convert them from relatively inert compounds to reactive ones that damage DNA. The normal function of these enzymes is to convert ingested toxins to harmless compounds for easy excretion. Unfortunately, their action on some chemicals renders them highly mutagenic.

20–55 False. Infectious agents are thought to participate in the formation of roughly 15% of human cancers. In most cases, the specific mechanism is unknown, but there are strong associations, for example, between hepatitis viruses type B and type C and liver cancer, and between *Helicobacter pylori* infection and stomach cancer.

20–56 False. Although it is popular to think so, there is scant evidence to support those ideas. However, we certainly know of specific instances—such as 2-naphthylamine and asbestos—where industrial products cause human cancers.

20–57 True. Useful therapies selectively target cancer cells and leave normal cells relatively unaffected. This selective action always depends on a key difference between normal cells and cancer cells. For example, most anticancer drugs and ionizing radiation damage DNA. These treatments preferentially kill cancer cells because the cancer cells have a diminished capacity to survive the damage; they have deactivated the machinery responsible for cell-cycle arrest to allow orderly repair of the damage.

20–58 True. It is fairly common for cancer cells to undergo further mutations that make them dependent on the hyperactivity of an oncogene. It is this phenomenon—oncogene dependence—that often makes it possible to kill cancer cells, yet avoid hurting the normal cells that depend on the function of the proto-oncogene from which the oncogene has evolved.

20–59 False. Although there are likely to be some cancer cells in a hypermutable population that are resistant to a single drug, it is unlikely that cells will be resistant to multiple drugs. For a combination therapy to work, the drugs must be designed so that a single mutation cannot render cells resistant. For example, if the multiple drugs in a cocktail can all be pumped out of the cell by an amplified ABC transporter, then treatment of a cancer cell population will select for cells that have amplified that gene … and the treatment will fail. If, on the other hand, a single mutation cannot render a cell resistant to all of the drugs in the cocktail, the treatment has the potential for eliminating all the cancer cells and producing a cure.

THOUGHT PROBLEMS

20–60 These data are consistent with the idea that cancer is a multistep process in which cancer-causing changes accumulate over time. The 25-year delay between exposure and cancer reflects the time it takes for lung cells to accumulate a sufficient number of changes to become cancerous. From other studies it is known that cigarette smoke contains chemical carcinogens that contribute to the progression from normal to cancerous cells.

Your uncle's suggestion that there is a genetically predisposed fraction of the population that is prone to lung cancer does not match the data. If a fixed fraction of the population were genetically predisposed, the incidence of lung cancer would be relatively constant over time. It would not be expected to track with per capita smoking.

20–61 The promyelocytes of APL are blocked at an intermediate stage in their

development, at a point where they still divide and increase in number. It is this unchecked increase in number that causes problems for the cancer patient. Normally, such precursor cells divide only a few times before they terminally differentiate into a nondividing blood cell. By triggering the differentiation of promyelocytes into terminally differentiated neutrophils, which no longer divide, treatment with all-*trans*-retinoic acid eliminates the problems caused by unchecked proliferation.

APL arises by one of a few types of translocation that fuses the retinoic acid receptor (RAR) gene on chromosome 17 with a gene on another chromosome to make a hybrid protein that interferes with the normal developmental program. It is not yet clear how the fusion protein blocks development, although it likely does so by interfering with the function of the normal RAR. In some way, treatment with all-*trans*-retinoic acid allows APL cells to move through the block.

Reference: Warrell RP Jr, de Thé H, Wang Z-Y & Degos L (1993) Acute promyelocytic leukemia. *N. Engl. J. Med.* 329, 177–189.

20–62 The products of oncogenes are the only feasible targets for such small molecules. The product of an oncogene has a dominant, growth-promoting effect on the cell. Thus, if the growth-promoting oncogene product were inhibited, the cell might return to a more normal state. This is the underlying rationale for searching for drugs that inhibit oncoproteins.

By contrast, the products of tumor suppressor genes are not targets for anticancer drug development. Tumor suppressor genes cause cancer by *not* making their product. Thus, there is no abnormal product to be inhibited in cancer cells that arise by mutation of tumor suppressor genes.

20–63

A. The one-hour incubation allows the binding reactions—test compound to kinase and ATP analog to kinase—to come to equilibrium. Making the measurements at equilibrium gives a much more reproducible assay and allows comparisons between test compounds to be made on an equal footing.
B. At equilibrium, some of the phage-attached kinases will be bound to the test compound and some will be bound to the ATP-analog-coated magnetic beads. The relative proportions depend on how strongly the test compound binds and its concentration. In the presence of a weakly binding test compound, most of the phage will be attached to the magnetic beads, which will yield a high count in the plaque assay. In the presence of the same concentration of a strongly binding test compound, most of the phage will be attached to the test compound and will be washed away at the end of the incubation. Thus, strongly binding test compounds will give a low count in the plaque assay.
C. The assay is set up to measure competition between the ATP analog and the test compound for the ATP-binding site on the protein kinase. Thus, it is expected that the test compounds that score well in this assay will bind at or near the ATP-binding cleft on the kinase. This assay can also identify molecules that bind to other sites (allosteric sites) but alter the conformation of the ATP-binding site, so that the kinase no longer binds ATP. One might imagine other sites where a useful inhibitor might bind to a kinase to inhibit its function—at a key protein–protein interface, for example—but those types of inhibitors will not be picked up in this assay system.
D. Although all protein kinases bind ATP in an evolutionarily conserved binding site, the binding sites are not identical. The amino acid differences in and around the binding site provide slightly different binding surfaces that can be exploited to develop compounds that selectively bind to one kinase but not others.

References: Griffin JD (2005) Interaction maps for kinase inhibitors. *Nat. Biotechnol.* 23, 308–309.

Fabian MA, Biggs WH, Treiber DK et al. (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* 23, 329–336.

CALCULATIONS

20–64

A. The K_d values can be estimated directly from the graph in Figure 20–15, as the concentrations of test compounds that give a half-maximal response. The K_d values for BIRB-796, VX-745, and SB203580 are about 0.3 nM, 3 nM, and 15 nM, respectively.

B. The phage concentration is 17 pM, well below the $K_{d(test)}$ values for the compounds tested, the lowest of which is 0.3 nM (300 pM). Thus, the estimates in part A are valid. Phage concentration = (10^{10} phage/mL) (mole/6 × 10^{23} phage) (10^3 mL/L) (10^{12} pmol/mole) = 17 pmol/L = 17 pM.

Reference: Fabian MA, Biggs WH, Treiber DK et al. (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* 23, 329–336.

DATA HANDLING

20–65 The highly rearranged karyotypes and their similarity from tumor to tumor suggest that the cancer cells themselves are being transmitted from devil to devil. It is extremely unlikely that a viral infection could induce the same set of complicated rearrangements in different animals. Most importantly, the existence of a chromosome 5 inversion in one Tasmanian devil, which is not present in chromosome 5 of its tumor cells, argues strongly that the tumors are not generated from the host devil's own cells. It appears that this cancer has arisen from a rogue line of cancer cells, from a tumor of unknown origin, that has acquired the capability for parasitic existence. This is one of just two examples of natural transmission of cancer by tumor cells, the other being a venereal disease in dogs. A special case of such transmission occurs occasionally during organ transplantation in humans. But the requirements for organ transplantation—matching tissue and immune suppression—highlight just how unusual natural transmission is. The cancer cells responsible for facial tumors in Tasmanian devils must somehow evade the new host's immune defenses.

Reference: Pearse A-M & Swift K (2006) Allograft theory: Transmission of devil facial-tumour disease. *Nature* 439, 549.

20–66

A. These results, which were reported in 1933, were the first indication that the tendency to form breast tumors was maternally transmitted in mice. The results of the experiments in Table 20–2 cannot be readily explained on the basis of standard types of chromosomal mutations, be they recessive, dominant, or X-linked. If you assume that the "high" strains are homozygous for a recessive mutation and the "low" strains are homozygous for the wild-type allele, all the F1 progeny would be heterozygous and unaffected. For a dominant mutation, the males, as well as the females, should transmit the mutations to their offspring. X-linked mutations would be expected to give affected males in every other generation. Although it was unknown at the time these experiments were carried out, the pattern of inheritance shown in Table 20–2 matches the expectations

for mitochondrial mutations, which are inherited via the egg cytoplasm. (See Problem 14–110 for more discussion of these patterns of inheritance.)

B. The key to understanding how the tendency to form breast cancers is inherited is the foster-mother experiment. Although presented as an accident in this problem, it represented a critical insight at the time. Because foster mothers could pass this trait on to mice with which they shared no genetic connection, inheritance could not be due to chromosomal (or mitochondrial) mutations. The link between mothers and daughters was identified as the milk. The so-called milk factor was later identified as a virus, which we now call mouse mammary tumor virus (MMTV).

References: Little CC (1933) The existence of non-chromosomal influence in the incidence of mammary tumors in mice. *Science* 78, 465–466.

Bittner JJ (1936) Some possible effects of nursing on the mammary gland tumor incidence in mice. *Science* 84, 162.

Paigen K (2003) One hundred years of mouse genetics: An intellectual history. I. The classical period (1902–1980). *Genetics* 163, 1–7.

20–67

A. Iressa™, which is being used clinically in the treatment of non-small-cell lung carcinomas, and Gleevec® have similar specificities. Iressa has fewer off-target binding interactions, and binds only one of those with an affinity than is within a factor of 10 of the main target. BIRB-796 binds to more off-target proteins than Gleevec or Iressa, and it binds three of them with only 10-fold lower affinity. Staurosporine, which is the least specific of all, is a potent inhibitor of many protein kinases.

B. The clustering of the binding targets on the kinome is expected. After all, the kinases that are most closely related are the closest together on the tree. Closely related kinases should have more similar binding sites, and thus might be expected to bind an inhibitor similarly. Indeed, it is the similarity of all kinases, and especially of closely related kinases, that makes drug development for them such a challenge.

C. There is no way that you (or anyone) could predict from these data that BIRB-796 would bind Abl(T315I). Resistant variants arise by such subtle changes that direct measurements must be made.

D. This high-throughput screen can be adapted to look for drugs that bind resistant variants of protein kinases. If the particular mutant versions responsible for resistance are identified, they can be included in the screen to find drugs that are active against them. It is hoped that screens such as the one described here can be used to identify a second generation of drugs that will prove beneficial in the treatment of cancers resistant to the primary drug.

Reference: Fabian MA, Biggs WH, Treiber DK et al. (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* 23, 329–336.

MEDICAL LINKS

20–68 Technological advances in our ability to detect cancers since 1970 mean that we can diagnose them at earlier stages in the course of the disease. Thus, even in the absence of better treatment regimes, a patient might be expected to survive somewhat longer now than in the past because they will be at a slightly earlier stage of the disease at the 5-year mark.

Reference: Weinberg RA (2006) The Biology of Cancer, p. 726. New York: Garland Science.

20–69 Individuals who are heterozygous for a mutation in the *Brca1* gene are susceptible to cancer of the breast and ovary because Brca1 is an especially important tumor suppressor in these tissues. The loss of the remaining functional copy of the *Brca1* gene—by mutation, chromosome loss, or epigenetic silencing—drives the affected cell toward the cancer phenotype. As a consequence, the cancer cells that arise in these tissues cannot carry out homologous recombination because they are missing Brca1. By contrast, the one good copy of the gene that is present in the patient's normal cells makes sufficient Brca1 to make the cells proficient for homologous recombination. Thus, when these patients are treated with olaparib, their cancer cells die because they cannot use homologous recombination to repair the double-strand breaks that arise from the inhibition of PARP. Their normal cells, however, which have a good copy of *Brca1*, repair the breaks just fine.

Reference: Fong PC, Boss DS, Yap TA et al. (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N. Engl. J. Med.* 361, 123–134.

MCAT STYLE

20–70

B. A rearrangement that deleted the inhibitory domain of a protein kinase would lead to increased kinase activity. Many oncogenes are hyperactive kinases. Choice A is incorrect because increased GTPase activity would decrease Ras activity, thereby reducing the proliferative signal. Oncogenic versions of Ras typically have decreased GTPase activity, which locks them into their active state. Choice C is incorrect because deleting the promoter would be expected to inactivate an oncogene, and oncogenes are hyperactive versions of normal genes. Choice D is not correct because tumor suppressors contribute to cancer only when they are inactivated.

20–71

B. Rare rearrangements that occurred in somatic cells *in utero* would be shared between the twins because they have a connected blood circulatory system. Choice C is incorrect because the rearrangement appeared before cells became cancerous, so it could not have been due to the genomic instability of the cancer cells. Choices A and D are not correct because the normal cells from the twins do not have the rearrangement. If the rearrangement were inherited from one or both parents, all the twins' normal cells would have been expected to carry the rearrangement.

20–72

D. The appearance of cancer at different times in the twins indicates that the rearrangement alone is insufficient to cause cancer, and suggests that additional mutations must occur to complete the conversion of the rearrangement-containing cells to cancerous cells. Since mutations occur randomly, their timing varies. The other choices list features that have been found in one or more cancers, but none of them can be inferred from the simple observation that cancer appears in the twins at different times.

20–73

C. Many cancer cells are able to survive and proliferate because oncogenic signaling pathways suppress apoptosis. Thus, loss of oncogenic signals could cause cells to undergo apoptosis. Choice A is unlikely because cancer cells are generally not recognized by the immune system. Choice B could be correct in the case of some kinds of chemotherapy that cause

DNA damage and exploit the defective DNA repair typical of cancer cells; however, it is unlikely for an inhibitor of a protein kinase. Choice D is not correct because the Warburg effect refers to the altered sugar metabolism in cancer cells, an unlikely cause of cell death.

20–74

C. Choices II and III both describe how some cancer cells survive targeted anticancer drugs. Choice I is incorrect because mutations that inactivate Mek or Erk would block oncogenic signaling—rather than promote it—thereby suppressing tumor growth.

20–75

A. Extensive biochemical analysis has demonstrated that vemurafenib binds to wild-type B-Raf, causing a conformational change in the other B-Raf in a dimer, which activates it and increases downstream signaling through the MAP kinase pathway, which promotes conversion of normal cells into cancer cells. Since B-Raf (V600E) does not dimerize, it is strongly inhibited by vemurafenib, which helps explain the extraordinary selectivity of vemurafenib for tumors that express the B-Raf (V600E) mutant. Choice B is incorrect because inhibition of Ras would reduce downstream signaling, which would decrease the signal for proliferation. Choice C is not correct because creating a tumor suppressor would reduce cancer formation, not promote it. Choice D is incorrect because it suggests that vemurafenib would increase apoptosis in normal cells, which would not promote cancer.

References: Poulikakos PI, Zhang C, Bollag G, Shokat KM & Rosen N (2010) RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* 464, 427–430.

Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJ, Stokoe D, Gloor SL, Vigers G, Morales T, Aliagas I, Liu B, Sideris S, Hoeflich KP, Jaiswal BS, Seshagiri S, Koeppen H, Belvin M, Friedman LS & Malek S (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 464, 431–435.

Answers to Problems in *Molecular Biology of the Cell*

A Modern Photograph of *Mola mola*.

Answers to Problems in *Molecular Biology of the Cell*, Sixth Edition

CHAPTER 1

1–1 False. The clusters of human hemoglobin genes arose during evolution by duplication from an ancient ancestral globin gene; thus, they are examples of paralogous genes. The human hemoglobin α gene is orthologous to the chimpanzee hemoglobin α gene, as are the human and chimpanzee hemoglobin β genes, and so on. All the globin genes, including the more distantly related gene for myoglobin, are homologous to one another.

1–2 True. In single-celled organisms, the genome is the germ line and any modification is passed on to the next generation. In multicellular organisms, most of the cells are somatic cells and make no contribution to the next generation; thus, modification of those cells by horizontal gene transfer would have no consequence for the next generation. The germline cells are usually sequestered into the interior of multicellular organisms, minimizing their contact with foreign cells, viruses, and DNA, thereby insulating the species from the effects of horizontal gene transfer.

1–3 True. Bacterial genomes seem to be pared down to the essentials: most of the DNA sequences encode proteins, a few encode functional RNAs, a small amount of DNA is devoted to regulating gene expression, and there are very few extraneous, nonfunctional sequences. By contrast, only about 1.5% of the DNA sequences in the human genome is thought to code for proteins. Even allowing for large amounts of regulatory DNA, much of the human genome is composed of DNA with no apparent function.

1–4 On the surface, the extraordinary mutation resistance of the genetic code argues that it was subjected to the forces of natural selection. An underlying assumption, which seems reasonable, is that resistance to mutation is a valuable feature of a genetic code, one that would allow organisms to maintain sufficient information to specify complex phenotypes. This reasoning suggests that it would have been a lucky accident indeed—roughly a one-in-a-million chance—to stumble on a code as error-proof as our own.

But all is not so simple. If resistance to mutation is an essential feature of any code that can support the complexity of organisms such as humans, then the only codes we *could* observe are ones that are error resistant. A less favorable frozen accident, giving rise to a more error-prone code, might limit the complexity of life to organisms that would never be able to contemplate their genetic code. This is akin to the anthropic principle of cosmology: many universes may be possible, but few are compatible with life that can ponder the nature of the universe.

Beyond these considerations, there is ample evidence that the code is not static, and thus could respond to the forces of natural selection. Deviant versions of the standard genetic code have been identified in the

mitochondrial and nuclear genomes of several organisms. In each case, one or a few codons have taken on a new meaning.

Reference: Freeland SJ & Hurst LD (1998) The genetic code is one in a million. *J. Mol. Evol.* 47, 238–248.

1–5 There are several approaches you might try.

1. Analysis of the amino acids in the proteins would indicate whether the set of amino acids used in your organism differs from the set used in Earth organisms. But even Earthly organisms contain more amino acids than the standard set of 20; for example, hydroxyproline, phosphoserine, and phosphotyrosine all result from modifications after a protein has been synthesized. Absence of one or more of the common set might be a more significant result.
2. Sequencing DNA from the "Europan" organism would allow a direct comparison with the database of sequences that are already known for Earth organisms. Matches to the database would argue for contamination. Absences of matches would constitute a less strong argument for a novel organism; it is a typical observation that about 15% to 20% of the genes identified in complete genome sequences of microorganisms do not appear to be homologous to genes in the database. Sufficiently extensive sequence comparison should resolve the issue.
3. Another approach might be to analyze the organism's genetic code. We have no reason to expect that a novel organism based on DNA, RNA, and protein would have a genetic code identical to Earth's universal genetic code.

1–6 Whether it is sunlight or inorganic chemicals, "to feed" means "to obtain free energy and building materials from." In the case of photosynthesis, photons in sunlight are used to raise electrons of certain molecules to a high-energy, unstable state. When they return to their normal, ground state, the released energy is captured by mechanisms that use it to drive the synthesis of ATP. Similarly, lithotrophs at a hydrothermal vent obtain free energy by oxidizing one or more of the reduced components from the vent (for example, $H_2S \rightarrow S + 2\ H^+$), using some common molecule in the environment to accept the electrons (for example, $2\ H^+ + ½\ O_2 \rightarrow H_2O$). Lithotrophs harvest the energy released in such oxidation–reduction (electron-transfer) reactions to drive the synthesis of ATP. For both lithotrophs and phototrophs, the key to success is the evolution of a molecular mechanism to capture the available energy and couple it to ATP synthesis.

For all organisms, be they phototrophs, organotrophs, or lithotrophs, their ability to obtain the free energy needed to support life depends on the exploitation of some nonequilibrium condition. Phototrophs depend on the continual flux of radiation from the sun; organotrophs depend on a supply of organic molecules, provided ultimately by phototrophs, that can be oxidized for energy; and lithotrophs depend on a supply of reduced inorganic molecules, provided, for example, by hydrothermal vents, that can be oxidized to produce free energy.

1–7 Four (**Figure A1–1**). All could have split from the common ancestor at the same time. Bacteria–archaea could have split from eukaryotes, followed by the separation of bacteria from archaea. Bacteria–eukaryotes could have split from archaea, followed by the separation of bacteria from

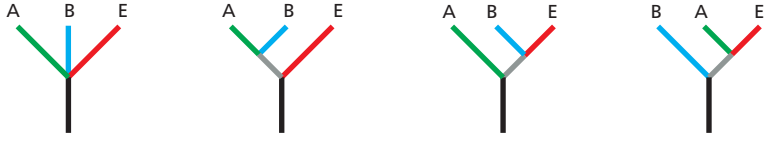


Figure A1–1 The four possible relationships for the evolution of archaea (A), bacteria (B), and eukaryotes (E) (Answer 1–7).

eukaryotes. Archaea–eukaryotes could have split from bacteria, followed by the separation of archaea from eukaryotes. Although horizontal transfers across these divisions make interpretations problematic, it is thought that archaea–eukaryotes first split from bacteria, and then archaea and eukaryotes split.

1–8 It is unlikely that any gene came into existence perfectly optimized for its function. It is thought that highly conserved genes such as ribosomal RNA genes were optimized by more rapid evolutionary change during the evolution of the common ancestor to archaea, bacteria, and eukaryotes. Since ribosomal RNAs (and the products of most highly conserved genes) participate in fundamental processes that were optimized early, there has been no evolutionary pressure (and little leeway) for change. By contrast, less conserved—more rapidly evolving—genes have been continually presented with opportunities to fill new functional niches. Consider, for example, the evolution of distinct globin genes, whose products are optimized for oxygen delivery to embryos, fetuses, and adult tissues in placental mammals.

1–9 Complexity is a logical explanation for the difference in rates of horizontal gene transfer (and it may even be right, although there are other possibilities). Successful transfer of an "informational" gene would require that the new gene product fit into a preexisting, functional complex, perhaps supplanting the original related protein. For a new protein to fit into a complex with other proteins, it would need to have binding surfaces that would allow it to interact with the right proteins in the appropriate geometry. If a new protein had one good binding surface, but not others, it would most probably disrupt the complex and put the recipient at a selective disadvantage. By contrast, a gene product that carries out a metabolic reaction on its own would be able to function in any organism. So long as the metabolic reaction conferred some advantage on the recipient (or at least no disadvantage), the gene transfer could be accommodated.

Reference: Jain R, Rivera MC & Lake JA (1999) Horizontal gene transfer among genomes: The complexity hypothesis. *Proc. Natl Acad. Sci. USA* 96, 3801–3806.

1–10 Like most questions about evolutionary relationships, this one was decided by comparing sequences of genes such as those for ribosomal RNA. These comparisons showed that fungi are more similar in gene sequence to animals than to plants, and probably split from the animal–plant lineage after plants separated from animals. Thus, fungi are thought never to have had chloroplasts, and fungi and plants are thought to have invented cell walls independently, as is suggested by the use of cellulose in plant cell walls and chitin in fungal cell walls.

1–11

A. The data in the phylogenetic tree (see Figure Q1–2) refute the hypothesis that plant hemoglobin genes arose by horizontal transfer. Looking at the more familiar parts of the tree, we see that the vertebrates (fish to human) cluster together as a closely related set of species. Moreover, the relationships in the unrooted tree shown in Figure Q1–2 are compatible with the order of branching we know from the evolutionary relationships among these species: fish split off before amphibians, reptiles before birds, and mammals last of all in a tightly knit group. Plants also form a distinct group that displays accepted evolutionary relationships, with barley, a monocot, diverging before bean, alfalfa, and lotus, which are all dicots (and legumes). The sequences of the plant hemoglobins appear to have diverged long ago in evolution, at or before the time that mollusks,

insects, and nematodes arose. The relationships in the tree indicate that the hemoglobin genes arose by descent from some common ancestor.

B. Had the plant hemoglobin genes arisen by horizontal transfer from a parasitic nematode, then the plant sequences would have clustered with the nematode sequences in the phylogenetic tree in Figure Q1–2.

1–12 Three general hypotheses have been proposed to account for the differences in rate of evolutionary change in different lineages. The individual hypotheses discussed below are not mutually exclusive and may all contribute to some extent.

The *generation-time* hypothesis proposes that rate differences are a consequence of different generation times. Species such as rat with short generation times will go through more generations and more rounds of germ-cell division, and hence more rounds of DNA replication. This hypothesis assumes that errors during DNA replication are the major source of mutations. Tests of this hypothesis in rat versus human tend to support its validity.

The *metabolic-rate* hypothesis postulates a higher rate of evolution for species with a higher metabolic rate. Species with high metabolic rates use more oxygen; hence, they generate more oxygen free radicals, a major source of damage to DNA. This is especially relevant for mitochondrial genomes, because mitochondria are the major cellular site for oxygen utilization and free-radical production.

The *efficiency-of-repair* hypothesis proposes that the efficiency of repair of DNA damage differs in different lineages. Species with highly efficient repair of DNA damage would have a reduced fraction of damage events that lead to mutation. There is evidence in cultured human and rat cells that such differences in repair exist, in the expected direction, but it is unclear whether such differences exist in the germ lines of these organisms.

Reference: Li WH (1997) Molecular Evolution, pp. 228–230. Sunderland, MA: Sinauer Associates, Inc.

CHAPTER 2

2–1 False. The pH of the solution will be very nearly neutral, essentially pH 7, because the few H^+ ions contributed by HCl will be outnumbered by the H^+ ions from dissociation of water. No matter how much a strong acid is diluted, it can never give rise to a basic solution. In fact, calculations that take into account both sources of H^+ ions and also the effects on the dissociation of water give a pH of 6.98 for a 10^{-8} M solution of HCl.

2–2 False. Many of the functions that macromolecules perform rely on their ability to associate and dissociate readily, which would not be possible if they were linked by covalent bonds. By linking their macromolecules noncovalently, cells can, for example, quickly remodel their interior when they move or divide, and easily transport components from one organelle to another. It should be noted that some macromolecules are linked by covalent bonds. This occurs primarily in situations where extreme structural stability is required, such as in the cell walls of many bacteria, fungi, and plants, and in the extracellular matrix that provides the structural support for most animal cells.

2–3 True. The difference between plants and animals is in how they obtain their food molecules. Plants make their own using the energy of sunlight, plus CO_2 and H_2O, whereas animals must forage for their food.

2–4 True. Oxidation–reduction reactions refer to those in which electrons are removed from one atom and transferred to another. Since the number

of electrons is conserved (no loss or gain) in a chemical reaction, oxidation—removal of electrons—must be accompanied by reduction—addition of electrons.

2–5 False. The equilibrium constant for the reaction A ⇌ B remains unchanged; it is a constant. Linking reactions together can convert an unfavorable reaction into a favorable one, but it does so not by altering the equilibrium constant, but rather by changing the concentration ratio of products to reactants.

2–6 True. A reaction with a negative $\Delta G°$, for example, would not proceed spontaneously under conditions where there is already an excess of products over those that would be present at equilibrium. Conversely, a reaction with a positive $\Delta G°$ would proceed spontaneously under conditions where there is an excess of substrates compared to those present at equilibrium.

2–7 False. The oxygen atoms that are part of CO_2 do not come from the oxygen atoms that are consumed as part of the oxidation of glucose (or of any other food molecule). The electrons that are abstracted from glucose at various stages in its oxidation are finally transferred to oxygen to produce water during oxidative phosphorylation. Thus, the oxygen used during oxidation of food in animals ends up as oxygen atoms in H_2O.

One can show this directly by incubating living cells in an atmosphere that contains molecular oxygen enriched for the isotope ^{18}O, instead of the naturally occurring isotope, ^{16}O. In such an experiment, one finds that all the CO_2 released from cells contains only ^{16}O. Therefore, the oxygen atoms in the released CO_2 molecules do not come directly from the atmosphere, but rather from the organic molecules themselves and from H_2O.

2–8 Organic chemistry in laboratories—even the very best—is rarely carried out in a water environment because of low solubility of some components and because water is reactive and usually competes with the intended reaction. The most dramatic difference, however, is the complexity. It is critical in laboratory organic chemistry to use pure components to ensure a high yield of the intended product. By contrast, living cells carry out thousands of different reactions simultaneously with good yield and virtually no interference between reactions. The key, of course, is that cells use enzyme catalysts, which bind substrate molecules in an active site, where they are isolated from the rest of the environment. There the reactivity of individual atoms is manipulated to encourage the correct reaction. It is the ability of enzymes to provide such special environments—miniature reaction chambers—that allows the cell to carry out an enormous number of reactions simultaneously without cross-talk between them.

2–9

A. Ethanol in 5% beer is 0.86 M. Pure ethanol is 17.2 M [(789 g/L) × (mole/46 g)], and thus 5% beer would be 0.86 M ethanol (17.2 M × 0.05).
B. At a legal limit of 80 mg/100 mL, ethanol will be 17.4 mM in the blood [(80 mg/0.1 L) × (mmol/46 mg)].
C. At the legal limit (17.4 mM), ethanol in 5% beer (0.86 M) has been diluted 49.4-fold (860 mM/17.4 mM). This dilution represents 809 mL in 40 L of body water (40 L/49.4). At 355 mL per beer, this equals 2.3 beers (809 mL/355 mL).
D. It would take nearly 4 hours. At twice the legal limit, the person would contain 64 g of ethanol [(0.16 g/0.1 L) × (40 L)]. The person would metabolize 8.4 g/hr [(0.12 g/hr kg) × (70 kg)]. Thus, to metabolize 32 g of ethanol (the amount in excess of the legal limit) would require 3.8 hours [(32 g) × (hr/8.4 g)].

2–10 Assuming that the change in enzyme activity is due to the change in protonation state of histidine, the enzyme must require histidine in the protonated, charged state. The enzyme is active only below the p*K* of histidine (which is typically around 6.5 to 7.0 in proteins), where the histidine is expected to be protonated.

2–11 The functional groups on the three molecules are indicated and named in **Figure A2–1**.

2–12 The instantaneous velocities are H_2O = 3.8 × 10^4 cm/sec, glucose = 1.2 × 10^4 cm/sec, and myoglobin = 1.3 × 10^3 cm/sec. The calculation for a water molecule, which has a mass of 3 × 10^{-23} g [(18 g/mole) × (mole/6 × 10^{23} molecules)], is shown below.

$$v = (kT/m)^{1/2}$$

$$v = \left(\frac{1.38 \times 10^{-16}\ \text{g cm}^2}{\text{K sec}^2} \times 310\ \text{K} \times \frac{1}{3 \times 10^{-23}\ \text{g}}\right)^{1/2}$$

$$v = 3.78 \times 10^4\ \text{cm/sec}$$

When these numbers are converted to km/hr the results are fairly astounding. Water moves at 1360 km/hr, glucose at 428 km/hr, and myoglobin at 47 km/hr. Thus, even the largest (slowest) of these molecules is moving faster than the swiftest human sprinter. And water molecules are traveling at Mach 1.1! Unlike a human sprinter, or a jet airplane, these molecules make forward progress only slowly because they are constantly colliding with other molecules in solution.

Reference: Berg HC (1993) Random Walks in Biology, Expanded Edition, pp. 5–6. Princeton, NJ: Princeton University Press.

2–13 It seems counterintuitive that polymerization of free tubulin subunits into highly ordered microtubules should occur with an overall increase in entropy (decrease in order). But it is counterintuitive only if one considers the subunits in isolation. Remember that thermodynamics refers to the whole system, which includes the water molecules. The increase in entropy is due largely to the effects of polymerization on water molecules. The surfaces of the tubulin subunits that bind together to form microtubules are fairly hydrophobic, and constrain (order) the water molecules in their immediate vicinity. Upon polymerization, these constrained water molecules are freed up to interact with other water molecules. Their new-found disorder much exceeds the increased order of the protein subunits, and thus the net increase in entropy (disorder) favors polymerization.

2–14 The whole population of ATP molecules in the body would turn over (cycle) 1800 times per day, or a little more than once a minute. Conversion of 3 moles of glucose to CO_2 would generate 90 moles of ATP [(3 moles glucose) × (30 moles ATP/mole glucose)]. The whole body contains 5 × 10^{-2} mole ATP [(2 × 10^{-3} mole/L) × 25 L]. Since the concentration of ATP does not change, each ATP must cycle 1800 times per day [(90 moles ATP/day)/(5 × 10^{-2} mole ATP)].

2–15 The human body operates at about 70 watts—about the same as a light bulb.

$$\frac{\text{watts}}{\text{body}} = \frac{10^9\ \text{ATP}}{60\ \text{sec cell}} \times \frac{5 \times 10^{13}\ \text{cells}}{\text{body}} \times \frac{\text{mole}}{6 \times 10^{23}\ \text{ATP}} \times \frac{50\ \text{kJ}}{\text{mole}} \times \frac{10^3\ \text{J}}{\text{kJ}}$$

$$= \frac{69.4\ \text{J/sec}}{\text{body}} = \frac{69.4\ \text{watts}}{\text{body}}$$

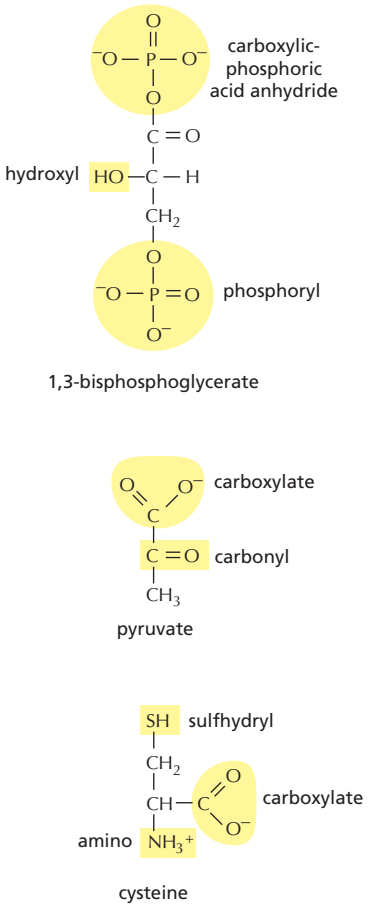


Figure A2–1 The functional groups in 1,3-bisphosphoglycerate, pyruvate, and cysteine (Answer 2–11).

2–16 You would need to expend 2070 kJ in climbing from Zermatt to the top of the Matterhorn, a vertical distance of 2818 m. Substituting into the equation for work

$$\text{work} = 75\ \text{kg} \times \frac{9.8\ \text{m}}{\text{sec}^2} \times 2818\ \text{m} \times \frac{\text{J}}{\text{kg m}^2/\text{sec}^2} \times \frac{\text{kJ}}{10^3\ \text{J}}$$
$$= 2070\ \text{kJ}$$

This is equal to about 1.5 Snickers™ (2070 kJ/1360 kJ), so you would be well advised to plan a stop at Hörnli Hut to eat another one.

In reality, the human body does not convert chemical energy into external work at 100% efficiency, as assumed in this answer, but rather at an efficiency of around 25%. Moreover, you will be walking laterally as well as uphill. Thus, you would need more than 6 Snickers to make it all the way.

Reference: Frayn KN (1996) Metabolic Regulation: A Human Perspective, p. 179. London: Portland Press.

2–17 In the absence of oxygen, the energy needs of the cell must be met by fermentation to lactate, which requires a high rate of flow through glycolysis to generate sufficient ATP. When oxygen is added, the cell can generate ATP by oxidative phosphorylation, which generates ATP much more efficiently than glycolysis. Thus, less glucose is needed to supply ATP at the same rate.

CHAPTER 3

3–1 True. In a β sheet, the amino acid side chains in each strand are alternately positioned above and below the sheet, and the carbonyl oxygens alternate from one side of the strand to the other. Thus, each strand in a β sheet can be viewed as a helix in which each successive amino acid is rotated 180°.

3–2 False. Intrinsically disordered regions of proteins typically have amino acid sequences with low hydrophobicity and high net charge. Low hydrophobicity reduces the effect of the hydrophobic force, which normally tends to drive the protein into a more condensed and ordered structure. A high net charge (either positive or negative) pushes similarly charged regions of the protein away from one another. By contrast, an amino acid sequence with high hydrophobicity and low net charge would tend to collapse into a defined structure.

3–3 True. Chemical groups on such protruding loops can often surround a molecule, allowing the protein to bind to it with many weak bonds.

3–4 True. Because an enzyme has a fixed number of active sites, the rate of the reaction cannot be further increased once the substrate concentration is sufficient to bind to all the sites. It is the saturation of binding sites that leads to an enzyme's saturation behavior.

3–5 False. The turnover number is constant since it is V_{max} divided by enzyme concentration. For example, a twofold increase in enzyme concentration would give a twofold higher V_{max}, but it would give the same turnover number: $2\ V_{max}/2\ [E] = k_3$.

3–6 True. The term cooperativity embodies the idea that changes in the conformation of one subunit are communicated to the other subunits in any given multimeric assembly, so that all of these subunits are in the same conformation. Usually, these subunits are identical; however, in hemoglobin, for example, there are four subunits of two somewhat different kinds.

3–7 True. Each cycle of phosphorylation–dephosphorylation hydrolyzes one molecule of ATP; however, it is not wasteful in the sense of having no benefit. Constant cycling allows the regulated protein to switch quickly from one state to another in response to stimuli that require rapid adjustments of cellular metabolism or function. This is the essence of effective regulation.

3–8 Since there are 20 possible amino acids at each position in a protein 300 amino acids long, there are 20^{300} (which is 10^{390}) possible proteins. The mass of one copy of each possible protein would be

$$\text{mass} = \frac{110\ \text{d}}{\text{aa}} \times \frac{300\ \text{aa}}{\text{protein}} \times 10^{390}\ \text{proteins} \times \frac{\text{g}}{6 \times 10^{23}\ \text{d}}$$

$$\text{mass} = 5.5 \times 10^{370}\ \text{g}$$

Thus, the mass of protein would exceed the mass of the observable universe (10^{80} g) by an enormous amount; more precisely by a factor of about 10^{290}!

3–9 Generally speaking, an identity of at least 30% is needed to be certain that a match has been found. Matches of 20% to 30% are problematical and difficult to distinguish from background "noise." Searching for distant relatives with the whole sequence usually drops the overall identity below 30% because the less conserved portions of the sequence dominate the comparison. Thus, searching with shorter, conserved portions of the sequence gives the best chance for finding distant relatives.

3–10 The close juxtaposition of the N- and C-termini of this kelch domain identifies it as a "plug-in" type domain. "In-line" type domains have their N- and C-termini on opposite sides of the domain.

3–11

A. These data are consistent with the hypothesis that the springlike behavior of titin is due to the sequential unfolding of Ig domains. First, the fragment contained seven Ig domains and there are seven peaks in the force-versus-extension curve. In addition, the peaks themselves are what you might expect for sequential unfolding. Second, in the presence of a protein denaturant, conditions under which the domains will already be unfolded, the peaks disappear and the extension per unit force increases. Third, when the domains are cross-linked, and therefore unable to unfold, the peaks disappear and extension per unit force decreases.

B. The spacing between peaks, about 25 nm, is almost exactly what you would calculate for the sequential unfolding of Ig domains. The folded domain occupies 4 nm, but when unfolded, its 89 amino acids would stretch to about 30 nm (89 × 0.34 nm), a change of 26 nm.

C. The existence of separate, discrete peaks means that each domain unfolds when a characteristic force is applied, implying that each domain has a defined stability. The collection of domains unfolds in order from least stable to most stable. Thus, it takes a little more force each time to unfold the next domain.

D. The sudden collapse of the force at each unfolding event reflects an important principle of protein unfolding; namely, its cooperativity. Proteins tend to unfold in an all-or-none fashion. A small number of hydrogen bonds are crucial for holding the folded domain together (**Figure A3–1**). The breaking of these bonds triggers cooperative unfolding.

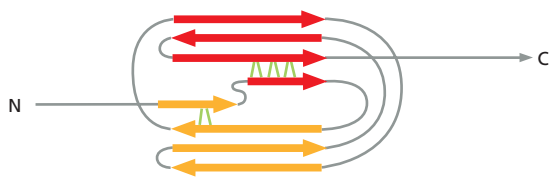


Figure A3–1 Hydrogen bonds that lock the domain into its folded conformation (Answer 3–11). The indicated hydrogen bonds (*green lines*), when broken, trigger unfolding of the domain. If you compare this topological diagram with the three-dimensional structure in Figure Q3–2A, you can pick out the two short β strands that are involved in forming these hydrogen bonds.

Reference: Rief M, Gutel M, Oesterhelt F, Fernandez JM & Gaub HE (1997) Reversible folding of individual titin immunoglobulin domains by AFM. *Science* 276, 1109–1112.

3–12

A. The relative concentrations of the normal and mutant Src proteins are inversely proportional to the volumes in which they are distributed. The mutant Src is distributed throughout the volume of the cell, which is

$$V_{cell} = (4/3)\pi r^3 = 4\pi(10 \times 10^{-6}\ m)^3/3 = 4.1888 \times 10^{-15}\ m^3$$

Normal Src is confined to the 4-nm-thick layer beneath the membrane, which has a volume equal to the volume of the cell minus the volume of a sphere with a radius 4 nm less than that of the cell:

$$V_{layer} = V_{cell} - 4\pi(r - 4\ nm)^3/3$$

$$= V_{cell} - 4\pi[(10 \times 10^{-6}\ m) - (4 \times 10^{-9}\ m)]^3/3$$

$$= (4.1888 \times 10^{-15}\ m^3) - (4.1838 \times 10^{-15}\ m^3)$$

$$V_{layer} = 0.0050 \times 10^{-15}\ m^3$$

Thus, the volume of the cell is 838 times greater than the volume of a 4-nm-thick layer beneath the membrane ($4.1888 \times 10^{-15}\ m^3/0.0050 \times 10^{-15}\ m^3$).

Even allowing for the interior regions of the cell from which it would be excluded (nucleus and organelles), the mutant Src would still be a couple of orders of magnitude less concentrated in the neighborhood of the membrane than the normal Src.

B. Its lower concentration in the region of its target X at the membrane is the reason why mutant Src does not cause cell proliferation. This notion can be quantified by a consideration of the binding equilibrium for Src and its target:

$$Src + X \rightarrow Src - X$$

$$K = \frac{[Src - X]}{[Src][X]}$$

The lower concentration of the mutant Src in the region of the membrane will shift the equilibrium toward the free components, reducing the amount of complex. If the concentration is on the order of 100-fold lower, the amount of complex will be reduced up to 100-fold. Such a large decrease in complex formation could readily account for the lack of effect of the mutant Src on cell proliferation.

3–13 The antibody binds to the second protein with an equilibrium constant, K, of $5 \times 10^7\ M^{-1}$.

A useful shortcut to problems of this sort recognizes that $\Delta G°$ is related to log K by the factor –2.3 RT, which equals –5.9 kJ/mole at 37°C. Thus, a factor of ten increase in the equilibrium constant (an increase in log K of 1) corresponds to a decrease in $\Delta G°$ of –5.9 kJ/mole. A 100-fold increase in K corresponds to a decrease in $\Delta G°$ of –11.9 kJ/mole, and so on. For each factor of ten increase in K, $\Delta G°$ decreases by –5.9 kJ/mole; for each factor of ten decrease in K, $\Delta G°$ increases by 5.9 kJ/mole. This relationship allows a quick estimate of changes in equilibrium constant from free-energy changes and vice versa. In this problem, you are told that $\Delta G°$ increased by 11.9 kJ/mole (a weaker binding gives a less negative $\Delta G°$). According to the relationship developed above, this increase in $\Delta G°$ requires that K decrease by a factor of 100 (a decrease by 2 in log K); thus, the equilibrium constant for binding to the second protein is $5 \times 10^7\ M^{-1}$.

The solution to the problem can be calculated by first determining the free-energy change represented by the binding to the first protein:

$$\Delta G^\circ = -2.3\ RT \log K$$

Substituting for K,

$$\Delta G^\circ = -2.3\ (8.3 \times 10^{-3}\ \text{kJ/K mole})\ (310\ \text{K}) \log (5 \times 10^9)$$

$$\Delta G^\circ = -5.92\ \text{kJ/mole} \times 9.7$$

$$\Delta G^\circ = -57.4\ \text{kJ/mole}$$

The free-energy change associated with binding to the second protein is obtained by adding 11.9 kJ/mole to the free-energy change for binding to the first protein, giving a value of –45.5 kJ/mole. Thus, the equilibrium constant for binding to the second protein is

$$\log K = (-45.5\ \text{kJ/mole})/(-5.92\ \text{kJ/mole})$$

$$= 7.7$$

$$K = 5 \times 10^7\ \text{M}^{-1}$$

3–14 The calculated values of fraction of tmRNA bound versus SmpB concentration are shown in **Table A3–1**. Also shown are rule-of-thumb values, which are easier to remember. Since the fraction bound = [SmpB]/([SmpB] + K_d), substitution of K_d values for [SmpB] gives the solution. For example, when [SmpB] = 10^2, fraction bound equals 100/101 = 0.99.

These relationships are useful not only for thinking about K_d, but also for enzyme kinetics. The rate of a reaction expressed as a fraction of the maximum rate is

$$\text{rate}/V_{max} = [S]/([S] + K_m)$$

which has the same form as the equation for fraction bound. Thus, for example, when the concentration of substrate, [S], is tenfold above the Michaelis constant, K_m, the rate is 90% of the maximum, V_{max}. When [S] is 100-fold below K_m, the rate is 1% of V_{max}.

The relationship also works for the fractional dissociation of an acidic group, HA, as a function of pH. When the pH is 2 units above pK, 99% of the acidic group is ionized. When the pH is 1 unit less than pK, 10% is ionized.

TABLE A3–1 Calculated values for fraction bound versus protein concentration (Answer 3–14).

[Protein]	Fraction Bound (%)	Rule of Thumb
$10^4\ K_d$	99.99	99.99
$10^3\ K_d$	99.9	99.9
$10^2\ K_d$	99	99
$10^1\ K_d$	91	90
K_d	50	50
$10^{-1}\ K_d$	9.1	10
$10^{-2}\ K_d$	0.99	1
$10^{-3}\ K_d$	0.099	0.1
$10^{-4}\ K_d$	0.0099	0.01

3–15 At [S] = 0, the rate equals $0/K_m$ and the rate is therefore zero. At [S] = K_m, the ratio of [S]/([S] + K_m) equals 1/2 and the rate is 1/2 V_{max}. At infinite [S] the ratio of [S]/([S] + K_m) equals 1 and the rate is equal to V_{max}.

3–16

A. An enzyme composed entirely of mirror-image amino acids would be expected to fold stably into a mirror-image conformation; that is, it would look like the normal enzyme when viewed in a mirror.

B. A mirror-image enzyme would be expected to recognize the mirror image of its normal substrate. Thus, "D" hexokinase would be expected to add a phosphate to L-glucose and to ignore D-glucose.

This experiment has actually been done for HIV protease. The mirror-image protease recognizes and cleaves a mirror-image substrate.

Reference: Milton RC, Milton SC & Kent SB (1992) Total chemical synthesis of a D-enzyme: the enantiomers of HIV-1 protease show reciprocal chiral substrate specificity. *Science* 256, 1445–1448.

3–17 This simple question required decades of research to provide a complete and satisfying answer. At the simplest level, hemoglobin binds oxygen efficiently in the lungs because the concentration (partial pressure) of

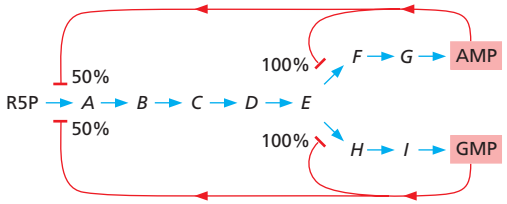


Figure A3–2 Pattern of inhibition in the metabolic pathway for purine nucleotide synthesis (Answer 3–18).

oxygen is highest there. In the tissues, the concentration of oxygen is lower because it is constantly being consumed in metabolism. Thus, hemoglobin will tend to release (bind less) oxygen in the tissues. This natural tendency—an effect on the binding equilibrium—is enhanced by allosteric interactions among the four subunits of the hemoglobin molecule. As a consequence, much more oxygen is released in the tissues than would be predicted by a simple binding equilibrium.

3–18 One reasonable proposal would be for excess AMP to feedback inhibit the enzyme for converting *E* to *F*, and excess GMP to feedback inhibit the step from *E* to *H*. Intermediate *E*, which would then accumulate, would feedback inhibit the step from R5P to *A*. Some branched pathways are regulated in just this way. Purine nucleotide synthesis is regulated somewhat differently, however (**Figure A3–2**). AMP and GMP regulate the steps from *E* to *F* and from *E* to *H*, as above, but they also regulate the step from R5P to *A*. Regulation by AMP and GMP at this step might seem problematical since it suggests that a rise in AMP, for example, could shut off the entire pathway even in the absence of GMP. The cell uses a very clever trick to avoid this problem. Individually, excess AMP or GMP can inhibit the enzyme to about 50% of its normal activity; together they can completely inhibit it.

CHAPTER 4

4–1 True. The human karyotype comprises 22 autosomes and the two sex chromosomes, X and Y. Females have 22 autosomes and two X chromosomes for a total of 23 different chromosomes. Males also have 22 autosomes, but have an X and a Y chromosome for a total of 24 different chromosomes.

4–2 True. All the core histones are rich in lysine and arginine, which have basic—positively charged—side chains that can neutralize the negatively charged DNA backbone.

4–3 False. By using the energy of ATP hydrolysis, chromatin remodeling complexes can catalyze the movement of nucleosomes along DNA, or even dissociate a nucleosome completely from the DNA.

4–4 True. Humans and mice diverged from a common ancestor long enough ago for roughly two out of three nucleotides to have been changed by random mutation. The regions that have been conserved are those with important functions, where mutations with deleterious effects were eliminated by natural selection. Other regions have not been conserved because natural selection cannot operate to eliminate changes in nonfunctional DNA.

4–5 True. Duplication of chromosomal segments, which may include one or more genes, allows one of the two genes to diverge over time to acquire different, but related functions. The process of gene duplication and divergence is thought to have played a major role in the evolution of biological complexity.

4–6 In *all* samples of double-stranded DNA, the numbers of As and Ts (hence their percentages) are equal since they always pair with each other. The same is true for G and C. Results such as this one stood out as odd in the days before the structure of DNA was known. Now it is clear that, while all cellular DNA is double stranded, certain viruses contain single-stranded DNA. The genomic DNA of the M13 virus, for example, is single stranded. In single-stranded DNA, A is not paired with T, nor G with C, and so the A = T and C = G rules do not apply.

4–7 The segment of DNA in Figure Q4–1 reads, from top to bottom, 5′-ACT-3′. The carbons in the ribose sugar are numbered clockwise around the ring, starting with C1′, the carbon to which the base is attached, and ending with C5′, the carbon that lies outside the ribose ring.

4–8 Because C always pairs with G in duplex DNA, their mole percents must be equal. Thus, the mole percent of G, like C, is 20%. The mole percents of A and T account for the remaining 60%. Since A and T always pair, each of their mole percents is equal to half this value: 30%.

4–9 The intermediate chromosome and the sites of the inversions are indicated in **Figure A4–1**.

4–10 With the stated assumptions, the DNA is compacted 27-fold in 30-nm fibers relative to the extended DNA. The total length of duplex DNA in 50 nm of the fiber is 1360 nm [(20 nucleosomes) × (200 bp/nucleosome) × (0.34 nm/bp) = 1360 nm]; 1360 nm of duplex DNA reduced to 50 nm of chromatin fiber represents a 27-fold condensation [(1360 nm/50 nm) = 27.2]. This level of packing represents 0.27% (27/10,000) of the total condensation that occurs at mitosis, still a long way from what is needed.

4–11 The biological outcome associated with histone methylation depends on the site that is modified. Each site of methylation has different surrounding amino acid context, which allows the binding of distinct reader complexes. It is the binding of different downstream effector proteins that gives rise to different biological outcomes.

4–12 A dicentric chromosome is unstable because the two kinetochores have the potential to interfere with one another. Normally, microtubules from the two poles of the spindle apparatus attach to opposite faces of a single kinetochore in order to separate the individual chromatids at mitosis. If a chromosome contains two centromeres, half of the time the microtubules from one of the poles will attach to the two kinetochores associated with one chromatid, while the microtubules from the other pole will attach to the two kinetochores associated with the other chromatid. Division can then occur satisfactorily. The other half of the time, the microtubules from each pole will attach to kinetochores that are associated with different chromatids. When that happens, each chromatid will be pulled to opposite spindle poles with enough force to snap it in two. Thus, two centromeres are bad for a chromosome, causing chromosome breaks—rendering it unstable.

4–13 Colonies are clumps of cells that originate from a single founder cell and grow outward as the cells divide repeatedly. In the red colony of

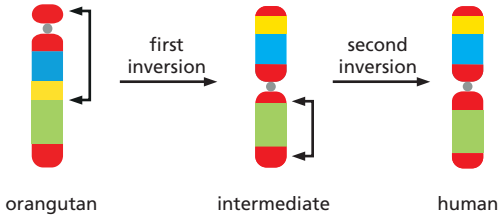


Figure A4–1 Inversions and intermediate chromosome in the evolution of chromosome 3 in orangutans and humans (Answer 4–9).

Figure Q4–3, the *Ade2* gene has been inactivated by its position next to the telomere. The inactivation is inherited, but at a low frequency the gene is reactivated. This gives rise to white cells whose descendants are also white (producing the white sectors), even though the gene has not moved away from the telomere. This pattern shows that the inactivation of a telomere-proximal gene is passed on to daughter cells in a way that is not completely stable, and that both the off and the on stare are heritable. An epigenetic mechanism is thought to be involved, based on the tendency of a condensed chromatin state to be inherited following DNA replication.

4–14 The *Hox* gene clusters are packed with complex and extensive regulatory sequences that ensure the proper expression of individual *Hox* genes at the correct time and place during development. Insertions of transposable elements into the *Hox* clusters are eliminated by purifying selection, presumably because they disrupt proper regulation of the *Hox* genes. Comparison of the *Hox* cluster sequences in mouse, rat, and baboon reveals a high density of conserved noncoding segments, supporting the presence of a high density of regulatory elements.

Reference: Lander ES, Linton L, Birren B et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921.

CHAPTER 5

5–1 True. Each time the genome is copied in preparation for cell division, there is a chance that mistakes (mutations) will be introduced. The rate of mutation for humans is estimated to be 1 nucleotide change per 10^{10} nucleotides each time the DNA is replicated. Since there are 6.4×10^9 nucleotides in each diploid cell, an average of 0.64 random mutations will be introduced into the genome each time it is copied. Thus, the two daughter cells from a cell division will often differ from one another and from the parent cell that gave rise to them. Even genomes that are copied perfectly, giving rise to identical daughter cells, will often be altered in subsequent replication cycles. The proportion of identical cells depends on the exact mutation rate.

5–2 True. If the replication fork moves forward at 500 nucleotide pairs per second, the DNA ahead of it must rotate at 48 revolutions per second (500 nucleotides per second/10.5 nucleotides per helical turn) or 2880 revolutions per minute. The havoc this would wreak on the chromosome is prevented by a DNA topoisomerase that introduces transient nicks just in front of the replication fork. This action confines the rotation to a short, single-strand segment of DNA.

5–3 True. The two ends of a single parental strand of DNA will be copied in the same direction. At the fork at one end of a replication bubble, this will correspond to the leading strand; at the fork at the other end, it will correspond to the lagging strand.

5–4 True. Consider a single template strand, with its 5′ end on the left and its 3′ end on the right. No matter where the origin is, synthesis to the left on this strand will be continuous (leading strand), and synthesis to the right will be discontinuous (lagging strand). Thus, when replication forks from adjacent origins collide, a rightward-moving (lagging) strand will always meet a leftward-moving (leading) strand.

5–5 False. Repair of damage to a single strand by base excision repair or nucleotide excision repair, for example, depends on just the two copies of genetic information contained in the two strands of the DNA double

helix. By contrast, precise repair of damage to both strands of a duplex—a double-strand break, for example—requires information from a second duplex, either a sister chromatid or a homolog.

5–6 The variation in frequency of mutants in different cultures exists because of variations in the time at which the mutations arose. For example, cultures with only one mutant bacterium must have acquired the mutation in the last generation; cultures with two mutants likely acquired a mutation in the next-to-last generation and produced two mutant daughter cells; cultures with four mutants likely acquired the mutation in the third-to-last generation and the mutant cell divided twice. Cultures with large numbers of mutant cells acquired a mutation early in growth and those cells divided many times. To understand this variability, it is best to think of the mutation rate (1 mutation per 10^9 bp per generation) as a probability: a 10^{-9} chance of making a mutation each time a nucleotide pair is copied. Thus, sometimes a mutation will occur before 10^9 nucleotides have been copied and sometimes after.

Analysis of the variation in frequencies among cultures grown in this way (which is known as fluctuation analysis) is a common method for determining rates of mutation. Luria and Delbrück originally devised the method to show that mutations preexist in populations of bacteria; that is, they do not arise as a result of the selective methods used to reveal their presence.

Reference: Luria SE & Delbrück M (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28, 491–511.

5–7 Mismatch repair normally corrects a mistake in the new strand, using information in the old, parental strand. If the old strand were "repaired" using the new strand that contains a replication error as the template, then the error would become a permanent mutation in the genome, with the "correct" information being erased in the process. Therefore, if repair enzymes did not distinguish between the two strands, there would be only a 50% chance that any given replication error would be corrected.

Overall, such indiscriminate repair would introduce the same number of mutations as would be introduced if mismatch repair did not exist. In the absence of repair, a mismatch would persist until the next replication. When the replication fork passed the mismatch, and the strands were separated, properly paired nucleotides would be inserted opposite each of the nucleotides involved in the mismatch. A normal, nonmutant duplex would be made from the strand containing the original information; a mutant duplex would be made from the strand that carried the misincorporated nucleotide. Thus, the original misincorporation event would lead to 50% mutants and 50% nonmutants in the progeny. This outcome is equivalent to that of indiscriminate repair: averaged over all misincorporation events, indiscriminate repair would also yield 50% mutants and 50% nonmutants among the progeny.

5–8 While the process may seem wasteful, it provides an elegant solution to the difficulty of proofreading during primer formation. To start a new primer on a piece of single-strand DNA, one nucleotide must be put in place and then linked to a second and then to a third and so on. Even if these first nucleotides were perfectly matched to the template strand, such short oligonucleotides bind with very low affinity and it would consequently be difficult to distinguish the correct from incorrect bases by proofreading. The task of the primase is to "just get anything down that binds reasonably well and don't worry about accuracy." Later, these sequences are removed and replaced by DNA polymerase, which uses the accurately synthesized DNA of the adjacent Okazaki fragment as its primer. DNA polymerase has the advantage—which primase lacks—of

putting the new nucleotides onto the end of an already existing strand. The newly added nucleotide is held firmly in place, and the accuracy of its base-pairing to the next nucleotide on the template strand can be accurately assessed. Therefore, as DNA polymerase fills the gap, it can proofread from the start of the new DNA strand that it makes. What appears at first glance as energetically wasteful is really just a necessary price to be paid for accuracy.

5–9 Clearly, DNA polymerases must be able to extend a mismatched primer occasionally; otherwise no mismatches would be present in the newly synthesized DNA. Most mismatches are removed by the 3′-to-5′ proofreading exonuclease associated with the DNA polymerase. When the exonuclease does not remove the mismatch, the polymerase can nonetheless extend the growing chain. In reality, DNA polymerase and the proofreading exonuclease are in competition with each other. In the case of bacteriophage T7 DNA polymerase, numbers are available that illustrate this competition. Normally, T7 DNA polymerase synthesizes DNA at 300 nucleotides per second, while the exonuclease removes terminal nucleotides at 0.2 nucleotides per second, suggesting that 1 in 1500 (0.2/300) correctly added nucleotides are removed by the exonuclease. When an incorrect nucleotide has been incorporated, the rate of removal increases tenfold to 2.3 nucleotides per second and the rate of polymerization decreases 3×10^4-fold to 0.01 nucleotide per second. Comparison of these rates for a mismatched primer suggests that about 1 in 200 (0.01/2.3) mismatched primers will be extended by T7 DNA polymerase.

Reference: Johnson KA (1993) Conformational coupling in DNA polymerase fidelity. *Annu. Rev. Biochem.* 62, 685–713.

5–10 As always, you come through with flying colors. Although you were initially bewildered by the variety of structures, you quickly realized that H forms were just like the bubbles except that cleavage occurred within the bubble instead of outside it. Next you realized that by reordering the molecules according to the increasing size of the bubble (and flipping some structures end-for-end), you could present a convincing visual case for bidirectional replication away from a unique origin of replication (**Figure A5–1**). The case for bidirectional replication is clear since unidirectional replication would give a set of bubbles with one end in common. Replication from a unique origin is likely, but not certain, because you cannot rule out the possibility that there are two origins on either side of and equidistant from the restriction site used to linearize the DNA. Repeating the experiment using a different restriction nuclease will resolve this issue and define the exact position of the origin(s) on the viral DNA. Your advisor is pleased.

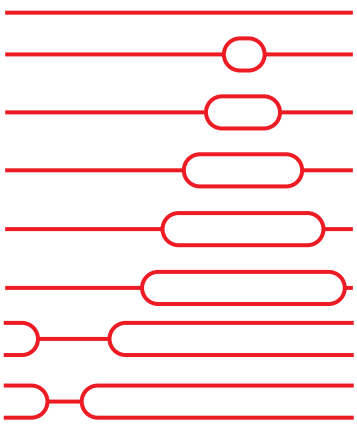


Figure A5–1 Bidirectional replication from a unique origin (Answer 5–10).

5–11

A. The regions of the tracks that are dense with silver grains correspond to those segments of DNA that were replicated when the concentration of ^{3}H-thymidine was high. The less-dense regions mark segments of DNA that were replicated when the concentration of ^{3}H-thymidine was low.

B. The difference in the arrangements of the dark and light sections of the tracks derives from the difference in the labeling schemes in the two experiments. In the first experiment (see Figure Q5–2A), ^{3}H-thymidine was added immediately after release of the synchronizing block. Thus, replication was initiated at origins in the presence of ^{3}H-thymidine, giving a continuous dark section on both sides of the origin. When the concentration of label was lowered, replication proceeded in both directions away from the origin, leaving light sections at both ends of the dark sections. In the second experiment (see Figure Q5–2B), replication began at origins in the absence of ^{3}H-thymidine so that the origin was unlabeled.

Addition of a high concentration of label followed by a low concentration gave rise to a dark section with a light section at one end. Adjacent dark sections are part of the same replicating DNA molecule; they are linked by the unlabeled (therefore invisible) segment that contains the replication origin.

C. The approximate rate of fork movement can be estimated from the labeling times and the lengths of the labeled sections. In the first experiment, segments roughly 100 μm in length were labeled during the 45-minute labeling period. Because two replication forks were involved in synthesizing each labeled segment, each replication fork synthesized about 50 μm of DNA in 45 minutes. Therefore, the rate of fork movement is about 1.1 μm/min (50 μm/45 min). In the second experiment, segments roughly 50 μm in length were labeled; however, each was synthesized by only one replication fork. Thus, the rate of fork movement was also about 1.1 μm/min.

This information is not sufficient to estimate the time required to replicate the entire genome. The missing information is the number of active origins of replication and their distribution. Assuming that all origins are activated at the same time and all forks move at the same rate, the minimum time required to replicate the genome (regardless of its size) is fixed by the distance between the two origins that are farthest apart.

Reference: Huberman JA & Riggs AD (1968) On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol.* 32, 327–341.

5–12 At many sites in vertebrate cells, the sequence 5′-CG-3′ is selectively methylated on the cytosine base. Spontaneous deamination of methyl-C produces T. A special DNA glycosylase recognizes a mismatched base pair involving T in the sequence TG, and removes the T. This DNA repair mechanism is clearly not 100% effective, as methylated C nucleotides are common sites for mutation in vertebrate DNA. Over time, the enhanced mutation rate of CG dinucleotides has led to their preferential loss, accounting for their underrepresentation in the human genome.

5–13 If the inaccurately repaired breaks were randomly distributed around the genome, then 2% of them would be expected to alter crucial coding or regulatory information. Thus, the functions of about 40 genes (0.02 × 2000) would be compromised in each cell, although the specific genes would vary from cell to cell. Because not all genes are expressed in every cell, gene mutations in some cells would be without consequence. In addition, because the human genome is diploid, the effect on cell function of mutations in expressed genes would be mitigated by the remaining allele. For most loci, one functional allele (50% of normal protein) is adequate for normal cell function; however, for some loci, 50% is not adequate. Thus, the mutations would be expected to compromise the functions of some cells.

Reference: Lieber MR, Ma Y, Pannicke U & Schwarz K (2003) Mechanism and regulation of human non-homologous DNA end-joining. *Nat. Rev. Mol. Cell Biol.* 4, 712–720.

5–14 The double Holliday junction that would result from strand invasion is shown in **Figure A5–2**. Two representations are shown, both correct. The upper one looks simpler because the invading duplex has been rotated so that the marked 5′ end is on the bottom. This arrangement minimizes the number of lines that must cross, which is why most recombination diagrams are shown in this way. The lower representation is perfectly correct, but it looks more complicated. Note that both drawings represent exactly the same molecular structure. DNA synthesis uses the 3′ end of the invading duplex as a primer and fills the single-strand gap by 5′-to-3′ synthesis, as indicated.

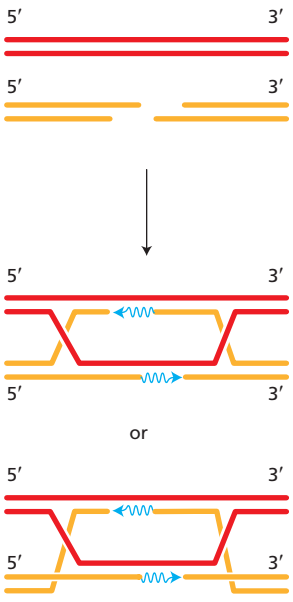


Figure A5–2 Double Holliday junction (Answer 5–14). New DNA synthesis is indicated by *wavy blue lines*.

5–15 A large percentage of the human genome is made up of repetitive elements such as *Alu* sequences, which are scattered among the chromosomes. If, for example, recombination were to occur between two such sequences that were on different chromosomes, a translocation would be generated. Unrestricted recombination between such repeated elements would quickly rearrange the genome beyond recognition. Different rearrangements in different individuals would lead to large numbers of nonviable progeny, putting the species at risk.

This calamity is avoided through the action of the mismatch repair system. Repeated sequences around the genome differ by a few percent of their sequence. When recombination intermediates form between them, many mismatches are present in the heteroduplex regions. When the mismatch repair system detects too high a frequency of mismatches, it aborts the recombination process in some way. This surveillance mechanism ensures that sequences that successfully recombine are nearly identical, as expected for sequences at the same locus on homologous chromosomes.

5–16 Cre-mediated recombination between oppositely oriented LoxP sites inverts the sequence between the sites, whereas recombination between LoxP sites in the same orientation deletes the sequence from the genome, releasing it as a circle (**Figure A5–3**). Since the circle likely lacks an origin of DNA replication, it will be lost as the cells divide. The easiest way to work out the products is to align the LoxP sites and then follow the crossover between them.

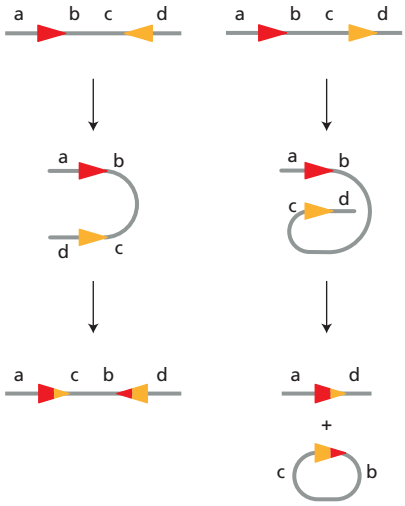


Figure A5–3 Products of Cre-mediated recombination between oppositely oriented and directly repeated LoxP sites (Answer 5–16).

CHAPTER 6

6–1 True. Errors in DNA replication have the potential to affect future generations of cells, while errors in transcription have no genetic consequence. Errors in transcription lead to mistakes in a small fraction of RNAs, whose functions are further monitored by downstream quality control mechanisms. They are not passed on to progeny cells. In contrast, errors in DNA replication change the gene and, thereby, affect all the copies of RNA (and protein) made in the original cell and all its progeny cells.

These considerations are reflected in the intrinsic error rates for RNA and DNA polymerases: RNA polymerases typically make 1 mistake in copying 10^4 nucleotides, while DNA polymerases make about 1 error per 10^7 nucleotides. Such significant differences in error rates suggest that natural selection is stronger against errors in replication than against errors in transcription.

6–2 False. Although intron sequences are mostly dispensable, they must be removed precisely. An error of even one nucleotide during removal would shift the reading frame in the spliced mRNA molecule and produce an aberrant protein.

6–3 False. Wobble pairing occurs between the third position in the codon and the first position in the anticodon.

6–4 False. Because correct base-pairing is only about 10- to 100-fold more stable than incorrect matches, additional mechanisms, beyond the simple thermodynamics of base-pairing, must be used to reach the accuracy of protein synthesis routinely achieved in the cell. Two such mechanisms are induced fit, where the ribosome folds around the correct base pairs, and kinetic proofreading, which introduces delays that allow poorly matched bases to dissociate.

6–5 False. Although only a few types of reactions are represented among the

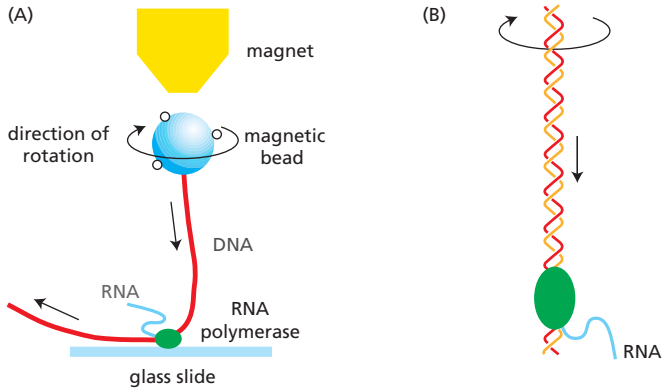


Figure A6–1 Rotation of DNA duplex due to movement relative to RNA polymerase (Answer 6–7). (A) Direction of rotation of the magnetic bead. (B) Direction of rotation of the DNA duplex.

ribozymes in present-day cells, ribozymes that have been selected in the laboratory can catalyze a wide variety of biochemical reactions, with reaction rates that approach those of proteins. In light of these results, it is unclear why ribozymes are so underrepresented in modern cells. It seems likely that the availability of 20 amino acids versus 4 bases affords proteins a greater number of catalytic strategies than ribozymes, as well as endowing them with the ability to bind productively to a wider range of substrates (for example, hydrophobic substrates, which ribozymes have difficulty with).

6–6 The RNA polymerase must be moving from right to left in Figure Q6–1. If the RNA polymerase does not rotate around the template as it moves, it will overwind the DNA ahead of it, causing positive supercoils, and underwind the DNA behind it, causing negative supercoils. If the RNA polymerase were free to rotate about the template as it moved along the DNA, it would not overwind or underwind the DNA, and no supercoils would be generated.

Reference: Liu LF & Wang JC (1987) Supercoiling of the DNA template during transcription. *Proc. Natl Acad. Sci. USA* 84, 7024–7027.

6–7 The bead would rotate clockwise from the perspective of the magnet, as shown in **Figure A6–1A**. As shown in **Figure A6–1B**, the motion of the helix relative to a fixed RNA polymerase causes the helix to rotate.

Reference: Harada Y, Ohara O, Takatsuki A, Itoh H, Shimamoto N & Kinosita K (2001) Direct observation of DNA rotation during transcription by *Escherichia coli* RNA polymerase. *Nature* 409, 113–115.

6–8 Statement C is the only one that is necessarily true for exons 2 and 3. It is also the only one true for exons 7 and 8. While the conditions given in A and B could be the case, they need not be. However, because the encoded protein sequence is the same in segments of the mRNA that correspond to exons 1 and 10, neither choice of alternative exons (2 versus 3, or 7 versus 8) can be allowed to alter the reading frame. To maintain the normal reading frame—whatever that is—the alternative exons must have a number of nucleotides that when divided by 3 (the number of nucleotides in a codon) gives the same remainder.

Because the sequence of the α-tropomyosin gene is known, the actual state of affairs can be checked. Exons 2 and 3 both contain the same number of nucleotides, 126, which is divisible by 3 with no remainder. Exons 7 and 8 also contain the same number of nucleotides, 76, which, when divided by 3, leaves a remainder of 1.

6–9 The only codon assignments consistent with the observed changes, and with the assumption that single-nucleotide changes were involved, are

GUG for valine, GCG for alanine, AUG for methionine, and ACG for threonine. It is unlikely that you would be able to isolate a valine-to-threonine mutant in one step because that would require two nucleotide changes. Typically, two changes would be expected to occur at a frequency equal to the product of the frequencies for each of the single changes; hence, the double mutant would be very rare.

6–10 Single-nucleotide deletions near the beginning of the gene (2) would be the most harmful since they would change the reading frame early in the coding sequence. As a result, the encoded protein would contain a nonsensical and likely truncated sequence of amino acids. In contrast, a reading frameshift that occurs toward the end of the coding sequence, as described in 1, will result in a largely correct protein that may be functional.

Deletion of three consecutive nucleotides, as in scenario 3, leads to the deletion of one amino acid, if it cleanly deletes a codon, or to the deletion of one amino acid and the substitution of another, if the deletion overlaps two adjacent codons. Importantly, deletion of three nucleotides would not alter the reading frame. The deleted amino acid (or altered amino acid) may or may not be important for the folding or activity of the protein. In many cases such mutations are silent; that is, they have insignificant consequences for the organism.

Substitution of one nucleotide for another, as in scenario 4, is often completely harmless, because it does not change the encoded amino acid. In other cases it may change an amino acid, and the consequences may be deleterious or benign, depending on the location and functional significance of that particular amino acid. Often, the most deleterious kind of single-nucleotide change creates a new stop codon, which gives rise to a truncated protein.

6–11 A broken mRNA when translated would produce a truncated protein that could be harmful to the cell. A protein fragment can retain some of the functions of the whole protein, allowing it, for example, to bind to a target protein but trap it in an unproductive complex. Alternatively, a protein fragment can display new, aberrant binding surfaces that allow it to bind to novel partners, interfering with their function. Finally, the deletion could remove the portion of the protein that normally controls its activity. In this case, the truncated protein might be locked into its active state, with dire consequences for the cell.

6–12 In a well-folded protein, the majority of hydrophobic amino acids will be sequestered in the interior, away from water. Exposed hydrophobic patches thus indicate that a protein is abnormal in some way. Some proteins initially fold with exposed hydrophobic patches that are used in binding to other proteins, ultimately burying those hydrophobic amino acids as well. As a result, hydrophobic amino acids are usually not exposed on the surface of a protein, and any significant patch is a good indicator that something has gone awry. The protein may have failed to fold properly after leaving the ribosome, it may have suffered an accident that partly unfolded it at a later time, or it may have failed to find its normal partner subunit in a larger protein complex.

6–13 Molecular chaperones fold like any other protein. Molecules in the act of synthesis on ribosomes are bound by hsp70 chaperones. And incorrectly folded molecules are helped by hsp60-like chaperones. That they function as chaperones when they have folded correctly makes no difference to the way they are treated before they reach their final, functional conformation. Of course, properly folded hsp60-like and hsp70 chaperones must already be present to help fold the newly made chaperones. At cell

division, each daughter cell inherits a starter set of such chaperones from the parental cell.

6–14 RNA has the ability to store genetic information like DNA and the ability to catalyze chemical reactions like proteins. Having both of these essential features of "life" in a single type of molecule makes it easier to understand how life might have arisen from nonliving matter. The use of RNA molecules as catalysts in several fundamental reactions in modern-day cells supports this idea. Nevertheless, it is not yet possible to specify a plausible pathway from the "primordial" soup to an RNA world. Because RNA molecules are highly susceptible to chain breakage (see below), many have speculated that there may have been an "RNA-like" precursor molecule to RNA—one that likewise had catalytic and informational properties, but was more stable.

The deoxyribose sugar of DNA makes the molecule much less susceptible to breakage. The hydroxyl group on carbon 2 of the ribose sugar is an agent for catalysis of the adjacent 3′-5′ phosphodiester bond that links nucleotides together in RNA. Its absence from DNA eliminates that mechanism of chain breakage. In addition, the double-helical structure of DNA provides two complementary strands, which allows damage in one strand to be repaired accurately by reference to the sequence of the second strand. Finally, the use of T in DNA instead of U, as in RNA, builds in a protection against the effects of deamination—a common form of damage. Deamination of T produces an aberrant base (methyl C), whereas deamination of U generates C, a normal base. The cell's job of recognizing damaged bases is much easier when the damage produces an abnormal base.

6–15 The complement of this hairpin RNA could also form a similar hairpin, as shown in **Figure A6–2**. The two structures would be identical in the double-stranded regions that involved standard G-C and A-U base pairs. They would differ in the sequence of the single-stranded regions. Because G-U base pairs can form in RNA, unlike C-A base pairs, one hairpin would be predicted to contain an additional base pair, as shown.

Figure A6–2 Hairpins formed by an RNA strand and by its complement (Answer 6–15). An RNA *(black)* and its complement *(blue)* are shown as double-stranded RNA in the middle. The structures formed by each strand are shown *above* and *below* the duplex. The nonstandard G-U base pair in the lower hairpin is highlighted with a *dashed box*.

6–16 The RNA molecule will not be able to catalyze its own replication. As a single molecule with a single catalytic site, it cannot be both template and catalyst simultaneously. (To visualize the critical difficulty, try to imagine how the active site of the RNA could copy itself.) Once a second molecule—either template or catalyst—was generated, then replication could begin.

Reference: Bartel DP & Szostak JW (1993) Isolation of new ribozymes from a large pool of random sequences. *Science* 261, 1411–1418.

CHAPTER 7

7–1 True. Both the helix–loop–helix motif and the leucine zipper motif are structural motifs that allow transcription regulators to dimerize, so that each member of the pair can position an α helix in the major groove of the DNA.

7–2 False. Even specialized cells must constantly respond to changes in their environment, which they do in many cases by altering the pattern of gene transcription.

7–3 True. In unmethylated regions of the genome, spontaneous deamination of C (a very common event) gives rise to the novel DNA base, uracil, which can be accurately recognized and repaired. By contrast,

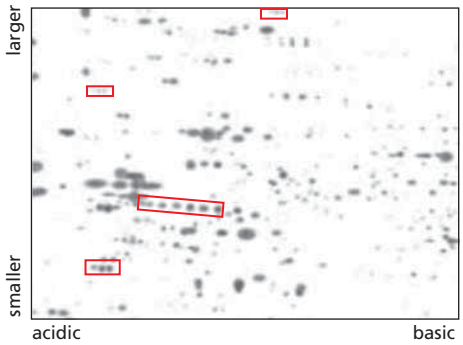


Figure A7–1 Protein spots in a two-dimensional gel that might differ by the number of attached phosphates (Answer 7–5). A few sets of horizontal spots that could be related by phosphorylation are boxed. Not all such sets of proteins are indicated. (Original image courtesy of Tim Myers and Leigh Anderson, Large Scale Biology Corporation.)

deamination of 5-methyl C gives rise to a T, a normal DNA base, which is more difficult for the cell's repair machinery to recognize as incorrect. As a consequence, methylated CG dinucleotides in the germ line have tended to be lost during evolution, leaving the CG islands found in modern genomes.

7–4 True. There are several epigenetic mechanisms of inheritance that allow cells to retain a memory of the gene expression patterns in their parent cells, including transcription regulators that activate their own transcription, DNA methylation, and chromatin structure, to name a few.

7–5 Each added phosphate alters the charge by one unit, but has relatively little effect on the molecular mass. As a consequence, proteins that differ only in the number of attached phosphates will appear to be the same size, but will have different isoelectric points, forming a set of horizontal spots, as shown for a few proteins in **Figure A7–1**. It is important to keep in mind that a horizontal array of spots does not prove that the proteins are related by phosphorylation; they could be different proteins with the same molecular mass and slightly different isoelectric points, or they could be the same protein with a different type of modification that affects the charge of the protein. Treatment of the proteins with a protein phosphatase before separation by gel electrophoresis could be used to resolve the issue.

7–6 Although it is true that cancer cells differ from their normal precursors, they typically differ in their expression of only relatively few genes (oncogenes and tumor suppressor genes). When the overall patterns of mRNAs in cancer cells are compared with the patterns of mRNAs in normal tissues, they match for the great majority of mRNAs. This RNA signature allows a tumor to be definitively assigned to a particular tissue type.

7–7 The two basic components of genetic switches are *cis*-regulatory DNA sequences and the transcription regulators that bind to them.

7–8 Under the specified conditions (equal concentrations of DNA and transcription regulator), the protein would occupy its recognition site equally well in the eukaryotic nucleus and in the bacterium. A nonmathematical way of thinking about this is to imagine a small volume of eukaryotic nucleus, equal in size to that of the bacterium and containing the binding site. That small volume in the eukaryotic nucleus is directly comparable to the interior of the bacterium. In those equal volumes, the ability of the transcription regulator to find its binding site is equivalent. So long as the *concentrations* of the DNA and transcription regulator are the same, the total volume will make no difference. This means, of course, that the total *number* of transcription regulator molecules is 500 times higher in a single nucleus than in a single bacterium.

Reference: Ptashne M (1986) A Genetic Switch: Gene Control and Phage λ, p. 114. Oxford, UK: Blackwell Scientific Press.

7–9 Bending proteins can help to bring together distant DNA regions that normally would contact each other only rarely (**Figure A7–2**). Such proteins act to increase the local concentration of transcription regulators in the neighborhood of RNA polymerase by bringing them closer together. Bending proteins are found in both prokaryotes and eukaryotes and are involved in many examples of transcriptional regulation.

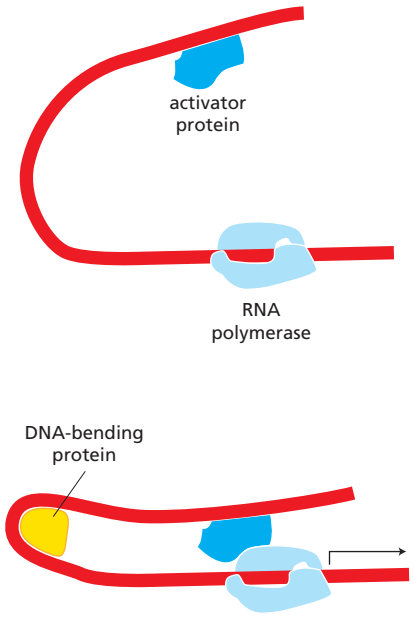


Figure A7–2 Role of a bending protein in bringing together distant transcription regulators (Answer 7–9).

7–10 In a sense, the DNA acts as a tether, holding the proteins in close proximity so that inherently weak interactions between them can occur readily.

7–11 The induction of a transcription activator that stimulates its own synthesis creates a positive feedback loop that can, depending on the stability of protein A, its affinity for its *cis*-regulatory sequence, and other parameters, lead to cell memory. The continued self-stimulated synthesis of activator A can, in principle, last for many cell generations, serving as a constant reminder of an event in the distant past. By contrast, the induction of a transcription repressor that inhibits its own synthesis creates a negative feedback loop that guarantees a transient response to the transient stimulus. Because repressor R shuts off its own synthesis, the cell will quickly return to the state that existed before the transient signal.

7–12 The affected individuals have one deleted gene and one inactive gene due to imprinting. Individuals who carry the deletion will produce affected offspring only if they mate to the sex in which imprinting occurs. Thus, females who carry the deletion are at risk for affected progeny only if the gene is paternally imprinted. Similarly, males who carry the deletion are at risk for affected offspring only if the gene is maternally imprinted. In the pedigree shown in Figure Q7–3A, it is only females who carry the deletion that have affected children. Thus pedigree A must involve paternal imprinting. Similarly, in pedigree B, it is only males who carry the deletion that have affected children; thus this pedigree must involve maternal imprinting.

Reference: Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM & Veres RC (2000) Genetics: From Genes to Genomes, pp. 408–410. Boston: McGraw-Hill Companies.

7–13 The most reasonable hypothesis is that the defective *β-galactosidase* gene is being transcribed and processed into piRNAs. Clusters of piRNAs are transcribed into a single large RNA from which many individual piRNAs are processed by cleavage and trimming. According to this hypothesis, some of the *β-galactosidase* sequences, which would be transcribed along with the piRNAs, are processed just like normal piRNAs. The β-galactosidase piRNAs would then silence expression of the normal *β-galactosidase* gene in the same way that piRNAs silence expression of genes on transposable elements.

If this hypothesis is correct, then you should be able to find *β-galactosidase* sequences in the population of piRNAs. This is indeed what is observed.

Reference: Ronsseray S, Josse T, Boivin A, Anxolabéhère D (2003) Telomeric transgenes and trans-silencing in *Drosophila*. *Genetica* 117, 327–335.

CHAPTER 8

8–1 False. A monoclonal antibody recognizes a specific antigenic site, but this does not necessarily mean that it will bind only to one specific protein.

There are two complicating factors. First, antigenic sites that are similar, but not identical, can bind to the same antibody with different affinities. If too much antibody is used in an assay, the antibody may bind to one protein with high affinity and to others with low affinity. Second, it is not uncommon for different proteins to have the same antigenic site; that is, the same cluster of five or six amino acid side chains on their surfaces. This is especially true of members of protein families, which have similar amino acid sequences, and are often identical in functionally conserved regions.

8–2 False. There are 6×10^{23} molecules per mole; hence, only 0.6 molecules in a yoctomole. The limit of detection is one molecule, or 1.7 yoctomole. No instrument can detect less than one molecule (it is either present in the instrument or it is not).

Reference: Castagnola M (1998) Sensitive to the yoctomole limit. *Trends Biochem. Sci.* 23, 283.

8–3 True. If each cycle doubles the amount of DNA, then 10 cycles equal a 2^{10}-fold amplification (which is 1024), 20 cycles equal a 2^{20}-fold amplification (which is 1.05×10^6), and 30 cycles equal a 2^{30}-fold amplification (which is 1.07×10^9). (It is useful to remember that 2^{10} is roughly equal to 10^3 or 1000. This simple relationship allows you to estimate the answer to this problem rapidly without resorting to your calculator. It comes in handy in a variety of contexts.)

8–4 True. Without quantitative details, it would be impossible to know the interaction is likely to occur in cells at all, and if it does, whether it is stable interaction with a long half-life or a dynamic interaction with rapid binding and dissociation.

8–5 True. This is a fundamental premise in the analysis of biochemical reactions. It applies equally to molecular complexes (such as *A*:*B*, which forms when proteins *A* and *B* bind to each other, and disappears when *A*:*B* dissociates) and metabolic reactions (for example, when metabolite B forms by chemical modification of metabolite A, and disappears when it is converted to metabolite C).

8–6 False. A protein with a rapid degradation rate will reach its new steady state concentration more quickly. The rate of approach to the new steady state is inversely related to the protein's half-life.

8–7 Cells in a tissue are bound together by protein-mediated attachments to one another and to an extracellular matrix containing collagen. Treatment with trypsin, collagenase, and EDTA disrupts these attachments. Trypsin is a protease that will cleave most proteins, but generally only those portions of a native protein that are unstructured. The triple-helical structure of collagen, for example, is a poor substrate for trypsin. Collagenase, which is a protease specific for collagen, digests a principal component of the extracellular matrix. EDTA chelates Ca^{2+}, which is required for the cell-surface proteins known as cadherins to bind to one another to link cells together. Removal of Ca^{2+} prevents this binding and thereby loosens cell–cell attachments.

The treatment does not kill the cells because all the damage occurs to extracellular components, which the cells can replace. So long as the plasma membrane is not breached, the cells will survive.

8–8 The rate of sedimentation of a protein is based on size *and* shape. The nearly spherical hemoglobin will sediment faster than the more rod-shaped tropomyosin, even though tropomyosin is the larger protein. Shape comes into play because molecules that are driven through a solution by centrifugal force experience the equivalent of frictional drag.

A spherical protein, with its smaller surface-to-volume ratio, will experience less drag than a rod, and therefore will sediment faster. You can demonstrate this difference using two sheets of paper. Crumple one into a sphere and roll the other into a tube. Now drop them. The crumpled ball will hit the ground faster than the tube. In this demonstration, the centrifugal force is replaced by gravity and the friction in solution is replaced by friction with air. The underlying principles are the same.

8–9 Although it is invaluable, hybridoma technology is labor intensive and time consuming, requiring several months to isolate a hybridoma cell line that produces a monoclonal antibody of interest. Also, there is no guarantee that the cell line will produce a monoclonal antibody with the specific properties you are after. It is much simpler—a few days' work—to add an epitope tag to your protein, using recombinant DNA technology, and use a commercially available, well-tested antibody to that epitope. The possibility that the tag may alter the function of the protein is a critical concern, but you can add the epitope easily to either the N- or C-terminus and test for the effect on the protein's function. In most cases, a tag at one or the other end of the molecule will be compatible with its function.

8–10 You would need 10^5 copies of a 120-kd protein in a mammalian cell (and 100 copies in a bacterial cell) to be able to detect it on a gel. The calculation comes in two parts: how many cell-equivalents can be loaded onto the gel, and how many copies of a protein can be detected in the band. As shown below for mammalian cells, 100 μg corresponds to 5×10^5 mammalian cells and to 5×10^8 bacterial cells.

$$\frac{\text{cells}}{\text{gel}} = \frac{100\ \mu\text{g}}{\text{gel}} \times \frac{\text{mg}}{1000\ \mu\text{g}} \times \frac{\text{mL}}{200\ \text{mg}} \times \frac{\text{cm}^3}{\text{mL}} \times \frac{(10^4\ \mu\text{m})^3}{(\text{cm})^3} \times \frac{\text{cell}}{1000\ \mu\text{m}^3}$$

$$= 5 \times 10^5\ \text{cells/gel}$$

There are 5×10^{10} 120-kd proteins in a 10-ng band.

$$\frac{\text{molecules}}{\text{band}} = \frac{10\ \text{ng}}{\text{band}} \times \frac{\text{nmol}}{120{,}000\ \text{ng}} \times \frac{6 \times 10^{14}\ \text{molecules}}{\text{nmol}}$$

$$= 5 \times 10^{10}\ \text{molecules/band}$$

Thus, if you can detect 5×10^{10} proteins in a band and can load the equivalent of 5×10^5 mammalian cells per gel, there must be 10^5 copies of the protein per cell ($5 \times 10^{10}/5 \times 10^5$) in order for it to be detectable as a silver-stained band on a gel. For a bacterial cell, there need to be 100 copies of the protein per cell ($5 \times 10^{10}/5 \times 10^8$).

8–11 An m/z difference of 80 corresponds to a phosphate. Addition of a phosphate to the hydroxyl of a serine, threonine, or tyrosine would add three O atoms (48), one P atom (31), and two H atoms (2), in place of one H atom (1) from the hydroxyl group, for a net addition of 80. Under conditions used in mass spectrometry analysis, there is no charge on the phosphate.

8–12 The correct PCR primers are primer 1 (5′-GACCTGTGGAAGC) and primer 8 (5′-TCAATCCCGTATG). The first primer will hybridize to the bottom strand and prime synthesis in the rightward direction. The second primer will hybridize to the top strand and prime synthesis in the leftward direction. (Remember that complementary strands in DNA are antiparallel to one another.)

The middle two primers in each list (primers 2, 3, 6, and 7) would not hybridize to either strand. The remaining pair of primers (4 and 5) would

hybridize, but they would prime synthesis in the wrong direction—that is, outward, away from the central segment of DNA. Incorrect choices, like these, have been made at one time or another in most laboratories that use PCR. The confusion generally arises because the conventions for writing nucleotide sequences have been ignored. By convention, nucleotide sequences are written 5′ to 3′ with the 5′ end on the left. For double-stranded DNA the 5′ end of the top strand is on the left.

8–13 In PCR amplification, a double-stranded fragment of the correct size is first generated in the third cycle (**Figure A8–1**).

8–14 A gain-of-function mutation increases the activity of the protein product of the gene, making it active in inappropriate circumstances, or giving it a novel activity. The change in activity often has a phenotypic consequence even when the normal protein is present, which is why such mutations are usually dominant. A dominant-negative mutation gives rise to a mutant gene product that interferes with the function of the normal gene product, causing a loss-of-function phenotype even in the presence of a normal copy of the gene. This ability of a single defective allele to determine the phenotype is the reason why such an allele is said to be dominant.

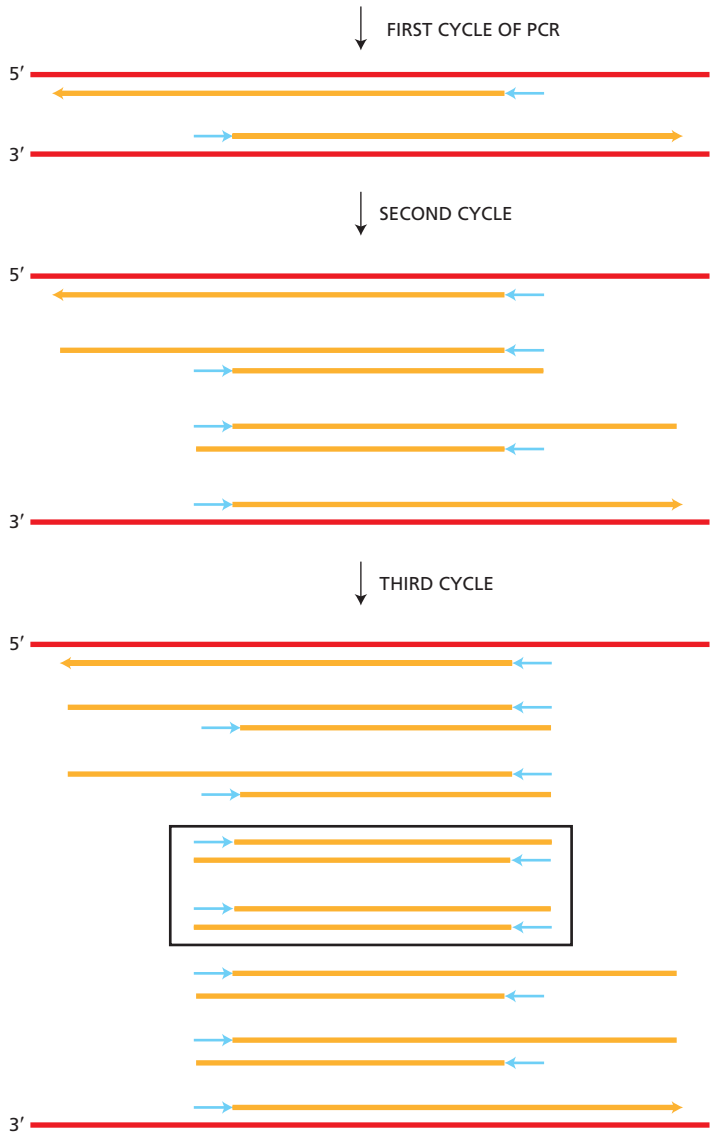


Figure A8–1 The PCR products generated during the first three cycles (Answer 8–13). DNA that has been synthesized during a cycle is shown as *orange lines*. The positions of the primers in new and old products are shown as *blue arrows*. The first products of the correct size are *boxed* in cycle 3.

8–15 This statement is largely true. Diabetes is one of the oldest diseases described by humans, dating back at least to the time of the ancient Greeks. The term "diabetes" itself comes from the Greek word for siphon, which was used to describe one of the main symptoms—increased production of urine: "The disease was called diabetes, as though it were a siphon, because it converts the human body into a pipe for the transflux of liquid humors." If there were no human disease, the role of insulin would not have come to our attention as soon as it did. It is difficult to overstate the case for the role of disease in focusing our efforts toward a molecular understanding. Even today, the quest to understand and alleviate human disease is a principal driving force in biomedical research.

8–16 These results are what you would expect if the mRNA were alternatively spliced. The numbers of reads for exon 4 versus the rest of the mRNA suggest that about half the mRNA was spliced to include all five exons, whereas the rest was spliced to skip exon 4. Thus the mRNA produced from this gene includes two relatively abundant species.

8–17 Of the network motifs in Figure Q8–5, A, B, and C are positive feedback loops, whereas the motif in D is a negative feedback loop. You can analyze these motifs step by step. For motif A, when expression of gene *X* is activated, repressor *X* turns off gene *Y*, which eliminates expression of repressor *Y*, thereby turning on gene *Z*, which activates expression of activator *Z*, which enhances expression of gene *X*, completing the positive feedback loop. For motif B, when expression of gene *X* is activated, repressor *X* turns off gene *Y*, which eliminates expression of activator *Y*, thereby turning off gene *Z*, which eliminates expression of repressor *Z*, which enhances expression of gene *X*, completing the positive feedback loop. The other motifs can be analyzed in the same way.

When you complete this analysis, you should notice a pattern. In network motifs, as in the English language, two negatives make a positive, but an odd number of negatives is still negative. Applying this simple rule, you will see that motifs A, B, and C each contain two inhibitory steps; hence, they are positive feedback loops. Motif D contains a single negative step; hence, it is a negative feedback loop.

8–18 If the perturbed system were exactly at the boundary between the two regions of attraction—the two stable states—it would be balanced on a knife edge. The slightest random fluctuation would drive the system to one or the other of the two stable states.

8–19

A. Comparison of repression levels with each individual operator (Figure Q8–7, constructs 4, 6, and 7) shows that only O_1 gives rise to a significant level of repression. O_2 and O_3 give the same level of expression (no repression) as a construct with no operators (construct 8).
B. So long as the combination of operators contains O_1, the dimeric operator causes significant repression; however, repression is only slightly elevated (less than 2-fold) relative to construct 4, which contains only operator O_1. Thus with a dimeric repressor, the activity of O_1 is not substantially enhanced by the presence of O_2 or O_3. By contrast, additional operators greatly increase repression by the tetrameric repressor by 10-fold (construct 3 versus construct 4) to 50-fold (construct 1 versus construct 4). These results suggest that the presence of multiple binding sites allows the tetrameric repressor—but not the dimeric repressor—to bind two sites at the same time, creating a loop in the DNA. Such a loop might be a more effective way to exclude RNA polymerase from the promoter, thereby increasing repression.
C. The ability of the tetrameric repressor to bind O_3 when it is in the presence of O_1 is an example of cooperative binding. The tight binding

of the repressor to O_1 increases the local concentration in the neighborhood of O_3, which increases the effectiveness with which the repressor can bind O_3.

Reference: Oehler S & Müller-Hill B (2010) High local concentration: a fundamental strategy of life. *J. Mol. Biol.* 395, 242–253.

CHAPTER 9

9–1 False. Although it is not possible to see DNA by light microscopy in the absence of a stain, chromosomes are clearly visible under phase-contrast or Nomarski differential-interference-contrast microscopy when they condense during mitosis. Condensed human chromosomes are more than 1 μm in width—well above the resolution limit of 0.2 μm.

9–2 True. Longer wavelengths correspond to lower energies. Because some energy is lost during absorption and re-emission, the emitted photon is always of a lower energy (longer wavelength) than the absorbed photon.

9–3 In a dry lens, a portion of the illuminating light is internally reflected at the interface between the cover slip and the air. By contrast, in an oil-immersion lens, there is no interface because glass and immersion oil have the same refractive index; hence, no light is lost to internal reflection. In essence, the oil-immersion lens increases the width of the cone of light that reaches the objective, which is a key limitation on resolution.

9–4 The main refraction in the human eye occurs at the interface between air (refractive index 1.00) and the cornea (refractive index 1.38). Because of the small differences in refractive index between the cornea and the lens and between the lens and the vitreous humor, the lens serves to fine-tune the focus in the human eye.

9–5 Humans see poorly under water because the refractive index of water (1.33) is very close to that of the cornea (1.38), thus eliminating the main refractive power of the cornea. Goggles improve underwater vision by placing air in front of the cornea, which restores the normal difference in refractive indices at this interface. The image is still distorted by the refractive index changes at the water–glass and glass–air interfaces of the goggles, but the distortion is small enough that the image can still be focused onto the retina, allowing us to see clearly.

9–6 Resolution refers to the ability to see two small objects as separate entities, which is limited ultimately by the wavelength of light used to view the objects. Magnification refers to the size of the image relative to the size of the object. It is possible to magnify an image to an arbitrarily large size. It is important to remember that magnification does not change the limit of resolution.

9–7 Fluorescently tagged antibodies and enzyme-tagged antibodies each have the advantage of amplifying the initial signal provided by the binding of the primary antibody. For fluorescently tagged secondary antibodies, the amplification is usually several-fold; for enzyme-linked antibodies, amplification can be more than 1000-fold. Although the extensive amplification makes enzyme-linked methods very sensitive, diffusion of the reaction product (often a colored precipitate) away from the enzyme limits the spatial resolution.

9–8 The wavelengths at which the chromophore is excited and at which it emits fluorescent light depend critically on its molecular environment. Using a variety of mutagenic and selective procedures, investigators have generated mutant fluorescent proteins that fluoresce throughout the

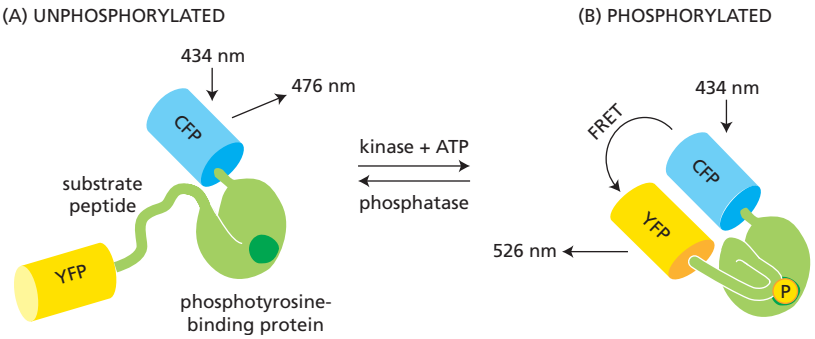


Figure A9–1 Conformational change in FRET reporter protein upon tyrosine phosphorylation (Answer 9–9).

visible range. These modified fluorescent proteins have a variety of different amino acids around the chromophore, which subtly influence its ability to interact with light.

Reference: Service RF (2004) Immune cells speed the evolution of novel proteins. *Science* 306, 1457.

9–9 The increase in FRET depends on phosphorylation of the protein, since no increase occurs in the absence of Abl protein or ATP, or when the phosphate is removed by a tyrosine phosphatase (see Figure Q9–4B). Thus, phosphorylation must cause CFP and YFP to be brought closer together. A reasonable explanation is that addition of phosphate to the tyrosine in the substrate peptide allows that segment of the protein to fold back to bind to the adjacent phosphotyrosine-binding domain, thereby decreasing the separation of the CFP and YFP domains (**Figure A9–1**).

CHAPTER 10

10–1 True. The hydrophobic interior of the lipid bilayer acts as a barrier to the passage of the hydrophilic lipid head groups that must occur during flip-flop. The energetic cost of this movement effectively prevents spontaneous flip-flop of lipids, so that it occurs extremely rarely in the absence of specific catalysts, known as phospholipid translocators.

10–2 False. The carbohydrate on internal membranes is directed away from the cytosol toward the lumen of an internal membrane-bounded compartment. Remember that the lumen of an internal compartment is topologically equivalent to the outside of the cell.

10–3 False. In addition to lipid rafts, which are microdomains with distinct lipid compositions, the apical and basolateral surfaces of epithelial cells, which are separated by intercellular tight junctions, also have different lipid compositions.

10–4 The same forces that dictate that certain lipids will form a bilayer, as opposed to micelles, operate in the repair of a tear in the bilayer. The tear will heal spontaneously because a bilayer is the most energetically favorable arrangement. The lipids that make up a bilayer are cylindrical in shape and therefore do not readily form a micelle (or a hemi-micelle), which would require cone-shaped lipids.

10–5 Vegetable oil is converted to margarine by reduction of double bonds (by hydrogenation), which converts unsaturated fatty acids to saturated ones. This change allows the fatty acids chains in the lipid molecules to pack more tightly against one another, increasing the viscosity, turning oil into margarine.

10–6 A raft 70 nm in diameter would have an area of 3.8×10^3 nm^2 (3.14×35^2), and a lipid molecule 0.5 nm in diameter would have an area of

$0.20\ nm^2$ (3.14×0.25^2). Thus, there would be about 19,000 lipid molecules per monolayer of raft ($3.8 \times 10^3/0.20 = 19{,}000$), and about 38,000 molecules in the raft bilayer. At a ratio of 50 lipids per protein, a raft would accommodate about 760 protein molecules. The true ratio of lipids to proteins in a raft is unknown.

10–7

A. Sequence A is the actual membrane-spanning α-helical segment of glycophorin, a transmembrane protein from red blood cells. It is composed predominantly of hydrophobic amino acids, although it does contain the uncharged polar amino acids threonine (T) and serine (S), which are not uncommon in membrane-spanning α helices.

Sequence B is unlikely to be a membrane-spanning segment because it contains three prolines (P), which would disrupt an α helix and thereby expose polar groups to the hydrophobic environment of the lipid bilayer.

Sequence C is also unlikely to be a transmembrane segment because it contains three charged amino acids, glutamic acid (E), arginine (R), and aspartic acid (D), whose presence in the hydrophobic lipid bilayer would be energetically unfavorable.

10–8 Your friend's suggestion is based on an important difference between inside-out and right-side-out vesicles. The contaminating right-side-out vesicles will carry carbohydrate on their exposed surface and, therefore, should be retained on a lectin affinity column. Inside-out vesicles, by contrast, will lack carbohydrate on their exposed surface and, therefore, should pass through the column.

10–9 Transmembrane domains that are composed entirely of hydrophobic amino acid side chains obviously cannot interact with one another via hydrogen bonds or electrostatic attractions, two of the more important ways to link proteins together noncovalently. Nevertheless, they can interact specifically via van der Waals attractions. If their surfaces are complementary, they can fit together well enough to make a large number of van der Waals contacts, which can hold them together. It should be noted, however, that the transmembrane segment of glycophorin contains a few polar amino acids that may participate in the dimerization process.

10–10 Cytosolic membrane-binding proteins could induce protrusion of the membrane in several ways. For example, a protein that bound to a concave surface of the membrane, instead of the convex surface shown in Figure Q10–2B, would bend the membrane to induce a protrusion. Alternatively, a protein that bound phospholipids with small head groups in the cytosolic leaflet of the membrane, instead of large head groups as shown in Figure Q10–2C, or removed head groups from the phospholipids, would induce a concave curvature of the membrane, giving rise to a protrusion. For the third method of membrane-bending shown in Figure Q10–2A—inserting a segment of protein into the cytosolic leaflet—it is difficult to see how such a mechanism could be used to induce a protrusion.

Reference: Prinz WA & Hinshaw JE (2009) Membrane-bending proteins. *Crit. Rev. Biochem. Mol. Biol.* 44:278–291.

CHAPTER 11

11–1 True. Transporters bind specific molecules and undergo a series of conformational changes to move the bound molecule across a membrane. They can transport passively down the electrochemical gradient, or the

transporters can link the conformational changes to a source of metabolic energy such as ATP hydrolysis to drive active transport. By contrast, channels form aqueous pores that can be open or shut, but always transport downhill; that is, passively. Channels interact much more weakly with the solute to be transported, and they do not undergo conformational changes to accomplish transport. As a consequence, transport through channels cannot be linked to an energy source and is always passive.

11–2 False. Transporters *and* channels saturate. It is thought that permeating ions have to shed most of their associated water molecules in order to pass, in single file, through the narrowest part—the selectivity filter—of the channel. This requirement limits their rate of passage. Thus, as ion concentrations increase, the flux of ions through a channel increases proportionally, but then levels off (saturates) at a maximum rate.

11–3 True. It takes a difference of only a minute number of ions to set up the membrane potential.

11–4 The order is CO_2 (small and nonpolar) > ethanol (small and slightly polar) > H_2O (small and polar) > glucose (large and polar) > Ca^{2+} (small and charged) > RNA (very large and highly charged). This list nicely illustrates the two basic properties that govern the capacity of molecules to diffuse through a lipid bilayer: size (small > large) and polarity (nonpolar > polar > charged).

11–5 The equilibrium distribution of a molecule across a membrane depends on the chemical gradient (concentration) and on the electrical gradient (membrane potential). An uncharged molecule does not experience the electrical gradient and, thus, will be at equilibrium when it is at the same concentration on both sides of the membrane. A charged molecule responds to both components of the electrochemical gradient and will distribute accordingly. K^+ ions, for example, are nearly at their equilibrium distribution across the plasma membrane even though they are about 30-fold more concentrated inside the cell. The difference in concentration is balanced by the membrane potential (negative inside), which opposes the movement of cations to the outside of the cell.

11–6

A. Yes, they can normalize both the H^+ and Na^+ concentrations. For every three cycles of the Na^+–H^+ antiporter, which imports one Na^+ and exports one H^+, the Na^+-K^+ pump cycles once, exporting three Na^+ ions with each operation.

(You may have wondered how to deal with the hydrolysis of ATP that occurs with each cycle of the Na^+-K^+ pump. When ATP is hydrolyzed by H_2O, the products are ADP and $H_2PO_4^-$. $H_2PO_4^-$ has a p*K* of 6.86, which means that it is about 70% ionized into HPO_4^{2-} and H^+ at the intracellular pH of 7.2. It turns out that you do not need to worry about this H^+ because elsewhere in the cell other processes reconvert the products of hydrolysis back into ATP to maintain a steady-state concentration.)

B. The linked action of these two pumps moves $3H^+$ out for every $2K^+$ that are brought into the cell, thereby increasing both the internal K^+ concentration and the membrane potential.

11–7 Based on the scale bars in Figure Q11–1, each microvillus approximates a cylinder 0.1 μm in diameter and 1.0 μm in height. The ratio of the area of the sides of a cylinder, which represent new membrane (new surface area), to the top of a cylinder (which is equivalent to the plasma membrane that would have been present anyway, had the microvillus not been extruded) gives the increase in surface area due to an individual microvillus. The area of the sides of a cylinder ($2\pi rh$, where r is the radius

and h is the height) is 0.31 μm^2 (2 × 3.14 × 0.05 μm × 1.0 μm); the area of the top of the cylinder (πr^2) is 0.0079 μm^2 [3.14 × $(0.05)^2$]. Thus, the increase in surface area for one microvillus is 0.31 μm^2/0.0079 μm^2 or 40. This value overestimates the increase for the entire plasma membrane, since the microvilli occupy only a portion of the surface. The fraction of plasma membrane occupied by microvilli can be estimated from the cross section in Figure Q11–1. A conservative estimate is that about half the plasma membrane is covered by microvilli. Thus, microvilli increase the surface area in contact with the lumen of the gut by approximately 40/2 or 20-fold.

Reference: Adapted from Krstić RV (1997) Ultrastructure of the Mammalian Cell, p. 207. Berlin, Germany: Springer-Verlag.

11–8 Just as a falling body in air reaches a terminal velocity due to friction, an ion in water also reaches a terminal velocity due to friction with water molecules. An ion in water will accelerate for less than 10 nanoseconds before it reaches terminal velocity.

11–9 The volume of the hemisphere explored by the ball is 2.05×10^4 nm^3 [(2/3) × 3.14 × $(21.4\ nm)^3$]. This volume corresponds to 2.05×10^{-20} liters [$(2.05 \times 10^4\ nm^3) \times (cm/10^7\ nm)^3 \times (liter/1000\ cm^3)$]. One ball in this volume corresponds to 8.13×10^{-5} M or 81.3 μM [(1 molecule/2.05×10^{-20} liters) × (mole/6×10^{23} molecules)]. Thus, the local concentration of a tethered ball is about the same as the concentration of free peptide needed to inactivate the channel.

Reference: Zagotta WN, Hoshi T & Aldrich RW (1990) Restoration of inactivation in mutants of *shaker* potassium channels by a peptide derived from ShB. *Science* 250, 568–570.

11–10 The expected membrane potential due to differences in K^+ concentration across the resting membrane is

$$V = 58\ mV \times \log \frac{C_o}{C_i}$$

$$V = 58\ mV \times \log \frac{9\ mM}{344\ mM}$$

$$V = -92\ mV$$

For Na^+, the equivalent calculation gives a value of +48 mV.

The assumption that the membrane potential is due solely to K^+ leads to a value near that of the resting potential. The assumption that the membrane potential is due solely to Na^+ leads to a value near that of the action potential.

These assumptions approximate the resting potential and action potential because K^+ *is* primarily responsible for the resting potential and Na^+ *is* responsible for the action potential. A resting membrane is 100-fold more permeable to K^+ than it is to Na^+ because of the presence of K^+ leak channels. The leak channel allows K^+ to leave the cell until the membrane potential rises sufficiently to oppose the K^+ concentration gradient. The theoretical maximum gradient (based on calculations like those above) is lowered somewhat by the entrance of Na^+, which carries positive charge into the cell (compensating for the positive charges on the exiting K^+). Were it not for the Na^+-K^+ pump, which continually removes Na^+, the resting membrane potential would be dissipated completely.

The action potential is due to a different channel, a voltage-gated Na^+ channel. These channels open when the membrane is stimulated, allowing Na^+ ions to enter the cell. The magnitude of the resulting membrane potential is limited by the difference in the Na^+ concentrations across the membrane. The influx of Na^+ reverses the membrane potential locally,

which opens adjacent Na^+ channels and ultimately causes an action potential to propagate away from the site of the original stimulation.

Reference: Hille B (1992) Ionic Channels of Excitable Membranes, 2nd ed., pp. 23–58. Sunderland, MA: Sinauer.

11–11

A. There are two kinds of cation channel in the rat muscle membrane—a 4-pA channel and a 6-pA channel. You can tell that there are two different channels by the characteristic amount of current they carry. Note that the 6-pA channel cannot be confused with the simultaneous opening of two 4-pA channels, which would give a current of 8 pA.

B. The number of Na^+ ions flowing through the 4-pA channel each millisecond is 2.5×10^4. The 6-pA channel carries 1.5 times as many (3.8×10^4 Na^+ ions) each millisecond.

$$\frac{Na^+}{msec} = 4\ pA \times \frac{A}{10^{12}\ pA} \times \frac{C/sec}{A} \times \frac{1\ Na^+}{1.6 \times 10^{-19}\ C} \times \frac{sec}{10^3\ msec}$$

$$= 2.5 \times 10^4$$

Reference: Sakmann B (1992) Elementary steps in synaptic transmission revealed by currents through single ion channels. *Science* 256, 503–512.

CHAPTER 12

12–1 False. The interior of the nucleus and the cytosol communicate through the nuclear pore complexes, which allow free passage of ions and small molecules. The cytoplasm and the nucleus are said to be topologically equivalent because the outer and inner nuclear membranes are continuous with one another, so that the flow of material between the nucleus and cytosol occurs without crossing a lipid bilayer. By contrast, the lumen of the ER and the outside of the cell are each separated from the cytosol by a layer of membrane. Thus, they are topologically distinct from the cytosol, but they are topologically equivalent to each other.

12–2 True. Ribosomes all begin translating mRNAs in the cytosol. The mRNAs for certain proteins encode a signal sequence for the ER membrane. After this sequence has been synthesized, it directs the nascent protein, along with the ribosome and the mRNA, to the ER membrane. Ribosomes translating mRNAs that do not encode such a sequence remain free in the cytosol.

12–3 False. Individual nuclear pores mediate transport in both directions. It is unclear how pores coordinate this two-way traffic so as to avoid head-on collisions.

12–4 False. All eukaryotic cells contain peroxisomes.

12–5 In the absence of a sorting signal, a protein will remain in the cytosol.

12–6 If the equivalent of one plasma membrane transits the ER every 24 hours and individual membrane proteins remain in the ER for 30 minutes (0.5 hr), then at any one time, 0.021 (0.5 hr/24 hr) plasma membrane equivalents are present in the ER. Since the area of the ER membrane is 20 times greater than the area of the plasma membrane, the fraction of plasma membrane proteins in the ER is 0.021/20 = 0.001. Thus, the ratio of plasma membrane proteins to other membrane proteins in the ER is 1 to 1000. Out of every 1000 proteins in the ER membrane, only 1 is in transit to the plasma membrane.

As this calculation illustrates, the sorting of proteins to the plasma

membrane represents a substantial purification from the mix of proteins in the ER.

12–7

A. The portion of nucleoplasmin responsible for localization to the nucleus must reside in the tail. The nucleoplasmin head does not localize to the nucleus when injected into the cytoplasm, and it is the only injected component that is missing a tail.

B. These experiments suggest that the nucleoplasmin tail carries a nuclear localization signal and that accumulation in the nucleus is not the result of passive diffusion. The observations involving complete nucleoplasmin or the nucleoplasmin tails do not distinguish between passive diffusion and active import; they say only that the tail carries the important part of nucleoplasmin—be it a localization signal or a binding site. The key observations that argue against passive diffusion are the results with the nucleoplasmin heads. They do not diffuse into the cytoplasm when they are injected into the nucleus, nor do they diffuse into the nucleus when injected into the cytoplasm, suggesting that the heads are too large to pass through the nuclear pores. Since the more massive form of nucleoplasmin with tails does pass through the nuclear pores, passive diffusion of nucleoplasmin is ruled out.

Reference: Dingwall C, Sharnick SV & Laskey RA (1982) A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* 30, 449–458.

12–8 Each nuclear pore complex must transport about 1 histone molecule per second, on average, throughout a day:

$$\text{transport} = \frac{32 \times 10^6 \text{ octamers}}{\text{day}} \times \frac{8 \text{ histones}}{\text{octamer}} \times \frac{\text{day}}{8.64 \times 10^4 \text{ sec}} \times \frac{1}{3000 \text{ pores}}$$

$$= 0.99 \text{ histones/second/pore}$$

Because histones are synthesized and imported into nuclei only during S phase, which is typically about 8 hours long, the transport rate is about 3 histones per second per pore during S phase (and none during the rest of the cell cycle).

12–9

A. The concentration of FG-repeats in yeast nuclear pores is about 287 mM, almost 6 times higher than the concentration used *in vitro*. Thus, the concentration inside the pore is certainly sufficient to allow gel formation. The volume of a yeast nuclear pore ($v = \pi r^2 h$) is 28.8×10^3 nm^3 [$3.14 \times (35 \text{ nm}/2)^2 \ (30 \text{ nm}) = 28{,}849$ nm^3]; 5000 FG-repeats in this volume corresponds to a concentration of 287 mM.

$$\text{concentration} = \frac{5000 \text{ FG}}{28.8 \times 10^3 \text{ nm}^3} \times \frac{\text{mmole}}{6 \times 10^{20} \text{ FG}} \times \frac{10^{21} \text{ nm}^3}{\text{cm}^3} \times \frac{1000 \text{ cm}^3}{\text{L}}$$

$$= \frac{289 \text{ mmol}}{\text{L}} = 289 \text{ mM}$$

B. With a diffusion coefficient of 0.1 μm^2/sec, it would take importin-MBP-GFP about 4.5 msec to traverse a yeast nuclear pore.

$$t = \frac{x^2}{2D}$$

$$= (30 \text{ nm})^2 \times \frac{\text{sec}}{2(0.1 \ \mu\text{m}^2)} \times \frac{\mu\text{m}^2}{10^6 \text{ nm}^2} \times \frac{10^3 \text{ msec}}{\text{sec}}$$

$$= 4.5 \text{ msec}$$

This rate of diffusion appears fast enough to meet biological needs and matches reasonably well with the rates of 5 to 10 msec measured for the import of various proteins through nuclear pores.

References: Frey S & Görlich D (2007) A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes. *Cell* 130, 512–523.

Frey S & Görlich D (2009) FG/FxFG as well as GLFG repeats form a selective permeability barrier with self-healing properties *EMBO J.* 28, 2554–2567.

12–10 Normal cells that carry the modified *Ura3* gene make Ura3 that gets imported into mitochondria. It is therefore unavailable to carry out an essential reaction in the metabolic pathway for uracil synthesis. These cells might as well not have the enzyme at all, and they will grow only when uracil is supplied in the medium. By contrast, in cells that are defective for mitochondrial import, Ura3 is prevented from entering mitochondria and remains in the cytosol, where it can function normally in the pathway for uracil synthesis. Thus, cells with defects in import into the mitochondrial matrix can grow in the absence of added uracil because they can make their own.

Reference: Maarse AC, Blom J, Grivell LA & Meijer M (1992) MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. *EMBO J.* 11, 3619–3628.

12–11 The binding of methotrexate to the active site prevents the enzyme from unfolding, which is necessary for import into mitochondria. Evidently, methotrexate binds so tightly that it locks the enzyme into its folded conformation and prevents chaperone proteins from unfolding it.

Reference: Eilers M & Schatz G (1986) Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* 322, 228–232.

12–12 The pores formed by porins are large enough for all ions and metabolic intermediates, but not large enough for most proteins. The size cutoff for free passage through the pores of mitochondrial porins is roughly 10 kilodaltons.

12–13 As shown in **Figure A12–1**, elimination of the first transmembrane segment (by making it hydrophilic) would be expected to give rise to a protein with the N-terminal segment in the cytosol (unglycosylated), but with all other membrane-spanning segments in their original orientation. In the unmodified protein, the first transmembrane segment served as a start-transfer signal, oriented so that it caused the N-terminal segment to

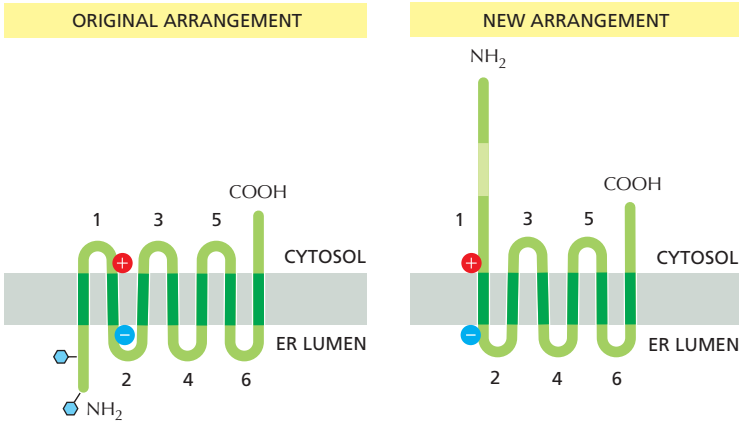


Figure A12–1 Arrangement of the original multipass transmembrane protein and of the new protein after the first hydrophobic segment was converted to a hydrophilic segment (Answer 12–13).

pass across the ER membrane. The next transmembrane segment is also a start-transfer signal, but oriented so that it passes C-terminal protein across the membrane until it reaches the next transmembrane segment, which serves as a stop-transfer signal. Two more pairs of similarly oriented start- and stop-transfer signals give rise to the final arrangement.

Elimination of the first start-transfer signal would permit the second start-transfer signal to initiate transfer. The arrangement of its flanking charged amino acids would orient it in the membrane so that its positively charged end faces the cytosol, just as it did in the original protein. It would then initiate transfer of C-terminal segments just as it did in the unmodified, original protein.

12–14 Symmetry of phospholipids in the two leaflets of the ER membrane is generated by a phospholipid translocator, called a scramblase, that rapidly flips phospholipids of all types back and forth between the monolayers of the bilayer. Because it flips phospholipids indiscriminately, the different types of phospholipid become equally represented in the inner and outer leaflets of the bilayer; that is, they become symmetrically distributed. The plasma membrane contains a different kind of phospholipid translocator, which is specific for phospholipids containing free amino groups (phosphatidylserine and phosphatidylethanolamine). These flippases remove these specific phospholipids from the external leaflet and transfer them to the internal leaflet of the plasma membrane, thereby generating an asymmetrical distribution.

CHAPTER 13

13–1 True. The cytosolic leaflets of the two membrane bilayers are the first to come into contact and fuse, followed by the noncytosolic leaflets. It is this pattern of leaflet fusion that maintains the topology of membrane proteins, so that protein domains that face the cytosol always do so, regardless of what compartment they occupy.

13–2 True. A misfolded protein is selectively retained in the ER by binding to chaperone proteins such as BiP and calnexin. Only after it has been released from such a chaperone protein—and thus approved as properly folded—does a protein become a substrate for exit from the ER.

13–3 True. The oligosaccharide chains are added in the lumens of the ER and Golgi apparatus, which are topologically equivalent to the outside of the cell. This basic topology is conserved in all membrane budding and fusion events. Thus, oligosaccharide chains are always topologically outside the cell, whether they are in a lumen or on the cell surface.

13–4 If the flow of membrane between cellular compartments were not balanced in a nondividing liver cell, some compartments would grow in size and others would shrink (in the absence of new membrane synthesis). Keeping all the membrane compartments the same relative size is essential for proper functioning of a liver cell. The situation is different in a growing cell such as a gut epithelial cell. Over the course of a single cell cycle, all the compartments must double in size to generate two daughter cells. Thus, there will be an imbalance in favor of the outward flow, which will be supported by new membrane synthesis equal to the sum total of all the cell's membrane.

13–5 The cell's SNAREs are all bound to the cytosolic surface of whatever membrane they are in. They function by juxtaposing the cytosolic surfaces of the two membranes to be fused. By contrast, an enveloped virus must fuse with a cell membrane by bringing together its external surface with

an external surface of a cell membrane. Thus, enveloped viruses cannot make use of a cell's SNAREs because they are located on the wrong side of the membrane. It is for this reason that enveloped viruses make their own fusion proteins, which are properly situated on their external surface.

13–6 The volume of a cylinder 1.5 nm in diameter and 1.5 nm in height is 2.65 nm^3 [$3.14 \times (0.75\ nm)^2 \times 1.5\ nm$], which equals 2.65×10^{-24} L [$2.65\ nm^3 \times (cm/10^7\ nm)^3 \times (L/10^3\ cm^3)$]. There are about 88 water molecules in this volume.

$$\frac{\text{water molecules}}{\text{cylinder}} = \frac{2.65 \times 10^{-24}\ \text{L}}{\text{cylinder}} \times \frac{55.5\ \text{mole}}{\text{L}} \times \frac{6 \times 10^{23}\ \text{molecules}}{\text{mole}}$$

$$= 88.2$$

In each monolayer in a circle of membrane 1.5 nm in diameter, there are about 9 phospholipids (PL) [$3.14 \times (0.75\ nm)^2 \times (PL/0.2\ nm^2) = 8.8$ PL]. Thus, there are about 5 water molecules per phospholipid in the area of close approach of the two membranes. This number is slightly less than half the number (10–12) estimated to be associated with phospholipid head groups under normal circumstances. This means that when a vesicle and its target membrane are drawn together in preparation for fusion, somewhat more than half of the water molecules that would normally bind to the membranes must be squeezed out.

Reference: Meuse CW, Krueger S, Majkrzak CF, Dura JA, Fu J, Connor JT & Plant AL (1998) Hybrid bilayer membranes in air and water: infrared spectroscopy and neutron reflectivity studies. *Biophys. J.* 74, 1388–1398.

13–7 To generate maximal alkaline phosphatase activity, vesicles from each strain must carry both v-SNAREs and t-SNAREs (see Figure Q13–2B, experiment 1). If either vesicle is lacking v-SNAREs or t-SNAREs, phosphatase activity is reduced to 30–60% of the maximum (see experiments 3, 4, 6, 7, 8, and 9). If both vesicles are missing either v-SNAREs (see experiment 2) or t-SNAREs (see experiment 5), phosphatase activity is very low, as it is if one vesicle is missing both SNAREs (see experiments 10 and 11). For a reasonable level of fusion, complementary SNAREs must be present on the vesicles. It does not matter which kind of SNARE is on vesicles from strain A so long as vesicles from strain B carry a complementary SNARE (compare experiments 3 and 4, experiments 6 and 7, and experiments 8 and 9).

You might have wondered why there is a low background of phosphatase activity, even where no fusion is expected (see experiments 2, 5, 10, and 11). If a few vesicles were to break, releasing small amounts of pro-Pase and protease, then a small amount of active alkaline phosphatase could be generated in the absence of vesicle fusion.

Reference: Nichols BJ, Ungermann C, Pelham HRB, Wickner WT & Haas A (1997) Homotypic vacuolar fusion mediated by t- and v-SNAREs. *Nature* 387, 199–202.

13–8 The modified PDI would be located outside the cell. If PDI were missing the ER retrieval signal, its gradual flow out of the ER to the Golgi apparatus would not be countered by its capture and return to the ER, as normally occurs. Similarly, it would be expected to leave the Golgi apparatus by the default pathway, mixed with the other proteins that the cell is secreting. It would not be expected to be retained anywhere else along the secretory pathway because it presumably has no signals to promote such localization.

Reference: Munro S & Pelham HR (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899–907.

13–9 The KDEL receptor binds its ligands more tightly in the Golgi apparatus, where it captures proteins that have escaped the ER, so that it can return them. The receptor binds its ligands more weakly in the ER, so those proteins that have been captured in the Golgi apparatus can be released upon their return to the ER. The basis for the different binding affinities is thought to be the slight difference in pH; the lumen of the Golgi apparatus is slightly more acidic than that of the ER, which is neutral.

Since the primary job of the KDEL receptor is to capture proteins that have escaped from the ER, it would be reasonable to design the system so that the receptors are found in the highest concentration in the Golgi apparatus. This is, in fact, the way it is in the cell. You would be correct if you predicted that the KDEL receptor does not have a classic ER retrieval signal; after all, the receptor is designed to spend most of its time in the Golgi apparatus, and a classic signal would ensure its efficient return to the ER. It does, however, have a "conditional" retrieval signal; upon binding to an ER protein in the Golgi apparatus, its conformation is altered so that a binding site for COPI subunits is exposed. That signal allows it to be incorporated into COPI-coated vesicles, which are destined to return to the ER.

Reference: Teasdale RD & Jackson MR (1996) Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* 12, 27–54.

13–10 The lysosomal enzymes are all acid hydrolases, which have optimal activity at the low pH (about 5.0) in the interior of lysosomes. If a lysosome were to break, the acid hydrolases would find themselves at pH 7.2, the pH of the cytosol, and would therefore do little damage to cellular constituents.

13–11 Adaptor proteins in general mediate the incorporation of specific cargo proteins into clathrin-coated vesicles by linking the clathrin coat to specific cargo receptors. Because melanosomes are specialized lysosomes, it would seem reasonable that the defect in AP3 affects the pathway for delivery of pigment granules from the *trans* Golgi network, which involves clathrin-coated vesicles. AP3 localizes to coated vesicles budding from the *trans* Golgi network, which is consistent with a function in transport from the Golgi to lysosomes. Interestingly, humans with the genetic disorder Hermansky–Pudlak syndrome have similar pigmentation changes, and they also have bleeding problems and pulmonary fibrosis. These symptoms are all thought to reflect deficiencies in the production of specialized lysosomes, which result from just a single biochemical defect.

References: Kantheti P, Qiao X, Diaz ME, Peden AA, Meyer GE, Carskadon SI, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL & Burmeister M (1998) Mutation in AP-3 delta in the *mocha* mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. *Neuron* 21, 111–122.

Zhen L, Jiang S, Feng L, Bright NA, Peden AA, Seymour AB, Novak EK, Elliott R, Gorin MB, Robinson MS & Swank RT (1999) Abnormal expression and subcellular distribution of subunit proteins of the AP-3 adaptor complex lead to platelet storage pool deficiency in the *pearl* mouse. *Blood* 94, 146–155.

CHAPTER 14

14–1 True. Although the three respiratory enzyme complexes can exist as independent entities in the mitochondrial inner membrane, the transfers

of electrons between the complexes mediated by the two mobile carriers—ubiquinone and cytochrome *c*—are facilitated by the formation of a larger structure.

14–2 True. The *c* subunits act like cogs in a gear wheel. When the supply of protons is limited, as in mitochondria, there are fewer subunits than when the proton gradient is high, as in chloroplasts.

14–3 True. Inheritance of organellar genomes is very different from the inheritance of nuclear genes, which is governed by Mendelian rules. A pattern of inheritance that does not obey Mendelian rules is unlikely to be due to a nuclear gene, which leaves the organellar genomes—the only other genomes in a cell.

14–4 In the presence of oxygen, yeast can generate about 15 times more ATP from each glucose molecule than they can in the absence of oxygen. Thus, to meet their energy needs, they need to process about 15-fold fewer glucose molecules; hence the dramatic drop in glucose consumption when O_2 is introduced.

14–5 It would take the heart 6 seconds to consume its steady-state levels of ATP. Because each pair of electrons reduces one atom of oxygen, the 12 pairs of electrons generated by oxidation of one glucose molecule would reduce 6 O_2. Thus, 30 ATP are generated per 6 O_2 consumed. At steady state, the rate of ATP production equals its rate of consumption. The time in seconds required to consume the steady-state level of ATP is

$$\text{time} = \frac{5\ \mu\text{mol ATP}}{\text{g}} \times \frac{6\ O_2}{30\ \text{ATP}} \times \frac{\text{min g}}{10\ \mu\text{mol}\ O_2} \times \frac{60\ \text{sec}}{\text{min}}$$
$$= 6\ \text{sec}$$

14–6 H^+ ions move much faster than Ca^{2+} ions through an aqueous solution because their "movement" is virtual; the H^+ ion that appears on one side of the cell is not the same one that started on the other side. H^+ ion movement, instead, depends on exchanges of hydrogen bonds for covalent bonds in a chain of water molecules. By contrast, a Ca^{2+} ion must actually diffuse through the medium.

The difference in the nature of the movements of these two ions is nowhere better illustrated than by their behavior in ice. As expected, the rate of diffusion of Ca^{2+} ions decreases considerably. Surprisingly, H^+ ions move even more rapidly. That is because H^+ movement depends on chains of water molecules. In ice, most of the water molecules are linked into chains, allowing H^+ ions to move very rapidly over long distances. In liquid water, the chains involve only a few water molecules, which means that there are periodic delays as H_3O^+ ions connect up with a new chain.

14–7 The rates of oxidation of the electron carriers, if measured rapidly enough, reveal their order in the respiratory chain. The carriers closest to oxygen will be oxidized first, and those farthest from oxygen will be oxidized last. This rationale allows you to deduce the order of electron flow through the carriers. Cytochromes *b* and c_1 are part of cytochrome *c* reductase and cytochromes *a* and a_3 are part of cytochrome oxidase.

$$\underset{b}{\text{cytochrome}} \rightarrow \underset{c_1}{\text{cytochrome}} \rightarrow \underset{c}{\text{cytochrome}} \rightarrow \underset{(a + a_3)}{\text{cytochrome}} \rightarrow O_2$$

14–8 In just the right amounts, an uncoupler such as dinitrophenol will promote weight loss by partially uncoupling electron flow from ATP synthesis, thereby decreasing the efficiency of oxidative phosphorylation. For example, if sufficient uncoupler were ingested to reduce the efficiency of oxidative phosphorylation to 50%, twice as many calories (from food

or internal stores, mainly fat) would have to be burned to generate the same amount of ATP. Dinitrophenol is no longer prescribed because its use led to several deaths; if oxidative phosphorylation is too efficiently compromised, not enough ATP will be generated to support essential cell functions and death is the result.

14–9 The number of protons in the matrix of an actively respiring liver mitochondrion at pH 7.5 (3.16×10^{-8} M H^+) is about 10.

$$\frac{H^+}{\text{mitochondrion}} = \frac{3.16 \times 10^{-8} \text{ mole } H^+}{\text{L}} \times \frac{6 \times 10^{23} \text{ } H^+}{\text{mole } H^+} \times \frac{(4/3)(3.14)(0.5\ \mu\text{m})^3}{\text{mitochondrion}} \times \frac{\text{L}}{10^{15}\ \mu\text{m}^3}$$

$$= 9.9$$

If the matrix of the mitochondrion started at pH 7 (10^{-7} M H^+), it originally held about 31 protons (31.4). Thus, to reach pH 7.5, about 21 protons would need to be pumped out. These are remarkable results. Regardless of the particulars of mitochondrial size and exact pH values, it is clear that only a few tens of protons are normally involved in establishing the proton-motive force. More than anything, these results emphasize the dynamic nature of proton pumping and ATP synthesis.

14–10

A. Presumably, the hydrolysis of an individual ATP molecule provides the driving force for the 120° rotation of the γ subunit, hence the corresponding revolution of the actin filament. Since a low concentration of ATP was used in these experiments, the pauses represent the variable times it takes for the next molecule of ATP to bind. Rotation through 120° corresponds to one αβ dimer, the unit of ATP hydrolysis (or of synthesis in ATP synthase's normal direction).

B. If three ATP molecules must be hydrolyzed to drive one complete rotation of the γ subunit, then in its normal operation, ATP synthase must synthesize three ATP molecules per rotation of the γ subunit.

Reference: Masaike T, Mitome N, Noji H, Muneyuki E, Yasuda R, Kinosita K & Yoshida M (2000) Rotation of F_1-ATPase and the hinge residues of the β subunit. *J. Exp. Biol.* 203, 1–8.

14–11

A. The energy of a mole of photons at any particular wavelength is the energy of one photon times Avogadro's number (N). Therefore, the energy of a mole of photons at a wavelength of 400 nm is

$$E = Nhc/\lambda$$

$$= \frac{6 \times 10^{23} \text{ photons}}{\text{mole}} \times \frac{6.6 \times 10^{-37} \text{ kJ/sec}}{\text{photon}} \times \frac{3 \times 10^{17} \text{ nm}}{\text{sec}} \times \frac{1}{400 \text{ nm}}$$

E = 297 kJ/mole for 400-nm light

This calculation for 680-nm and 800-nm light gives

E = 175 kJ/mole for 680-nm light

E = 149 kJ/mole for 800-nm light

B. If a square meter receives 1.3 kJ/sec of 680-nm light, which is worth 175 kJ/mole of photons, then the time it will take for a square meter to receive one mole of photons is

$$\text{time} = \frac{\text{sec}}{1.3 \text{ kJ}} \times \frac{175 \text{ kJ}}{\text{mole}}$$

time = 135 sec/mole

C. If it takes 135 seconds for a square meter of tomato leaf to receive a mole

of photons and eight photons are required to fix a molecule of CO_2, it will take just under 2 hours to synthesize a mole of glucose.

$$\text{time} = \frac{135 \text{ sec}}{\text{mole photons}} \times \frac{8 \text{ mole photons}}{\text{mole } CO_2} \times \frac{6 \text{ mole } CO_2}{\text{mole glucose}}$$

$$\text{time} = 6480 \text{ seconds or } 108 \text{ minutes}$$

The actual efficiency of photon capture is considerably less than 100%. Under optimal conditions for some rapidly growing plants, the efficiency of utilization of photons that strike a leaf is about 5%. However, even this value greatly exaggerates the true efficiency of utilization of the energy in sunlight. For example, a field of sugar beets converts only about 0.02% of the energy that falls on it during the growing season. Several factors limit the overall efficiency, including saturation of the photosystems far below maximum sunlight, availability of water, and low temperatures.

D. In contrast to the very low overall efficiency of light utilization, the efficiency of conversion of light energy to chemical energy *after photon capture* is 33%.

$$\text{efficiency} = \frac{\text{mole } CO_2}{8 \text{ mole photons}} \times \frac{\text{mole photons}}{175 \text{ kJ}} \times \frac{468 \text{ kJ}}{\text{mole } CO_2}$$

$$= 0.33 \text{ or } 33\%$$

14–12 Protons pumped across the crista membrane into the crista space can exit to the intermembrane space, which equilibrates with the cytosol, a huge H^+ sink. Both the mitochondrial matrix (pH 8) and the cytosol (pH 7.4) house many metabolic reactions that require a pH around neutrality. The largest H^+ concentration difference between the mitochondrial matrix and cytosol that is compatible with function is therefore relatively small (less than 1 pH unit). Much of the energy stored in the mitochondrial electrochemical gradient is instead due to the membrane potential (about 140 mV of the 200 mV potential difference is due to the membrane potential).

By contrast, chloroplasts have a smaller, dedicated compartment—the thylakoid space—into which H^+ ions are pumped. Much higher concentration differences can be achieved (more than 3 pH units), and nearly all of the energy stored in the thylakoid electrochemical gradient is due to the pH difference between the stroma and the thylakoid space.

14–13 Variegation occurs because the plants have a mixture of normal and defective chloroplasts. These sort out by mitotic segregation to give patches of green and yellow in leaves. Many of the green patches have cells that still retain defective chloroplasts in addition to the normal ones. As such patches grow, they can segregate additional cells that have only defective chloroplasts, giving rise upon cell division to an island of yellow cells in a sea of green ones. By contrast, yellow patches are due to cells that retain only defective chloroplasts. Thus, yellow cells cannot give rise to green cells by mitotic segregation; hence, there are no green islands surrounded by yellow.

CHAPTER 15

15–1 False. Most second messengers, including cyclic AMP, Ca^{2+}, and IP_3, are water-soluble and diffuse freely through the cytosol; however, second messengers such as diacylglycerol are lipid soluble and diffuse in the plane of the membrane.

15–2 False. GTP-binding proteins are uniformly on when GTP is bound and off when GDP is bound; thus, GEFs turn GTP-binding proteins on and GAPs

turn them off. The same is not true for protein kinases and phosphatases. Attachment of a phosphate will turn some target proteins on and others off. Indeed, attachment of a phosphate at one location in a protein can turn it on, while phosphorylation at a different location can turn the same protein off. Thus, while protein kinases throw the molecular switch, it is not always in the same direction.

15–3 True. Intracellular signaling pathways that involve enzymes or ion channels can significantly amplify a signal. Once activated, a protein kinase, for example, can phosphorylate hundreds of its target proteins. Similarly, activation of an ion channel can raise the cytosolic concentration of a critical ion by many fold.

15–4 False. Ligand binding usually causes a receptor tyrosine kinase to assemble into dimers, which, because of their proximity, activates the kinase domains. The receptors then phosphorylate themselves to initiate the intracellular signaling cascade. In some cases, the insulin receptor for example, the receptor exists as a dimer and ligand binding is thought to rearrange their receptor chains, causing the kinase domains to come together.

15–5 True. Protein tyrosine phosphatases, unlike serine/threonine protein phosphatases, remove phosphate groups only from selected phosphotyrosines on a subset of tyrosine-phosphorylated proteins.

15–6 False. Although there is some overlap in the cell–cell communication molecules used in plants and animals, there are many significant differences. For example, plants do not use the nuclear receptor family, Ras, JAK, STAT, TGFβ, Notch, Wnt, or Hedgehog proteins.

15–7 At a circulating concentration of hormone equal to 10^{-10} M, about 1% of the receptors will have a bound hormone molecule $\{[R–H]/[R]_{TOT} = 10^{-10}\ M/(10^{-10}\ M + 10^{-8}\ M) = 0.0099\}$. Half of the receptors will have a bound hormone molecule when the concentration of hormone equals the K_d; that is, at 10^{-8} M $\{[R–H]/[R]_{TOT} = 10^{-8}\ M/(10^{-8}\ M + 10^{-8}\ M) = 0.5\}$. Thus, the hormone concentration will have to rise 100-fold to elicit a response. The relationships between concentration of ligand (hormone, in this case), K_d, and fraction bound are developed in Answer 3–86, p. 486.

15–8

A. A telephone conversation is analogous to synaptic signaling in the sense that it is a private communication from one person to another, usually some distance away and sometimes very far away. It differs from synaptic signaling because it is (usually) a two-way exchange, whereas synaptic signaling is a one-way communication.
B. Talking to people at a cocktail party is analogous to paracrine signaling, which occurs between different cells (individuals) and is locally confined.
C. A radio announcement is analogous to an endocrine signal, which is sent out to the whole body (the audience) with only target cells (individuals tuned to the specific radio station) affected by it.
D. Talking to yourself is analogous to an autocrine signal, which is a signal that is sent and received by the same cell.

15–9 Cells with identical receptors can respond differently to the same signal molecule because of differences in the internal machinery to which the receptors are coupled. Even when the entire signaling pathway is the same, cells can respond differently if they express different effector proteins at the ends of the pathways.

15–10 Phosphorylation/dephosphorylation offers a simple, universal solution to the problem of controlling protein activity. In a signaling pathway, the

activities of several proteins must be rapidly switched from the off state to the on state, or vice versa. Attaching a negatively charged phosphate to a protein is an effective way to alter its conformation and activity. And it is an easy modification to reverse. It is a universal solution in the sense that one activity—that of a protein kinase—can be used to attach a phosphate, and a second activity—a protein phosphatase—can be used to remove it. About 2% of the protein-coding genes in the human genome encode protein kinases, which presumably arose by gene duplication and modification to create appropriate specificity. Because serines, threonines, and tyrosines are common amino acids on the surfaces of proteins, target proteins can evolve to have appropriate phosphorylation sites at places that will alter their conformations. Finally, phosphorylation/dephosphorylation provides a flexible response that can be adjusted to give rapid on/off switches or more long-lasting changes.

All of these attributes of phosphorylation/dephosphorylation are missing with allosteric regulators. While it is possible, in principle, for small molecules to turn proteins on or off, it is not a universal solution. Specific molecules would have to be "designed" for each target protein, which would require the evolution of a metabolic pathway for the synthesis and degradation of each regulatory molecule. Even if such a system evolved for one target protein, that specific solution would not help with the evolution of a system for other target proteins. In addition, regulation by binding of small molecules is very sensitive to the concentration of the regulator. For a monomeric target protein, the concentration of a small molecule would have to change by 100-fold to go from 9% bound to 91% bound—a minimal molecular switch (see Answer 3–86, p. 486). Few metabolites in cells vary by such large amounts.

15–11 The use of a scaffolding protein to hold the three kinases in a signaling complex increases the speed of signal transmission and eliminates cross-talk between pathways; however, there is relatively little opportunity for amplification of the signal from the receptor to the third kinase. Freely diffusing kinases offer the possibility for greater signal amplification since the first kinase can phosphorylate many molecules of the second kinase, which in turn can phosphorylate many molecules of the third kinase. The speed of signal transmission is likely to be slower, unless the concentration of kinases (and the potential for amplification) is high enough to compensate for their separateness. Finally, free kinases offer the potential for spreading the signal to other signaling pathways and to other parts of the cell. The organization that a cell uses for a particular signaling pathway depends on what the pathway is intended to accomplish.

15–12

1. If more than one effector molecule must bind to activate the target molecule, the response will be sharpened in a way that depends on the number of required effector molecules. At low concentrations of the effector, most target proteins will have a single effector bound (and therefore be inactive). At increasing concentrations of effector, the target proteins with the requisite number of bound effectors will rise sharply, giving a correspondingly sharp increase in the cellular response.
2. If the effector activates one enzyme and inhibits another enzyme that catalyzes the reverse reaction, the forward reaction will respond sharply to a gradual increase in effector concentration. This is a common strategy employed in metabolic pathways involved in energy production and consumption.
3. The above mechanisms give sharp responses, but a true all-or-none response can be generated if the effector molecule triggers a positive feedback loop so that an activated target molecule contributes to its own further activation. If the product of an activated enzyme, for example, binds

to the enzyme to activate it, a self-accelerating, all-or-none response will be produced.

15–13 The analysis of individual frog oocytes shows clearly that the response to progesterone is all-or-none, with no oocytes having a partially activated MAP kinase. Thus, the graded response in the population results from an all-or-none response in individual oocytes, with different mixtures of fully mature or immature oocytes giving rise to intermediate levels of MAP kinase activation (**Figure A15–1**). It is not so clear why individual oocytes respond differently to different concentrations of progesterone, although there is significant variability among oocytes in terms of age and size (and presumably in the number of progesterone receptors, the concentrations of components of the MAP kinase signaling module, and downstream targets).

Whether a graded response in a population of cells indicates a graded response in each cell or a mixture of all-or-none responses is a question that arises in many contexts in biology.

Reference: Ferrell JE & Machleder EM (1998) The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes. *Science* 280, 895–898.

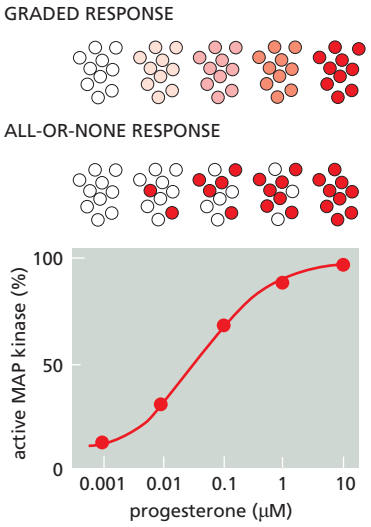


Figure A15–1 Graded or all-or-none responses in individual oocytes that give rise to a graded response in the population (Answer 15–13).

15–14 Any mutation that generated a regulatory subunit incapable of binding to the catalytic subunit would produce a permanently active PKA. When the catalytic subunit is not bound to the regulatory subunit, it is active.

Two general types of mutation in the regulatory subunit could produce a permanently inactive PKA. A regulatory subunit that was altered so that it could bind the catalytic subunit, but not bind cyclic AMP, would not release the catalytic subunit, rendering PKA permanently inactive. Similarly, a mutant regulatory subunit that could bind cyclic AMP, but not undergo the conformational change needed to release the catalytic subunit, would permanently inactivate PKA.

15–15 The time that the catalytic kinase subunit spends in its active conformation depends on the extent to which its regulatory subunits are modified. Each modification by phosphorylation or by Ca^{2+} binding nudges the equilibrium toward the active conformation of the kinase subunit; that is, each modification increases the time spent in the active state. By summing the inputs from multiple pathways in this way, phosphorylase kinase integrates the signals that control glycogen breakdown.

15–16 In both cases the signaling pathways themselves are rapid. If the pathway modifies a protein that is already present in the cell, its activity is changed immediately, leading to a rapid response. If the pathway modifies gene expression, however, there will be a delay corresponding to the time it takes for the mRNA and protein to be made and for the cellular levels of the protein to be altered sufficiently to invoke a response, which would usually take an hour or more.

15–17 Cells of flies with the heterozygous $Dsh^{\Delta}/+$ genotype probably make just half the normal amount of Dishevelled. Thus, underexpression of Dishevelled corrects the multi-hair phenotype generated by the overexpression of Frizzled. This relationship suggests that Frizzled acts upstream of Dishevelled; it is easy to imagine how underexpression of a downstream component could correct the overexpression of an upstream component. All this makes sense, as Frizzled is a Wnt receptor and Dishevelled is an intracellular signaling protein. However, if you knew nothing of the functions of Dishevelled and Frizzled, with only the genetic interactions as a guide, it would be possible to imagine more complex relationships (involving other unknown components) with Dishevelled acting upstream of Frizzled that could account for the phenotypes given in this problem. See if you can design such a pathway.

Reference: Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD & Luo L (2001) *Drosophila* Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. *Cell* 105, 81–91.

CHAPTER 16

16–1 False. The entry of Ca^{2+} through the voltage-sensitive Ca^{2+} channels in T tubules is not sufficient, by itself, to trigger rapid muscle contraction. Instead, this initial burst of Ca^{2+} opens Ca^{2+}-release channels in the sarcoplasmic reticulum, which flood the cytoplasm with Ca^{2+}, initiating rapid muscle contraction by binding to troponin C.

16–2 True. When ATP in actin filaments (or GTP in microtubules) is hydrolyzed, much of the free energy released by cleavage of the high-energy bond is stored in the polymer lattice, making the free energy of the ADP-containing polymer higher than that of the ATP-containing polymer. This shifts the equilibrium toward depolymerization so that ADP-containing actin filaments disassemble more readily than ATP-containing actin filaments.

16–3 False. The centrosome, which establishes the principal array of microtubules in most animal cells, nucleates microtubule growth at the minus end. Thus, the plus ends of the microtubules are near the plasma membrane, and the minus ends are buried in the centrosome at the center of the cell. This orientation of the array requires that plus-end directed motors be used to transport cargo to the cell periphery and that minus-end directed motors be used for cargo delivery to the center of the cell.

16–4 In cells, most of the actin subunits are bound to thymosin, which locks actin into a form that cannot hydrolyze its bound ATP and cannot be added to either end of a filament. Thymosin reduces the concentration of free actin subunits to around the critical concentration. Actin subunits are recruited from this inactive pool by profilin, whose activity is regulated so that actin polymerization occurs when and where it is needed. The advantage of such an arrangement is that the cell can maintain a large pool of subunits for explosive growth at the sites and times of its choosing.

16–5 Sketches representing sarcomeres at each of the arrows in Figure Q16–1 are shown in **Figure A16–1**. As illustrated in these pictures, the increase in tension with decreasing sarcomere length in segment I is due to increasing numbers of interactions between myosin heads and actin. In segment II, actin begins to overlap with the bare zone of myosin, yielding a plateau at which the number of interacting myosin heads remains constant. In segment III, the actin filaments begin to overlap with each other, interfering with the optimal interaction of actin and myosin and producing a decrease in tension. In segment IV, the spacing between the Z discs is less than the length of the myosin thick filaments, causing their deformation and a precipitous drop in muscle tension.

Reference: Gordon AM, Huxley AF & Julian FJ (1966) The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J. Physiol.* 184, 170–192.

16–6 A growth rate of 2 μm/min (2000 nm/60 sec = 33 nm/sec) corresponds to the addition of 4.2 αβ-tubulin dimers [(33 nm/sec) × (αβ-tubulin/8 nm) = 4.17 dimers/sec] to each of 13 protofilaments, or about 54 αβ-tubulin dimers/sec to the ends of a microtubule.

Reference: Detrich HW, Parker SK, Williams RC, Nogales E & Downing

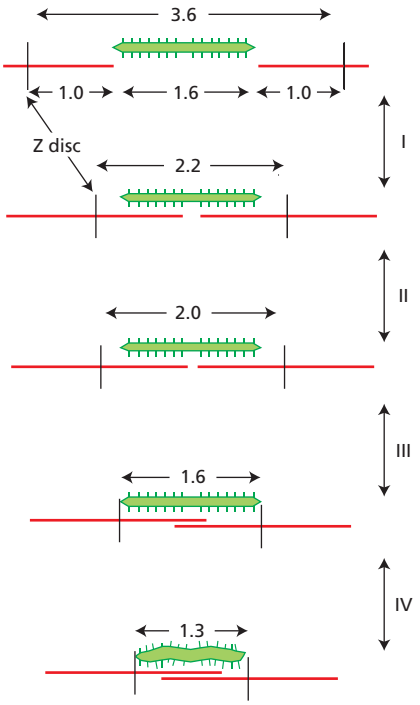


Figure A16–1 Schematic diagrams of sarcomeres at the points indicated by *arrows* in Figure Q16–1 (Answer 16–5). Numbers refer to lengths in micrometers.

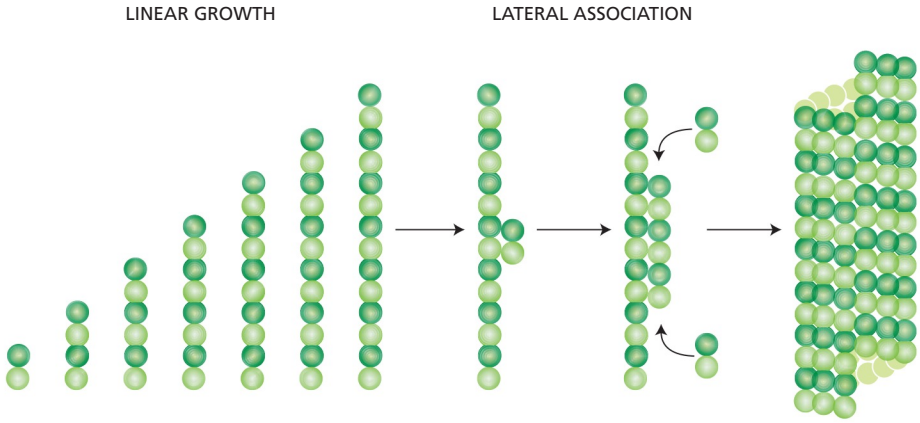


Figure A16–2 Rapid addition of αβ-tubulin dimers to nucleation structure (Answer 16–7).

KH (2000) Cold adaptation of microtubule assembly and dynamics. *J. Biol. Chem.* 275, 37038–37047.

16–7 Once the first lateral association has occurred, the next αβ-dimer can bind much more readily because it is stabilized by both lateral and longitudinal contacts (**Figure A16–2**). The formation of a second protofilament stabilizes both protofilaments, allowing the rapid addition of new αβ-tubulin dimers to form adjacent protofilaments and to extend existing ones. At some point the sheet of tubulin curls into a tube to form the microtubule.

Reference: Leguy R, Melki R, Pantaloni D & Carlier M-F (2000) Monomeric γ-tubulin nucleates microtubules. *J. Biol. Chem.* 275, 21975–21980.

16–8 The centrosome nucleates a three-dimensional, starburst array of microtubules that grow until they encounter an obstacle, ultimately the plasma membrane. Dynamic instability of the microtubules, coupled to the requirement for equal pushing of oppositely directed microtubules, eventually positions the centrosome in the middle of the cell. One way to think about the notion of equal and opposite forces is to realize that the microtubules are not absolutely rigid structures. Imagine pushing an object with a short steel rod versus a very long one; the short rod transmits force effectively, but the long rod will bend, delivering less force. The same principle may operate inside the cell, with microtubules of equal length delivering the same force. When all the oppositely directed microtubules emanating from a centrosome are the same length, the centrosome will be in the center of the cell.

16–9

A. The unidirectional movement of kinesin along a microtubule is driven by the free energy of ATP hydrolysis. ATP binding and hydrolysis are coupled to a series of conformational changes in the kinesin head that bring about the unidirectional stepping of the kinesin motor domains along the microtubule.

B. In the trace, the kinesin moves 80 nm in 9 sec, an average rate of about 9 nm/sec. This rate is about 100-fold slower than the *in vivo* rate because the experimental conditions (ATP concentration and force exerted by the interference pattern) were adjusted to slow the movements of kinesin so that individual steps could be observed.

C. As can be seen in Figure Q16–3B, the kinesin molecule took 10 steps to move 80 nm, indicating that the length of an individual step is about 8 nm.

D. Since the step length and the interval between β-tubulin subunits along a microtubule protofilament are both 8 nm, a kinesin appears to move by stepping from one β-tubulin to the next along a protofilament. Because kinesin has two domains that can bind to β-tubulin, it presumably keeps

one domain anchored as it swings the other domain to the next β-tubulin binding site—much like a person walking along a path of stepping-stones.

E. The data in Figure Q16–3B contain no information about the number of ATP molecules hydrolyzed per step. Other experiments by these same investigators suggest that hydrolysis of one ATP does not cause multiple steps. By lowering ATP concentrations to slow movement along the microtubule, the investigators showed the same sort of stepping pattern as in Figure Q16–3B, although on a longer time scale. If hydrolysis of a single ATP could cause multiple steps, a clustering of steps might have been expected under these experimental conditions. None of these experiments rule out the possibility that more than one ATP molecule might need to be hydrolyzed for each step.

Reference: Svoboda K, Schmidt CF, Schnapp BJ & Block SM (1993) Direct observation of kinesin stepping by optical trapping interferometry. *Nature* 365, 721–727.

16–10 The mitochondrion is about 12 times faster. It moves at 10^6 body lengths per day. The swimmer moves at 100 body lengths/1.75 min, which is 8.2×10^4 body lengths per day.

16–11 Cofilin binds preferentially to actin with bound ADP. When cofilin binds to ADP-containing actin filaments, it introduces strain into the filament by twisting it, which makes it easier for the filament to be severed and for ADP-actin subunits to dissociate. Because polymerization is faster than ATP hydrolysis, the newly added subunits are resistant to depolymerization by cofilin. Thus, cofilin efficiently dismantles older filaments in the cell, which contain more ADP-actin.

16–12 The building blocks—soluble subunits—of the three types of filaments are the basis for their polarity differences. The building blocks for actin filaments (an actin monomer) and microtubules (αβ-tubulin) have polarity—distinct ends—and thus form a polymer with distinct ends when they are linked together. By contrast, the building block of intermediate filaments is a symmetrical tetramer with identical ends. Thus, when these subunits are linked together, the ends of the resulting filament are also identical.

16–13 The unidirectional motion of a lamellipodium results from the nucleation and growth of actin filaments at the leading edge of the cell and depolymerization of the older actin meshwork more distally. Cofilin plays a key role in differentiating the new actin filaments from the older ones. Because cofilin binds cooperatively and preferentially to actin filaments containing ADP-actin, the newer filaments at the leading edge, which contain ATP-actin, are resistant to depolymerization by cofilin. As the filaments age and ATP hydrolysis proceeds, cofilin can efficiently disassemble the older filaments. Thus, the delayed ATP hydrolysis by filamentous actin is thought to provide the basis for a mechanism that maintains an efficient, unidirectional treadmilling process in the lamellipodium.

CHAPTER 17

17–1 False. Although a number of cells equivalent to an adult human is replaced about every three years, not all cells are replaced at the same rate. Blood cells and cells that line the gut are replaced at a high rate, whereas cells in most organs are replaced more slowly, and neurons are rarely replaced.

17–2 True. If the length of the cell cycle were shorter than it takes for the cell

to double in size, the cell would get progressively smaller with each division; if it were longer, the cells would get bigger and bigger.

17–3 True. The origin recognition complex serves as a scaffold at origins of replication in eukaryotic cells around which other proteins are assembled and activated to initiate DNA replication.

17–4 False. Equal and opposite forces that tug the chromosomes toward the two spindle poles would tend to position them at random locations between the poles. The poleward force on each chromosome is opposed by a polar ejection force that pushes the chromosome away from the pole. The ejection force is mediated by plus-end directed kinesin motors on chromosome arms that interact with interpolar microtubules and transport the chromosomes away from the spindle poles. This balance of forces tends to position the chromosomes at the midpoint between the poles—the metaphase plate.

17–5 False. At the start of meiosis, each diploid cell contains two sets of homologs: one from the mother and one from the father. During meiosis, these two sets of homologs are randomly assorted so that sperm and eggs will get one set of homologs, but each set will be a mixture of paternal and maternal homologs.

17–6 False. Organism senescence (aging) is distinct from replicative cell senescence, which occurs in the absence of telomerase. Aging is thought to depend largely on progressive oxidative damage to macromolecules. Strategies that reduce metabolism—for example, restricted caloric intake—decrease the production of reactive oxygen species, and can extend the life-span of experimental animals.

17–7 Enzymes for most metabolic reactions function in isolation; that is, their enzymatic competence does not depend on critical interactions with other proteins. So long as the enzyme folds properly and its small-molecule substrate is present, the reaction will proceed. By contrast, cell-cycle proteins must interact with many other proteins to form the complexes that are critical for coordinated progression through the cell cycle. The ability of many human cell-cycle proteins to interact with yeast components implies that the binding surfaces responsible for these interactions have been preserved through more than a billion years of evolution. That is remarkable.

17–8

A. The relationships between cell fluorescence and position in the cell cycle are indicated in **Figure A17–1A**. Because Hoechst 33342 binds to DNA, cellular fluorescence is proportional to DNA content. The peak with the lowest fluorescence corresponds to cells in G_1, which are diploid. The peak with the highest fluorescence corresponds to cells in G_2 and M, which have finished replication and are tetraploid (and thus have twice the fluorescence of G_1 cells). Cells in S phase, which are replicating their DNA, are between diploid and tetraploid and thus have intermediate levels of fluorescence.

B. The distributions of fluorescence for cells treated with agents that block the cell cycle in G_1, S, and M phases are shown in **Figure A17–1B, C, and D**. Cells blocked in either G_1 or in M form sharp distributions because all the cells have the same amount of DNA (diploid for G_1 and tetraploid for M). Cells treated with an inhibitor that blocks in S phase give a biphasic distribution. Cells that were in S phase at the time the inhibitor was added give a broad distribution because the cells are distributed through all stages of replication. Cells that were in other phases of the cell cycle, however, pile up at the beginning of S phase, giving a sharp peak with a DNA content very close to that of G_1 cells.

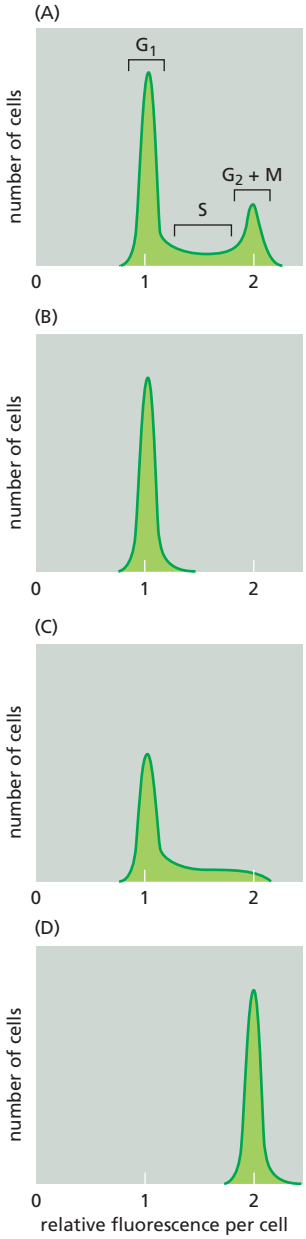


Figure A17–1 Relationships between fluorescence and the cell cycle (Answer 17–8). (A) Distribution of fluorescent cells among phases of the cell cycle for a normal population of dividing cells. (B) A cell population blocked in G_1. (C) A cell population blocked in S. (D) A cell population blocked in M.

17–9 Cohesins must be present during S phase because it is only while DNA is

being replicated that sister chromatids can be reliably identified by the cellular machinery that links them together. Once sister chromatids have separated, it is impossible for a nonspecific DNA-binding protein like cohesin to tell which chromosomes are sisters. And it would be virtually impossible for any protein to distinguish sister chromatids from homologous chromosomes. If sister chromatids are not kept together after their formation, they cannot be accurately segregated to the two daughter cells during mitosis.

Reference: Uhlmann F & Nasmyth K (1998) Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* 8, 1095–1101.

17–10 The dose of caffeine required to interfere with the DNA replication checkpoint mechanism is much higher than the amount imbibed by even the most excessive drinkers of coffee and colas. The concentration of caffeine in a cup of coffee is about 3.4 mM.

$$[\text{caffeine}] = \frac{100\ \text{mg}}{150\ \text{mL}} \times \frac{\text{g}}{1000\ \text{mg}} \times \frac{\text{mole}}{196\ \text{g}} \times \frac{1000\ \text{mL}}{\text{L}}$$

$$= 3.4 \times 10^{-3}\ \text{M} = 3.4\ \text{mM}$$

Since the concentration in a cup is less than the 10 mM required to interfere with the DNA replication checkpoint mechanism, you cannot get a higher concentration by drinking it and diluting it in the water volume of the body. If you assume for the purposes of calculation that the caffeine is not metabolized or excreted (but that all the liquid is), then you can ask how many cups of coffee would you need to drink (at 100 mg of caffeine per cup) to reach a concentration of 10 mM in 40 L of body water. The answer is: you would need to drink 784 cups of coffee!

17–11 There are 46 human chromosomes, each with two kinetochores—one for each sister chromatid—thus, there are 92 kinetochores in a human cell at mitosis.

17–12 Prophase (see Figure Q17–2E), prometaphase (see Figure Q17–2D), metaphase (see Figure Q17–2C), anaphase (see Figure Q17–2A), telophase (see Figure Q17–2F), and cytokinesis (see Figure Q17–2B).

17–13 A high frequency of trisomy does not mean that the chromosomes are difficult to segregate. For a trisomy to be present in a human infant requires that two conditions be met. First, the chromosomes must suffer nondisjunction during meiosis. Second, the chromosome complement of the fertilized egg has to be sufficient to support embryonic development. Down syndrome, which occurs at a frequency of one affected individual per 700 live births, and Edwards syndrome, which occurs at a frequency of one per 3000 live births, are the most common autosomal trisomies that meet both conditions. The most common trisomy involves chromosome 16, which occurs in more than 1% of pregnancies, but it is not compatible with normal development.

17–14 Since the assortment of homologs is a binary choice for each chromosome, the number of possible combinations is $2^{23,}$ which is 8.4×10^6. If recombination were allowed at any possible position between homologs, as it is in reality, the number of possible combinations would increase immeasurably.

CHAPTER 18

18–1 True. Adult tissues are maintained at a constant size, so that there must

be a balance between cell death and cell division. If this were not so, the tissue would grow or shrink.

18–2 True. Cytochrome *c* mediates apoptosis from signals within a mammalian cell—the intrinsic pathway of apoptosis. This has been confirmed directly by generating cytochrome *c*-deficient mouse embryo fibroblasts (MEFs) by reverse genetics. Although mice with knockouts of their cytochrome *c* genes die about midway through gestation because of problems with mitochondrial function, fibroblasts from such embryos can be cultured under special conditions and tested for sensitivity to various apoptotic signals. They are resistant to a variety of agents that induce the intrinsic pathway of apoptosis.

Reference: Li K, Li Y, Shelton JM, Richardson JA, Spencer E, Chen ZJ, Wang X & Williams RS (2000) Cytochrome *c* deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* 101, 389–399.

18–3 Overexpression of a secreted protein that binds to Fas ligand would protect tumor cells from attack by killer lymphocytes. By binding to the Fas ligand on the surface of killer lymphocytes, the secreted protein would prevent the Fas ligand from binding to the Fas death receptor on the surface of tumor cells, thereby insulating them from death-inducing interactions with killer lymphocytes. Secreted proteins that bind to Fas ligand are commonly known as decoy receptors. They play a normal role in modulating the killing induced by interactions between Fas ligand and Fas. When tumor cells overproduce such decoy receptors, they subvert this normal mechanism into a cellular defense against Fas-mediated killing.

Reference: Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, Huang A, Donahue CJ, Sherwood SW, Baldwin DT, Godowski PJ, Wood WI, Gurney AL, Hillan KJ, Cohen RL, Goddard AD, Botstein D & Ashkenazi A (1998) Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396, 699–703.

18–4 Somewhat surprisingly, cytochrome *c* seems not to be required for apoptosis in *C. elegans.* However, even if it were required, *C. elegans* mutants that were defective for cytochrome *c* would not have been isolated because they would not be viable. Cytochrome *c* is an essential component of the electron-transport chain in mitochondria. Without it, no production of ATP by oxidative phosphorylation would be possible, and such a mutant organism could not survive.

Reference: Ellis HM & Horvitz HR (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44, 817–829.

18–5 Upon microinjection of cytochrome *c*, both cell types undergo apoptosis. The presence of cytochrome *c* in the cytosol is a signal for the assembly of apoptosomes and the downstream events that lead to apoptosis. Cells that are defective for both Bax and Bak cannot release cytochrome *c* from mitochondria in response to upstream signals, but there is no defect in the downstream part of the pathway that is triggered by cytosolic cytochrome *c*. Thus, microinjection bypasses the defects in the doubly defective cells, triggering apoptosis.

Reference: Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB & Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292, 727–730.

18–6 The retention of the web cells in *Apaf1*$^{-/-}$ mice indicates that Apaf1 is essential for web-cell apoptosis, presumably in conjunction with

cytochrome *c*. The absence of web cells in *Casp9*$^{-/-}$ mice indicates that caspase-9 is not required for web-cell apoptosis. These observations suggest that Apaf1 may activate a different caspase in web cells, in addition to or instead of caspase-9.

Reference: Earnshaw WC, Martins LM & Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* 68, 383–424.

18–7 The two cells in Figure Q18–2 have released cytochrome *c*–GFP from all their mitochondria within a few minutes: within 6 minutes for the cell in Figure Q18–2A and within 8 minutes for the cell in Figure Q18–2B. The time after exposure to UV light at which the release occurred varied dramatically for the two cells: after 10 hours for the cell in Figure Q18–2A and after 17 hours for the cell in Figure Q18–2B. These observations indicate that individual cells release cytochrome *c* from all their mitochondria rapidly, but that release is triggered in different cells at widely varying times after exposure to apoptosis-inducing levels of UV light.

Reference: Goldstein JC, Waterhouse NJ, Juin P, Evan GI & Green DR (2000) The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2, 156–162.

18–8

A. One-eighth of Fas–Fas ligand complexes on the lymphocytes from an individual with ALPS would be expected to be composed entirely of normal Fas subunits. Since half the Fas protein in the lymphocytes is normal and there are three Fas subunits per complex, the probability of three normal Fas subunits coming together in a complex is $(\frac{1}{2})^3$.
B. In an individual heterozygous for a mutation that eliminates Fas expression, all the expressed Fas protein would be normal; thus, 100% of the Fas–Fas ligand complexes would be composed entirely of normal Fas subunits. The total number of Fas molecules, however, would be half the number present in an individual with two normal genes for Fas.
C. Fas mutations associated with ALPS are dominant because they reduce the number of normal Fas–Fas ligand complexes by a factor of eight in heterozygotes. Mutations that eliminate Fas expression are recessive because they reduce the number of Fas–Fas ligand complexes by only a factor of two.

Reference: Siegel RM, Chan FK-M, Chun HJ & Lenardo MJ (2000) The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat. Immunol.* 1, 469–474.

CHAPTER 19

19–1 False. Although cells can be readily dissociated by removing Ca^{2+} from the external medium, it is unlikely that Ca^{2+}-dependent cell–cell adhesions are regulated by changes in Ca^{2+} concentration. Cells have no way to control the Ca^{2+} concentration in their environment.

19–2 True. The barriers formed by tight-junction proteins restrict the flow of molecules between cells and the diffusion of proteins (and lipids) from the apical to the basolateral domain and vice versa.

19–3 False. The elasticity of elastin fibers derives from their lack of secondary structure: elastin forms random coils that are easily stretched. The set of hydrogen bonds that stabilizes an α helix is too strong, in aggregate, to be disrupted by the kinds of forces that deform elastin.

19–4 True. Tension—a mechanical signal—applied to an integrin can cause it to tighten its grip on intracellular and extracellular structures, including

not only cytoskeletal and matrix components, but also intracellular molecular signaling complexes. Similarly, loss of tension can loosen its hold, so that molecular signaling complexes fall apart on either side of the membrane. Thus, the tension on the integrin can trigger or inhibit molecular signaling.

19–5 This quote is correct in spirit, though incorrect in detail. Warren Lewis was trying to draw attention to the importance of the adhesive properties of cells in tissues at a time when the problem had been largely ignored by the biologists of the day. The quote is incorrect because a large fraction of our bodies is made up of connective tissue such as bone and tendon, whose integrity depends on the matrix itself rather than on the cells that inhabit it. It is not at all easy to dissociate cells from tissues, as anyone who has eaten a tough piece of steak can testify.

19–6 IgG antibodies contain two identical binding sites; thus, they are able to cross-link the molecules they recognize (this is the basis for immune precipitation). If whole antibodies were used to block aggregation, they might cross-link the cells rather than inhibit their aggregation. By contrast, monovalent Fab fragments cannot cross-link cells. They bind to the cell adhesion molecules and prevent them from binding to their partners, thus preventing cell aggregation (**Figure A19–1**).

Reference: Beug H, Katz FE & Gerisch G (1973) Dynamics of antigenic membrane sites relating to cell aggregation in *Dictyostelium discoideum*. *J. Cell Biol.* 56, 647–658.

19–7

A. Even though all of the claudin-4 has disappeared, the cells still express claudin-1, which is not affected by the toxin. Using antibodies specific for claudin-1, the authors showed that it remained intact at the sites of the tight junctions in the presence of the toxin.

B. Because the tight junction prevents molecules from penetrating the junction, added toxin will have access to only one side of the junction. Its inability to work from the apical side suggests that its binding sites on the claudin-4 molecules are accessible only from the basolateral side. If the toxin binds to monomers, as suggested above, then it could be that the monomers are delivered to the basolateral membrane domain, and therefore are accessible only from that side of the epithelial sheet. Alternatively, if the strands of claudin molecules are all oriented in the same way—that is, with their "top" surfaces all facing the apical side and their "bottom" surfaces all facing the basolateral side, as would be expected from symmetry principles—then a toxin-binding site on the bottom surface would only be accessible from the basolateral side.

Reference: Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y & Tsukita S (1999) *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J. Cell Biol.* 147, 195–204.

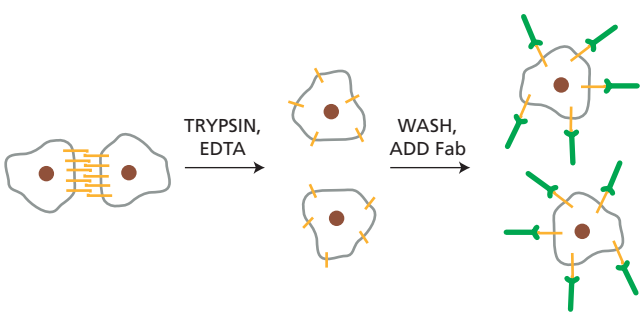


Figure A19–1 Fab antibody fragments block cell adhesion (Answer 19–6).

19–8 Mice that are homozygous for knockout of the gene for either nidogen-1 or nidogen-2 presumably have no phenotype because the two forms of nidogen can substitute for one another. Mice that are homozygous for the mutant form of laminin γ-1, which does not bind nidogen, have a much more severe phenotype than either of the individual nidogen gene knockouts because the mutation eliminates the ability of both nidogens to bind to laminin. As a result, these mice do not form proper basal laminae and die at birth with severe defects in kidney and lung. If this is the correct explanation for the genetic observations, then you would predict that mice that are homozygous for knockouts of both nidogen genes would have a very severe phenotype, comparable to that of the laminin γ-1 mutant. Such mice have been made and they do have a severe phenotype.

Reference: Sasaki T, Fassler R & Hohenester E (2004) Laminin: the crux of basement membrane assembly. *J. Cell Biol.* 164, 959–963.

19–9 This statement encapsulates our growing recognition of the diverse roles the basal lamina plays. Although it provides structural support for the cells that rest upon it, mechanical stability is only one of the several functions the basal lamina supplies. For example, during the regeneration of muscles or motor neurons, the neuromuscular junction is reestablished based on information contained in the basal lamina. Special molecules stuck in the basal lamina—like messages on a bulletin board—mark the site of the junction and allow it to be reconstituted exactly. Growing evidence indicates that similar processes occur during the original development of muscle and neuromuscular junctions.

Reference: Sanes JR (2003) The basement membrane/basal lamina of skeletal muscle. *J. Biol. Chem.* 278, 12601–12604.

19–10 The high level of activation when alanine was substituted for D723 in the β chain, or for R995 in the α chain, indicates that those residues are somehow important for holding the $\alpha_{IIb}\beta_3$ integrin in an inactive state. The "charge-swap" experiment, which showed that D723R paired with R995D was as inactive as the wild type, suggests strongly that these two residues form an electrostatic attraction—a salt bridge—that helps to hold $\alpha_{IIb}\beta_3$ integrin in its inactive configuration. It follows that inside-out signaling is probably triggered by breaking this salt bridge.

Reference: Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ & Ginsberg MH (1996) Breaking the integrin hinge: A defined structural constraint regulates integrin signaling. *J. Biol. Chem.* 271, 6571–6574.

19–11 The high density of negative charges on the polysaccharide components of proteoglycans causes the sugar chains to be extended, occupying a large volume. The negative charges on the proteoglycans trap an equal number of cations to maintain electrical neutrality. Electrostatic forces confine these charges—both the fixed negative charges on the polysaccharide and the mobile cations—to the volume occupied by the proteoglycan. The concentration of particles in the volume of the proteoglycan is higher than in the surrounding solution; hence, the water flows in to try to equalize the concentrations inside and outside. The proteoglycans thus trap water to form a hydrated gel by drawing in water molecules by osmosis. In the absence of the negative charges, the sugar chains would collapse into fibers or granules, dramatically altering the properties of the extracellular matrix.

19–12 Because the racemization of L-aspartate to D-aspartate occurs slowly, proteins that turn over rapidly will have very low levels of D-aspartate, if it can be detected at all. Proteins that are degraded and replaced more

slowly will be expected to have a higher percentage of D-aspartate, with the absolute level depending on the rate of turnover. What makes the observations on elastin remarkable is the age dependence of the D-aspartate levels. These observations have been interpreted to mean that our lifetime supply of elastin is made early on and never degraded. In addition, studies in humans that made use of the inadvertent metabolic ^{14}C-labeling due to atmospheric testing of nuclear weapons, led to the conclusion that elastin synthesis occurs almost exclusively during the fetal and postnatal periods of development. Experiments in mice support this idea.

Reference: Shapiro SD, Endicott SK, Province MA, Pierce JA & Campbell EJ (1991) Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. *J. Clin. Invest.* 87, 1828–1834.

19–13 If you soak the lettuce in tap water, it will take up water due to osmosis and become crisper. Soaking the lettuce in salt water or sugar water will have the opposite effect, sucking even more water out of the lettuce, making it even limper. Your day-old lettuce is long past the point at which photosynthesis can do it any good, and the bright light will dry it out even more.

19–14 At 0.1 MPa the hydraulic conductivity of a single aquaporin water channel is 4.4×10^{-23} m^3/s [$(4.4 \times 10^{-22}$ m^3/s MPa) $\times$ 0.1 MPa = 4.4×10^{-23} m^3/s]. Thus, the question becomes how many water molecules are in 4.4×10^{-23} m^3. There are 3.33×10^{28} water molecules/m^3 [(55.5 moles/L)$(10^3$ L/$m^3)(6 \times 10^{23}$ water molecules/mole)]. Therefore, 1.5×10^6 water molecules flow through a water channel each second at 1 atmosphere of pressure [$(4.4 \times 10^{-23}$ $m^3/s)(3.33 \times 10^{28}$ water molecules/$m^3)$].

Reference: Tyerman SD, Bohnert HJ, Maurel C, Steudle S & Smith JAC (1999) Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. *J. Exp. Bot.* 50, 1055–1071.

CHAPTER 20

20–1 False. It is not that DMBA is a specific mutagen, but rather that the *Ras* gene is converted to its activated, cancer-causing form by a particular A-to-T alteration that leads to a very specific amino acid change. DMBA causes mutations throughout the genome, but only those at the specific site in the *Ras* gene give rise to cells that have cancerous properties and thus are identified in the assay.

20–2 True. That is why oncogenes in their overactive, mutant form tend to drive cell growth and proliferation, and why the loss of tumor suppressor genes removes regulatory brakes on these pathways, which also promotes cell growth and proliferation.

20–3 True. Many cancers appear to be maintained by a small population of stem cells. These cells usually divide more slowly than the cells in the bulk of the tumor, and they are less sensitive to treatments aimed at rapidly dividing cells. If the stem cells are not killed, the cancer is likely to return.

20–4 False. Although it is popular to think so, there is scant evidence to support those ideas, except in very specific instances like 2-naphthylamine and asbestos.

20–5 The key difference in the incidences of colon cancer and osteosarcomas is the size of the population of cells at risk for the disease. Colon cancer arises from the population of proliferating cells in the colon, which

are present in roughly the same number throughout life. This population can accumulate mutations over time, giving rise to the age-dependent increase in cancer incidence. By contrast, the cells at risk for osteosarcomas are present in much greater numbers during adolescence, when cell proliferation is required to increase the size of the skeleton, than they are in young children or adults. It is in this large, proliferating population that an abnormal lineage of cancer cells is most likely to arise. In this case, it is the number of cells at risk that is the most important determinant of the frequency of cancer.

Reference: Knudson AG (2001) Two genetic hits (more or less) to cancer. *Nat. Rev. Cancer* 1, 157–162.

20–6 Development of most cancers requires a gradual accumulation of mutations in several different genes. In the ongoing presence of cigarette smoke, these mutations evidently accumulate at an increased rate (over their accumulation in the absence of cigarette smoke). By stopping smoking, an individual returns to the normal, slower rate of mutation accumulation. Thus, whatever mutations remain to be generated in a reformed smoker are generated at a slower rate than in a continuing smoker. The slower rate of accumulation of mutations translates into a lower cumulative risk.

Reference: Peto R, Darby S, Deo H, Silcocks P, Whitley E & Doll R (2000) Smoking, smoking cessation, and lung cancer in the UK since 1950: combination of national statistics with two case-control studies. *Br. Med. J.* 321, 323–329.

20–7

A. The key observation about these multiple, localized rearrangements is that they give copy numbers that are either 0 or 1. As can be seen by examination of the rearranged chromosome in the progressive rearrangements model, different segments are present in 0 copies (I), 1 copy (A, E, G, H, J), 2 copies (B, D, F), and 3 copies (C) (**Figure A20–1**). By contrast, in the chromosome catastrophe model, segments are present in either 0 copies (A, E, G, J) or 1 copy (B, C, D, F, H, I) (see Figure A20–1). Computer simulations indicate that it is virtually impossible for a sequence of rearrangements to produce a chromosome in which every segment is present either once or not at all.

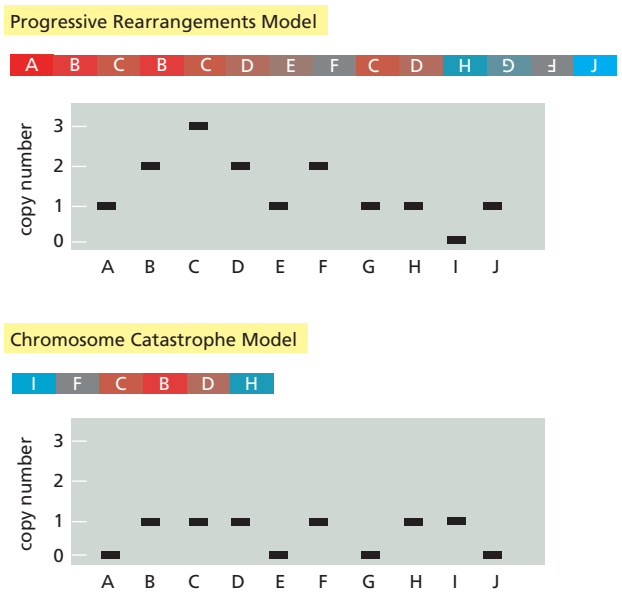


Figure A20–1 Copy number variation associated with the rearranged chromosomes generated by progressive rearrangement or chromosome catastrophe (Answer 20–7).

B. The authors of the paper suggest two possible explanations for how such shattered chromosomes might arise. One possibility is ionizing radiation, which commonly generates double-strand breaks. A pulse of ionizing radiation passing through a condensed mitotic chromosome could break the chromosome in multiple nearby places, giving rise to ends that could be rejoined in random order. A second possibility is that the damage is triggered by the fusion of two chromosomes that have lost their telomeres. When the two centromeres of such dicentric chromosomes are pulled to opposite daughter cells during anaphase, they form a so-called anaphase bridge. It is unclear how these bridges are resolved, but they appear to induce the formation of micronuclei containing fragmented DNA in the daughter cells. This fragmentation could account for the multiple, localized chromosome rearrangements observed. Which, if either, of these explanations is correct must await further experimentation.

C. These rearrangements certainly have the capacity to be driver events. Rearrangements could inactivate one copy of a tumor suppressor gene by deleting it completely or partially, or by splitting it into two pieces. Similarly, rearrangements could activate an oncogene by placing it near a more active promoter, or by fusing it with another gene to give a hybrid protein with oncogenic properties. Examples of both kinds of event were found among the set of cancers examined by the authors of the paper.

Reference: Stephens PJ, Greenman CD, Fu B et al. (2011) Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell* 144, 27–40.

20–8 The promyelocytes of APL are blocked at an intermediate stage in their development, at a point where they still divide and increase in number. It is this unchecked increase in number that causes problems for the cancer patient. Normally, such precursor cells divide only a few times before they terminally differentiate into a nondividing blood cell. By triggering the differentiation of promyelocytes into terminally differentiated neutrophils, which no longer divide, treatment with all-*trans*-retinoic acid eliminates the problems caused by unchecked proliferation.

APL arises by one of a few types of translocation that fuses the retinoic acid receptor (RAR) gene on chromosome 17 with a gene on another chromosome to make a hybrid protein that interferes with the normal developmental program. It is not yet clear how the fusion protein blocks development, although it likely does so by interfering with the function of the normal RAR. In some way, treatment with all-*trans*-retinoic acid allows APL cells to move through the block.

Reference: Warrell RP Jr, de Thé H, Wang Z-Y & Degos L (1993) Acute promyelocytic leukemia. *N. Engl. J. Med.* 329, 177–189.

20–9 The products of oncogenes are the only feasible targets for such small molecules. The product of an oncogene has a dominant, growth-promoting effect on the cell. Thus, if the growth-promoting oncogene product were inhibited, the cell might return to a more normal state. This is the underlying rationale for searching for drugs that inhibit oncoproteins.

By contrast, the products of tumor suppressor genes are not targets for anticancer drug development. Tumor suppressor genes cause cancer by *not* making their product. Thus, there is no abnormal product to be inhibited in cancer cells that arise by mutation of tumor suppressor genes.

20–10 Individuals who are heterozygous for a mutation in the *Brca1* gene are susceptible to cancer of the breast and ovary because Brca1 is an especially important tumor suppressor in these tissues. The loss of the remaining functional copy of the *Brca1* gene—by mutation, chromosome loss, or epigenetic silencing—drives the affected cell toward the cancer

phenotype. As a consequence, the cancer cells that arise in these tissues cannot carry out homologous recombination because they are missing Brca1. By contrast, the one good copy of the gene that is present in the patient's normal cells makes sufficient Brca1 to make the cells proficient for homologous recombination. Thus, when these patients are treated with olaparib, their cancer cells die because they cannot use homologous recombination to repair the double-strand breaks that arise from the inhibition of PARP. Their normal cells, however, which have a good copy of *Brca1*, repair the breaks just fine.

Reference: Fong PC, Boss DS, Yap TA et al. (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N. Engl. J. Med.* 361, 123–134.

20–11 The highly rearranged karyotypes and their similarity from tumor to tumor suggest that the cancer cells themselves are being transmitted from devil to devil. It is extremely unlikely that an infectious agent such as a virus or a microorganism could induce the same set of complicated rearrangements in different animals. Most importantly, the existence of a chromosome 5 inversion in one Tasmanian devil, which is not present in chromosome 5 of its tumor cells, argues strongly that the tumors are not generated from the host devil's own cells. It appears that this cancer has arisen from a rogue line of cancer cells, from a tumor of unknown origin, that has acquired the capability for parasitic existence. This is one of just two known examples of natural transmission of cancer by tumor cells, the other being a venereal disease in dogs. A special case of such transmission occurs occasionally during organ transplantation in humans. But the requirements for organ transplantation—matching tissue and immune suppression—highlight just how unusual natural transmission is. The cancer cells responsible for facial tumors in Tasmanian devils must somehow evade the new host's immune defenses.

Reference: Pearse A-M & Swift K (2006) Allograft theory: Transmission of devil facial-tumour disease. *Nature* 439, 549.

CHAPTER 21

21–1 True. The maternal mRNAs and proteins that were deposited in the egg are responsible for the early stages of development, until—at some point around the blastula stage—the maternal mRNAs and proteins are destroyed and the embryo's genome is activated.

21–2 False. The basic body plan and axes set up in miniature during gastrulation are preserved into adult life, despite the complex developmental rearrangements that occur along the way.

21–3 True. During the blastula stages, cells have the potential to give rise to all or almost all the cell types of the adult body, but a cell's developmental options are progressively restricted as it becomes committed to one germ layer, then one organ, then one cell type. Cell determination starts early and progressively narrows the options as the cell steps through a programmed series of intermediate states, ultimately forming one of the highly specialized cell types of the adult body. Although some cell types in the adult retain some degree of pluripotency, their range of options is generally narrow.

21–4 True. A small number of highly conserved signaling pathways, including transforming growth factor-β (TGFβ), receptor tyrosine kinase (RTK), Wnt, Hedgehog, and Notch pathways, govern most of the known inductive events in animal development.

21–5 False. The coding DNA for genes involved in development has been highly conserved between species. It is changes to the noncoding, regulatory DNA that seems to be most important for the developmental differences between species. The regulatory elements determine when, where, and how strongly the gene is to be expressed; thus, they can alter the logic of gene-regulatory networks and change the outcome of development.

21–6 False. Although there are examples where cells change their maturation state in a way that depends on cell division, this is not the general rule. For example, neuroblasts in the developing *Drosophila* embryo carry on with their normal timetable of maturation and differentiation even when cell division is artificially blocked. It seems that most developing cells can change their state without a requirement for cell division.

21–7

1. Cell proliferation, which produces many cells from one.
2. Cell–cell interactions, which coordinate the behavior of each cell with that of its neighbors.
3. Cell specialization, or differentiation, which creates cells with different characteristics at different positions.
4. Cell movement, which rearranges the cells to form structured tissues and organs.

21–8 The three germ layers are the ectoderm, endoderm, and mesoderm. The ectoderm gives rise to the epidermis and the nervous system. The endoderm gives rise to the gut tube and its appendages such as lung, pancreas, and liver. The mesoderm gives rise to muscles, connective tissue, blood, kidney, and various other components.

21–9 In the early stages of the *Drosophila* embryo, a rapid series of nuclear divisions occurs without cell division, giving rise to a syncytium containing many nuclei in a common cytoplasm. The initial patterning of the *Drosophila* embryo occurs in this syncytium by direct diffusion of transcriptional regulators and mRNA molecules.

21–10 The patterns represented by flags from Japan and France could be created by a gradient of a single morphogen, while that of Norway would require gradients of two morphogens.

The pattern for the Japanese flag is the easiest to understand. If cells in the center of the red circle secreted a morphogen, its concentration would decrease in a circular pattern as it diffused away from its source. Cells near the source, which experienced a concentration above a certain threshold, would turn red; those that were farther away would experience a concentration below the threshold and turn white. The pattern for the French flag could be generated by a morphogen secreted by a line of cells at one end of the field, with a high concentration generating red cells, an intermediate concentration giving rise to white cells, and a low concentration producing blue cells. The more complex pattern of the Norwegian flag could be generated by gradients of two morphogens secreted by lines of cells at right angles to each other.

More complex patterns could be generated by additional morphogens or by morphogens that interacted with one another. The famous mathematician Alan Turing modeled the behaviors that could be generated by two interacting morphogens and showed that complex, biologically relevant patterns could be produced.

Reference: Kondo S & Miura T (2010) Reaction-diffusion model as a framework for understanding biological pattern formation. *Science* 329, 1616–1620.

21–11 The behavior of the two precursor cells reveals interactions between them that require cell contact, and that Lin12 is critical for the communication

between them. In the absence of Lin12, a cell cannot receive the signal required to change its fate from an AC to a VU cell and thus remains an AC. To become a VU cell requires a signal that involves Lin12, and, by implication, the signal must come from the AC. The genetic mosaics provide the most conclusive evidence: cells without Lin12 become ACs, while cells with hyperactive *Lin12* become VU cells. If Lin12 were required as the signaling molecule to bring about the AC-to-VU change in fate, then cells without Lin12 in the genetic mosaic would become VU cells, being capable only of receiving the signal, not sending it. These experiments do not speak to the specific role of Lin12, but Lin12 shares structural features with Notch, suggesting that it, too, is a cell-surface receptor.

In wild-type worms, the two precursor cells exchange signals, with small initial differences in signal-to-receptor strength becoming magnified until the signaling is all one way: from the precursor that will become the AC to the precursor that will become the VU cell via the Lin12 receptor. This example is a classic case of lateral inhibition.

Reference: Seydoux G & Greenwald I (1989) Cell autonomy of *lin-12* function in a cell fate decision in *C. elegans*. *Cell* 57, 1237–1245.

21–12 The results of these experiments are consistent with the morphogenetic substance being maternal mRNA encoding alkaline phosphatase. Puromycin, a translation inhibitor, would be expected to block expression, which it does. Actinomycin D, a transcription inhibitor, would not be expected to block expression, and it does not. Since treatment with actinomycin D gave a negative result, the author of the study checked several other transcription inhibitors: each gave the same result. In addition, these inhibitors blocked transcription of other genes. The results with cytochalasin B showed that expression of alkaline phosphatase was timed to occur in a way that was independent of cell division in the early embryo.

Reference: Whittaker JR (1977) Segregation during cleavage of a factor determining endodermal alkaline phosphatase development in ascidian embryos. *J. Exp. Zool.* 202, 139–153.

21–13 These observations point to the dominant role of *cis*-regulatory sequences in the diversification of the *Hox* genes. Because *HoxA3* and *HoxD3* can substitute for one another, the two Hox proteins must be functionally equivalent. However, that equivalence is manifest only when the genes are placed in the context of specific regulatory sequences. It is the *cis*-regulatory sequences at the *HoxA3* locus that allow the transplaced *HoxD3* gene to be expressed in the proper amounts and appropriate cells to build correct pharyngeal structures. Similarly, the *cis*-regulatory sequences at the *HoxD3* locus permit the transplaced *HoxA3* gene to construct a normal axial skeleton. It is these same regulatory sequences that prevent the transplaced *HoxA3* and *HoxD3* genes from carrying out their "normal" functions.

Reference: Greer JM, Puetz J, Thomas K & Capecchi M (2000) Maintenance of functional equivalence during paralogous Hox gene evolution. *Nature* 403, 661–665.

21–14 Removing introns would be expected to reduce the delay because it reduces the time needed to transcribe the gene, one component of the delay in *Hes7* expression. If the model were correct, you might expect that removing introns would reduce the delay, thereby speeding up the oscillations in *Hes7* expression. Everything else being equal, a faster oscillation time might be expected to give rise to more closely spaced somites.

The results of the experiments are even more informative. Removing one intron did not have much effect, presumably because it did not alter the delay sufficiently. Removing two introns, however, has just the

expected effect: somite formation occurred faster than normal, so that somites were more closely spaced. And the mice had more vertebrae than normal! Removal of all three introns stopped the clock completely, because oscillations in *Hes7* expression were abolished. Evidently, the delay must be within certain limits for the system to work at all.

Reference: Harima Y, Takashima Y, Ueda Y, Ohtsuka T & Kageyama R (2013) Accelerating the tempo of the segmentation clock by reducing the number of introns in the *Hes7* gene. *Cell Rep.* 3, 1–7.

21–15 In the absence of Delta, each cell continues to oscillate because of the feedback inhibition by unstable Her7 on its own transcription and the inherent delay in its transcription and translation. However, the oscillations in adjacent cells are no longer coupled. Even though they start out in lock step, as shown by the well-formed initial somites (Figure Q21–2B), they eventually fall out of phase due to random stochastic differences between the cells. In the presence of Delta, however, a reciprocal pattern of signaling is established in adjacent cells that keeps the cells in synchrony (Figure Q21–2C). In reality, this relatively simple diagram (Figure Q21–2D) is difficult to analyze intuitively to convince yourself that the scheme operates to keep adjacent cells oscillating together; however, mathematical modeling of the system is persuasive.

Reference: Soza-Ried C, Öztürk E, Ish-Horowicz D & Lewis J (2014) Pulses of Notch activation synchronise oscillating somite cells and entrain the zebrafish segmentation clock. *Development* 141, 1780–1788.

21–16 Because the extra mass of wing tissue is composed of normal-looking cells, Dpp must stimulate both cell division, to increase the number of cells, and cell growth so that the extra cells are the right size.

References: Edgar BA & Lehner CF (1996) Developmental control of cell cycle regulators: a fly's perspective. *Science* 274, 1646–1652.

Prober DA & Edgar BA (2001) Growth regulation by oncogenes—new insights from model organisms. *Curr. Opin. Genet. Dev.* 11, 19–26.

Zecca M, Basler K & Struhl G (1995) Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. *Development* 121, 2265–2278.

21–17 In vertebrates, neurons manage self–non-self discrimination using cadherin-like transmembrane proteins that are expressed in different combinations from the *Protocadherin* locus, which encodes 58 related cadherin-like proteins. Homophilic recognition results in self-avoidance of dendrites emanating from the same neuron. Dendrites from different neurons express different protocadherins and thus evade repulsion.

CHAPTER 22

22–1 False. The daughters of a stem-cell division in the crypt make independent decisions whether to remain stem cells or commit to terminal differentiation. On average, about 50% of the daughters remain stem cells, while the remainder differentiate.

22–2 False. There are many different types of stem cell, each specialized for the genesis of different classes of terminally differentiated cells.

22–3 False. Hepatocytes of the liver and the β cells of the pancreas are differentiated cells that can divide to replace lost cells. Renewal occurs predominantly by division of these differentiated cells, even though both liver and pancreas maintain small populations of stem cells.

22–4 True. Excessive action of osteoclasts, which break down bone matrix, or deficient action of osteoblasts, which build up bone matrix, can weaken bones, causing them to become brittle.

22–5 The pattern of labeling expected for stem cells would be bursts of cells with labeled nuclei, which would increase in number over short periods and then disappear with time. This labeling pattern was seen not only in cells of the squamous epithelia, but also in the crypts of the small and large intestine. The two other patterns of labeling are not consistent with the expectations for stem cells. This is obvious for the pattern characterized by no labeling at all, but what about rare labeled cells that persist? Renewal by stem-cell differentiation implies a flux through a pathway, as occurs in gut, skin, and the blood system: cells are born and die and must be replaced. A labeled stem cell will progressively lose its label as it divides to produce progeny cells.

Reference: Messier B & Leblond CP (1960) Cell proliferation and migration as revealed by radioautography after injection of thymidine-H^3 into male rats and mice. *Am. J. Anat.* 106, 247–285.

22–6 It is clear from the images in Figure 22–1 that most crypts become monoclonal—express only one fluorescent protein—over time. The small number of stem cells and the constant competition for contact with Paneth cells almost guarantees that crypts will become monoclonal. On average, it takes about three months for an intestinal crypt in mice to become monoclonal.

Reference: Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD & Clevers H (2010) Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143, 134–144.

22–7 These results support the hypothesis that most, if not all, new β cells are generated from preexisting β cells. If all new β cells were generated from stem cells, the frequency of cells expressing HPAP would be expected to decline from 30% to less than 5% (30%/6.5) in 12 months, since like their stem cell parents, none of the newly formed β cells should express HPAP. Such a decrease is not supported by the data in Figure Q22–2. On the other hand, if all new β cells were derived from preexisting β cells, then the percentage of HPAP-expressing cells should remain fairly constant at around 30%, a result that better matches the data in Figure Q22–2.

References: Dor Y, Brown J, Martinez OI & Melton DA (2004) Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429, 41–46.

Rais Y, Zviran A, Geula S et al. (2013) Deterministic direct reprogramming of somatic cells to pluripotency. *Nature* 502, 65–70.

22–8 If spleen colonies arose from single transplanted cells, then all the cells in a colony should show the same genome rearrangement. By contrast, if colonies arose from multiple cells, then only a portion of the cells in the colony would have any one rearrangement. The likelihood that multiple cells in an aggregate would all have the same rearrangement is vanishingly small. The outcome of such experiments showed clearly that single cells give rise to spleen colonies.

Reference: Becker AJ, McCulloch EA & Till JE (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* 197, 452–454.

22–9 Since 10 cells in the enriched population were required to generate a

spleen colony, as shown by the intercept for the enriched cells in Figure Q22–3, and only 1 in 10 transplanted cells lodges in the spleen, it is likely that the enriched population consists nearly entirely of stem cells. To be sure the enriched cells were true stem cells, you would need to know whether the putative stem cells could repopulate all the various blood cell types. The authors of this study demonstrated that point in additional experiments. The curves in Figure Q22–3 are separated by about a factor of 1000, suggesting that about 1 in 1000 bone marrow cells is a hematopoietic stem cell.

Reference: Spangrude GJ, Heimfeld S & Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. *Science* 241, 58–62.

22–10 In general, there are three different ways to deliver the OSKM transcription regulators: as DNA, RNA, or protein. Each method has its challenges. Various DNA-based methods include integrating vectors that can subsequently be excised from the genome and vectors such as plasmid DNA and adenoviral vectors that do not integrate into the genome. Transfections of mRNAs encoding the OSKM transcription regulators have also proven successful; however, RNA transfections tend to elicit antiviral responses, which decrease reprogramming efficiencies. Several modifications—ensuring that all mRNAs have guanine caps, substituting 5-methyl cytidine for cytidine and pseudouridine for uridine, and blunting the interferon response—significantly enhanced reprogramming, so that up to 2% of fibroblasts were converted to iPS cells. Introduction of proteins also works, but the challenge is to generate and purify the proteins in the quantities required.

Reference: Warren L, Manos PD, Ahfeldt T et al. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified RNA. *Cell Stem Cell* 7, 618–630.

CHAPTER 23

23–1 True. There are about 10^{14} bacterial, fungal, and protozoan cells in the human body and about 10^{13} human cells.

23–2 False. The microbiomes—the combined genomes of the microbiota—vary considerably among individuals, even between close relatives and identical twins.

23–3 False. While many pathogens cause disease only by entering host cells, extracellular pathogens do not need to; they exert their deleterious effects by secreting toxins or by injecting effector proteins directly into host cells.

23–4 False. Most DNA viruses replicate their genomes in the nucleus, but most RNA viruses replicate theirs in the cytosol. (Influenza is one of the RNA viruses that replicates in the nucleus, where it practices its unusual habit of cap-snatching to initiate mRNA synthesis.)

23–5 True. Antibiotics are not effective against viral infections. They are directed at components of bacteria, in order to interfere with their proliferation or kill them. The use of antibiotics to treat viral diseases can contribute to the growing problem of antibiotic resistance and can modify the make-up of the microbiota. There is a class of drugs known as antivirals that are effective against viral infections. These drugs operate on the same principle as antibiotics, targeting components unique to the virus. For example, the antivirals used to treat AIDS patients target the reverse

transcriptase that copies the HIV RNA genome into DNA, and the HIV protease that is necessary to process components of the virion.

23–6 Pathogens must be able to: (1) colonize the host; (2) find a nutritionally compatible niche in the host's body; (3) avoid, subvert, or circumvent the host's adaptive immune response; (4) replicate, using host resources; and (5) exit one host and spread to another.

23–7 Microbiota transplantation is effective because it restores the proper mix of microbial species that normally populate the gut and protect against colonization by *Clostridium difficile*. Antibiotic treatments disturb the gut microbiota, reducing the diversity of species normally present. Analysis of the 16S ribosomal RNA genes in gut samples showed that patients with recurrent infections treated by antibiotics had decreased diversity and number of phyla, and lacked the two predominant phyla: Bacteroidetes and Firmicutes. It is not clear how the normal gut microbiota protects against germination of the *C. difficile* spores and recolonization (reinfection), but it does so very effectively.

Reference: Austin M, Mellow M & Tierney WM (2014) Fecal microbiota transplantation in the treatment of *Clostridium difficile* infections. *Am. J. Med.* 127, 479–483.

23–8 The three mechanisms of horizontal gene transfer are natural transformation by released naked DNA, transduction by bacteriophage, and sexual exchange by conjugation.

23–9 YopJ blocks the TAK1 signaling pathway by preventing phosphorylation of TAK1 (Figure Q23–1, lane 3). Inactive YopJ does not prevent phosphorylation (lane 2). How YopJ prevents TAK1 phosphorylation is not defined by these experiments. However, because YopJ is a serine/threonine acetylase, it is reasonable to suppose that YopJ interferes with TAK1 phosphorylation by acetylation of serines or threonines in TAK1. In principle, such acetylation could alter the conformation of TAK1, preventing its phosphorylation. In reality, YopJ is much more precise: it acetylates the very residues that are normally phosphorylated, thereby chemically blocking TAK1 activation.

Reference: Paquette N, Conlon J, Sweet C, Rus F, Wilson L, Pereira A, Rosadini CV, Goutagny N, Weber ANR, Lane WS, Shaffer SA, Maniatis S, Fitzgerald KA, Stuart L & Silverman N (2012) Serine/threonine acetylation of TGFβ-activated kinase (TAK1) by *Yersinia pestis* YopJ inhibits innate immune signaling. *Proc. Natl Acad. Sci. USA* 109, 12710–12715.

23–10 Because SopE and SptP both target the monomeric GTPase, Rac, it is reasonable to predict that SopE might act as a GEF (guanine nucleotide exchange factor) to activate Rac by promoting release of GDP and uptake of GTP, and that SptP might act as a GAP (GTPase-activating protein) to inactivate Rac by promoting hydrolysis of GTP to GDP. The sequential action of two simultaneously injected proteins might be accounted for in two general ways. The later acting protein (SptP) might need to be activated by a cellular process, delaying the onset of its function. Alternatively, the earlier acting protein (SopE) might be deactivated in some way with time, allowing the activity of SptP to predominate at later times. As the authors of this study showed, SopE is inactivated over time by proteasomal degradation, permitting SptP to return Rac to its inactive, GDP-containing form at later times, thereby stopping membrane ruffling.

Reference: Kubori T & Galán JE (2003) Temporal regulation of *Salmonella* virulence effector function by proteasome-dependent protein degradation. *Cell* 115, 333-342.

23–11 What Snow showed very clearly was that the cases were clustered around a single public water pump. He made a reasonable hypothesis that the water from the central pump was the source of the cholera, but he could find nothing suspicious-looking in the water. Most scientists remained skeptical because Snow offered no more than a correlation between the location of disease and a water pump; he carried out no further experiments to test his conclusion. Anyone who believed in bad air might look at the distribution of victims and conclude that there was a centralized source of bad air, a huge cesspit perhaps. They might also have argued that victims near the periphery of the distribution likely got their water from nearby public water pumps, not the central one; hence, they would not have been expected to get the disease.

It is important to remember that in 1854 Louis Pasteur had not yet formulated the germ theory of disease, and Robert Koch had yet to see bacteria in the microscope, grow them in culture, and re-inoculate them into a host to prove their ability to cause disease. Thus, Snow's inability to convince his skeptics is understandable. It was, in fact, Koch who solved the puzzle of cholera in the 1880s. He identified a characteristic comma-shaped bacillus associated with the disease, and showed that he could infect guinea pigs with it and cause cholera. He found these bacteria in the water supply used by the infected patients, just as Snow would have predicted.

23–12 It is still unclear what mechanisms account for the seasonal variability of influenza epidemics. Several suggestions have been made, although none is completely satisfactory. Explanations have been proposed in three broad categories, as summarized below.

1. *Seasonal variations in contact rates*. Contact rates between infected and susceptible individuals increase during the winter and rainy season, with more time spent indoors. But the increase is remarkably small—1–2 hours more in winter and 0.5 hour in the rainy season—relative to the 21–22 hours we normally spend indoors. Also, in the southwestern US, hot temperatures drive people indoors in the summer, without an accompanying increase in flu rates. Nevertheless, there are strong correlations between influenza transmission and crowding—on airplanes, at festivals, among soldiers—suggesting that contact rates are an important factor.
2. *Seasonal variations in virus survival*. The transmission of influenza by aerosols and direct contact means that the virus must survive exposure to environmental conditions. Virus survival increases with decreasing temperature, decreasing absolute humidity, and decreasing ultraviolet radiation. Since these three factors vary together, with less accommodating conditions in the summer, they offer a tidy explanation for the winter peaks of influenza in temperate zones, but are less satisfying for the tropics, where temperature does not vary much and the highest humidity—the least favorable condition—coincides with the peak of flu activity in the rainy season.
3. *Seasonal variations in human resistance*. Seasonal changes in the physiological functions that enable an individual to avoid or mitigate infection after exposure to the virus could underlie the peaks of influenza. For example, temperature and humidity can affect the nasal passages and their susceptibility to infection. Similarly, in temperate zones, there are seasonal variations in vitamin D levels, which are important for human immune function. Low levels of vitamin D coincide with winter in temperate zones and with the rainy season in the tropics.

Which of these potential explanations, or combinations of them, actually accounts for the seasonal variations in influenza infections is unclear. Distinguishing the causal relationships from casual associations has thus far proved to be an insurmountable challenge.

Reference: Tamerius J, Nelson MI, Zhou SZ, Viboud C, Miller MA & Alonso WJ (2011) Global influenza seasonality: reconciling patterns across temperate and tropical regions. *Environ. Health Perspect.* 119, 439–445.

23–13 Genome segmentation allows rapid testing of mutations generated by different viruses. For example, when two different influenza viruses—with distinct mutational histories—infect the same cell, they can generate hybrid virus progeny that carry 2^8 different combinations of RNA segments. Independent mutation of segments followed by random reassortment in mixed infections of the same cell offers a rapid means to explore new capabilities. In 2009, the H1N1 strain of influenza virus emerged that derived genes (RNA segments) from pig, avian, and human influenza viruses.

Reference: Weber M & Weber F (2014) Segmented negative-strand RNA viruses and RIG-I: divide (your genome) and rule. *Curr. Opin. Microbiol.* 20, 96–102.

23–14 Because pig cells carry cell-surface carbohydrate chains with both types of sialic acid–galactose linkage, avian, human, and swine influenza viruses can infect them. If different influenza viruses simultaneously infect the same pig cell, their different RNA segments can be reassorted to generate new combinations with properties that the human population has not previously experienced—a potentially dangerous situation.

23–15 Microorganisms produce antimicrobial compounds as weapons in their competition with other microorganisms. Surveys of soil bacteria that have never been exposed to the antibiotics used in modern medicine reveal that the bacteria are commonly resistant to several of the antibiotics in current use. In developing clinically useful compounds from natural products, we have simply taken advantage of the bacteria's evolutionary "research" for our own benefit.

23–16 Many naturally occurring bacteria secrete an enzyme that hydrolyzes antibiotics with a structure like penicillin. These enzymes, which are known as β-lactamases, are widespread in nature and are thought to have evolved in response to biological warfare among microorganisms.

Reference: Florey HW (1944) Penicillin: a survey. *Br. Med. J.* 2, 169–171.

23–17 The whole point of antibiotics is to kill the microorganism but leave the host untouched. In fact, several substances that killed bacteria in culture had been discovered before penicillin, but they were all very toxic to human cells. Thus, Chain's control was crucial. If penicillin was harmless to mice, it was very likely to be harmless to humans as well. It is now known that penicillin interferes with bacterial cell wall synthesis, a process unrelated to any process in human cells.

Reference: Florey HW (1944) Penicillin: a survey. *Br. Med. J.* 2, 169–171.

CHAPTER 24

24–1 False. Developing T cells whose T cell receptors interact strongly with a self-peptide–MHC complex are induced to die in the thymus, in the process of negative selection, although some are positively selected to become natural regulatory T cells, which exit the thymus, along with other positively selected T cells.

24–2 False. A subpopulation of epithelial cells in the thymus expresses the transcriptional regulator AIRE, which induces small amounts of transcription

from many genes that encode proteins not normally expressed in the thymus.

24–3 True. In humans, the combinatorial joining of gene segments in immunoglobulin heavy and light chains could generate about 1.5×10^6 different antigen-binding sites. By contrast, the random losses and gains of nucleotides that occur when the gene segments are joined increases diversity up to 10^8-fold.

24–4 Trees, like all living organisms, maintain an active defense system against invaders, a form of innate immunity that allows them to grow and thrive in soil that contains the very microbes that will cause their decay when they die. Plants have a variety of Toll-like receptors that function as pattern recognition receptors in innate immune responses against various pathogens. Plants also secrete defensins that disrupt the membranes of many pathogens. Bruce Beutler, in his Nobel lecture, recalled a conversation with his father.

"I remembered a walk my father and I took through a grove of redwoods in Sequoia National Park. I was perhaps 10 or 12 years of age. 'Why is it that trees don't simply rot?' I asked him, aware that plants had none of the lymphoid or myeloid cells that confer immunity to vertebrates. He explained there were tannins and perhaps other molecules in trees that made them resistant to decay. 'But they rot after they die, and the tannins are still there,' I countered. The discussion went on, venturing into infections of live plants such as potatoes and wheat, and I tentatively concluded that plants must have some form of immunity that was actively maintained in the sense that it depended on their vitality. But at least for the two of us, not much seemed to be known about it."

Reference: Bruce A. Beutler. Nobel Lecture, Physiology or Medicine, 2011.

24–5 In each proteolytic cleavage that generates two active products, the small, freely diffusing product serves as an attractant for neutrophils, which engulf the complement-coated pathogen. The larger product, which can carry out the next cleavage in the sequence, remains bound to the pathogen surface where the reaction began. Thus, the late components of the membrane attack complexes remain attached to the membrane where the reaction began. In both cases, the activated molecules typically have a short life span, ensuring that the attack does not spread to host cells.

24–6 In the first round of replication, the G:U mismatch would be converted to a G-C (the normal base pair) in one daughter duplex and to an abnormal A-U base pair in the other daughter duplex. In the next replication cycle, the A-U base pair would give rise to an A-T base pair and an A-U base pair. Ultimately, the A-U, if not repaired by other processes, would be diluted out and lost to view. In the final analyzed bacterial progeny, the original G-C would become A-T, or as stated in the problem, G→A and C→T. So the results of the bacterial experiments are consistent with the idea that the AID-induced mutations arose by deamination of C to U in DNA.

Reference: Petersen-Mahrt SK, Harris RS & Neuberger MS (2002) AID mutates *E. coli* suggesting a DNA deamination mechanism for antibody diversification. *Nature* 418, 99–103.

24–7

A. The only peptide that sensitizes the target cells for lysis by incubation with cytotoxic T cells includes residues 365–380 of the 1968 strain of influenza. The corresponding peptide from the 1934 strain does not sensitize the target cells, indicating that the two amino acid changes—DA to

ET—at positions 372 and 373 are critical. The fact that peptide 369–382 from the 1968 strain does not sensitize the target cells suggests amino acids 365–368 are also likely to be critical for sensitization. These experiments constituted a remarkably clear demonstration of the importance of peptide fragments of a protein antigen in T cell recognition.

Only this one peptide—and not others—works in this assay because the assay uses one specific clone of cytotoxic T cells, which expresses one specific T cell receptor. That receptor has one binding specificity: the identified peptide in the binding groove of one particular class I MHC molecule. If you were able to isolate all the different cytotoxic T cells that are stimulated during an influenza virus infection, you would expect to find, among the collection, cells that respond to different viral peptides in the grooves of different class I MHC proteins.

B. Transport of both class I and class II MHC proteins to the cell surface depends on their assembly with a peptide, usually derived from intracellular cytosolic proteins with class I MHC molecules and endocytosed extracellular proteins with class II MHC molecules. Thus, all the MHC molecules on the cell surface should already have a peptide in their peptide-binding grooves. It is thought that the experiment worked because some of the originally bound peptides exchanged with extracellular peptide, which was provided at high concentration, and because only a small number of specific peptide–MHC complexes are required on the surface of a target cell for a cytotoxic T cell to recognize the target and kill it.

Reference: Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D & McMichael AJ (1986) The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44:959–968.

24–8

A. T cells develop in the thymus, passing through a series of stages that lead to the different kinds of T cells. Only T cells that undergo positive selection ultimately mature and exit the thymus; the rest die. Positive selection depends on a weak interaction between the T cell receptor and the self MHC molecules bound with self peptides that are displayed on antigen-presenting cells (APCs) in the thymus. In the thymus of an d-type/k-type heterozygous mouse, the APCs would have both d-type and k-type class I MHC molecules on their surface, both of which would display self peptides, triggering maturation of cytotoxic T cells that can interact with either type of class I MHC protein, and some will interact strongly if a foreign peptide is bound to the MHC protein. Thus, cytotoxic T cells from infected heterozygous mice should be able to lyse both infected d-type and infected k-type cells.

B. Once again, since developing T cells learn to recognize peptides in association with self MHC proteins in the thymus, one would expect that the cytotoxic T cells that develop in the transplanted d-type thymus would recognize peptides only in association with class I MHC molecules of the d-type, even though the cells in the rest of the mouse express both d-type and k-type MHC types. Thus, the cytotoxic T cells would be expected to lyse infected d-type cells but not infected k-type cells.

Reference: Rolf M. Zinkernagel. Nobel Lecture, Physiology or Medicine, 1996.

24–9 From Figure Q24–3B, it appears that dendritic cells contact in the neighborhood of 100 T cells per 10 minutes, or about 600 per hour. At this rate, it would take about 100 minutes for 100 dendritic cells to scan 10^5 T cells, or 1000 minutes to scan 10^6 T cells. The authors point out that their methodology may underestimate the true rate of encounters between T cells and dendritic cells because they are unable to detect contacts with thin

dendritic processes, which are invisible using this technique. Other studies have estimated that dendritic cells may scan T cells at up to 10 times this rate, which would reduce the average time to find the specific T cells to 10 to 100 minutes. In either case, it is clear that dendritic cells in lymph nodes are capable of efficiently scanning the T cell repertoire and initiating a fairly rapid T-cell-mediated response.

References: Bousso P & Robey E (2003) Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat. Immunol.* 4, 579–585.

Miller MJ, Hejazi AS, Wei SH, Cahalan MD & Parker I (2004) T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. *Proc. Natl Acad. Sci. USA* 101, 998–1003.

24–10 Positive selection for weak-binding T cells serves an essential purpose: it identifies T cells whose receptors can bind to self MHC molecules. Since foreign antigens are presented to T cells as peptides bound to self MHC molecules, only those T cells that can recognize such complexes will be immunologically useful. Developing T cells that cannot recognize peptide–self-MHC complexes at all would be useless. The thymus gets rid of potentially dangerous T cells—ones that might trigger an autoimmune response—by inducing those T cells that bind self-peptide–MHC complexes strongly to either kill themselves or become regulatory T cells.

24–11 These results show clearly that CD4 proteins promote the T cell response to peptide–MHC complexes on an APC. In the presence of functional CD4, T cells respond to as few as one or two peptide–MHC complexes by taking up Ca^{2+}. When the antibody is bound to the CD4, preventing it from binding to the MHC molecule, T cells do not respond until there are 25–30 peptide–MHC complexes at the interface. Thus, CD4 dramatically increases the sensitivity of T cells to respond to their specific antigen on the surface of an APC.

Reference: Irvine DJ, Purbhoo MA, Krogsgaard M & Davis MM (2002) Direct observation of ligand recognition by T cells. *Nature* 419, 845–849.

Credits

Chapter 1

Figure 1–1 Data courtesy of Steve Freeland.

Chapter 2

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Chapter 3

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Chapter 4

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Chapter 5

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Chapter 6

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Chapter 8

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Answers

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Table 1 Constants

Symbol	Constant	Value
c	Speed of light in vacuum	3.0×10^{17} nm/sec
e	Charge on a proton or an electron	1.6×10^{-19} coulomb (C)
F	Faraday constant	96 kJ/V mole, 96 kC/mole
g	Earth's gravitational acceleration	9.81 m/sec^2
h	Planck's constant	6.6×10^{-37} kJ sec/photon
k	Boltzmann constant	1.38×10^{-23} J/K
N	Avogadro's number	6.02×10^{23} molecules/mole
R	The gas constant	8.3×10^{-3} kJ/K mole

Table 2 Variables

Symbol	Units	Definition
ΔE_0	V	Standard electromotive potential (T = 298 K, all concentrations at 1 M)
ΔE	V	Electromotive potential
E_0	V	Standard reduction potential (T = 298 K, all concentrations at 1 M)
E	kJ/photon	Energy of a photon at a particular wavelength
$\Delta G°$	kJ/mole	Standard free-energy change (T = 298 K, all concentrations at 1 M)
ΔG	kJ/mole	Free-energy change
K	(variable)	Ratio of the molar concentrations of products to reactants at equilibrium
λ	nm	Wavelength
M_r	no units	Relative molecular mass (mass of molecule relative to 1/12 mass of carbon atom)
n	no units	Number of electrons transferred during a redox reaction
v	sec^{-1}	Frequency
pH	no units	Negative $\log_{10}$ of molar concentration of H^+
pK	no units	The pH at which an ionizable group is half dissociated
T	K	Absolute temperature
V	V	Membrane potential
z	no units	Valence (charge) on solute

Table 3 Units

Symbol	Name	Measure of	Conversion factors
A	ampere	electrical current	1 C/sec
Å	Ångström	length	10^{-10} m, 0.1 nm
Bq	becquerel	radioactivity	1 disintegration/sec, 60 dpm
C	coulomb	electrical charge	1 A sec, 6.2 x 10^{-18} electrons, J/V
°C	centigrade degree	temperature	K – 273
Ci	curie	radioactivity	3.7 × 10^{10} Bq, 2.22 × 10^{12} dpm
cm	centimeter	length	10^{-2} m, 10^{7} nm
cpm	counts/min	radioactivity	dpm × counting efficiency[a]
d	dalton	molecular mass	1.66 × 10^{-24} g (1/12 mass of a carbon atom)
dpm	disintegrations/min	radioactivity	0.016 Bq, cpm/counting efficiency[a]
dyne	dyne	force	g cm/sec^2, 10 μN
erg	erg	energy	g cm^2/sec^2, dyne cm
g	gram	mass	6.02 × 10^{23} daltons
Gy	gray	ionizing radiation	J/kg, m^2/sec^2
J	joule	energy	1 kg m^2/sec^2, 10^{7} ergs, 0.239 cal
K	Kelvin	temperature	°C + 273
kb	kilobase	nucleotides	1000 bases or base pairs
kcal	kilocalorie	energy	4.18 kilojoules
kd	kilodalton	molecular mass	1000 d
kJ	kilojoule	energy	0.24 kilocalories
L	liter	volume	1000 mL
m	meter	length	100 cm, 10^{9} nm
M	molar	concentration	moles solute per liter of solution
μg	microgram	mass	10^{-6} g
min	minute	time	60 sec
mL	milliliter	volume	1 cm^3, 10^{-3} L
mole	mole	number	6.02 × 10^{23} molecules
mV	millivolt	electrical potential	10^{-3} volts
N	newton	force	1 kg m/sec^2, 1 J/m, 10^{5} dynes
nm	nanometer	length	10^{-9} m, 10 Å
Pa	pascal	pressure	1 N/m^2, 9.87 × 10^{-6} atmosphere (atm)
S	siemens	electrical conductance	1 A/V
sec	second	time	3600 sec/hour; 86,400 sec/day
V	volt	electrical potential	1 W/A, 1 J/C, 1000 mV
W	watt	power	1 J/sec, 1 V A

[a]See table of radioactive isotopes for efficiency of counting of specific isotopes (Table 6, page 965).

Table 4 Prefixes

Symbol	Name	Value	Symbol	Name	Value
d-	deci-	10^{-1}	da-	deca-	10^{1}
c-	centi-	10^{-2}	h-	hecto-	10^{2}
m-	milli-	10^{-3}	k-	kilo-	10^{3}
μ-	micro-	10^{-6}	M-	mega-	10^{6}
n-	nano-	10^{-9}	G-	giga-	10^{9}
p-	pico-	10^{-12}	T-	tera-	10^{12}
f-	femto-	10^{-15}	P-	peta-	10^{15}
a-	atto-	10^{-18}	E-	exa-	10^{18}
z-	zepto-	10^{-21}	Z-	zetta-	10^{21}
y-	yocto-	10^{-24}	Y-	yotta-	10^{24}

Table 5 Geometric Formulas

Figure	Area	Surface area	Volume
square	l^2		
circle	πr^2		
ellipse	$\pi r_1 r_2$		
cube		$6\,l^2$	l^3
cylinder		$2\,\pi rh + 2\,\pi r^2$	$\pi r^2 h$
sphere		$4\,\pi r^2$	$4/3\,\pi r^3$
cone			$1/3\,\pi r^2 h$

Table 6 Radioactive Isotopes

Isotope	Emission	Half-life	Counting efficiency[a]	Maximum specific activity[b]
^{14}C	beta	5730 years	96%	0.062 Ci/mmol
^{3}H	beta	12.3 years	65%	29 Ci/mmol
^{35}S	beta	87.4 days	97%	1490 Ci/mmol
^{125}I	gamma, Auger, and conversion electrons	60.3 days	78%	2400 Ci/mmol
^{32}P	beta	14.3 days	100%	9120 Ci/mmol
^{131}I	beta and gamma	8.04 days	100%	16,100 Ci/mmol

[a] Maximum efficiency for an unquenched sample in a liquid scintillation counter. Most real samples are quenched to some extent.
[b] This value assumes one atom of radioisotope per molecule. If there are two radioactive atoms per molecule, the specific activity will be twice as great, and so on.

Table 7 The Genetic Code†

1st Position (5′ end)	2nd Position				3rd Position (3′ end)
↓	T	C	A	G	↓
T	F	S	Y	C	**T**
	F	S	Y	C	**C**
	L	S	*	*	**A**
	L	S	*	W	**G**
C	L	P	H	R	**T**
	L	P	H	R	**C**
	L	P	Q	R	**A**
	L	P	Q	R	**G**
A	I	T	N	S	**T**
	I	T	N	S	**C**
	I	T	K	R	**A**
	M	T	K	R	**G**
G	V	A	D	G	**T**
	V	A	D	G	**C**
	V	A	E	G	**A**
	V	A	E	G	**G**

Table 8

Amino acids		Codons†			
A	Alanine	GCT	GCC	GCA	GCG
C	Cysteine	TGT	TGC		
D	Aspartic acid	GAT	GAC		
E	Glutamic acid	GAA	GAG		
F	Phenylalanine	TTT	TTC		
G	Glycine	GGT	GGC	GGA	GGG
H	Histidine	CAT	CAC		
I	Isoleucine	ATT	ATC	ATA	
K	Lysine	AAA	AAG		
L	Leucine	CTT	CTC	CTA	CTG
		TTA	TTG		
M	Methionine	ATG			
N	Asparagine	AAT	AAC		
P	Proline	CCT	CCC	CCA	CCG
Q	Glutamine	CAA	CAG		
R	Arginine	CGT	CGC	CGA	CGG
		AGA	AGG		
S	Serine	TCT	TCC	TCA	TCG
		AGT	AGC		
T	Threonine	ACT	ACC	ACA	ACG
V	Valine	GTT	GTC	GTA	GTG
W	Tryptophan	TGG			
Y	Tyrosine	TAT	TAC		
*	STOP	TAA	TAG	TGA	

† Codons are shown as DNA instead of RNA (Ts in place of Us) to facilitate the conversion of DNA sequences into protein sequences.