Antisense and Ribozyme Methodology

Edited by

Ian Gibson

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Laboratory Companion

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Preface

The discovery of new drugs is a difficult, expensive, and long term activity, and often ends in tears after clinical failures. Approximately 80 % of applied research programs in this area fail at various technical hurdles. A rational drug discovery program involves hypothesis, identifying molecular targets involved in the physiology of the disease, and developing a *selective* agent to interact with this target. Eventually, despite success *in vitro*, clinical trials must ensue. At this point considerations of selectivity, safety, and pharmacokinetics are important. Further development requires animal cell models, and here relevance to the human condition is of the essence. All of these considerations are prevalent now in the field of antisense and ribozyme technology. The inhibition of restenosis in rats and pigs, and of leukemia cell proliferation and tumor growth in mice have been recorded. Further tests against the human immunodeficiency virus-1, the cytomegalovirus (CMV), and the human papilloma virus are now undergoing clinical trials. As the molecular and cellular biology of these biological systems are investigated, new therapeutic targets emerge. This monograph attempts to underline the approach to the targeting of nucleic acid informational systems via antisense DNA molecules and ribozymes (RNA). Key stages in disease processes may then be inhibited. Specific nucleic acid sequences may be targeted, and this should enable the normal sequence to escape the effect of the antisense DNA or ribozyme.

The work in this field has, however, thrown up discrepancies in that non-antisense mechanisms gave biological effects on, for example, cellular proliferation. This has led on the one hand to a wide skepticism of the positive claims with antisense or ribozymes, especially when effects could not be repeated, or controls gave similar effects. A careful analysis of conditions of the experiments, the use of a wide range of controls and, most importantly, attempts to show specific effects on target m-RNA and protein levels have been highlighted as essential stages in any analysis. The latter may be particularly difficult due to long turnover times of the key protein. As secondary structures for RNA become more predictable from current crystallization studies, then the discrepancies may yet find an explanation.

The field is certainly evolving at a fast rate, and groups are vying with each other to increase the specificity of targeting, the stability of the molecules, and maximum uptake into cells. This monograph

appears at an early stage in the testing of such molecules, and is written with the aim of giving the research worker a focal point from where they can start to carry out experiments. It does not pretend to have the 'answer' to the problems, but with the help of active practitioners in the field takes a view on 'the best practice'.

Chapter 2 is the work of Dr David Tidd of the Department of Biochemistry, University of Liverpool who is a leading expert on chimeric antisense molecules. Chapter 3 is the work of Dr. George Sczakiel who is an expert on ribozyme construction and development. Chapter 4 is the work of a PhD student at the University of East Anglia in Norwich, Ciara Twomey, and myself, who are both involved in the development of delivery systems of ribozymes into cells.

An active research field such as this is almost certain to render some of these developments redundant in a year or so. However, if this monograph serves to excite and enthuse others to enter the field, then it will have worked.

Norwich, 1997 Ian Gibson

A note on the layout of this book:

In order to facilitate the use of this book as a methodological source for your bench work, a wide page format has been chosen.
Due to the type of durable binding used, the book has the advantage of lying flat on your bench top for convenient use. In addition, a wide margin leaves room for your own notes and provides some key notes and pictograms to assist you in finding the relevant information:

a pipette symbol marks the start of a step by step protocol section, a grey bar runs down the margin of the whole protocol section

this symbol draws your attention to potential hazards and safety suggestions

comments on the key steps in methodology are highlighted by a key symbol

a "good idea" symbol marks useful hints for optimization of methodology

this pictogram indicates discussions of alternative approaches

the tool indicates troubleshooting guides that should help you in finding out what could or did go wrong and in solving and avoiding problems

suggestions for monitoring quality and reliability of the experimental procedure are highlighted by the magnifying glass

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CHAPTER 1

Antisense and Ribozyme Methodology

1.1 The Potential

Antisense and ribozyme methodologies are being considered in the context of the development of new medical therapies, where key events in disease initiation or progression involve nucleic acid sequences (Milligan *et al.*, 1993; Wagner, 1994). The base-pairing rules leading to the concept of complementarity between nucleotide sequences features prominently in key processes in living cells. DNA synthesis, RNA synthesis, the interaction between tRNA molecules and messenger RNA sequences, and the dramatic catalytic intra-strand cleavage of RNA indicate the importance of base pairing interactions, which ensure accurate information transfer in key biological processes. These mechanisms often lead to wider biological considerations. For example, the importance of RNA in catalytic events has led to speculation about the origin of life forms based on the early evolution of RNA sequences.

principles of antisense methodology

It would appear logical, then, to attempt to interrupt normal informational flow by, for example, inducing base pairing between mRNA and a complementary DNA or RNA sequence. This might be predicted to lead to the blockage of mRNA or viral RNA function (Zamecnik and Stephenson, 1978; Goodchild, 1989) (Fig. 1). Another possibility might be to form a triplex structure involving a double-stranded DNA and a third strand which binds in the major groove of the duplex (Fig. 2). This again would be predicted to block gene expression in cells by blocking transcription, or preventing the interaction of regulatory proteins with specific DNA sequences. If such interactions could be made specific, inducing permanent inactivity of one gene and not another, even to the extent of discriminating between single base differences, then the potential would be enormous. Sequence recognition of DNA by triplex-forming oligonucleotides is limited to interactions between oligopyrimidines or oligopurines and homopurines in the DNA target.

naturally occurring antisense RNA

Naturally occurring *antisense RNAs* complementary to a mRNA can be transcribed from the opposite strand of the same template DNA, utilizing another promoter. This has been shown to occur with natural transcripts in both eukaryotes and prokaryotes (Nepveu and Marcu, 1986; Adelman *et al.*, 1987; Kindy *et al.*, 1987; Simons, 1991).

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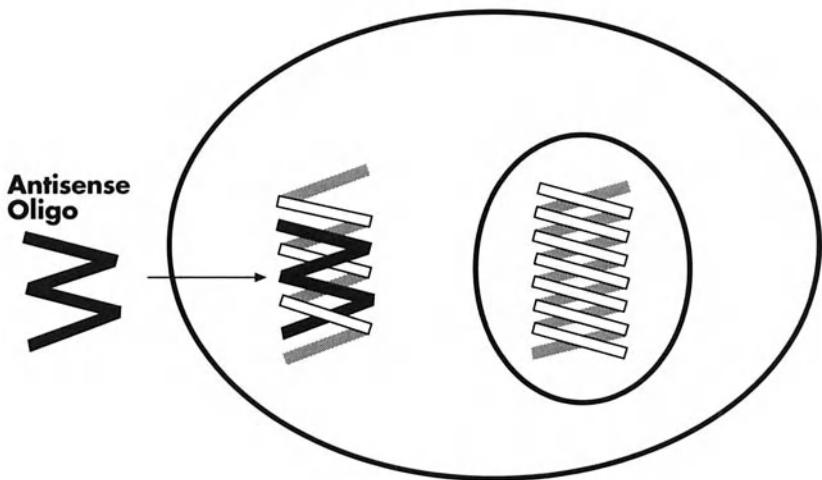

Fig. 1 Antisense oligodeoxynucleotide binds to complementary sequence in target m-RNA preventing translation. Taken from *Oligodeoxynucleotides: Antisense inhibitors of Gene Expression* (Cohen J, ed) Macmillan Press, 1989

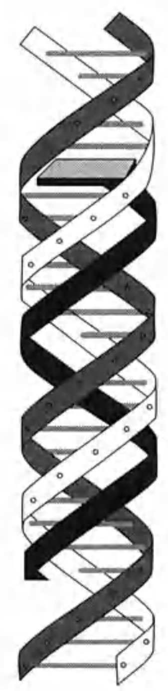

Fig. 2 Triple helix with third strand binding to DNA in major groove

RNA:RNA complexes, for example, could interfere with the processing of primary transcripts. Such complexes have been shown to be unwound by a specific nuclease, and modified (Bass and Weintraub, 1988; Wagner *et al.*, 1989). This mechanism would clearly affect the transport of messages to the cytoplasm by interfering with translation or accelerating the degradation of the mRNA. In prokaryotes, RNA:RNA interactions have been documented in plasmids, where they are involved in control of replication incompatibility and conjugation in the transposon IS10; they are involved in the control of transcription in phage development, and in bacteria they are a mechanism for controlling chromosomal gene expression (Inouye, 1988).

discrimination of single base differences

Potentially, this methodology should allow discrimination between nucleic acid sequences differing by one base, and such claims have been made (Chang *et al.*, 1991; Saison-Behmoaras *et al.*, 1991). The wild-type protein would remain unaffected, but cells containing the aberrant protein product could be targeted via the mRNA sequence. This could be achieved with a small piece of synthesized DNA some 15–16 bases long, since there ought to be no overlap within a genome with an exactly similar sequence of base pairs in any other part of the genome. Such discrimination, of course, depends on the cellular conditions prevailing in the binding reaction of the two nucleotide sequences, and on the biochemical machinery necessary for the resultant inactivation of the mRNA.

Another method to target the relevant mRNA involves the utilization of plasmids or genetic constructs which are capable of producing RNA molecules complementary to mRNA sequences. Attempts to improve on the production of such antisense RNA and to effect entry of the constructs into cells bringing about successful transfection are being widely researched in the field of gene therapy (Bielinska *et al.*, 1996).

persistence of phenotype

In both these approaches, however, the pre-existing level of mRNA and/or protein may mean that the phenotype persists for some time even in the presence of the complementary antisense sequence. By adjusting levels of antisense RNA or DNA, it is also possible to tackle this problem. Recently, the use of modified oligodeoxynucleotides containing C-5 propynylpyrimidine-2′-deoxyphosphorothioate has shown that they are effective with high or low expression levels of RNA (Flanagan *et al.*, 1996). There are, however, other factors to be considered in taking a strategic approach to the use of this technology. For example, is it necessary to know how the antisense achieves its effect, or is it enough just to have an effect? This monograph is an attempt to introduce the laboratory workers to the problems, and to help them circumvent some of those which have arisen in the application of antisense and ribozyme technology.

1.2 Antisense Technology

1.2.1 Problems

difficulties of access into cells

Whilst interesting and exciting results have been achieved with antisense technology utilizing cell cultures or organisms (Stein and Chang, 1993), there remain some formidable hurdles. First we will discuss problems with the addition of short DNA oligomers. The entry of oligodeoxynucleotides into cells is poor, and has required research into improving this aspect of their development. The easy penetration of the cell membrane in tissue culture or in the whole organism has yet to be achieved. Several processes have been shown to be involved, including receptor-mediated endocytosis, pinocytosis, etc. Once through the cell membrane, it is crucial to ensure that the antisense molecule reaches the target molecule. This will become a greater problem when attempting treatment of whole organisms. The methods of delivery of antisense molecules are being avidly researched, but as yet no one method has emerged as the leader (see below).

1.2.2 Resistance to Nucleases

Another problem yet to be overcome is the stability of the antisense molecule both in tissue culture and in the whole organism, where active nucleases may rapidly destroy the molecule. These problems have been addressed with limited success by various approaches.

chemical modification to improve stability

The DNA molecule has been and can be chemically modified to tackle this problem of stability, and also the problem of entry into the cell (Bielinska *et al.*, 1990; Fisher *et al.*, 1993). Oligonucleotides with a phosphodiester backbone are sensitive to both exonucleases and endonucleases, and have short half lives in cells or organisms. By substituting linkages in the backbone with a thioate group, for example, the stability is considerably improved. Stability can also be improved by the addition of methylphosphonate groupings, or even by forming chimeras utilizing two different substitutions at different linkage points in the backbone, e.g., methylphosphonate-phosphodiester chimeras (Fig. 3). However, in terms of entry into the cell other modifications to the antisense molecule are essential.

peptide nucleic acids

A recent innovation in this research field involves the use of peptide nucleic acid (PNA) oligomers to form sequence-specific stable complexes with complementary duplex DNA. In PNA, an oligonucleotide mimic of the regular DNA nucleobases is attached via a linker to an *N*-(2-aminoethyl) glycine backbone. They bind as homopyrimidine PNAs to duplex DNA, and form a stable P loop structure in a PNA_2-DNA triplex with a displaced DNA strand. The stability is conferred both by Hoogsteen pairing and by hydrogen bonding between the peptide NH group on the PNA. The latter forms a Hoogsteen pair with the oxygen on the phosphate group of the oligonucleotide. The resultant triplex inhibits restriction endonuclease action on duplex

	X	Y	Z
Phosphodiester	O	A,G,C or T	H
Methylphosphonate	CH_2	A,G,C or T	H

Fig. 3 Modifications of oligodeoxynucleotides

DNA, and inhibits RNA polymerase mediated transcription activity *in vitro* (Larsen and Nielsen, 1996). The C5 propyne oligodeoxynucleotides mentioned above demonstrate good stability and enhanced affinity for mRNA.

1.2.3 Entry into Cells

Poor uptake into cells of all these compounds occurs both in primary cultures and in standard cell lines. By modifying phosphorothioated and phosphodiester oligomers with 3′ or 5′ cholesterol groups, better uptake has been achieved. On the other hand, this did not increase the oligomers' ability to form nucleotide sequence homologies. Both phosphodiester and phosphorothioated structures show sequence-dependent undesired side reactions. Chimeric antisense molecules show reasonable uptake properties, however, and also maintain specificity, i.e., the ability to discriminate a single base difference. Uptake can also be improved with liposomes or immunoliposome vectors which carry the antisense DNA sequences (Gareis *et al.*, 1991; Wagner *et al.*, 1993). Methods are also now used to deliver DNA via specific cell surface receptors, e.g., those for folic acid, utilizing vari-

ous vectors (Ch'ng *et al.*, 1989). Other receptors have included those for transferrin and macroglobulin or asialoglycoprotein.

Delivery of the oligomer to the required site of activity, following uptake into cells, would appear to be a difficult problem to overcome. This problem will need to be solved before antisense molecules become beneficial therapeutic agents. The use of fluorescent tags on the oligomers has been used as an indicator of cell localization. DNA fragments are often seen within cytoplasmic vesicles or endosomes, and may escape from these into the cytoplasm. This does not seem, however, to occur to any great extent. Transient permeabilization of the plasma membrane with, e.g., streptolysin delivers the oligomers and brings about specific antisense effects, suggesting release from the endosome or circumvention of these bodies (Giles *et al.*, 1995a). There is also evidence for oligomer presence in the nucleus, particularly following the microinjection of antisense DNA molecules. Therapeutic use of the molecules will certainly require the penetration of the cellular membrane barrier and accurate targeting to the substrate.

use of fluorescent tags

1.2.4 How Antisense Works

There is a preference for modified oligodeoxynucleotides which function via the RNase H enzyme, which destroys the DNA:RNA complex formed following the addition of antisense DNA. Another mechanism which, it is claimed, operates in the cell is that where the ribosome cannot function on the mRNA strand because the antisense DNA molecule physically restricts its movement. The chimeras and phosphodiester compounds utilize the RNase H mechanism, but the thioated compounds seem to function via different mechanisms, including association with proteins (Khaled *et al.*, 1996). It is, of course, not essential to know the mechanism provided the effect on the phenotype or gene expression is achieved. Nevertheless, there could be serious side-effects on the expression of other genes if there is a lack of specificity.

action of RNase H

1.2.5 Success

Despite these apparent difficulties, antisense technology (i.e., the use of small DNA molecules to target genes in plant, bacterial, or animal cells, and to modify or inhibit gene expression) has achieved widespread application and interest. Its application in clinical trials is now underway in treating leukemias or HIV viral replication.

Finally, the delivery of genetic constructs which synthesize the antisense RNA molecules have also yet to achieve high levels of success. For example, recombinant adeno-associated viral vectors, in association with plasmids and replication-competent adenoviruses or adenovirus polylysine-DNA complexes, achieve good transfection or delivery to cells, but the concern remains that they might enter other undesirable cells and produce their antisense RNA product, with resultant phenotypic effects.

Viral transduction and, in particular, retroviral transduction may carry a permanently integrated copy of foreign DNA into the transfected cell. This is a popular choice used in gene therapy trials but again is less than 100 percent effective. Where safer methods of plasmid introduction are used, there is often poor expression of the introduced genes, and certainly poor integration.

All these considerations illustrate that before we can be sure of clinical efficiency there are several important biological barriers to conquer.

1.3 Ribozymes

1.3.1 What Are They?

definition of ribozymes

Associated with antisense DNA approaches to therapy, others have preferred to utilize small RNA molecules which cleave other RNA molecules, and mRNA sequences in particular. These apparently catalytic RNA molecules (ribozymes) occur naturally. There are three major classes of ribozymes:

(i) Group I introns, which occur in the gene for nuclear 26 s rRNA in *Tetrahymena thermophila*

(ii) Group II introns, which occur in organellar genes of lower eukaryotes and plants

(iii) Small plant pathogenic RNA molecules, occurring in satellite RNAs and virusoids, which play some role in viral replication

hammerhead ribozymes

This ability to cleave RNA sequences has resulted in the design of 'hammerhead' ribozymes. The hammerhead structure consists of three base-paired stems with a consensus sequence, resembling a hammer. Cleavage of the phosphodiester bond occurs at a unique site and generates 2′, 3′-cyclic phosphate and 5′-hydroxyl termini. The structure in three dimensions, however, resembles a wishbone. The three stems diverge at an acute angle and the third points in the opposite direction. The core is held together by base-pairing interactions. There is intense activity at this time in the unearthing of RNA structures and the various conformations and chemical links making up their organization.

Ribozymes differ from antisense DNA molecules, and cleave their target following binding. With both antisense DNA and ribozymes, the recycling of the molecules could be important for further action on fresh substrates.

1.3.2 Problems

The problems with ribozymes mirror those with antisense molecules. Entry into cells, targeting of sequences, stability, and delivery to the appropriate compartment in the cell where the substrate is to be found have been addressed to maximize success with ribozymes. They can be introduced to cells by endogenous delivery, and occasionally by exogenous delivery. Delivery problems, as in the case of antisense DNA using exogenous methods, have been circumvented with liposomes, microinjection, or electroporation. Specificity of action, however, where there is a need to discriminate between molecules reflecting a single base difference, has been claimed with ribozymes (Funato *et al.*, 1994).

difficulties of access into cells

1.3.3 Stable Ribozymes

improving stability

The stability of ribozymes has been improved following modifications with 2′-fluor or 2′-amino groups, and also by utilizing DNA-RNA chimeras. Ribozymes complexed to lipofectin (Karikó *et al.*, 1994) or DOTAP (Heidenrich *et al.*, 1996) may be protected against nuclease digestion by a variety of adaptations, e.g., chemical modification, or the absence of nucleases in the endosomal components which contain the ribozymes.

The endogenous expression of ribozymes has been boosted by inserting ribozymes into the untranslated regions of genes with strong promoters transcribed by RNA polymerase II, e.g., SV40 or retroviral long terminal repeats. Another approach has involved inserting the ribozyme into the anti-codon loop of tRNA, which is transcribed by RNA polymerase III. These constructs result in the production of high levels of ribozyme transcripts. This is necessary for *in vivo* activity, where the ratio of ribozyme:substrate needs to be large to be effective. Other requirements involves the maintenance of catalytic activity and the accessibility of the target. A further strategy has involved the production of ribozyme transcription units which localize to splicing or translational complexes, and which are involved with the processing and transportation of the targeted RNA substrates.

1.3.4 Designing Ribozymes

The construction of the ribozyme and the site for attack in the substrate have also raised problems. The length of the hybrid arms is critical, and here we need these to be long enough to maintain specificity, but short enough to allow dissociation so that the ribozyme can function again. If they are too long, they may create mismatched complexes. Also, the target of the ribozyme requires effective dissociation following cleavage. Careful planning is needed to meet the requirement of effective cleavage and catalytic activity. Antisense

effects are always possible when the two molecules, ribozyme and substrate, interact, and the identification of cleavage products and the use of mutant ribozymes becomes necessary to distinguish between these possibilities. The design of ribozymes that attack multiple sites in the target has also been shown to lead to effective biological effects, but cross reactions with other genes or antisense effects have not always been investigated. Recently, Heidenreich *et al.* (1996) have shown that ribozymes can function via cleavage in isolated cell nuclei. Chemical stabilization of the ribozyme to nuclease digestion can lead to increased catalytic activity. DNA-RNA ribozymes can also, like antisense DNA molecules, cause target RNA degradation via RNase H activity.

The number of success stories of ribozymes eliminating gene expression or effects on viral replication or cell physiology are increasing. This makes it a valuable tool, despite the paucity of our knowledge in most of these cases of the mechanism of action of the ribozyme, and whether cross-reactions with other gene products occur. The addition of the ribozymes to whole plant or animal systems is currently in progress, and in particular hairpin ribozymes are being developed against HIV sequences and leukemic cells *in vitro*, followed by transfer back to the patients.

repair of mutant DNA

Finally, another property of the ribozyme suggests that this technology may have great potential in the field of gene therapy. The ability of trans-splicing ribozymes to repair mutant RNAs means it is possible to 'edit' genetic information in mRNAs synthesized in mammalian cells. One advantage of this repair mechanism is that the 'new' gene sequence remains within its natural host environment, ensuring that gene regulatory mechanisms are intact (Jones *et al.*, 1996). This approach has heralded a technique which may yet make way for the development of safe therapeutic ribozymes in the treatment of a variety of inherited genetic diseases.

1.4 Ribozymes or Antisense DNAs?

recognition of mismatches

Each of these has its proponents, who argue success with one or other in modifying gene activity. Each has had successes in distinguishing between matched and mismatched substrates. An antisense DNA molecule distinguishes a mismatched target through reduced binding. A ribozyme has two opportunities to distinguish. Firstly, it shows reduced affinity to the mismatch, and secondly, the mismatch complex cleaves more slowly than the matched sequences. Since the target sequence is known, it is possible to design against the mismatch situation. Whilst this remains to be tested in a biological system, it does occur *in vitro*, where a mismatch occurring within 1 or 2 bases of the cleavage site can be distinguished. In biological situations, it is necessary to show that cleavage does occur, however, and that ribozyme effects are not purely antisense. Intuitively, ribozymes should behave like enzymes and be available for further activity, although

again a stable antisense DNA might also be capable of several reactions. Whether both or either succeed as therapeutic agents will depend on the results of current investigations into uptake, stability, and targeting of substrates in the cell and organism, and a straightforward competition between the two in the same biological situation.

choice of sequence

Another feature which is limiting in both cases is the choice of sequence. Ribozymes are restricted to key NUX sites (see section 3.5) in a known sequence for their cleavage activity, but antisense DNA has been targeted to open loops in the DNA, which is based on computational considerations of free energy, etc. (Giles *et al.*, 1995b). Even with ribozymes, however, the NUX sequence may be associated with a particular secondary structure. The oligomer *itself* must be chosen so that *its* secondary structure is limited. Antisense DNA molecules have been targeted to cap sites, translation initiation sites, intron-exon splice junctions and sequences, and the 3′ untranslated ends. Again, success with all these approaches has been claimed, and it is not possible to pick the champion at this stage, although most would favor the translation initiation site.

1.5 The Choice Today!!

I intend here to take a bold approach, and to illustrate the best practice for the use of antisense or ribozymes to enable the researcher to apply the technology. This does not mean that alternatives are not available and equally effective. I have selected the chimeric antisense DNA molecule as the example. The approach with these molecules tackles the question of effective RNase H activity and the best uptake into cells. The use of phosphorothioated oligodeoxynucleotides, whilst more popular, has been shown to involve non-sequence-specific mechanisms, e.g., by an affinity for proteins like protein kinase C or fibroblast growth factor. Other effects of these compounds on, for example, cell proliferation and immune activation, may involve sequence-specific mechanisms if they combine GC-CG dinucleotides or a run of 4 G's.

In the case of ribozymes, I have selected the particular approach of one group of workers which has been shown to be effective, and combines in its approach questions of stability and target availability as in the case with antisense DNA.

Finally, whilst there is no one delivery method which is yet the standard, I have selected several methods to introduce antisense RNA or ribozyme sequences into cells. The entry of antisense DNA (or ribozymes) via liposomes is also described, as are methods involving vectors. Since this monograph attempts to set workers up with the techniques which are currently in use but still in the progress of development, I believe a selective approach is justified. Each active research group has its favorite method of delivery, synthesis and choice of biological systems.

A final concern in the approaches involves the continuous choice of controls to demonstrate that there is inhibition of gene activity. The aim is always to record specific effects by known mechanisms rather than non-specific biological effects. Wagner (1994) has illustrated the basic rules from our current knowledge in antisense experiments.

controls

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Antisense and Ribozyme Methodology
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CHAPTER 2

Design and Synthesis of Antisense DNA Molecules

2.1 Introduction

sensitivity to RNase H

It is generally considered desirable to design antisense oligonucleotide structures which retain the capacity to direct ribonuclease H (RNase H), the ubiquitous enzyme activity which catalyses the hydrolysis of the RNA strand in RNA:DNA heteroduplexes (Giles *et al.*, 1995*a*,*b*; Toulmé and Tidd, 1997). In this way, inactivation of the target mRNA is irreversible, and a single antisense oligonucleotide molecule may act in a catalytic fashion in mediating the destruction of several mRNA molecules by the enzyme, through repetitive hybridization to form substrate complexes and dissociation from cleaved product complexes. However, there may be a problem of specificity in the application of this strategy, in that an antisense base sequence of sufficient length to specify a unique species of mRNA (usually 15 or more bases) could have the potential, under constant physiological conditions, to form imperfectly matched complexes with regions of partial complementarity in non-targeted mRNAs. These could be of adequate stability and lifetime to result in cleavage of the mRNAs by RNase H (Giles and Tidd, 1992*a*; Giles *et al.*, 1993). Therefore, what is required is the ability to control the stringency of hybridization of the antisense oligonucleotide under physiological conditions, so that significant hybrid formation and cleavage by RNase H will only occur at the fully complementary site within the targeted mRNA. This may be achieved through incorporation of an appropriate number of helix-destabilizing structural modifications within the antisense molecule which, in combination with the disruptive effects of base mismatches, will prevent hybridization at sites of partial complementarity in non-targeted mRNAs, but which will have minimal effect at the target.

specificity

helix-destabilizing modifications

Helix-destabilizing structural modifications are best incorporated starting at both termini of the oligonucleotide, where they may have the additional effect of protecting the molecule against exonuclease attack (Tidd and Warenius, 1989). A number of oligonucleotide analogue structures are suitable for this purpose, not all of which support RNase H activity themselves. Therefore, it is the remaining central section of the molecule which is responsible for generating RNase H-mediated antisense effects against the target mRNA. In this section, we have concentrated on the nuclease-resistant, RNase H-inactive,

non-ionic methylphosphonate modification developed by Miller and T'so (1987), where a direct phosphorus-to-carbon linked methyl group replaces the acidic hydroxyl of the internucleoside linkage (Fig. 3).

Substantial reductions in melting temperature (T_m) for half dissociation of hybrids formed between antisense oligodeoxynucleotides and complementary RNA molecules were observed with progressive inward replacement of phosphodiester linkages in the former by methylphosphonate groups (Tidd, 1990; Giles and Tidd, 1992b). Methylphosphonodiester-phosphodiester chimeric antisense oligodeoxynucleotides elicited enhanced initial rates of cleavage of target RNA molecules by RNase H, relative to the parent all-phosphodiester oligomers, in physiological cell-free systems, probably through the accelerated dissociation of less stable enzyme-product complexes (Giles and Tidd, 1992b). In addition, through increased stringency of hybridization, these chimeric molecules achieved greater specificity for cleavage at the target site relative to non-targeted sites of partial complementarity, compared with corresponding unsubstituted antisense oligodeoxynucleotides (Giles and Tidd, 1992a; Giles *et al.*, 1993). Single base discrimination for RNase H-mediated destruction of target mRNA was observed in living human leukemia cells loaded with methylphosphonodiester-phosphodiester chimeric antisense oligodeoxynucleotides by reversible plasma membrane permeabilization with streptolysin O (Giles *et al.*, 1995c). This level of specificity was not attained with corresponding all-phosphodiester antisense oligodeoxynucleotides.

improved specificity

Only a limited number of oligodeoxynucleotide analogue structures retain the ability to direct RNase H, and amongst these the most important is the relatively nuclease-resistant phosphorothioate modification, in which a non-bridging oxygen atom of the internucleoside linkage is replaced by sulfur. Thus phosphorothioates may also be utilized as the central, RNase H-active section of methylphosphonate chimeric antisense oligodeoxynucleotides, to give molecules with even greater resistance to degradation by endonucleases (Giles *et al.*, 1995b). However, the phosphorothioate modification is itself helix-destabilizing, and the combined effects of methylphosphonate and phosphorothioate on overall hybridization potential may preclude the use of such chimeric antisense oligodeoxynucleotides in certain applications (Giles *et al.*, 1995b). For these reasons, then, we have chosen the methylphosphonate-phosphodiester chimeras as best practice for antisense DNA experiments involving addition from the media.

phosphorothioates

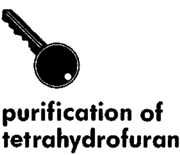
2.2 Synthesis of Methylphosphonodiester-Phosphodiester Chimeric Oligodeoxynucleotides

2.2.1 Materials and Chemicals

Methylphosphonate oligodeoxynucleotide analogues are synthesized in analogous fashion to normal phosphodiester oligodeoxynucleotides on automatic DNA synthesizers, except that 5′-O-dimethoxytrityl-2′-deoxynucleoside-3′-O-methylphosphonamidite synthons (available commercially from Glen Research Corporation, Sterling, VA, USA; UK supplier Cambio Ltd., Cambridge) replace the 5′-O-dimethoxytrityl-2′-deoxynucleoside-3′-O-(β-cyanoethyl) phosphoramidites. Methylphosphonamidites are less reactive than phosphoramidites, and coupling times need to be extended to 1 min (a reaction time of 5 min is recommended by the manufacturer). Apart from that, the same cycle and reagents as used for phosphodiester oligodeoxynucleotide synthesis may be employed. Hogrefe *et al.* (1993a) have recommended further modifications to the synthesis cycle and reagents for efficient synthesis of methylphosphonate oligodeoxynucleotides on a large scale. However, in our hands, for syntheses on the scale of at least 1–10 μmole, such modifications did not result in any improvement in final yield of pure product with an Applied Biosystems Model 381A Synthesizer using the slower cycle, Version 1.23 software, 1 μmole cycle and the "Improved 10-micromole synthesis cycle Model 381" (ABI User Bulletin No. 15, August 12, 1988).

2.2.2 Solutions

Methylphosphonamidites are made up in solution at a concentration of 0.1 M. Thymidine and 6-*N*-benzoyl-protected deoxyadenosine methylphosphonamidites are dissolved in the regular anhydrous acetonitrile supplied for oligodeoxynucleotide synthesis. However, 2-*N*-isobutyryl-protected deoxyguanosine and 4-*N*-benzoyl-protected deoxycytidine methylphosphonamidites are insoluble and liable to precipitate in this solvent, and the manufacturer recommends that these be dissolved in anhydrous tetrahydrofuran. In our experience, coupling efficiencies for these two methylphosphonamidites were sub-optimal even when dissolved in the most anhydrous commercially available tetrahydrofuran, suggesting that further purification of the solvent was required. On the other hand, the deoxyguanosine methylphosphonamidite gave good coupling efficiencies when dissolved in a 1:1 v/v mixture of anhydrous acetonitrile and analytical grade dichloromethane dried over 4A molecular sieve. Deoxycytidine methylphosphonamidite is also available in the more rapidly deprotectable 4-*N*-isobutyryl form, which is also less susceptible to trans-



purification of tetrahydrofuran

amination reactions with ethylenediamine during deprotection (see below). The compound is readily soluble in anhydrous acetonitrile and gives good coupling efficiencies in this solvent.

2.2.3 Maximizing Product Purity

A batch of several different methylphosphonodiester-phosphodiester chimeric oligodeoxynucleotides is synthesized by first installing the methylphosphonamidite solutions on the synthesizer, and running the syntheses of the 3'-methylphosphonate end sections of each consecutively, before replacing the methylphosphonamidites with phosphoramidite solutions, and continuing the syntheses of the central phosphodiester sections, and so on. It is essential that the final trityl group is left on the oligodeoxynucleotide, to aid in purification of the full length product. Coupling efficiencies of methylphosphonamidites are significantly lower than those of phosphoramidites, and failure sequences can be quite abundant in the crude deprotection reaction mixture.

2.2.4 Deprotection of Chimeric Oligodeoxynucleotides

Unlike the normal phosphodiester linkage, the methylphosphonodiester internucleoside linkage is highly susceptible to base-catalyzed hydrolysis, and the usual conditions for deprotecting phosphodiester and phosphorothioate oligodeoxynucleotides with concentrated ammonia at 55 °C for 16 h cannot be employed. The deprotection procedure for methylphosphonate oligodeoxynucleotide analogues recommended by Agrawal and Goodchild (1987) has consistently given the best results with methylphosphonate-containing chimeric oligodeoxynucleotides.

1. The controlled pore glass support from a 1 μmole synthesis is placed in 3 ml concentrated ammonia solution in a sealed screw cap vial and left at room temperature for 2 h, to cleave the oligodeoxynucleotide from the support, and to remove the protecting group from cytosine residues and thereby prevent formation of 4-*N*-ethylamino adducts by transamination during full deprotection with ethylenediamine (Hogrefe *et al.*, 1993*b*).
2. The supernatant is transferred to a 50 ml round bottomed flask, and 0.1 ml pyridine added to prevent detritylation during removal of ammonia. The ammonia is blown off in a stream of air or nitrogen while warming the flask in a waterbath at 40 °C, prior to evaporation to dryness on a rotary evaporator with a high vacuum oil pump and liquid nitrogen trap.

3. The residue is suspended in 5 ml ethylenediamine-absolute ethanol 1:1 (v/v), the flask sealed, and left at room temperature for 16 h. The extended incubation time required for full deprotection, relative to that recommended for all-methylphosphonate oligodeoxynucleotides, may reflect a slower rate of deprotection of the phosphodiester oligodeoxynucleotide section in the chimeric molecules under these conditions.
4. The solvent is removed on a rotary evaporator and the residue dissolved in 10 ml 0.1 % pyridine-0.1 M triethylammonium acetate pH 8.0, 1:1 v/v (Py/TEAA).

It is recommended that the triethylammonium acetate used for purification and HPLC analysis of oligodeoxynucleotides is prepared from triethylamine that has been refluxed for 4 h with potassium hydroxide pellets and redistilled. The presence of pyridine has been shown to prevent the slow detritylation of 5′-dimethoxytrityl-oligodeoxynucleotides which may occur in aqueous solution even at neutral or slightly alkaline pH.

2.2.5 Failed Sequences

A sample of the crude oligodeoxynucleotide solution (50 µl) is mixed with 1 ml perchloric acid 70 %-absolute ethanol (1:1 v/v), and the absorbance of the released dimethoxytrityl cation recorded at 498 nm to give a rough estimate of the trityl yield of the synthesis (dimethoxytrityl cation millimolar absorptivity at 498 nm = 71.7). Another sample is subjected to analysis by reverse phase HPLC (see below) to check the ratio of trityl-on product (the longest retained major peak), to failure sequences achieved in the synthesis.

fraction of failure sequences

2.3 Primary Purification by Reversed-Phase, Solid-Phase Extraction on C18 Sep-Pak Cartridges

2.3.1 Equipment

C18 Sep-Pak solid phase extraction cartridges (Waters Chromatography Division of Millipore) are siliconized, to prevent loss of oligodeoxynucleotide through non-specific adsorption, by treatment with 3 ml "Repelcote(VS)" (BDH/Merck) for 5 min. The cartridges are then washed successively with 10 ml dichloromethane, 10 ml acetonitrile and 10 ml Py/TEAA. All glassware (Pasteur pipettes, beakers, etc.) contacting the oligodeoxynucleotide solutions during purification are also siliconized with Repelcote, and then washed consecutively with dichloromethane, acetonitrile and water.

prevention of non-specific absorption

2.3.2 Method

1. The crude, deprotected oligodeoxynucleotide solution in Py/TEAA is applied to the siliconized C18 Sep-Pak cartridge, and allowed to drip through slowly under gravity using a polypropylene disposable syringe barrel (e.g., Plastipak, Becton Dickinson) as reservoir (oligonucleotides may adsorb to other types of plastic).
2. A sample (50 µl) of the effluent is mixed with 1 ml perchloric acid-70 %/absolute ethanol (1:1 v/v) for trityl analysis at 498 nm. If not all trityl-containing material has bound to the cartridge, the effluent may be diluted further with an excess of Py/TEAA and reapplied to the same cartridge, or applied to a second cartridge to be treated in parallel.
3. When all trityl is bound, the cartridge(s) is washed with 10 ml Py/TEAA, and developed with a step gradient of acetonitrile in Py/TEAA starting from 10 ml 5 % acetonitrile (1:1, v/v).

spectrophotometric monitoring

variation of Sep-Pak cartridges

elution levels

Cartridge effluents are monitored spectrophotometrically at 270–290 nm (off the absorption maximum of pyridine) against a Py/TEAA blank, to give an indication of the elution of failure sequences, and 200 µl samples of the effluents are assayed for trityl as above, to test for elution of the product oligodeoxynucleotide. The characteristics of C 18 Seip-Pak cartridges tend to vary slightly from batch to batch, and the degree of substitution of phosphodiester linkages with the mor lipophilic, non-ionic methylphosphonate groups in the chimeric oligodeosynucleotide will affect retention, with more highly substituted molecules requiring higher concentrations of the acetonitrile organic modifier to effect their elution from the cartridge. Generally, failure sequences will begin to elute at around 10 % acetonitrile, and the trityl-on product will begin to appear in the effluent at or above 15 % acetonitrile. Trityl-on methylphosphonodiester/phosphorothiodieste oligodeoxynucleotides are even more highly retained than their phosphodiester counterparts. Once substantial amounts of failure sequences begin to elute, it is advisable to hold the acetonitrile concentration of the eluent constant, until the absorbance of the effluent at 270–290 nm reduces to a low constant value, before increasing the concentration further.

2.3.3 Purification of the Oligodeoxynucleotide

1. When trityl is detected in the effluent, the fraction is diluted with TEAA and reapplied to the cartridge, which is then washed with 10 ml TEAA (the presence of pyridine to protect against premature detritylation is no longer required) and 10 ml water.

2. Detritylation is performed on the cartridge by allowing 10 ml 0.5 % trifluoroacetic acid/water (1:1, v/v) to drip through over 30 min before neutralizing with 10 ml TEAA and washing with 2×10 ml water.
3. The partially purified chimeric oligodeoxynucleotide product is eluted from the cartridge with 3 ml 50 % acetonitrile/water (1:1, v/v), and the total number of A_{260} units recovered is determined from the absorbance at 260 nm of a diluted sample.

Samples of the eluate are analyzed by weak anion exchange HPLC and by reverse phase HPLC (see below). A certain degree of cleavage of methylphosphonodiester internucleosi linkages may occur during deprotection, to yield fragments carrying a 5'-dimethoxytrityl group which will copurify with the product during trityl selection on C18 Sep-Pak cartridges. While reverse phase HPLC is useful for distinguishing between trityl-on oligodeoxynucleotide and trityl-off failure sequences, this technique may give inadequate or no separation between trityl-off product and some trityl-off oligodeoxynucleotide fragments. In this case, weak anion exchange HPLC, where the separation is not based solely on the overall negative charge on the molecule, has provided a useful cross-check of the purity of chimeric oligodeoxynucleotide preparations. It has generally been found that this technique is more discriminating than reverse phase HPLC.

2.3.4 Further Purification

1. Further clean-up of the product is achieved by diluting the oligodeoxynucleotide solution x10 with TEAA, and applying it to a new siliconized C18 Sep-Pak cartridge, which is then eluted with a step gradient of acetonitrile in TEAA.
2. The major oligodeoxynucleotide-containing fractions are rapidly identified by running UV absorption spectra between 220–300 nm, and their purity is assessed by HPLC analysis.
3. Appropriate fractions are pooled, and rapidly desalted by diluting them with TEAA and applying the solution to a siliconized C18 Sep-Pak cartridge, which is then washed exhaustively with water.
4. The desalted product is eluted with 3 ml 50 % acetonitrile/water (1:1, v/v).

This technique may also be used to concentrate and desalt rapidly fractions collected from HPLC purification of the oligodeoxynucleotides, as discussed below.

The Sep-Pak purification scheme is generally adequate to provide a product which is sufficiently pure for most purposes, especially if the

second stage, trityl-off separation, is reiterated with exhaustive elution at an acetonitrile concentration just slightly less than that required to start eluting the product. At this stage the oligodeoxynucleotide is in the form of its triethylammonium salt. The triethylammonium cation is quite toxic to mammalian cells, and for biological applications it is advisable to convert the oligodeoxynucleotide to the sodium salt.

1. This may be achieved by passing the oligodeoxynucleotide solution through a small column of Dowex 50WX8–400 ion-exchange resin (Aldrich Chemical Company), sodium form, held in a 10 ml polypropylene syringe barrel with a plug of siliconized glass wool, and eluting with water until all UV-absorbing material is recovered.
2. The Dowex 50 resin is supplied in the hydrogen form, and is converted into the sodium form by washing the column with 1 M sodium chloride solution until the effluent is no longer acidic, followed by a water wash to remove the salt.
3. The final desalted oligodeoxynucleotide solution is concentrated in a Savant Speed Vac Concentrator, adjusted to a volume of 1 ml with water, and cleared of all residual small molecule contaminants by passage through a gel filtration NAP 10 column (Pharmacia), eluting with 1.5 ml water.
4. The final product solution is evaporated to dryness in a Speed Vac concentrator and stored with desiccant at -20 °C.

2.4 Analysis and Purification by HPLC

2.4.1 Analysis of Chimeric Oligodeoxynucleotides by HPLC

Methylphosphonodiester-phosphodiester and methylphosphonodiester-phosphorothiodiester chimeric oligodeoxynucleotides may be analyzed by reverse phase HPLC on Brownlee Aquapore RP-300 7 micron (Applied Biosystems) and Asahipak C8P-50 (UK supplier Prolabo/Rhone-Poulenc) columns. A generally useful, steep gradient from 5–70 % acetonitrile (HPLC grade) in 0.1 M triethylammonium acetate, pH 7.0, in 20 min, at a flow rate of 1 ml/min, serves to separate 5′-dimethoxytrityl-oligodeoxynucleotides from failure sequences in all applications. Shallower gradients over shorter concentration ranges may be used for more stringent analysis of final products. The triethylammonium acetate buffer is best prepared from triethylamine that has been refluxed with potassium hydroxide pellets for 4 h, and redistilled to remove UV-absorbing impurities that can interfere with the analyses. The lifetime of the silica-based Aquapore column is enhanced by installing a column dry-packed with silica pre-column gel, 37–53 microns (Whatman), upstream of the sample injector, to saturate the buffer with silicate prior to contact with the analytical column. In the case of analyzing fluorescein-tagged oligodeoxynucleotides (see below), where optimum signal from the fluorescence

preparation of buffer

enhancing lifetime of HPLC column

detector is achieved by using a slightly alkaline triethylammonium acetate buffer pH of 8.0, the use of a silica-packed column in the solvent delivery line is highly recommended.

Excellent separations of chimeric oligodeoxynucleotides have been achieved by weak anion exchange HPLC on HRLC MA7 plasmid columns (Bio-Rad) at a column temperature of 65 °C, using a 60 min gradient from 0–1.5 M potassium chloride in 20 mM potassium phosphate/50 % formamide/water (1:1, v/v), pH 7.5, and a flow rate of 1 ml/min. In the case of chimeric oligodeoxynucleotides with only 4 phosphodiester linkages, and therefore carrying only 4 negative charges, the composition of the weak buffer was changed to 1 mM potassium phosphate/50 % formamide/water (pH 7.5) to achieve retention on the column. The HRLC MA7 Plasmid column has now been superseded by the HRLC MA7Q anion exchange column. The use of "HiPerSolv" grade (BDH/Merck), low UV-absorbing potassium dihydrogen orthophosphate and potassium chloride is recommended for preparation of buffers, as well as spectrophotometric grade formamide. However, UV-absorbing impurities in these constituents can still interfere with the analyses by concentrating on the column at low ionic strength and then eluting during running of the gradient as the salt concentration is increased. Such problems may be largely eliminated by installing a column dry-packed with Partisil-10 SAX, strong ion exchanger (Whatman) in the solvent line on the instrument, upstream of the sample injector. The packing material in this column is replaced periodically as it becomes exhausted.

alternative purification of chimeric oligodeoxynucleotides

low UV-absorbing reagents

The C8P-50 and HRLC columns have the advantage that the packing materials are polymer based, and may be regenerated by flushing with a 0.2 M solution of sodium hydroxide, followed by 0.2 M acetic acid, whereas such a treatment would be incompatible with silica-based chromatographic supports. Therefore, these columns are suitable for "dirty" applications, such as the analysis of oligonucleotides in cell and tissue extracts, which would rapidly and irreversibly poison the latter. Column performance is monitored periodically with a cocktail of oligodeoxynucleotide standards, and the regeneration procedure applied when a deterioration in the separation is observed.

regeneration of columns

2.4.2 Purification of Chimeric Oligodeoxynucleotides by HPLC

Where highly pure chimeric oligodeoxynucleotides are required, the product may be subjected to a final purification step on the analytical HRLC anion exchange column. This is particularly appropriate for phosphorothioate-containing oligodeoxynucleotides, where the preparation will contain substantial amounts of material seen as an apparent "n-1" peak on anion exchange HPLC analysis, but which co-elutes with the true product on reverse phase HPLC analysis and during C18 Sep-Pak purification. This impurity is a mixture of full length oligodeoxynucleotides in which one of the internucleoside linkages is phosphodiester rather than the desired phosphorothiodiester, as a

final purification

result of failure in the sulfurisation step and oxidation occurring during deprotection (Zon, 1993).

preparative HPLC

For preparative HPLC, the injector should be fitted with a large volume sample loop. Significant amounts of oligodeoxynucleotide may be purified relatively quickly by running the HPLC isocratically and injecting the oligodeoxynucleotide in batches of 5–10 A_{260} units. The same conditions and buffers as used for the analytical separation may be used for preparative HPLC: "weak buffer", 20 mM potassium phosphate/50 % formamide/water (v/v) pH 7.5; "strong buffer", 1.5 M potassium chloride/20 mM potassium phosphate/50 % formamide/water (v/v) pH 7.5; flow rate 1 ml/min; column temperature 65 °C. In order to determine the optimal isocratic conditions for preparative separation, the nominal percentage of strong buffer in the 60 min 0–100 % strong buffer gradient at which the product elutes during an analytical separation is first noted, and the column equilibrated at this constant eluent composition. The oligodeoxynucleotide should elute in the void volume of the column at this concentration under isocratic conditions, and this is verified by injection of an analytical sample of about 0.05 A_{260} units. The column is then re-equilibrated at progressively reduced percentages of strong buffer in the eluent until injection of an analytical sample of the oligodeoxynucleotide produces a product peak retention time of about 20 min. This eluent composition should suffice for preparative separation, since the retention time reduces with the vastly greater amounts of oligodeoxynucleotide injected. The HPLC UV detector is offset from the oligodeoxynucleotide absorption maximum to 290 nm and the range set to 2 absorbance units full scale. The separation may be fine tuned by reducing the percentage of strong buffer still further during the preparative runs. Inadequately purified fractions may be rerun under the improved separation conditions after being rapidly concentrated and desalted. This is achieved by dilution x10 with TEAA to permit binding of the oligodeoxynucleotide to a siliconized C18 Sep-Pak cartridge, which is then washed exhaustively with water and the product eluted with 3 ml 50 % acetonitrile/water (1:1, v/v). Product fractions are pooled desalted on a C18 Sep-Pak cartridge, and converted to the sodium salt prior to removal of all small molecule contaminants by passage through a NAP 10 gel filtration column, as described above.

2.4.3 Re-Use of Columns

regeneration of column

After completion of the preparative separation, the column must be regenerated by flushing with 0.2 M sodium hydroxide followed by 0.2 M acetic acid, before being used again for analytical work, since memory effects can be quite serious.

An alternative approach, which is not strictly HPLC, is to load up to 120 A_{260} units on to the HRLC column all at once, under isocratic conditions of 100 % weak buffer and a flow rate of 1 ml/min, using multiple injections if necessitated by the volume of sample. A manually operated step gradient is then cautiously applied until UV-absorbing

material appears in the effluent, when the elution is left to run isocratically until a constant baseline is re-established. The process is then continued in this fashion until all that was applied to the column has eluted. However, in using this approach, the UV absorption trace is uninterpretable as a chromatograph, since fluctuations in the effluent concentration of pure product give the appearance of multiple peaks, particularly when increasing the concentration of strong buffer over the range required to elute all product from the column. The column is regenerated and then used analytically to identify the range of fractions containing pure oligodeoxynucleotide.

2.5 Synthesis of Chimeric Oligodeoxynucleotides with Fluorescein Attached

It is desirable to have a readily detectable, fluorescent reporter group attached to oligodeoxynucleotides for monitoring intracytoplasmic delivery (see below) and metabolism against a background of UV-absorbing biomolecules. This function is adequately fulfilled by fluorescein, which, at the same time, does not appear to affect unduly the biochemical and biological properties of antisense oligonucleotides.

The most straightforward approach to labeling oligonucleotides with fluorescein is to derivatize post-synthetically to an amino-linker group introduced at the 5′-terminus as the last cycle on the synthesizer. A number of amino-linker phosphoramidites are available commercially, but for creating a linkage to methylphosphonodiester-phosphodiester and methylphosphonodiester-phosphorothiodiester chimeric oligodeoxynucleotides, a degree of selectivity is required. Aminolink 2 (Applied Biosystems), for example, is protected on phosphorus with a methoxy group, and the extended treatment with ammonia at room temperature required to achieve complete deprotection at this function may lead to loss of oligodeoxynucleotide product through hydrolysis of methylphosphonodiester internucleoside linkages. On the other hand, use of the β-cyanoethyl-protected 5′-Amino-Modifier C6-TFA (Glen Research, UK supplier Cambio) is compatible with the deprotection conditions for methylphosphonate-containing chimeric oligodeoxynucleotides, as described above.

efficiency of coupling

In order to confirm that efficient coupling of amino-linker to oligonucleotide has been achieved, an interrupt is programmed into the DNA synthesizer to occur immediately prior to the capping step of this cycle. The capping step is omitted by jumping to the next command, and 2 further cycles are executed for coupling of an arbitrary deoxynucleoside phosphoramidite. The trityl fraction of the first supplementary cycle, which ideally should be colorless, gives an indication of the efficiency of coupling of amino-linker. If coupling has been less than ideal, the detritylation step of the final cycle may be omitted, as for trityl-on oligodeoxynucleotide synthesis. In this way, oligodeoxynucleotide molecules that failed to couple with amino-linker may be

removed during initial C18 Sep-Pak clean-up of deprotected product, through a reversal of the strategy normally used to select trityl-on product from failure sequences.

problems in separation

In the absence of a 5′-dimethoxytrityl group, the complete separation of 5′-amino oligodeoxynucleotide from all end-capped failure sequences cannot be achieved on C18 Sep-Pak cartridges. However, significant enhancement of the purity of the preparation is accomplished by exhaustive elution of the cartridge with a concentration of acetonitrile in TEAA just slightly less than that required to displace the product. This is determined with an exploratory step gradient, monitoring fractions by weak anion exchange HPLC, following which product-containing fractions are diluted with TEAA and returned to the cartridge. The 5′-amino oligodeoxynucleotide is eluted with an appropriate concentration of acetonitrile in TEAA, desalted on a new C18 Sep-Pak as described above, and dried down in a Speed Vac Concentrator.

The millimolar absorptivity, ε_{260}, of an oligonucleotide at 260 nm, may be estimated by summing the values for each base, 8.8 for T; 7.3 for C; 11.7 for G; 15.4 for A, as given by Sproat and Gait (1984). This provides a useful working means of converting A_{260} units into approximate numbers of µmoles.

coupling of fluorescein

1. For coupling of fluorescein, 5′-amino oligodeoxynucleotide (1 µmole, very approximately 200 A_{260} units of a 20-mer) is dissolved in 1 ml 200 mM sodium bicarbonate/sodium carbonate buffer, pH 9.0, in a 1.5 ml plastic microcentrifuge tube (Eppendorf), heated at 90 °C for 5 min, and allowed to cool.
2. A solution of 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS, Boehringer Mannheim) in dimethylformamide (7 µmole in 0.1 ml) is added, mixed, and the pH of the solution immediately checked with a semi-micro electrode.
3. If necessary, the pH is readjusted to 9.0 by addition of one or two grains of solid sodium carbonate. Reaction is complete in less than 30 min.
4. A sample of the reaction mixture is analyzed by weak anion exchange HPLC, the column effluent being monitored with UV and fluorescence detectors connected in series (excitation 494 nm, emission detection centered on 530 nm, Wratten 15 520 nm cut-off emission filter). The reaction proceeds with the disappearance of the 5′-amino oligodeoxynucleotide UV absorption peak and the appearance of a UV absorption/fluorescence peak at longer retention time, consistent with an increase in overall charge on the molecule due to the negative charges on fluorescein and removal of the positive charge at the amino function through formation of the amide.

5. If unreacted 5′-amino oligodeoxynucleotide remains at 30 min it is necessary to add more FLUOS solution to achieve further reaction. Failure to observe additional conversion of 5′-amino oligodeoxynucleotide to product may mean that the reaction is inhibited by some form of intermolecular secondary structure. In this case complete reaction is secured by adding fresh FLUOS solution and heating the reaction mixture to 90 °C.

completion of reaction

6. To remove the bulk of the carboxyfluorescein impurity, the reaction mixture is applied to a NAP 10 gel filtration column, previously washed with dimethylformamide/water 1:1 v/v, and the oligodeoxynucleotide eluted with the same solvent.
7. The fluorescein-tagged product is diluted x10 with TEAA, and further purified on a C18 Sep-Pak cartridge using the standard approach, except that to ensure efficient removal of residual fluorescein impurity, the wash solutions of acetonitrile in TEAA are heated to 90 °C and pushed through the cartridge by syringe.

2.6 Summary

In this chapter, we have described the synthesis of antisense molecules which combine stability, excellent hybridization properties and, as we shall describe in a later chapter, uptake properties which are rarely bettered. Consideration of the target site depends, of course, on the consideration of the secondary structure of the target mRNA, which is never a sure prediction even when the sequence is known. The translation site is then the favored one since more successful work has been done with antisense DNA directed to this target sequence than any other. We will return to these questions in the next chapter where we look at the use of ribozymes.

We have not ventured to discuss in this section the use of genetic constructs which synthesize antisense molecules, but will feature this when we discuss the intracellular synthesis of ribozymes.

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Antisense and Ribozyme Methodology
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CHAPTER 3

The Design and Synthesis of Hammerhead Ribozymes

3.1 Introduction

definition of ribozymes

The term 'ribozyme' was introduced for ribonucleic acids (RNAs) that perform two functions: firstly, the recognition of a defined RNA motif via base-specific interactions and, subsequently, the catalysis of the hydrolysis of a specific phosphodiester bond, resulting in RNA strand breakage. Several classes of ribozymes have been identified in recent years, which differ in size and structure. These include large catalytic RNAs such as the group I and group II intron ribozymes, as well as RNase P (reviewed by Cech, 1993) and the small hairpin ribozyme (Prody *et al.*, 1986), the hepatitis δ ribozyme (Kuo *et al.*, 1988) and the hammerhead ribozyme (for a review see Bratty *et al.*, 1993). The latter was identified in two plant pathogens, the avocado sunblotch viroid (ASBVd; Forster and Symons, 1987) and the peach latent mosaic viroid (PLMVd; Hernandez and Flores, 1992), as well as in an RNA transcript of a satellite DNA found in newt. All of these ribozymes, except RNase P, were originally found to cleave intramolecularly (in *cis*) and only one catalytic cycle has to be assumed to explain their natural function. Conversely, RNase P, which is involved in the maturation of pre-tRNA, is the only natural catalytic RNA that can act intermolecularly (in *trans*) and perform several catalytic cycles, like a real enzyme.

classes of ribozymes

structure and mode of action

The catalytically active RNA structure of various ribozymes can be formed by one RNA strand, i.e., in *cis*. For most of the above listed classes of ribozymes, an active ribozyme-substrate complex can also be assembled by two or, in the case of the hammerhead ribozyme, even three strands (Uhlenbeck, 1987; Haseloff and Gerlach, 1988). Such trans-cleaving hammerhead ribozymes contain sequences that recognize the substrate via 'antisense sequences' as well as sequences that form the main portion of the catalytic core which catalyses the hydrolysis of the cleavable motif. After dissociation of the complex formed between the ribozyme and the cleaved substrate, the trans-cleaving ribozyme is left unchanged, like a conventional enzyme. The minimal catalytic cycle of a trans-cleaving ribozyme is schematically depicted in Fig. 4. It should be noted, however, that trans-cleaving ribozymes can be active *in vitro* as well as in living cells. This observation has made it attractive to think of trans-cleaving ribozymes as sequence-specific nucleases that can be used in molecular biology

and molecular medicine as tools to inhibit gene expression as well as viral functions (for reviews see Marschall *et al.*, 1994; James and Al-Shamkhani, 1995; Sczakiel and Nedbal, 1995). When considering the application of ribozymes one faces two options: firstly, the exogenous use of ribozymes, usually chemically synthesized short-chain hammerhead ribozymes that can be stabilized by various chemical modifications (for a review see Marschall *et al.*, 1994); and secondly, recombinant genes for the endogenous expression of ribozymes can be applied. In this case, the size of a trans-cleaving ribozyme is not limited. Recombinant genes encoding hairpin ribozymes and hammerhead ribozymes have been used as potent inhibitors of aberrant gene expression or viral replication in a variety of biological systems (for reviews see Marschall *et al.*, 1994; James and Al-Shamkhani, 1995; Steinecke *et al.*, 1992; Rhodes and James, 1990).

This article is focused on hammerhead ribozymes that cleave in *trans*. It will provide an introduction to the design of ribozymes and the selection of target sites. Finally, some theoretical considerations, as well as strategies for the synthesis and the application of ribozymes in mammalian cells, will be summarized.

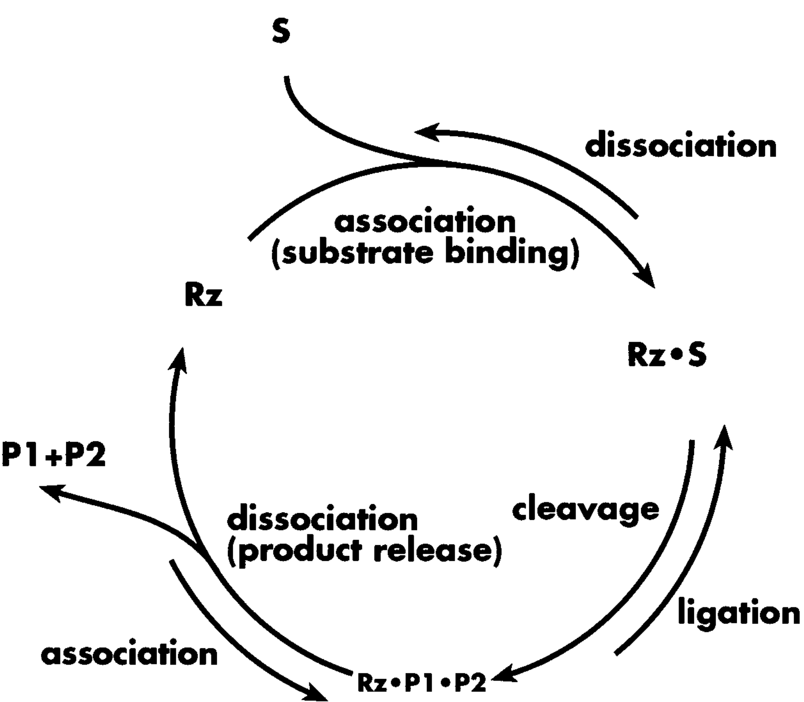


Fig. 4 Minimal catalytic cycle of a trans-cleaving ribozymes. Abbreviations: S, substrate (target); Rz, ribozyme; P1 and P2, cleavage products (=cleaved substrate)

3.2 The Design of Hammerhead Ribozymes

Ribozyme function consists of two ordered steps, sequence-specific binding of the substrate via antisense arms, and hydrolysis of the cleavable motif (Fig. 5). Both steps contribute to efficacy in living cells. The antisense arm-substrate interactions determine the effectiveness of the formation of the ribozyme-substrate complex. It seems reasonable to assume that accessibility of complementary sequences of the ribozyme and its target is a necessary prerequisite for efficient pairing. For long-chain hammerhead ribozymes, Ellis and Rogers (1993) provided experimental evidence that the rate-limiting step of ribozyme activity is the formation of the ribozyme-substrate complex, but not the cleavage step. The chemical step, i.e., the rate of the cleavage reaction, is determined by the sequence of the cleavable motif. It is known that certain invariant bases within the sequence of the active ribozyme-substrate complex, as well as specific sequence requirements for the cleavable motif, are necessary for catalysis. Therefore, a series of constraints on the selection of potential target sites for *trans*-cleaving ribozymes have to be considered.

3.3 Improving the Reactions

3.3.1 Accessibility of the Target – Substrate Binding

stability of ribozyme-substrate complex

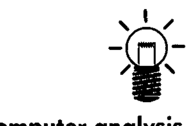
Parameters that adequately describe the association of two complementary RNA strands depend on the chain length. For short-chain complementary sequences ($<$ bp), intramolecular interactions (e.g., secondary structures) are often not very stable, and are therefore thought to be resolvable during duplex formation. For hammerhead ribozymes with short antisense arms, consisting of up to ten complementary bases within both antisense arms, efficient formation of the ribozyme-substrate complex is strongly influenced by complex stability (melting temperature). This means that the antisense arms have to be sufficiently long to guarantee stability of the ribozyme-substrate complex. Conversely, for long-chain complementary sequences and ribozymes with more than 30 complementary bases, the stability of the ribozyme-substrate complex is sufficiently high *in vitro* at physiological temperature, pH value and ionic strength and, presumably, also in living cells. However, in this case, the formation of the catalytically active complex is kinetically controlled, and the intramolecular RNA structures seem to have a great influence on the rate of pairing. For long-chain hammerhead ribozymes ($>$100 nt), the association rate constant (KASS) *in vitro* is of the order of $10^5\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ (Homann *et al.*, 1993) and often even smaller, whereas KASS values for short-chain (oligomeric) RNA strands are of the order of $10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$.

3.3.2 Finding the Target

accessibility of RNA sequence

To monitor the local accessibility of a given RNA sequence, one can perform experimental analyses such as nuclease mapping, chemical probing, or thermodynamic as well as kinetic selection for effective annealing. These strategies provide information on the accessibility of a given target sequence for proteins, low molecular weight compounds, and even nucleic acids. On the other hand, theoretical analyses and the use of computer algorithms that are based on specific assumptions can be performed with less expense. Such programs are aimed to increase the probability of applying antisense and ribozyme inhibitors more successfully than on a purely random basis.

3.3.3 Theoretical Considerations



computer analysis

Pre-selection of potential target regions is usually based on computer-supported analyses of the entire target sequence. These algorithms attempt to provide information on the extent of local folding, on the energy difference of defined sequence stretches in the single-stranded versus the double-stranded state, and on structural elements that could be important for RNA duplex formation, i.e., the formation of the ribozyme-substrate complex.

For example, the calculation of the so-called 'local folding potential' (Sczakiel *et al.*, 1993) can be used to monitor the extent of intramolecular interactions within a given sequence stretch, which is equivalent to its local thermodynamic stability. Thus, the local folding potential is regarded as being correlated with the efficiency of intermolecular interactions. To determine the local folding potential of a given stretch of bases (window), the program calculates the most stable structure, and plots the corresponding value for the free energy (ΔG) versus the sequence position. Subsequently, the window is shifted for a given distance (step width, e.g., 1 nt) along the target sequence, and the next ΔG value is calculated. Thus the entire target sequence is scanned and a plot of ΔG values versus the position of the window is generated. Local maxima correspond to potentially unstable local sequence stretches, and indicate potential target regions for complementary RNA. The target motif for hammerhead ribozymes should be chosen so that its antisense sequences forming helix I or helix III are directed against a target region with a low folding potential (local maximum of the ΔG value).

prediction of structure

Currently, a number of programs are available that predict the secondary structure, or even include tertiary interactions of a given DNA or RNA sequence. For long-chain sequences, such predictions are not regarded as being extremely reliable. However, for short-chain sequences ($<\sim 100$ nt), an extensive computer analysis of possible secondary structures can help to identify sequence stretches that are less involved in intramolecular interactions, and which might thus represent more promising target sites for complementary RNA.

In a simplified view, formation of an RNA duplex consists of two steps: firstly, the resolution of the structures of each of the two complementary strands, which is an energy-consuming step, and secondly, the formation of the duplex, i.e., a gain of energy. This implies that the energy difference between both steps is critical for the efficiency of binding and, accordingly, a program has been developed for its calculation (James and Cowe, 1996).

However, it should be noted that there are a number of alternative theoretical approaches to increase the probability of choosing effective local target regions. Based on calculations of ΔG values for short-chain ribozyme-substrate structures, and their relationship with structural elements of the catalytically active ribozyme-substrate complex, Denman (1993) proposed a computer-based method to predict active *trans*-cleaving hammerhead ribozymes.

choosing targets

3.3.4 Experimental Approaches

The accessibility of a target sequence is a key parameter for efficacy of *trans*-cleaving ribozymes. Accessibility, however, can be defined in two alternative ways. On the one hand, accessibility means absence of stable local structures of the target as well as the ribozyme in regions that are needed for the formation of the ribozyme-substrate complex, i.e., for the formation of helix I and helix III of the hammerhead (Fig. 5). In this case, computer-based strategies, as well as chemical or enzymatic probing, seem to be appropriate to obtain information on target accessibility. For long-chain complementary RNA, however, there is a significant correlation between the kinetics of annealing between the target strand and the antisense strand, and the extent of antisense-mediated inhibition in living cells (Rittner *et al.*, 1993a). Thus, it seems reasonable to assume that the rate of annealing between a long-chain *trans*-cleaving ribozyme and its target influences the efficacy of the ribozyme.

assessment of accessibility

Two experimental approaches have been established that are suitable for screening large pools of related complementary sequences, as well as ribozymes, for fast and efficiently annealing species. For short-chain sequences that correspond to short and usually chemically synthesized ribozymes, an array of systematically permutated sequences or an array of oligomeric sequences that scan along a given target sequence are fixed onto a membrane, and are subsequently hybridized with a complementary strand. Strong hybridization signals indicate efficiently annealing pairs of complementary sequences (Southern *et al.*, 1992).

3.3.5 Kinetic Studies

For long-chain sequences (< nt), an alternative procedure has been developed. This is based on measuring the annealing kinetics of series of related complementary RNAs or ribozymes (Rittner *et al.*,

annealing kinetics

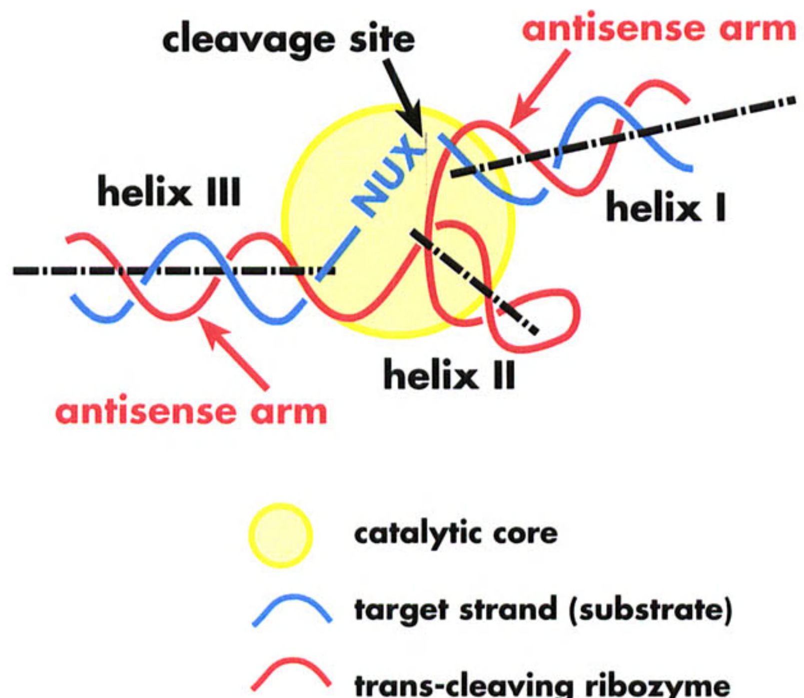


Fig. 5 Complex formed between a trans-cleaving hammerhead ribozyme and its target (substrate)

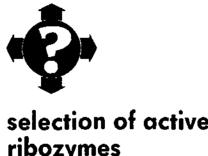
1993a). Briefly, a parental antisense RNA or ribozyme, preferentially an asymmetric hammerhead ribozyme, is end-labeled and subjected to limited alkaline hydrolysis, thereby generating a pool of successively shortened and end-labeled antisense or ribozyme species. This pool is incubated together with a large excess of target RNA in solution, and aliquots are withdrawn to follow the time course of the annealing reaction. The gel electrophoretic analysis of the single-strand fraction and the double-strand fraction at different time points allows identification of fast-annealing species versus slow-annealing species. In fact, we observed that extension of an approximately 60 nt long asymmetric HIV-1-directed hammerhead ribozyme by 5 nt could increase its annealing rate up to ten-fold (Homann and Sczakiel, unpublished).

functional assay of selectivity

In the case of the cellular *bcr-abl* fusion sequence, selectivity has to be defined as efficient destruction of the malignant *bcr-abl* transcript versus inefficient or almost no destruction of the wild type transcripts of *abl* and *bcr*, that are both necessary for normal cell proliferation. Thus, it can be important to increase the selectivity of a ribozyme by increasing the selectivity of the binding step. The aim is to select for ribozymes that anneal rapidly with the target (*bcr-abl*) but slowly or not at all with related non-target sequences (*bcr* or *abl*). In case of *bcr-abl*-directed asymmetric hammerhead ribozymes, addition of five to ten nucleotides led to a hundred-fold increase of the annealing

rate, and to efficient and specific destruction of target RNA *in vitro* (Kronenwett *et al.*, 1996). Selectivity has also been achieved with a ribozyme with or without DNA arms (James *et al.*, 1996 and unpublished).

An elegant approach to select for *in vivo* active ribozymes from pools of ribozyme species that share the same catalytic core but have random sequences forming their antisense arms was described recently (Lieber and Strauss, 1995). The selection procedure included ribozymes directed against all potential cleavage sites located on the target molecule. Both steps of ribozyme action, substrate binding and the cleavage step, were included in the selection. Interestingly, the most active selected ribozymes had short antisense arms, i.e., 7 to 8 nt on either side.



selection of active ribozymes

3.4 Length of Arms

3.4.1 Choosing Antisense Arms of Hammerhead Ribozymes

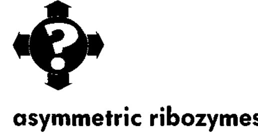
The two antisense arms of a hammerhead ribozyme should be sufficiently long to form a stable ribozyme-substrate complex via approximately 15 or more Watson-Crick base pairs. However, there are no general rules on the exact length and symmetry of both antisense arms. Biologically successful examples of the use of ribozymes as inhibitors of gene expression and viral functions have been described for all kinds of combinations. Even ribozymes with antisense arms of several hundred nucleotides in length have been reported, though catalytic turnover is not conceivable in this case (Sczakiel and Goody, 1994). Usually, the selection of antisense arms is influenced by technical considerations that include the location of the cleavable motif or the location of restriction sites. Since the antisense arms serve for substrate binding, they can be used for optimization of the kinetics of substrate binding (see above).

length of ribozyme arms

Recently, size constraints were described for endogenously expressed HIV-1-directed hammerhead ribozymes that required antisense arms of a minimal length of 33 nt on either side in order to be active as inhibitor (Crisell *et al.*, 1993).

3.4.2 Arms of Different Lengths

An alternative to the conventional 'symmetric' design is the so-called 'asymmetric' design (Fig. 6; Tabler *et al.*, 1994). So far, one has not observed a reduced activity *in vitro* or reduced efficacy in living cells of asymmetric hammerhead ribozymes when compared with their symmetric analogues. The asymmetric design of hammerhead ribozymes has a number of specific advantages. Cloning strategies for recombinant ribozyme genes can be performed with less expense,



asymmetric ribozymes

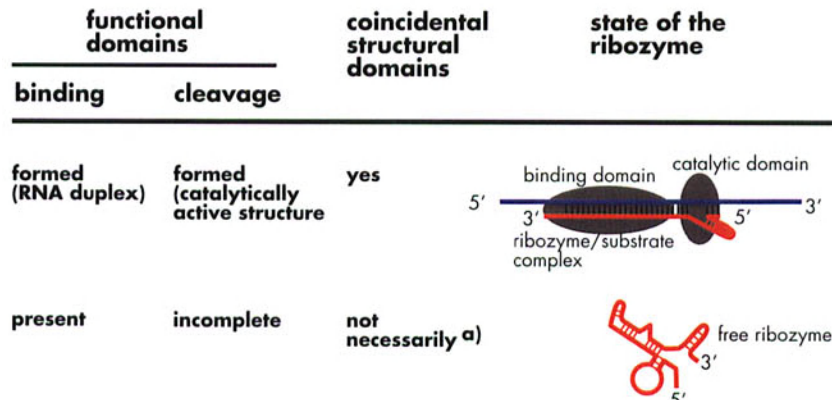


Fig. 6 Asymmetric design and domain structure of hammerhead ribozymes. In the case of asymmetric hammerhead ribozymes, a structural domain can be formed by antisense sequences (binding domain) that does not interact intramolecularly with sequences forming the catalytic domain in functional as well as structural terms

and pre-constructed plasmids that contain the catalytic domain and a 3 nt 5′ arm (helix I) can be used to insert directly PCR-amplified sequences that add the 3′ arm (Tabler *et al.*, 1994). Further, the long-chain 3′ arm can be used to apply the above-described protocols for increasing the substrate binding properties.

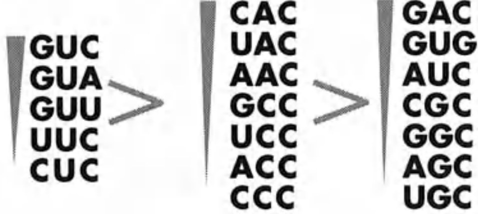
3.5 Cleavage of the Target Motif

sequence specificity

The sequence motif recognized and cleaved by the hammerhead ribozyme is usually referred to as the 'NUX' motif where 'N' represents either G, U, A, or C and 'X' represents either U, A, or C. Despite the fact that the first position does not seem to require a specific base, the 'NUX' motif has not so far been reduced to the 'UX' motif. Systematic studies with permutated cleavable motifs and corresponding trans-cleaving hammerhead ribozymes showed that certain base triplets are cleaved relatively fast, whereas others were cleaved significantly more slowly or were not cleaved at all under the same experimental conditions (Fig. 7). All studies indicate that a 'G' residue at the third position is not tolerated. Efficient cleavage of the triplets 'GUC', 'UUC', and 'GUA' was reported by all studies. However, variations of the relative cleavage efficiencies occurred as well. The 'AUC' triplet served as the best cleavable motif in one study (Zoumadakis and Tabler, 1995), whereas cleavage of this motif could not be measured at all by Perriman *et al.* (1992), and only at suboptimal levels by Shimayama *et al.* (1995). The observed variations of the cleavage efficiencies may be partially explained by differences of the neighboring sequences, which were not the same among the constructs compared in Fig. 4. Zoumadakis and Tabler (1995) speculated that bases upstream (5′) of the cleavable triplet could have a significant influence

Ruffner et al., 1990

Perriman et al., 1992



Zoumadakis & Tabler, 1995

Fig. 7 Cleavage efficiency of base triplets by hammerhead ribozyme

on the cleavage efficiency, which is stronger than the influence of bases downstream (3'). On the other hand, a recent study by Shimayama *et al.* (1995) showed that even in the presence of mismatches in the NUX motif, cleavage was still surprisingly efficient. Similarly, mutations in the vicinity of the NUX motif reduced but did not abolish ribozyme activity (Zoumadakis *et al.*, 1994).

3.6 Synthesis of Ribozymes

RNA molecules can be synthesized chemically with appropriate expense provided that the total length does exceed much more than 45 to 50 bases. Considering that normally 22 nt of a trans-cleaving hammerhead ribozyme are required for formation of the catalytic core, this means that the maximal length of the antisense arms is of the order of 12+12 to 14+14 nt or, in the case of an asymmetric design (Tabler *et al.*, 1994), 3+25 nt. Longer hammerhead ribozymes have to be synthesized enzymatically by run-off transcription *in vitro*, or endogenously after transfer of recombinant ribozyme genes into living cells.

3.6.1 Chemical Synthesis of Short Hammerhead Ribozymes

Oligoribonucleotides can be synthesized on solid-phase using phosphoramidite chemistry with the usual DNA synthesizers. All necessary compounds are commercially available. Alternatively, purified oligoribonucleotides can be purchased from commercial suppliers.

chemical modification

The synthesis of ribozymes by chemical means allows one to introduce specific chemical modifications into the base, ribose, or phosphate moieties that do not disturb the ribozyme activity. Thereby, some of the disadvantages in the application of synthetic RNA such as rapid degradation, limited membrane permeability, or stability of the ribozyme-substrate complex can be avoided (e.g., Paolella *et al.*, 1992; for a review see Marschall *et al.*, 1994). In principle, the existing repertoire that chemists have developed for the improved use of oligodeoxyribonucleotides (DNA) could be expanded to include RNA and ribozymes as well. The first stimulating results in the exogenous use of chemically synthesized hammerhead ribozymes strongly suggest that we should continue to explore ways of applying them biologically. For example, the synthesis of amelogenin, a protein that plays a major role in mammalian enamel biomineralization, could be inhibited in new-born mice after local injection of a chemically modified amelogenin mRNA-directed hammerhead ribozyme (Lyngstadaas *et al.*, 1995).

3.6.2 Enzymatic Transcription *in Vitro*

transcription from DNA

Long-chain ribozymes can be synthesized by transcription *in vitro* from template DNA. The template consists of the start signal (promoter) for an RNA polymerase such as the SP6, T3, or T7 RNA polymerase at its 5′ end, and a transcribed portion that contains the sequences of the ribozyme. To generate suitable templates, one can use recombinant DNA, usually plasmids, that have to be linearized in order to terminate transcription at the 3′ end of the ribozyme sequence. The recovery of transcription can be increased by using 5′-elongated templates. It is important to note that enzymatic transcription *in vitro* does not result in an exactly defined transcript length, but rather produces 3′ ends that may be shorter or extended by two to three nucleotides when compared with the template. However, the transcription reaction can be used to incorporate modified nucleotide triphosphates into the ribozyme, such as a 5′ CAP signal, radiolabels, fluorescent labels, modifications that increase the stability of RNA, or groups that can be used for post-transcriptional modifications and reactions (e.g., biotinyl residues).

PCR methodology

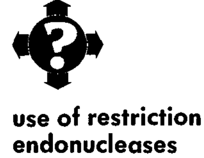
Another approach to generate appropriate templates makes use of the asymmetric design and PCR methodology. A cDNA fragment serving for substrate binding is selected, and two primers for PCR are synthesized, such that the 5′-primer contains the sequences that form three base pairs in helix I of the hammerhead, as well as the catalytic

domain (Fig. 6). The 3′-primer is a simple short primer that defines the length of helix III. The resulting PCR products can be used to synthesize RNA directly or can be used for the cloning of recombinant ribozyme genes (see below).

3.7 Endogenous Expression of Ribozyme Genes

Ribozymes can be expressed endogenously from recombinant ribozyme genes just like conventional recombinant genes in molecular biology. An expression cassette consists of a promoter that is active and either constitutive or inducible in the target cell, the ribozyme-coding sequence, and termination signals for transcription. Both polymerase II and polymerase III promoters have been used successfully to express ribozymes in eukaryotic cells *ex vivo* as well as *in vivo* (e.g., Dropulic *et al.*, 1992; Heinrich *et al.*, 1993; Inokuchi *et al.*, 1994; Thompson *et al.*, 1995).

Two additional experimental options are available to support the construction of recombinant genes for long-chain ribozymes. Firstly, cDNA fragments can be converted into ribozymes if they contain specific recognition sequences for restriction endonucleases. This strategy makes use of the fact that the recognition sequences for a series of restriction endonucleases contain a cleavable triplet for hammerhead ribozymes (Tabler and Tsagris, 1991). For example, the recognition sequence for *Sal* I (GTCGAC) contains the triplet GTC, which is GUC at the RNA level, and which represents a potential cleavable motif. This site can be used to insert an oligonucleotide consisting of the 22 nt that are necessary for formation of the catalytically active hammerhead structure (see Fig. 5).



use of restriction endonucleases

Alternatively, a strategy can be employed that, again (see above), makes use of the asymmetric design of hammerhead ribozymes and the PCR methodology (Tabler and Tsagris, 1996). A cDNA of the target serves as a template for PCR amplification of the sequences that form helix III with the substrate. The primers have to be chosen such that the PCR fragment can be cloned into one out of a set of 64 plasmids that already contain a promoter and the sequences that form three base pairs in helix I of the hammerhead, as well as the catalytic domain. By using this protocol, a recombinant ribozyme gene can be cloned in a single step. Similar methods can be used to clone genes synthesizing antisense RNA molecules which form complexes with mRNA sequences.

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CHAPTER 4

Delivery of Ribozymes and Antisense DNA Molecules into Mammalian Cells

4.1 Introduction

A variety of methods have been used to deliver ribozyme and antisense DNA molecules into living cells (Fig. 8). These are either by direct addition to the medium or via genetic constructs. The progress of antisense oligodeoxynucleotides and ribozymes as therapeutic agents is, however, hampered by poor transport and intracellular delivery. In this Chapter we address some of the techniques currently being used and being adapted to address these problems. We also look at some other problems which have still to be solved.

4.2 Exogenous Application

A number of reports on the use of chemically synthesized oligonucleotides indicate that small nucleic acids can be taken up by cells when added to the culture medium (Nestle *et al.*, 1994). If the medium concentration of chemically modified oligonucleotides exceeds values of 10 to 15 mM, biological effects may be specific for the type of chemical modification, but not specific for the target sequence. Despite the large number of publications on the exogenous use of antisense oligonucleotides, including a considerable number of convincing studies, this approach continues to be somewhat obscure, unpredictable, and often, reproducibility cannot be achieved. The exogenous use, i.e., addition to the culture medium or tissue, allows continuous delivery of ribozymes or antisense DNA molecules, but is restricted to small modified ribozymes or antisense molecules that are sufficiently stable and membrane permeable. For long-chain ribozymes and ribozyme encoding genes, however, this approach is regarded as inappropriate. One convincing study, however, involves the delivery of small antisense DNA molecules to cells following streptolysin treatment of the cells.

addition to culture medium

There is growing evidence to suggest that oligonucleotides are unable to gain significant access to the cytoplasm of almost all types of mammalian cells, if unaided by microinjection, electroporation, lipofection, or plasma membrane permeabilization (Bergan *et al.*,

uptake of oligonucleotides

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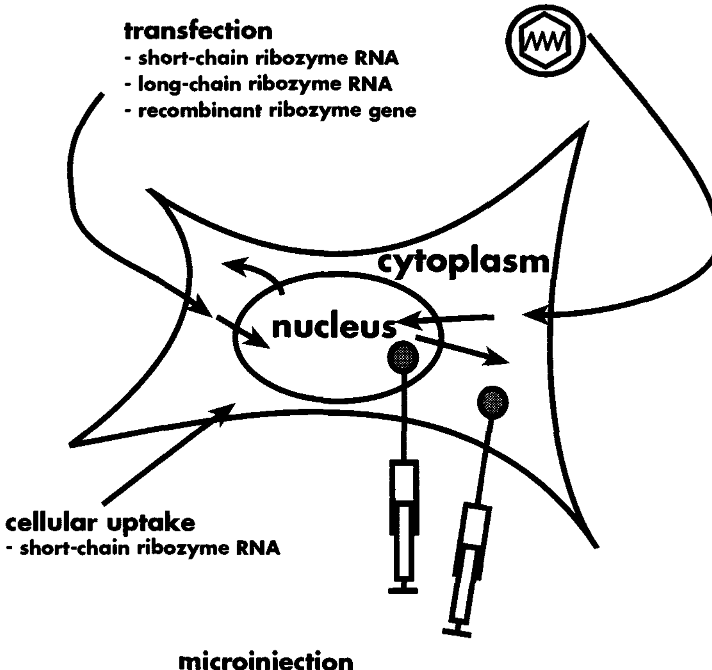


Fig. 8 Options for ribozyme and antisense DNA delivery to living cells

1993; Callis *et al.*, 1987; Harvey *et al.*, 1994; Spiller and Tidd, 1995; Giles *et al.*, 1995a,*b*,*c*). When presented exogenously to cells, fluorescence-tagged oligonucleotides are seen to be taken up by the cells to a limited extent by processes of endocytosis and fluid phase pinocytosis, but the internalized molecules remain in endosomes or vesicles within the cells, still bounded by a membrane which separates them from the target mRNAs in the cytoplasm and nucleus. On the other hand, when introduced directly into the cytoplasm by physical microinjection, oligonucleotides rapidly accumulate in the nucleus (Chin *et al.*, 1990). Therefore, it is apparent that artificial manipulations to effect intracytoplasmic delivery must generally be undertaken if true antisense effects of oligonucleotides are to be achieved in living cells.

4.2.1 Intracytoplasmic Delivery of Antisense Oligodeoxynucleotides by Reversible Plasma Membrane Permeabilization with Streptolysin O

use of streptolysin

Barry *et al.* (1993) originally concluded that oligonucleotides could be introduced into cells by permeabilization with streptolysin O, on the basis of antisense effects observed in a transformed mouse kidney cell line. It was subsequently confirmed, using a fluorescein-tagged oligodeoxynucleotide, that reversible plasma membrane permeabilization with streptolysin O did indeed permit entry of the oligomer into the cytoplasm of human leukemia cells, to the extent that intense nuclear accumulation was observed (Spiller and Tidd, 1995) analogous to that produced by direct intracytoplasmic microinjection (Chin *et al.*, 1990). A two-parameter flow cytometric technique for optimizing permeabilization was established which has proved invaluable, since not only do different cell lines vary in their sensitivity to the treatment, but different batches of streptolysin O (Sigma) can exhibit profound variation in potency.

preparation of streptolysin O solution

Streptolysin O is dissolved in permeabilization buffer [137 mM NaCl; 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); 1,4 piperazine diethanesulfonic acid (PIPES), pH 7.4; 5.6 mM glucose; 2.7 mM KCl; 2.7 mM EGTA; 1 mM Na-ATP; 0.1 % bovine serum albumin] and activated with dithiothreitol (5 mM) for 2 h at room temperature. The batch is tested for activity with cells before being divided into aliquots and stored frozen at -20 °C.

optimization of permeabilization

Permeabilization efficiency is dependent on the number of cells per tube and the volume of streptolysin O solution, as well as its concentration, and so optimization should be performed on the same scale as the anticipated antisense experiment. The following is an example of an experiment to determine the optimum streptolysin O concentration.

1. Cells are washed once in permeabilization buffer, and samples of 10^6 cells are suspended in 0.1 ml of permeabilization buffer containing streptolysin O at a range of concentrations, and fluorescein-tagged oligodeoxynucleotide at 1 μM. The optimum concentration of streptolysin O may vary over the range of 10–200 U/ml, depending on the batch.
2. The suspensions are incubated at 37 °C for 20 min before resealing is induced by addition of 1 ml tissue culture medium containing 10 % fetal bovine serum.
3. Incubation at 37 °C is continued for 30 min.
4. Samples of the cultures are diluted 10-fold with culture medium containing propidium iodide (10 μg/ml) and left on ice for 10 min to stain dead cells before being analyzed by flow cytometry.

The flow cytometer is fitted with a 515–548 nm band-pass filter (green fluorescence photomultiplier) and a 620 nm long-pass filter (red fluorescence photomultiplier). Electronic compensation is applied to correct for spillover, and all measurements are made on a minimum of 5000 cells. For each streptolysin O concentration, the percentage of cells in 3 sub-populations is tabulated according to the following criteria: (1) background fluorescence signal in both channels: viable cells which were not permeabilized during treatment with streptolysin O; (2) both red and green fluorescence signals: dead cells; and (3) green fluorescence signal only: viable cells which were successfully permeabilized for entry of oligodeoxynucleotide during treatment with streptolysin O and subsequently resealed their plasma membranes.

In practice, there is always a trade-off between failure to permeabilize the cell population completely and significant levels of cell kill. Choice of the optimum streptolysin O concentration will depend upon consideration of the aims of the antisense experiment. It remains to be shown that this method is equally effective for ribozyme delivery.

4.3 Microinjection

precise control

The delivery of ribozymes or antisense DNA to a specific sub-cellular localization can, at least partially, be achieved by microinjection (Sczakiel *et al.*, 1993). The advantages of this technique include the delivery of defined concentrations and, correspondingly, amounts of material. Further, the stoichiometry between the target or its genetic information and the ribozyme is defined when both are co-microinjected. Comparison of the efficacy of related ribozymes or antisense molecules that differ, for example, in the kind of chemical modification or their length is not influenced by uptake or extracellular stability when being microinjected. Further, this conceptual advantage of the microinjection method can be used to deliver directly and to compare different kinds of inhibitors for the same target. For example, ribozyme-mediated inhibition can be compared with inhibition of the same target (e.g., viral functions in an infected cell) by low molecular weight inhibitors.

comparison of different inhibitors

Technically, microinjection is easiest with frog oocytes. However, in many cases mammalian tissue culture cells or primary cells are a more relevant biological system. Microinjection either into the nucleus or into the cytoplasm can be performed. Beside the advantages of microinjection, reproducibility and error range are sometimes unsatisfactory, which seems to be not only due to the cell culture conditions but also to the complexity of this technique. A standard deviation of microinjection experiments in the range of 15 % to 30 % is probably the best one can reach. Similar conclusions have been made with the microinjection of antisense DNA molecules.

4.4 Other Methods Used in Nucleic Acid Transfection

transfection agents

Nucleic acids of almost all sizes can be used for transfection into living cells according to established protocols. For example, transfection of DNA (>50 nt) into human cells has been achieved successfully by using calcium phosphate co-precipitation (Chen and Okayama, 1987) or commercial transfection reagents (e.g., DOTAP; Rittner *et al.*, 1993). In the case of exogenous targets, such as many viral sequences, the target can be co-transfected together with a ribozyme. Transfection experiments with *in vitro* synthesized mRNA coding for the enzyme chloramphenicol acetyltransferase (CAT) showed that the transfected RNA was intact for up to two days, and biologically active as indicated by the detection of CAT activity in cell lysates (Rittner *et al.*, 1993). These results indicate that transfected ribozymes may be present intracellularly in an active form over a period of time that is long enough for mediating significant inhibitory effects. Further, this experiment shows that transfected RNA enters the cytoplasm, which is the location of a number of potential target RNA sequences. However, it remains open whether and to what extent transfected RNA may enter the nucleus.

When co-transfected with proviral infectious HIV-1 DNA into human cells, an *in vitro* synthesized long-chain hammerhead ribozyme led to surprisingly strong inhibition of HIV-1 replication, even though the excess of ribozyme (30 ng/ml) over proviral DNA (10 ng/ml) was remarkably low (appr. 3:1; Homann *et al.*, 1993) when considering the fact that the transfected HIV-1 DNA obviously gives rise to a significantly larger number of target transcripts.

4.5 Electroporation

Zimmerman *et al.* (1976) used electroporation to induce the fusion of plasma membranes, and Wong and Neumann (1982) first used the technique to introduce DNA into fibroblasts. The technique is now commonly used to aid the uptake of proteins and nucleic acids into a range of cell types (Keating and Matthews, 1995).

4.5.1 Method

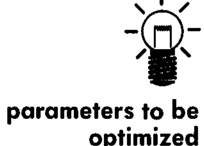
The following method is as optimized by Neumann *et al.* (1982) for the transfection of both circular and linear DNA into mouse LTK cells.

1. Trypsinize, wash and resuspend mouse LTK cells in HEPES buffered saline (HBS: 140 mM NaCl; 25 mM HEPES; 0.75 mM Na_2HPO_4, pH 7.1; at 20 °C) to a final concentration of 5×10^7 cells/ml.

2. Incubate the cell suspension in up to 20 μg DNA/0.4 ml for 10 min.
3. Sterilize the sample cell by treatment with 70 % alcohol. Gloves and mouth protection should be worn when transferring the solution in step 2 to the sterile cell.
4. Three successive pulses are applied at an interval of 3 sec. The sample is kept at 20 °C at a field strength of 8±0.5 kV/cm.
5. Ten minutes after the electrical impulses, aliquots of 100 μl of cell suspension are transferred to DMEM.
6. After 24 h, incubate the cell monolayers in HAT (Hypoxanthine, Aminopterin and Thymidine) medium under standard conditions.

4.5.2 Transfection: Optimization of Conditions

Although Neumann *et al.* (1982) recommend optimizing the technique for each cell line, and especially for cell size, it is noted that even for non-optimized conditions the yield obtained is comparable to other methods. This lesser dependence on cell type, compared with other methods, is thought to be due to the physical nature of the procedure (Chu *et al.*, 1987).



parameters to be optimized

The main parameters which should be optimized for each cell line are field strength, number of pulses, length of intervals, incubation period before addition of complete medium, and DNA concentration. Winterbourne *et al.* (1988) found field strength and pulse duration to be the critical parameters. Optimum results were obtained using the minimum number of pulses of longest possible duration and highest field strength compatible with cell viability. The maximum concentration of DNA which has minimal cytotoxicity can then be determined using the optimal electrical parameters (Herr *et al.*, 1994).

4.5.3 Mechanism of Uptake Following Electroporation

Electroporation results in the formation of pores in the cell membrane. These pores may form at the points of structural defects in the cell membrane, due to the interaction of the external electric field with lipid dipoles in a pore configuration (Neumann *et al.*, 1982). Generally, it is believed that nucleic acid diffuses into the cytoplasm following the formation of these pores in the plasma membrane by electroporation. However, efficient diffusion through such pores has been found to be limited to much smaller molecules than nucleic acid (Kinosita and Tsong, 1977), and Winterbourne *et al.* (1988) propose that the DNA enters the cell by electroporation-mediated injection of DNA into the cells, due to the difference between the electrophoretic mobility of cells and DNA. The DNA then passes end-on through the pores formed by electroporation. That is, uptake is not due to diffusion

alone, but is aided by the electrophoretic movement of the molecules involved.

4.5.4 Benefits and Drawbacks of Electrophoretic Mediated Uptake

high efficiency

A major point in favor of electroporation as a transfection procedure is that the majority, in fact up to 100 %, of the viable cells are transfected. Thus selection of transfectants is not normally required. Another positive aspect of electroporation relative to conventional uptake mechanisms is the state of the integrated nucleic acid. The protocol can be altered to give between one and many copies of the inserted nucleic acid per cell. This procedure has a lower mutation frequency than most uptake procedures. The lack of entrapment of oligonucleotides in endosomes also renders this method more useful than many of the conventional uptake systems. Furthermore the capacity to transfect large nucleic acid fragments, up to 150 kb, and integration into the host genome, render the technique versatile (Thomas and Capecchi, 1987). Electroporation can be used equally well for transient or stable transfection, since it gives similar transfection efficiencies. The major drawback of this method is that a large proportion of the cell population is usually susceptible to electrophoresis-mediated nucleic acid cytotoxicity (Winterbourne *et al.*, 1988).

4.6 Diethylaminoethyl-Dextran (DEAE-Dextran) and DNA Transfection

McCutchan and Pagano (1968) reported a transfection with diethylaminoethyl-dextran-mediated SV40 DNA uptake. The method has since been used to gauge the infectivity of a range of viruses, including SV40, polyoma, adenovirus, HSV-1 and AAV1. In 1981, DEAE-dextran was first used to facilitate the uptake of plasmid DNA (Sompayrac and Danna, 1981). The method is still commonly used.

4.6.1 Methods for Transfection of Adherent Cells

The following is based on the method described by Sompayrac and Danna (1981) for transfection of BSC-1 monkey cells.

1. Plate cells in log phase in 35 mm Petri dishes and culture in 3 ml of appropriate serum-supplemented medium.
2. Incubate the cultures at 37 °C until slightly subconfluent, then wash twice in serum-free medium.

3. Dissolve 200 μg/ml of DEAE-dextran (mol. wt. 2×10^6) in Dulbecco's minimum essential medium (DMEM) and 0.05 M Tris-HCl (pH 7.3). Slowly add 1.5 μg of 10^5 cells. Shake DEAE-dextran constantly while adding DNA.
4. Add 0.7 ml of DEAE-DNA solution to cells, ensure it is spread well over the cells and incubate at 37 °C in a CO_2 incubator for 8 h.
5. Wash the cells in serum-free DMEM and incubate at 37 °C in appropriate serum-supplemented medium.
   An assay for transient transfection efficiency, in this case a standard plaque assay for infectivity of viral DNA, can be carried out over the next 2–10 days.
6. One day after infection, trypsinize cells and replate them on to subconfluent monolayers of BSC-1 cells in 60 mm Petri dishes, each Petri dish receiving a sufficient number of cells to produce approximately 50 plaques. Incubate the cells at 37 °C overnight.
7. Replace the medium with 5 ml of DMEM supplemented with 2–5 % serum and 1 % noble agar.
8. Incubate the cells at 37 °C for 6–8 days.
9. Add an overlay of 3 ml of 0.1 % neutral red in DMEM containing 0.5 % noble agar. Return plates to incubator overnight.
10. Count plaques.

The main difference between the above method and the standard procedure is step 4. Traditionally this incubation would have been carried out for 30 min at room temperature (McCutchan and Pagano, 1968). This assay is optimized for the maximum specific infectivity of DNA rather than for the percentage of cells infected. Longer incubation periods were not used, since the DEAE-dextran at the concentration used in standard assays would be cytotoxic. Thus, Sompayrac and Danna (1981) decreased the DEAE-dextran concentration to 200 μg/ml, and increased the incubation period to 8 h. These protocol alterations resulted in the transfection efficiency of BSC-1 cells increasing from ~3.5 % with the standard assay, to ~25 %. When the incubation period was further extended to 16 h, up to 50 % of the cells were infected; however, the reproducibility of this result was poor.

4.6.2 Possible Alterations of the Above Protocol

Glycerol/DMSO Shock

The transfection efficiency of some cell types is increased following treatment with a glycerol/DMSO shock, as described in the calcium phosphate coprecipitation method (see below). The procedure is as

outlined for calcium phosphate with two exceptions: the optimum time for harvest of cells is 72 h post-transfection, and between 8 and 25 μg/60 mm culture plate of plasmid DNA should be used. Lopata *et al.* (1984) saw a 50-fold increase in the level of expression of the transfected gene in mouse L cells following either a glycerol (15 %, 0.5–2 min) or a DMSO (10 %, 2–30 min) shock. Sussman and Milman (1984) reported that DMSO shock resulted in 80 % of recipient cells expressing the transfected DNA, a 15-fold increase over that obtained without the shock treatment.

Chloroquine Treatment

Chloroquine was found to increase the fraction of cells expressing the DEAE-dextran transfected gene by approximately 20-fold. (Luthman and Magnusson, 1983). Banerji *et al.* (1983) followed the same procedure, and found a 2–5 fold increase in expression of the transfected gene. Chloroquine should be added to the growth medium in step 5 of the above procedure. The optimal concentration varies between cell lines and 100 μM can be used as a starting concentration. 2–5 h later the chloroquine-containing medium should be replaced with the standard, serum-supplemented culture medium, and the incubation continued as usual. The mechanism of action is as described for the calcium phosphate co-precipitation method.

Sodium Butyrate Treatment

Sodium butyrate treatment of cells, following transfection by either calcium phosphate or DEAE-dextran, was carried out by Reeves *et al.* (1985). They found a marked increase in the expression of the transfected gene. Furthermore, several alterations in the structure of the minichromosomes formed by the transfected genes were noted. The new form resembled those believed to be active chromatin.

Carbon Dioxide Shock

Carbon dioxide shock was found by Sussman and Milman (1984) to increase transfection efficiency. The same step, which was described as somewhat inconvenient, failed to increase transfection efficiency according to Lopata *et al.* (1984).

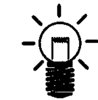
4.6.3 Transfection of Cells Growing in Suspension

The protocol listed below is based on that successfully used by Fujita *et al.* (1986) in transfecting the cell lines Raji, Jurkat and EL-4 cells in suspension.

1. 2×10^7 cells in logarithmic phase should be collected by centrifugation (1500 r.p.m., 5 min, room temperature).

2. Wash the pellet twice in TBS (25 mM Tris-HCl; 137 mM NaCl; 5 mM KCl; 0.7 mM $CaCl_2$; 0.5 mM $MgCl_2$; 0.6 mM NaH_2PO_4 ; pH 7.4) and collect cells as in step 1.
3. Resuspend the pellet in 1 ml TBS containing 10 μg of plasmid DNA and 500 μg of DEAE-dextran. Incubate in a laminar air flow cabinet at room temperature for 30 min, tapping the cells occasionally to prevent clumping.
4. Collect the cells by centrifugation (1000 r.p.m., 5 min, room temperature) and wash twice in TBS.
5. Resuspend the cells in appropriate serum-supplemented medium and incubate at 37 °C at a suitable CO_2 level for 24–48 h. Cells should be split as necessary to prevent them entering stationary phase.
6. Assays of transient transfection can then be carried out.

4.6.4 Transfection Optimization



DNA concentration

The optimal concentration of DNA to be transfected varies between cell lines, and therefore must be determined experimentally for each system. Selden *et al.* (1986) noted that increasing the DNA concentration does not always result in a higher level of expression. In their system, with 30–50 % confluent mouse L cells, transfectant expression was linear with plasmid DNA concentration up to ~4 μg/10 cm culture dish. Between 1 and 10 μg of DNA should be used in initial experiments.

cell concentration

The optimal cell concentration at which transfection should be performed is very variable. Generally, transfections are carried out when cells are 20–50 % confluent for adherent cells, and 2×10^7 in suspension cultures. Selden *et al.* (1986) recommend that cells which are more sensitive to toxic effects of DEAE-dextran should be plated at higher densities, as should certain primary cultures.

DEAE-dextran concentration

The optimal concentration of DEAE-dextran varies between cell lines from 100–1000 μg/ml. Since DEAE-dextran can be cytotoxic, especially over long incubation periods, the balance of the increased efficiency and cytotoxicity must be determined for each cell line. Generally, between 100–500 μg ml is suitable for most cell lines, both adherent and in suspension.

The majority of adherent cell types should be incubated for 4–8 h in step 4. However, some cell types are transfected better with a shorter incubation, sometimes as little as 30 min.

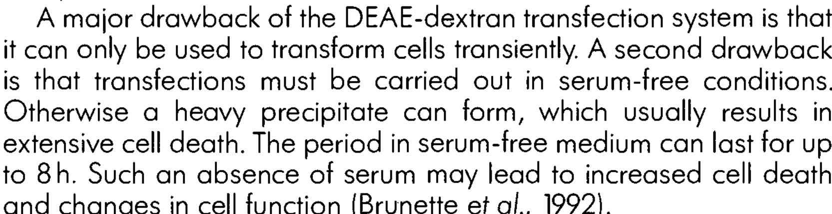
The onset of transfectant expression can vary from 24 h to several days after the transfection incubation (step 4). The duration of this expression is also variable, ranging from less than one day to several weeks.

4.6.5 Mechanism of DEAE-Dextran Uptake and Intracellular Distribution

The mechanism by which DNA uptake is facilitated by DEAE is poorly understood. It is thought that DEAE helps binding of the nucleic acid to the plasma membrane. Increasing resistance to nucleases is another proposed mechanism. It is generally accepted that the DEAE-nucleic acid complex enters the cell by endocytosis. All of the supercoiled plasmid DNA which reaches the nucleus following DEAE transfection of mammalian cells is rapidly assembled into nucleosome-containing minichromosomes (Reeves *et al.*, 1985).

4.6.6 Benefits and Drawbacks

The main factor in favor of DEAE-dextran is that it is a simple, reproducible technique. The standard method gives a low transfection efficiency of approximately 3–5 %. However, alterations of this protocol, as listed above, have resulted in uptake into up to 25 % of BSC-1 monkey cells (Sompayrac and Danna, 1981). Another point in favor of this method is that it can successfully transfect some cell lines which cannot be transfected by the calcium phosphate coprecipitation method, such as BSC-1 cells. (Sompayrac and Danna, 1981) The method is versatile, in that it can be used to transfect monolayers and some cells growing in suspension (Banerji *et al.*, 1983; Fujita *et al.*, 1986).



A major drawback of the DEAE-dextran transfection system is that it can only be used to transform cells transiently. A second drawback is that transfections must be carried out in serum-free conditions. Otherwise a heavy precipitate can form, which usually results in extensive cell death. The period in serum-free medium can last for up to 8 h. Such an absence of serum may lead to increased cell death and changes in cell function (Brunette *et al.*, 1992).

4.7 Calcium Phosphate Transfection

The calcium phosphate-DNA coprecipitation technique was first described when Graham and van der Eb (1973) used the method to introduce adenovirus DNA into human cells. Wigler *et al.* (1978) later adapted the technique for stable transfection of mouse cells with exogenous DNA. Today, calcium phosphate coprecipitation is the most commonly used method of DNA transfection. The same standard method is also used as an RNA transfection system (Kleinschmidt and Pederson, 1990).

4.7.1 Method

Note: HEPES Buffered Saline Solution (HeBS) and calcium chloride should be filter sterilized, stored at 4 °C, and warmed to room temperature before use.

1. The cells are split into 10 cm dishes such that confluence has almost been reached on harvesting. The medium should be replaced with 9 ml of complete medium 2–4 h prior to transfection.
2. 10–50 µg of nucleic acid is diluted into 450 µl of sterile water.
3. Calcium chloride is then added, to a final concentration on mixing with the HEPES buffered saline of 125 mM.
4. The mixture is then added dropwise, using a sterile Pasteur pipette, to 500 µl 2xHEPES buffered saline (pH 7.12). Some protocols recommend bubbling the HeBS whilst adding the nucleic acid-calcium chloride mixture, followed by immediate vortexing of the solution. The precipitate, which forms immediately, should be translucent. An opaque solution indicates the HeBS is not at the correct pH.
5. The solution should be left to stand for 20 min at room temperature. During this incubation a precipitate will form.
6. Add 1 ml of the precipitate formed to 10 ml of prewarmed culture medium, and mix the solution gently before replacing the medium.
7. Incubate the cells at 37 °C and appropriate carbon dioxide levels. It is important to ensure the optimal CO_2 level during incubation, as pH is critical throughout this procedure. The incubation period is traditionally 4–5 h; however, more recent protocols have successfully used up to 24 h (Kleinschmidt and Pederson, 1990). After this incubation, the precipitate should appear as small grains covering the cells. If the medium is too acidic, no precipitate will form. If it is too alkaline, there will be large spheres of precipitate in the medium (Gorman, 1985).
8. After this period, the cells should be washed twice in medium and resuspended in 10 ml of complete medium.
9. Harvest the cells up to 48 h later. Transient expression can be tested at this stage.

In the original method by Graham and van der Eb (1973), the DNA is diluted directly into HeBS. This solution is then added to calcium chloride. This is in stark contrast to most modern protocols, which stress the importance of adding these solutions in the order listed above.

4.7.2 Possible Alterations to Above Method

Stable Transfection

In stable transfection assays, the cells are not harvested as above. Instead, the incubation period in step 9 is reduced to between 12 and 24 h. The growth medium is then removed and selective medium added. The cells are sustained in this selective medium until clones have developed, which is a matter of 2–3 weeks. After this time the colonies are counted, cells trypsinized, and chosen colonies analyzed. An example of such an application is given by Wigler *et al.* (1978). The nucleic acid should be precipitated with ethanol, in order to sterilize it, for use in stable transfection assays.

Glycerol Treatment

increased transfection efficiency

Exposure to glycerol can increase transfection efficiency. Frost and Williams (1978) found that treatment with 20 % glycerol resulted in a 3–6 fold enhancement of transfection of 293 cells, while a 10–50 fold enhancement was seen in HeLa cells. Furthermore, no increase in toxicity was seen. Glycerol treatment should be included after step 7 above. The cells should be washed in serum-free medium and 0.5 ml of 10–20 % glycerol in HeBS added to each 25 mm^2 flask. The flask should be incubated at 37 °C for 30 sec – 3 min. The first washing medium of step 8 should be added to the glycerol solution, to act as an immediate stop solution. The optimal incubation period varies between cell lines, and the glycerol solution should be removed before the cells start to shrink. Gorman (1985) notes that where the incubation with calcium phosphate has been extended to 18 h, the addition of glycerol seems to have less effect. Kingston (1989) recommends an incubation period of 4–6 h when followed by glycerol shock.

Dimethylsulfoxide Treatment (DMSO)

The above procedure can be used with 10–20 % DMSO in place of glycerol, but this procedure does not appear to be as successful as glycerol shock. Frost and Williams (1978) saw a 10–20 fold increase in transfection of HeLa cells. However, no such increase was associated with the majority of transfections into 293 cells. Generally, this method is thought to be successful in some cell types only.

Chloroquine Treatment

Treatment of the cells with chloroquine diphosphate has been found to increase the transfection efficiency of viral DNA by increasing the fraction of cells transfected (Luthman and Magnusson, 1983). They increased the calcium phosphate-mediated transfection efficiency of polyoma virus DNA approximately six-fold. It may act by inhibiting lysosomal degradation of the DNA following endocytosis. Alterna-

tively, the chloroquine may protect the oligonucleotides from nuclease degradation by steric block, since chloroquine is known to bind tightly to DNA (Cohen and Yielding, 1965). Chloroquine should be added to the culture medium, with the DNA precipitate, to a final concentration of 200 μg/ml in DMEM. The exact concentration of chloroquine should be optimized for each cell type. This method should not be used in conjunction with an increased incubation period with calcium phosphate precipitate, as chloroquine diphosphate can be extremely cytotoxic over long periods. Most cell lines cannot survive more than 4 h incubation with chloroquine. In initial studies, the cells should be watched closely and the medium changed immediately should the cells start to deteriorate.

Sodium Butyrate Treatment

Treatment with sodium butyrate immediately following the uptake of exogenous DNA increases both transient and stable expression of the transfectant. Gorman and Howard (1983) found transient expression increased 3–4 fold to 40 % following sodium butyrate treatment in monkey kidney CV1 cells, while stable expression in the same cell line increased from 1–10 %. This increase in transient expression appears to involve two mechanisms: the number of expressing cells, and an increase in enhancer-dependent transcription of particular plasmids containing SV40, polyoma or papilloma virus control regions. Sodium butyrate, to a final concentration of between 2 and 10 mM, should be added to the medium in step 8 above and incubated overnight as usual. The butyrate medium solution should then be removed and the cells re-fed. Other chemical treatments, such as cytochalasin D, colchicine and Colcemid, have also been used successfully to increase transfection efficiency (Farber and Eberle, 1976).

Song and Lahiri (1995) developed a method for efficient and equal calcium phosphate transfection of large batches of cells. In brief, cells are pooled and resuspended in medium. Calcium phosphate-DNA precipitate, formed in the standard way, is added directly to all of the cells. This solution was mixed prior to incubation at room temperature for 30 min before the cells were divided evenly on to a set number of plates. The cells are cultured in 5 ml of medium and harvested 48 h later. They found that this method not only reduced variability of transfection efficiency between plates, but also gave improved promoter activity.

4.7.3 Method Optimization

The main parameters which should be optimized for each cell line are nucleic acid concentration, incubation period and inclusion of glycerol, chloroquine or sodium butyrate treatment.

nucleic acid concentration

The optimal concentration of nucleic acid is generally higher than in DEAE-mediated transfections. This concentration varies from 10–50 μg between cell lines. The use of carrier nucleic acid is another

consideration. Where a low concentration of nucleic acid is used, a carrier should be added to increase the concentration to a final concentration of 20 µg/ml. Generally, plasmid DNA is not thought to require a carrier. The nature of the nucleic acid should also be considered. DNA from any source, whether eukaryotic, bacterial or viral, can be transfected by this method, as long as appropriate regulatory sequences for expression in mammalian cells are present (Spandidos, 1984). Supercoiled DNA results in efficient gene transfer. Impurities in the DNA preparation can reduce transfection efficiency, so the preparation should be purified twice by cesium chloride centrifugation (Kingston, 1989). Calf thymus DNA, salmon sperm DNA, or DNA isolated from eukaryotic cells can be used as carrier DNA. The last, when prepared in the laboratory, usually gives a 2–3 fold higher transfection efficiency than either of the commercially available DNA preparations (Spandidos, 1984).

incubation period

The optimal incubation period with the precipitate is also highly variable between cell lines. While long incubations are required for any uptake into some cell types, other cell lines cannot survive such an exposure. In initial experiments, cells should be closely observed for signs of deterioration. The trypan blue exclusion assay can be used when one is unsure of cell state. The optimal incubation period can be determined in this way.

In stable transfections, the incubation in non-selecting medium, step 9, should allow expression of the transfected gene. The optimal period varies between cell lines, ranging from 12–24 h. As such, the effect of a range of incubation periods on the rate of transformation should be examined.

glycerol treatment

Kingston (1989) suggests carrying out a crude initial experiment to determine whether glycerol treatment is beneficial to the transfection. He suggests starting with 10 % glycerol for 3 min. If the procedure increases transfection efficiency, fine tuning experiments can then be carried out. The same principle could be applied to chloroquine and sodium butyrate treatment.

The precipitate which forms in step 5 above should be in solution. An uneven precipitate can result in low transfection efficiency. In this case, the nucleic acid should be mixed more slowly with the HeBS, and vortexed throughout. An alternative technique involves passing the donor DNA 5 times through a 19-gauge syringe needle to produce lower molecular weight DNA. The carrier DNA can also be sheared in this way if required; however, the average molecular weight should not be less than 10 kb (Spandidos, 1984).

4.7.4 Calcium Phosphate-Mediated Uptake and Intracellular Distribution

Graham and van der Eb (1973) developed this method on the basis that divalent cations are known to be essential for uptake of DNA into bacterial cells. They found that, at sufficiently high concentrations, cal-

mechanism of uptake

cium ions enhanced the uptake of viral DNA into human KB cells. They proposed that the technique proceeds in two stages, adsorption and uptake, where adsorption is the association of the precipitate with the cell membrane, and uptake occurs by a calcium-requiring mechanism. The precipitation of DNA increases the concentration of DNA on the cell surface. Loyter *et al.* (1982) found that calcium phosphate enters the cells in the same way whether free or complexed to DNA, indicating the uptake pathway is non-specific. No precipitate-associated fluorescence was seen in ATP-depleted cells, indicating the uptake process is active. Thus these results indicate uptake may occur by pinocytosis. Other evidence presented in this chapter suggests that calcium phosphate induces phagocytosis, thus facilitating uptake into the cells.

On fluorescent staining of the precipitate, punctate cytoplasmic vesicles are seen in numerous cells, while very little nuclear staining was apparent. Nuclear staining is seen in 1–5 % of the cells with cytoplasmic staining. Although free nucleic acid and nucleic acid bound to calcium phosphate are thought to enter the cell in the same way, the free form is not seen in the nucleus. Thus the limited nuclear staining seen appears to be directed by the calcium phosphate treatment.

4.7.5 Benefits and Drawbacks of Calcium Phosphate-Mediated Uptake

The main benefits of calcium phosphate mediated nucleic acid uptake are as follows:

1. This method is very versatile as it can be used for either transient or stable transfection of cell lines. Furthermore, the method can be applied to both monolayers and some suspension cultures.
2. Calcium phosphate-DNA precipitates are resistant to nucleases present in serum, as well as those added externally (Loyter *et al.*, 1982) This resistance to nucleases should result in an increase in the amount of full length DNA available to act in the cell.
3. Where the cells are incubated with a mixture of different plasmids, the proportion of each plasmid taken up by the transfected cells will reflect the ratio in the original mixture.

The main drawbacks are as follows:

check pH of stock solutions

1. This method can be difficult to reproduce. Graham and van der Eb (1973) noted that this can be due to the highly pH-sensitive nature of the method. The optimal pH range is very narrow. Thus if the procedure becomes irreproducible, the pH of the stock solutions, namely HeBS, calcium chloride, and culture medium, should be checked. The CO_2 levels in the incubators should also be noted.

2. Loyter *et al.* (1982) found that approximately two-thirds of the cell-associated calcium phosphate-DNA complex was extracellular. EDTA and nuclease digestion studies, together with fluorescent labeling of the internalized complex, demonstrated that while at least 20.3 % of the DNA added was cell associated, only 7.3 % of the total DNA added was intracellular. Thus calcium phosphate coprecipitation may not be as efficient as it appeared at first. This should be taken into account when determining the efficiency of the method.
3. The method's parameters can vary greatly between cell lines, thus optimization is essential for each cell line.

4.8 Cationic Lipids

(See appendix for full chemical titles)

Cationic lipids were first used in cultured cells by Felgner *et al.* (1987) in the introduction of a transient expression vector into a range of mammalian cell types. Malone *et al.* (1989) developed the method for transfection with mRNA. Cationic lipids have since been routinely used for the delivery of antisense DNA (Felgner *et al.*, 1987; Bennett, 1992; Capaccioli *et al.*, 1993; Cumin *et al.*, 1993), and are also the favored vehicle for transport of ribozymes into cells (Sioud, 1994; Leopold *et al.*, 1995; Karikó *et al.*, 1994; Malone, 1995). A range of cell types, mammalian and otherwise, have been transfected using such lipids (Malone *et al.*, 1989; Karger and Komro, 1990). *In vivo* transfection has also been successfully carried out (French *et al.*, 1994; Felgner *et al.*, 1995).

4.8.1 Cationic Lipid Formulations

classification

There are a number of cationic lipids now commercially available. They can be classified according to their nucleic acid binding domain, which can be monoamine or polyamine, and their hydrophobic domain: oleic acid side chains, cholesterol, and shorter chain and/or saturated lipids. Those that are used most are classified, in this way, below:

Hydrophobic binding domain	*Monoamine*	*Polyamine*
Oleic acid	Lipofectin (DOTMA) DOTAP	Lipofectamine (DOSPA)
Shorter chain+/or saturated lipids	DMRIE-C	DOGS
Cholesterol	DC-Chol	

The above contain two lipid species: a cationic lipid, after which the compound is named, and a neutral phospholipid, DOPE. The activity relative to the chemical structure of such compound is still being researched (Felgner *et al.*, 1994). Capaccioli *et al.* (1993) compared unmodified oligodeoxynucleotide uptake by three different cationic lipids into human leukemia T cells, human colon adenocarcinoma, and murine fibroblasts. While an increase in uptake was seen with each of the three lipids, DOTAP was most effective in all three cell lines. Cell uptake mediated by DOTMA was slightly less effective in each cell line, while DOGS-mediated uptake was significantly less effective in two of the three cell lines tested. Gibco BRL found the efficiency of cationic lipid-mediated RNA transfection into BHK-21 cells decreased in the following order: DMRIE-C, Lipofectin, Cellfectin and Lipofectamine (Ciccarone *et al.*, 1995). DOTAP yielded transfection rates comparable to DOTMA in cultured primary rat hepatocytes, at both a lower concentration and considerably less cost. The manufacturers of lipofectamine found it to have a two- to thirty-fold greater DNA transfection efficiency than lipofectin in NIH3T3 cells (Hawley-Nelson *et al.*, 1995). However, they found lipofectin to be generally more efficient for transfection with RNA. Malone (1995) found that, in general, RNA transfection with monoamine-based, short side chain lipids was more effective. The danger of drawing general conclusions and applying them to studies on different cell lines is well demonstrated by the work of Leventis and Silvius (1990). They found that DOTB (1,2-dioleoyl-3-(4′-trimethylammonium) butanolyl-sn-glycerol) was the most effective of the cationic lipids tested in CV-1 cells. However DOTAP was five times more effective than DOTB in 3T3 cells. In conclusion, on reading through the literature it became apparent that some cationic lipids are more efficient in certain cell lines than others. It is also obvious that transfection systems for each cell line must be optimized for each cationic lipid individually. Other studies using the same transfection system should be seen only as guidelines.

choice of cationic lipid

4.8.2 Methods

The protocols for use of cationic lipids are very straightforward and are clearly outlined in the manufacturers' instructions. The protocol details for most cationic lipid types are similar. Those for DOTMA (Lipofectin)™ and DOSPA (Lipofectamine)™ are as follows:

The following protocols are based on use of 35 mm culture dishes, which are adequate for most applications. Where larger vessels are required, the manufacturer recommends increasing the volumes proportionally in the following ranges; in 60 and 100 mm culture plates use the following: lipid and nucleic acid diluted volumes of 300 and 800 μl respectively, cationic lipid volumes of 6–75 μl and 16–200 μl respectively, volume of nucleic acid 3–6 μl and 8–16 μl respectively. Total transfection medium volume should be 2.4 ml/60 mm plate and 6.4 ml/100 mm plate.

general comments

4.8.3 Lipofectin-Mediated Transient Transfection of Adherent Cells

1. Seed cells at 1–2×10^5 cells in 2 ml of the normal growth medium for that cell type, including serum.
2. Incubate at 37 °C until a certain confluence is reached; the optimal cell density for use with this protocol will vary between cell lines. Once the optimal concentration has been found, the cell concentration should be kept constant between experiments.
3. Prepare the following solutions for each transfection as follows:
   Solution A: 1–2 µg of nucleic acid in 100 µl of Opti-MEM 1 (Gibco BRL) reduced serum medium.
   Solution B: 2–20 µl of Lipofectin reagent in 100 µl of serum-free medium.
4. Combine solutions A and B to give solution C. Mix gently, then leave to stand for 10–15 min at room temperature.
5. Wash the cells once with 2 ml of serum-free medium.
6. Add 0.8 ml of serum-free medium to each Petri dish of solution C, giving solution D. Mix gently just prior to adding to the cells. The culture dish should be gently swirled whilst adding solution D.
7. Incubate the cells for 5–24 h at 37 °C in a CO_2 incubator.
8. Fresh, serum supplemented medium can be added in one of two ways:
   - Replace solution D with 2 ml of normal growth medium
   - Add 1 ml of growth medium containing twice the normal serum concentration required to solution D.

   The first option should be employed where toxicity of solution D is a problem.
9. Reincubate cells for a total of 48–72 h, then test cell extracts for transfectant activity.

4.8.4 Lipofectin-Mediated Stable Transfection of Adherent Cells

1. Seed 1–2×10^5 cells/mm tissue culture plate in 4 ml of the normal serum-supplemented growth medium.
2. Incubate at 37 °C in a CO_2 incubator for 18–24 h; the cells should then be 30–50 % confluent.
3. Prepare the following solutions for each transfection:
   Solution A: 2 µg of nucleic acid into 100 µl of Opti-MEM 1 medium.

Solution B: 5–20 µl of lipofectin into 100 µl of Opti-MEM 1 medium.

4. Mix solutions A and B gently and leave to stand at room temperature for 10–15 min, to produce solution C.
5. Wash cells once in 2 ml of Opti-MEM 1 medium.
6. Add 0.8 ml of Opti-MEM 1 medium solution C, giving solution D, and mix gently just prior to adding to cells. The culture dish should be gently swirled whilst adding the medium.
7. Incubate the cells for 5–24 h at 37 °C in a CO_2 incubator.
8. Replace solution D with 4 ml of normal serum-containing growth medium.
9. Incubate the cells for another 48 h at 37 °C in a CO_2 incubator.
10. Subculture cells at a ratio of at least 1:5 into selection medium.

4.8.5 Lipofectin-Mediated Transfection of Cells in Suspension

1. Wash cells once with Opti-MEM 1 medium and resuspend $2–3 \times 10^6$ in 0.8 ml of OPTI-MEM 1 in a six-well plate or a 35 mm culture plate.
2. Prepare the following solutions for each transfection:
   Solution A: 1–5 µg of nucleic acid into 100 µl of Opti-MEM 1 medium.
   Solution B: 2–25 µl of Lipofectin into 100 µl of Opti-MEM 1 medium.
3. Combine solutions A and B to give solution C. Mix gently, then leave to stand for 10–15 min at room temperature.
4. Add solution C to the cell suspension whilst swirling the culture gently.
5. Incubate the cells for 5–24 h at 37 °C in a CO_2 incubator.
6. Add 4 ml of the appropriate normal growth medium to each culture.
7. Reincubate cells for a total of 48–72 h, collect cells by centrifugation, and then test cell extracts for transfectant activity.

4.8.6 Lipofectamine-Mediated Transient or Stable Transfection of Adherent Cells

Note: In contrast to lipofectin, the protocol for lipofectamine-mediated transfection of adherent cells is the same for stable and transient transfection.

1. Seed 1–3×10^5 cells per 2 ml of serum-supplemented growth medium into a six-well plate or 35 mm tissue culture plate.
2. Incubate at 37 °C until a certain confluence is reached; the optimal cell density for use with this protocol will vary between cell lines. Once the optimal concentration has been found the cell concentration should be kept constant between experiments.
3. Prepare the following solutions for each transfection:
   Solution A: 1–2 μg of nucleic acid in 100 μl of Opti-MEM 1 reduced serum medium.
   Solution B: 2–25 μl of Lipofectamine reagent in 100 μl of Opti-MEM 1.
4. Combine solutions A and B to give solution C. Mix gently then incubate for 15–45 min at room temperature.
5. Wash the cells once with 2 ml of serum-free medium whilst solution C is incubating.
6. Add 0.8 ml of serum-free medium to solution C. Mix gently just prior to adding to the cells. The culture dish should be gently swirled whilst adding solution C.
7. Incubate the cells for 2–24 h at 37 °C in a CO_2 incubator.
8. Following incubation, fresh serum-supplemented medium can be added in one of two ways:
   - Remove solution C and replace with normal growth medium.
   - Add 1 ml of growth medium, containing twice the normal serum concentration required, to solution C.

   The first option should be employed where toxicity of solution C is a problem. If step 6 included serum-supplemented medium, add 1 ml of complete medium at this stage.
9. At 18–24 h following the start of transfection, replace the medium with fresh complete medium.
10. In transiently transfected cells, assay cell extracts for transfectant activity 24–72 h after the start of transfection. When DNA is stably expressed, at 72 h post transfection, passage the cells 1:10 into the selective medium for the transfected reporter gene.

A comparison of DMEM and Opti-MEM 1 medium, by Karger and Komro (1990), for the incubation of cells with the DNA-lipofectin complex found that Opti-MEM 1 resulted in greater transfectant expression than DMEM. The need to dilute stock lipofectin and stock DNA before mixing together was also proven in this study.

choice of culture medium

4.8.7 Lipofectamine-Mediated Transfection of Cells in Suspension

This protocol is similar to that for Lipofectin (section 4.8.5) with the exception of the stages listed below:

2. Solution A: 1–3 µg of DNA in 100 µl of Opti-MEM 1 medium. Solution B: 2–25 µl of Lipofectamine in 100 µl of Opti-MEM 1.
3. Solution A and B should be allowed to stand for 15–45 min.
5. Incubate cells at 37 °C for 2–24 h.
7. Incubate cells for a total of 24 to 72 h.

4.8.8 Optimizing Transfection

optimization of all parameters

It is essential to optimize the transfection protocol in order to minimize cytotoxicity while maximizing efficiency. All components of the transfection reaction, that is, lipid volume, nucleic acid concentration, cell number, and period of exposure, should be optimized individually. This should be done by varying one parameter per assay until its optimum value is determined, starting with the lipid concentration. Using this information the next parameter can be varied. The manufacturers provide guidelines for the initial step in optimizing transfection: the volume of cationic lipid should be varied from 2–25 µl, while all other parameters are kept as follows: for lipofectin 1–2 µg of nucleic acid, 6 h incubation, and cells which have not reached confluence (40–60 % confluence could be used as a starting point); for lipofectamine 1–2 µg of nucleic acid, 5 h incubation period, and cell concentrations as listed in the protocols.

evaluation of cytotoxicity

Trypan blue dye exclusion assay, together with microscopic examination, can be used to evaluate cytotoxicity. Alternatively, protein concentration can be used as an indication of cell yield. It has been noted, in the case of both lipofectin and lipofectamine, that lipid alone appears to be more cytotoxic than lipid-DNA complexes (Padilla, unpublished). Other researchers suggest that cytotoxicity of cationic lipids may be significantly enhanced by the addition of nucleic acid (Malone *et al.*, 1989). For this reason, the optimization procedure should include the nucleic acid, to give a truer indication of toxicity, even though it may render optimization more expensive. It is often possible to increase cell viability and therefore transfection efficiency dramatically by increasing cell concentration. However, cells should never be confluent at the time of transfection. High densities such as are required for transfection with cationic lipid often reduce cell viability, with this effect being heightened with time. It should be noted that the factors to be optimized are interlinked. For instance, Bennett (1992) noted that optimal transfection efficiency is achieved at an equal molar ratio of positive charge from cationic lipid and negative

charge from nucleic acid. Thus these factors should be looked at simultaneously, after determining the optimal individual concentrations previously. Similarly, in considering the period of exposure of cells to cationic lipid, the cell density should be taken into account.

efficiency of nucleic acid uptake

The efficiency of cationic lipid-mediated uptake of nucleic acids varies between cell lines, and even within cell lines between studies. The reason for such differences in sensitivity are unknown. However, the normal function, in terms of influx and efflux, of the cell is bound to affect uptake. Bennett (1992) found that cationic lipids do not appear to aid uptake of oligonucleotides into some cell lines such as the human promyelocytic leukemia cell line, HL60, or the human T-cell leukemia cell line Jurkat. Other studies suggest lipofectamine and lipofectin increased the transfection efficiency in Jurkat cells (Hawley-Nelson *et al.*, 1995). Other cell lines vary in their susceptibility to cationic lipid-mediated uptake, different concentrations being required to achieve target gene expression. This discussion demonstrates that transfection optimization must be carried out for each cell line and for each set of transfection conditions.

Optimal cationic lipid-DNA complex formation and the initial incubation of 2–24 h requires serum-free medium (Felgner *et al.*, 1987). This absence of serum may lead to increased cell death and changes in cell function (Brunette *et al.*, 1992). The manufacturer recommends that if a cell line cannot tolerate the absence of serum for 2–24 h, both the lipofectin and lipofectamine protocols should be altered as follows.

Lipid-nucleic acid complexes should be formed as usual in Opti-MEM 1 medium. The complexes can then be diluted in 5–10 % fetal bovine serum-supplemented medium, such as Opti-MEM 1 or Dulbecco's MEM. This medium must be free of antibacterial agents. The addition of 5 % serum should have little effect on transfection efficiency. The manufacturer recommends that, in the case of lipofectamine, the transfection conditions be reoptimised if changes in serum status are made, as the optimal lipid concentration may differ. This does not appear to be necessary where transfection is lipofectin-mediated. Brunnette *et al.* (1992) found in all four lipid formulations they tested, namely lipofectin, transfectace, DOTMA:Cholesterol (1:1), and L-PE:CEbA (6:4), oligonucleotide transfection efficiency was at least as high in the presence of serum as achieved using the standard, serum-free protocol. Similar results were found by Hawley-Nelson *et al.* (1995) with Lipofectin™ and LipofectACE™ in CHO-KI and BHK21 cells. Lipofectamine was also tested in this system. Although a decrease in efficiency was seen in the presence of serum, the transfection efficiency was still comparable to or better than other transfection lipids (Malone, 1995). Total cellular protein levels in all the transfection methods was higher in the presence of serum, indicating that serum diminishes growth inhibition and/or cytotoxic effects associated with transfection. Thus optimizing the serum state of the medium may also increase transfection efficiency. Studies which carry out extensive cationic lipid optimization experiments include Felgner *et al.* (1987), Malone *et al.* (1989), Capaccioli *et al.* (1993) and Leopold *et al.* (1995).

4.8.9 Cationic Lipid Uptake and Intracellular Distribution

mechanism of uptake

Cationic lipids form small, unilamellar vesicles (average size 100 nm) when formulated as outlined in the protocols. The surface of these vesicles is positively charged. As such, they interact spontaneously with both DNA (Felgner *et al.*, 1987) and RNA (Sullivan, 1994). The nucleic acid is not thought to be encapsulated in the liposome as with conventional liposomes. In fact it is thought that 2–4 liposomes may assemble with a single 5 kb plasmid (Felgner *et al.*, 1987). The lipids are thought to neutralize the negative charge of the complexed nucleic acid, thereby facilitating fusion with plasma membranes (Felgner *et al.*, 1995). Thus, when added to tissue culture cells, nucleic acid-cationic lipid complexes interact with the cell membrane, resulting in lipid fusion (Malone, 1995). Electron microscope studies of uptake of various cationic lipids has shown attachment of liposomes to the cell membrane. Although cationic lipid fusion is thought to be understood (Duzgunes *et al.*, 1989), the mechanism of uptake into the cell is still unresolved. The two leading hypotheses for uptake of nucleic acid-lipid complexes into the cell are transfection via direct cytoplasmic delivery, and transfection via an endocytotic pathway. Felgner *et al.* (1987) assume direct fusion of the lipid to the cell membrane. Legendre and Szoka (1992) concluded that membrane fusion and endocytosis may work in parallel as uptake pathways for cationic lipids in a number of mammalian cell lines.

intracellular distribution of internalized nucleic acids

The use of cationic lipids appears to affect the intracellular distribution of the internalized nucleic acid in the cases of both DNA and RNA. Studies using fluorescently labeled oligodeoxynucleotides, without any uptake aid or modification, found that intracellular oligodeoxynucleotide tends to be concentrated in small, punctate vesicles in the cytoplasm (Loke *et al.*, 1989). These results suggest receptor mediated endocytosis is the main oligodeoxynucleotide uptake pathway. There are some reports, however, of fluorescently labeled oligodeoxynucleotide appearing as diffuse cytoplasmic and nuclear staining (Wu Pong *et al.*, 1994). Where uptake of fluorescently labeled oligodeoxynucleotide was mediated by cationic lipid, Bennett (1995) found that most of the oligodeoxynucleotide was localized in the nucleus, while some was still found in cytoplasmic vesicles. Cumin *et al.* (1993) found that in the absence of lipofectin, radiolabeled phosphorothioated oligodeoxynucleotide failed to enter CHO cells. Lipofectin-mediated uptake of the same oligodeoxynucleotide resulted in distribution in proportions of 54 % in the nuclei and 35 % in the cytosol. Thus cationic lipids appear to facilitate a great decrease in the occurrence of cytoplasmic, punctate vesicles, and an increase in nuclear accumulation of oligodeoxynucleotide. The results of Capaccioli *et al.* (1993) suggest that DOTAP bypasses the normal receptor mediated endocytosis pathway of oligodeoxynucleotide uptake.

Fluorescently labeled RNA studies have also been carried out. Lange *et al.* (1993) found DOTAP-mediated uptake of fluorescein-

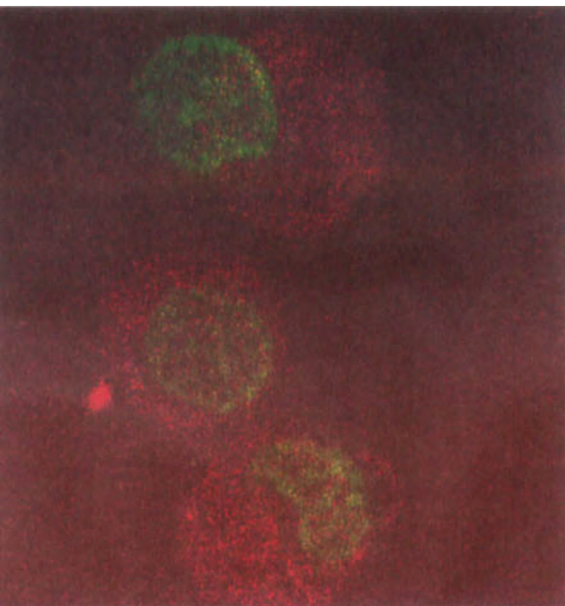


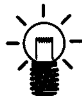
Fig. 9 Cationic lipid mediated uptake of a fluorescently labeled ribozyme into a 32D murine leukemic cell line. Green fluorescence is visible in the nucleus, red in the cytoplasm indicates ribozyme distribution

labeled ribozyme resulted in diffuse fluorescence of the whole cell, with spots of enhanced staining both in the cytoplasm and the nucleus. Similar whole cell diffuse fluorescence was seen with DOTAP-mediated ribozyme uptake (Sioud, 1994). Diffuse cytoplasmic fluorescence of fluorescein-labeled ribozyme is evident in Fig. 9. The nuclei are stained with DAPI. Much less fluorescence was found to be associated with the nuclear than the cytoplasmic regions (Twomey, unpublished). A radiolabeled lipofectin-mediated ribozyme uptake study found that most of the intact ribozyme was located in the cytoplasm up to three hours post incubation, though some nuclear accumulation was seen (Karikó *et al.*, 1994). There are reports of naked RNA, suitably protected against nucleases, being employed in uptake studies. In some cases these have been shown to enter the nuclei much more avidly than the cytoplasm. Further studies are being carried out in various laboratories to establish the effects of cationic lipids on RNA uptake. Preliminary *in vivo* data suggest that the distribution of oligonucleotides changes in the presence of cationic lipids (Bennett, 1995). There appears then to be some unpredictability about effective ribozyme uptake and distribution.

4.8.10 Benefits and Drawbacks of Cationic Lipids

Cationic lipids are effective in delivering nucleic acids to cells. Both DNA (Felgner *et al.*, 1987; Capaccioli *et al.*, 1993; Cumin *et al.*, 1993) and RNA (Malone *et al.*, 1989; Leopold *et al.*, 1995) uptake into cells

is augmented via cationic lipids. The basis of this phenomenon is thought to be due to a combination of increased uptake into cells and protection from nuclease activity. Nuclease degradation of DNA (Capaccioli *et al.*, 1993) and RNA (Karikó *et al.*, 1994), both in cells and in serum, decreases dramatically in cationic lipid-nucleic acid complexes. The maintenance of high intracellular and extracellular concentration of nucleic acids should promote increased effectiveness. There are a number of suggestions in the literature of other ways in which cationic lipids aid oligonucleotide action. Akhtar and Juliano (1992) suggest that oligonucleotide complexed to cationic lipid is released slowly following uptake, and is sustained over a period of several days. The use of cationic lipids also circumvents the problems associated with viral delivery systems. These include the possibility of recombination of the virus within the host genome (Sullivan, 1994) and induction of an immune response against intrinsic viral antigens, thus making repeat administration less effective (Felgner *et al.*, 1995). This technique is very versatile in terms of both the cell types and the nucleic acid to be transfected. Some cell types which were not previously transfectable by other methodologies can be transfected by cationic lipids (Felgner *et al.*, 1995). For instance, Malone *et al.* (1989) found UA37, a hemopoietic cell line, was effectively transfected by RNA-lipofectin but not by conventional DNA transfection protocols. Felgner *et al.* (1987) found the method to be equally effective for both stable and transient DNA expression. Cationic lipids are also versatile in that they can be modified to enhance cell specificity by, for example, supplementing lipofectin with hemagglutinin (Mazur *et al.*, 1994). The procedure has the added benefits of being simple, reliable and reproducible (Malone *et al.*, 1989). There are two main restrictions recognized regarding the use of cationic lipids in cells. Cationic lipids are cytotoxic. The degree of cytotoxicity varies greatly between cell lines. A toxicity profile must be prepared for specific cell types prior to use. This profile will establish the maximum concentration of cationic lipid which would give efficient transfection in that cell line. The second problem is the that cells must be in a serum-free environment during initial incubation with the lipid-nucleic acid complex. However, as discussed earlier, this problem can usually be circumvented.



use high nucleic acid concentrations

versatility

disadvantages

In conclusion, although transfection with cationic lipids is not as efficient as most viral-based vector systems, in many cases enough nucleic acid is transfected into the cells to be effective. As the discussion above outlines, the benefits appear to outweigh the drawbacks, at least in cell lines where toxicity is limited.

4.8.11 Future Developments

New cationic lipids are currently being developed. Early results suggest increased transfection efficiency compared with those in use at present (Geddes, 1995). Improvements may also arise from optimization in particle size and stability, and work is currently underway in these areas.

4.9 Vector-Mediated Delivery

Recombinant ribozyme or antisense genes can be delivered by a vector system such as retrovirus-derived amphotropic vectors or parvovirus-derived infectious particles. The advantages include relatively high yields of transduced cells, and a relatively high probability of stable integration of the vector DNA including the ribozyme gene. On the other hand, this approach is time-consuming and expensive.

One of the most promising methods for gene or nucleotide sequence transfer has involved the use of various ligands, e.g., asialo-orosomucoid or transferrin, which are covalently linked to polylysine. This is then complexed with DNA containing a reporter gene. Subsequent studies showed that the addition of adenovirus or fusenogenic peptides from influenza virus, for example, helped the DNA escape endosomal lysis.

A recent paper (Keller *et al.*, 1995) illustrates this technique, whereby the ligand (steel factor) contained in a vector-targeted gene transfers to hematopoietic cells expressing c-kit receptor. This paper describes the methods for determination of the expression of c-kit in a cell population by flow cytometric analysis. The incubation of cells, the use of biotinylated steel factor, and their subsequent analysis are also described in this paper. The method of cellular transfection estimation of protein concentration (Pierce, Rockford, IL) and luciferase activity are described.

The disadvantage of this vector is that although high transfection levels are achieved, expression is transient. Other vectors will be needed to ensure stable integration. Advantages are, of course, that the DNA enters via receptor-mediated endocytosis, and is therefore unlikely to create cytotoxicity. Other biotinylated ligands can be used, large amounts of DNA can be packaged to include regulatory sequences, and there will be no safety hazards as with retroviral vectors.

4.10 Conclusions

Comparison of transfection methods is complicated by differences independent of the actual transfection procedure such as cell type, nucleic acid source, and concentration. However, some direct comparisons have been made, and other studies are similar enough to be compared. A number of studies have compared the use of DEAE-dextran and calcium phosphate precipitation. The findings of these studies are summarized below.

The DEAE-dextran method is best suited to transfection at low concentrations of DNA, optimally less than 1 µg/60 mm Petri dish. 5–15 µg/60 mm of DNA is required for optimal calcium phosphate transfection (Graham and van der Eb, 1993). Luthman and Magnusson (1983) note that with limiting DNA concentration, the DEAE-

relative efficiency of different methods

dextran method results in up to 10-fold higher DNA infectivity than does the calcium phosphate procedure, while in transfections with larger quantities of DNA, the two methods result in similar DNA infectivity. However, using 8 µg of plasmid, Lopata *et al.* (1984) see a 5–10 fold higher increase in expression with DEAE-dextran than with calcium phosphate. The maximum uptake efficiency seen following calcium phosphate-mediated DNA uptake is 15 % (Chu *et al.*, 1987). Sussman and Milman (1984) found up to 80 % of the recipient cells expressed the transfected gene following DEAE-dextran mediated transfection. In a study which directly compared the poration efficiency of Streptolysin O with electroporation, Harvey *et al.* (1994) found streptolysin O to be more efficient at permeabilizing both hamster (CHO and xrs5 cells) and human cells (untransformed human fibroblasts). In comparing electroporation with calcium phosphate, the transfection efficiency with calcium phosphate is generally greater. While the two are similar for easily transfected cell lines, more difficult cells usually show greater transfection efficiency following calcium phosphate treatment. Malone *et al.* (1989) found DEAE is 100 to 1000-fold less efficient than lipofectin.

In conclusion, in choosing an uptake procedure a number of factors must be taken into consideration. Firstly, if transfection is to be carried out *in vivo*, of the methods discussed, only cationic lipids and viral-mediated transfection could be considered. While viral transfection is often not considered due to its hazardous nature, cationic lipids may be unsuitable due to cytotoxicity. The suitability of cationic lipids is also cell line dependent. In *ex vivo* transfection any of the methods described, including viral and cationic lipids, can be employed. From the discussion above, it is evident that factors such as the cell line and oligonucleotide concentration would affect the choice of transfection procedures. The subsequent use of the transfected cells may also influence which method is used. An example of this is where low mutation frequency and controlled copy number transfection is required, such as in gene expression studies, where electroporation seems to be the most appropriate transfection procedure. Thus each situation must be considered independently.

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CHAPTER 5

The Future

The question which of course requires an answer is whether it is best to progress with ribozymes or antisense in pursuit of new therapeutic treatments. Protagonists at this moment are equally divided but there is also a view which straddles both and says it is too early to decide, and that we ought to progress with both approaches. Will major problems like delivery to the key sites within the gene, or messenger RNA uptake into cells and selective gene targeting be overcome? New and more effective delivery systems are emerging quite rapidly (Bielenska *et al.*, 1996) and despite success *in vitro* with cationic lipids in reducing specific protein synthesis (Bennett, 1992), success *in vivo* is problematical and no efficient delivery system is at hand. A final question is, will we be able to target the appropriate gene or genes at the right time to reverse an emerging or emerged phenotype or to inhibit viral multiplication, and how do we choose this gene?

Since ribozymes are small RNA molecules it is easy to insert them into vectors. In fact, multiple ribozymes targeting several parts of an mRNA molecule can be inserted into the vector as substitutes for a single coding gene. Ribozyme sequences synthesized from plasmids or viral vectors must be delivered from transcription units which are designed to produce large numbers of such sequences, each retaining catalytic activity and able to access the target mRNA. A strategy for the development of ribozyme transcription units could utilize differences in the processing, transport, sub-cellular localization and/or protein binding properties of an mRNA and small RNA transcripts. RNA targets may be cleaved even when they have a range of sub-cellular locations or processing and transport characteristics. These units could produce high levels of ribozymes in the nucleus or cytoplasm, locate at splicing or translation complexes, or along pathways similar to the targeted RNA sequences. These considerations are all under investigation and are likely to lead to stable transcripts with excellent catalytic activity and co-localization of target and substrate in the cell. Similar considerations apply to the addition of antisense DNA molecules to cells, either directly or through plasmid/viral/genetic constructs. Entry into the cells, co-localization with the target, and improving levels of the antisense molecules (DNA or RNA) in the relevant cells are being actively researched.

The clinical use of ribozymes against HIV infection has received attention in cell cultures and primary hemopoietic cells, albeit some-

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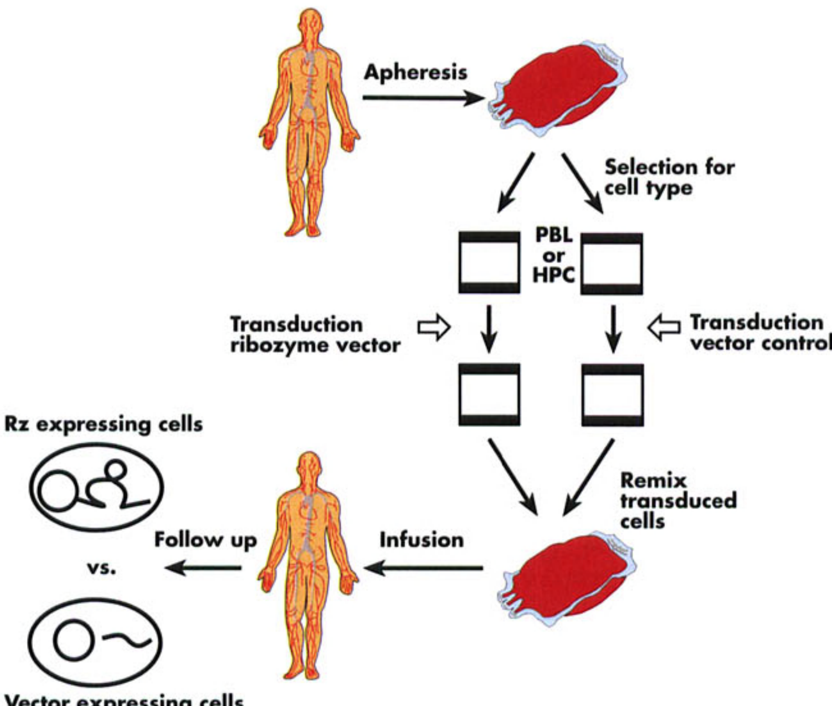

Fig. 10 Plan of treatment with patients with ribozymes. Peripheral blood cells (PBC) or hemopoetic progenitor cells (HPC) are taken from patients, subdivided and treated with a ribozyme containing vector or a control vector and returned to the patient. Those with or without ribozymes or vectors can be identified by molecular methodology. If both cell types are present at all stages the ribozymes cannot be causing toxic effects

what distant from the problem in treating patients. However, a clinical trial against peripheral blood cells harvested from patients who are HIV seropositive is planned. 50 % of the cells will be transduced with the control vector, and 50 % with a hairpin ribozyme expression vector. They will then be mixed and infused back into patients. Since the cells will be marked, those expressing ribozymes may survive longer than those marked via the vector. The clinical success of the ribozyme will involve protecting cells from HIV viral infection and in removing, transfecting and infusing the T lymphocytes back into patients, thereby augmenting their immune response (Fig. 10). Clinical trials are also in progress for the treatment of leukemias with ribozymes and antisense DNA molecules. Animal models have provided surprising successes despite our lack of understanding of mechanisms of action of uptake into cells.

Further researches *in vivo* are the targeting of specific organs, transport proteins, or growth factors, as well as specific mRNA sequences, in an effort to tackle tumorigenesis, drug resistance, or angiogenesis. Until the initial trials produce results, however, then questions of whether antisense or ribozymes should be used remain

unclear. The pace is quickening and no doubt before this book reaches the shelf the field will have moved on. Certainly questions of biological potency, safety, pharmacokinetics, tissue disposition and *in vivo* stability are under intensive investigation. The most significant advance, however, in moving these applied aspects forward may yet emanate from an understanding of secondary messenger structure of RNA molecules, and the conditions which cause RNA molecules to take up a particular shape or form. When this is understood, then some of the discrepancies in results may become easier to explain.

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Appendix

	Tradename	*Company*
DOTMA N-[1-(2,3-dioleyloxy)propy1]-n,n,n-trimethylammonium chloride	Lipofectin™	Gibco BRL
DMRIE-C 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol in water		Gibco BRL
DOSPA 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate	Lipofectamine™	Gibco BRL
DOTAP 1,2-dioleoxyloxy-3-(trimethylammonium) propane		Boehringer Mannheim
DOGS Dioctadecyl amido glycyl spermine		Promega
DC-Chol 3-β-(N-(N,N-dimethylaminoethane) carbamoyl cholesterol		Dept. of Pharmacology, University of Pittsburgh

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