Reverse Genetics of RNA Viruses

Reverse Genetics of RNA Viruses

Applications and Perspectives

Edited by

Anne Bridgen

WILEY-BLACKWELL

A John Wiley & Sons, Ltd., Publication

This edition first published 2013

Wiley-Blackwell is an imprint of John Wiley & Sons, formed by the merger of Wiley's global Scientific, Technical and Medical business with Blackwell Publishing.

Registered office: John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial offices: 9600 Garsington Road, Oxford, OX4 2DQ, UK
The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK
111 River Street, Hoboken, NJ 07030-5774, USA

For details of our global editorial offices, for customer services and for information about how to apply for permission to reuse the copyright material in this book please see our website at www.wiley.com/wiley-blackwell.

Library of Congress Cataloging-in-Publication Data

Reverse genetics of RNA viruses : applications and perspectives / [edited by] Anne Bridgen.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-0-470-97965-5 (cloth)
I. Bridgen, Anne, 1961–
[DNLM: 1. RNA Viruses–genetics. 2. Reverse Genetics–methods. 3. RNA, Viral–genetics. 4. Viral Proteins–genetics. 5. Viral Vaccines. QW 168]
579.2′5–dc23

2012016778

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Cover design: Gary Thompson

Set in 10.5/12.5pt Times by Aptara Inc., New Delhi, India.

First Impression 2013

I would like to dedicate this book to Professor Sir Kenneth Murray, FRS, FRSE, for his mentoring during the course of my PhD and his introduction to the powerful world of molecular biology.

Ken, you were an inspiration in the way in which you searched out important issues in science and tackled them, no matter how insurmountable the obstacles. Your groundbreaking work on the manipulation of hepatitis B virus and early development of an effective and safe vaccine has been much of the inspiration to my work in this field, and I thank you for this.

Contents

List of Contributors

Maria Armesto, Avian Viral Diseases, Institute for Animal Health, Compton Laboratory, Newbury, UK

Wendy S. Barclay, Department of Medicine, Imperial College London, UK

Kirsten Bentley, Avian Viral Diseases, Institute for Animal Health, Compton Laboratory, Newbury, UK

Erica Bickerton, Avian Viral Diseases, Institute for Animal Health, Compton Laboratory, Newbury, UK

Antonio V. Borderia, Institut Pasteur, Paris, France

Mark Boyce, Institute for Animal Health, Pirbright Laboratory, Pirbright, UK

Anne Bridgen, Croft Dhu, Newtonmore, Inverness-shire, UK

Paul Britton, Avian Viral Diseases, Institute for Animal Health, Compton Laboratory, Newbury, UK

Karl-Klaus Conzelmann, Max von Pettenkofer Institute and Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany

Iris J.C. Dautzenberg, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

W. Paul Duprex, Department of Microbiology, Boston University, School of Medicine and National Emerging Infectious Disease Laboratories, Boston, USA

Ruth A. Elderfield, Department of Medicine, Imperial College London, UK

Richard M. Elliott, Biomedical Sciences Research Complex, School of Biology, University of St Andrews, UK

Alexander Ghanem, Max von Pettenkofer Institute and Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany

Ian Goodfellow, Section of Virology, Department of Medicine, Imperial College London, UK

Lorian C.S. Hartgroves, Department of Medicine, Imperial College London, UK

Rob C. Hoeben, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

Sarah Keep, Avian Viral Diseases, Institute for Animal Health, Compton Laboratory, Newbury, UK

Alexander Ploss, Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Diseases, The Rockefeller University, New York, USA

Linda J. Rennick, Department of Microbiology, Boston University, School of Medicine and National Emerging Infectious Disease Laboratories, Boston, USA

Peter A.E. Sillevis Smitt, Department of Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands

Sanne K. van den Hengel, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden and Department of Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands

Diana J.M. van den Wollenberg, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

Marco Vignuzzin Institut Pasteur, Paris, France

Acknowledgements

I wish to thank all those who have participated in the planning and execution of this book. First and foremost to my publishing editor at Wiley, Nicky McGirr, for her support and encouragement in the planning stages of the book. Also to my writing editor Fiona Woods (Seymour) for her answering of endless questions and guidance along the way.

Thanks are due for the constructive criticism of those who refereed the book outline. Several suggestions made at this stage have been incorporated and have improved the final book.

Huge thanks are due to the chapter authors for giving their precious time to describe their viral system. This volume could not have been written without you so thanks indeed.

I also thank my many research colleagues in reverse genetics, most particularly Professor Richard Elliott in whose laboratory I first worked in reverse genetics and his group in Glasgow (now St Andrews), but also all those with whom I shared trials and tribulations, as well as successes, at meetings and seminars.

I thank my current colleagues and students, particularly Dr Michael Baron at the Institute of Animal Health, Pirbright and my doctoral student Mr Siddharth Bakshi at the University of Ulster for their patience while I was writing this book. And to Dr Michael Baron and Professor Rachel Fearns, Boston University School of Medicine, for proof reading my chapters (the introduction and perspectives chapters).

It has taken a lot longer and been a lot more work than I anticipated, and so I particularly thank my husband Dr Ian Bradbury for his support during the prolonged writing and editing, and his forbearance when I decided that writing in Chamonix was preferable to writing at home! Thank you, all of you.

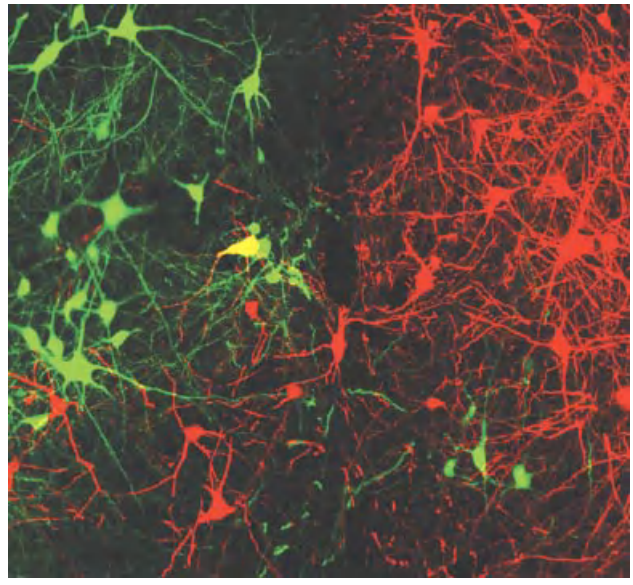

Plate 1 Examples of monosynaptic tracing of neuronal connections with RABV. Interneurones of the spinal cord which are directly connected to motor neurones of the left (green) and right (red) quadriceps muscle. The yellow interneurone is connected to both right and left motoneurones. G gene-deficient RABV expressing GFP (SADΔG-eGFP) or RFP (SADΔG-RFP) were injected into the right or left muscle, respectively, and there infect motor neurones expressing RABV G from an AAV vector. The G protein mediates a single transsynaptic spread of RABV to the postsynaptic interneurones.
Source: Kindly provided by Anna Stepien and Silvia Arber, University of Basel.

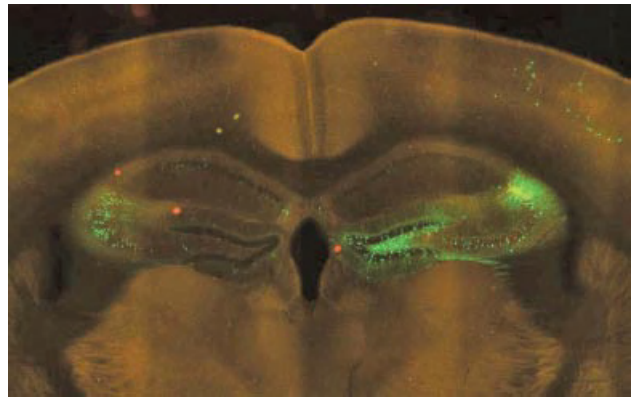

Plate 2 Direct connections of right and left hippocampal neurones in the mouse brain. The CA3 region in the right hippocampus (yellow) was injected with an AAV vector expressing TVA, td-tomato, and RABV G protein. Subsequent selective infection of TVA-expressing neurones with the RABV SADΔG-eGFP pseudotyped with EnvA is indicated by yellow. Green staining indicates neurones infected by transsynaptic spread of SADΔG-eGFP and reveals direct connections between left and right hemispheres.
Source: Kindly provided by Martin Schwarz, MPIMF Heidelberg.

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

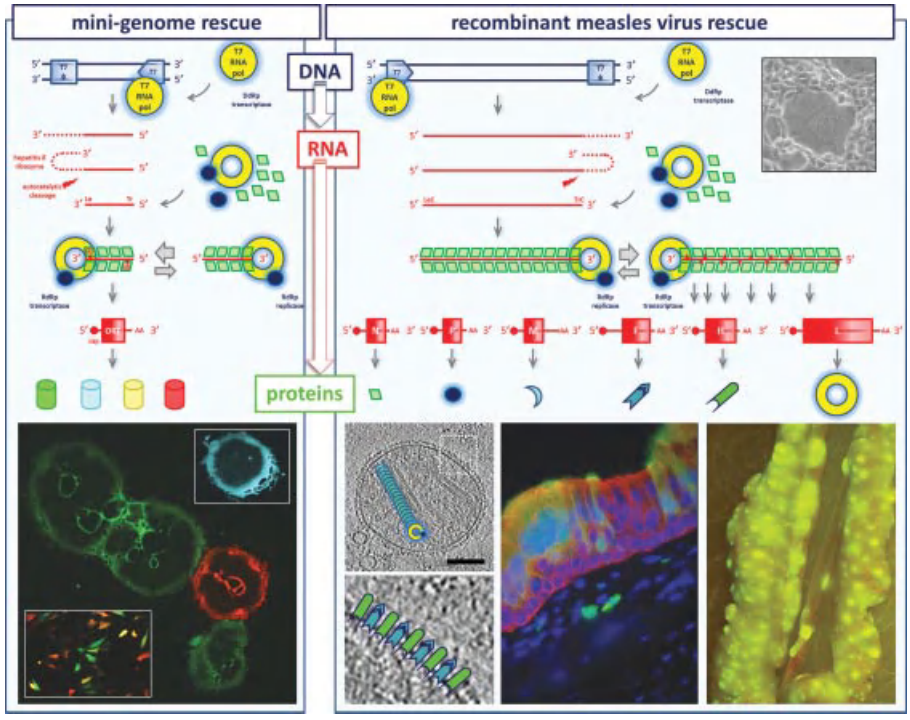

Plate 3 Schematic representations of the mini-genome (left) and recombinant measles virus (right) rescue systems used to study transcription and replication and to generate rMVs respectively. T7 bacteriophage DNA-dependent RNA polymerase (DdRp) is supplied by a recombinant host-range adapted vaccinia virus (MVA-T7). The transcriptase recognises the T7 promoter engineered into the plasmid backbone to generate either a negative sensed minigenome RNA or a full-length genome positive sensed transcript. Presence of T7 terminators (T7ϕ) leads to the detachment of the transcriptase from the DNA template. Nascent RNA transcripts contain a hepatitis δ ribozyme at the 3′ end (dashed red line). Formation of the secondary structure leads to the autocatalytic cleavage of the T7 transcript *in cis*. This generates minigenome or genome length transcripts which conform to the 'rule of six'. The minigenome is flanked by leader (Le) and trailer (Tr) sequences and the antigenome is flanked by leader complement (LeC) and trailer complement (TrC) sequences. Co-transfection of three helper plasmids which also contain T7 promoters and the open reading frames encoding the virus N (green rhombus), P (blue circle) and L (yellow ring) proteins allows the formation of either a negative sensed minigenomic (−)RNP or a positive sensed antigenomic (+)RNP. The L and P proteins function as a transcriptase on the minigenomic (−)RNP producing a single capped (red circle) and polyadenylated (AA) mRNA containing the open reading frame (ORF) of a reporter protein such as enhanced green fluorescent protein (green barrel), enhanced cyan fluorescent protein (cyan barrel), enhanced yellow fluorescent protein (yellow barrel) or HcRed (red barrel). The L and P proteins also function as a replicase generating (+)RNP minigenomes from the (−)RNP template. These (+)RNP minigenomes are in turn replicated to produce additional (−)RNP minigenomes. Expression of the fluorescent proteins is detected by UV microscopy in single cells (inset) when the system is driven exclusively by cotransfected plasmids or in multinucleated syncytia when the minigenome replication/transcription assay is driven by a superinfecting MV. In recombinant measles virus rescue the L and P proteins initially act as a replicase using the positive sensed antigenomic (+)RNP to generate the negative sensed genomic (−)RNP. This is the basic unit of infectivity of MV and the L and P proteins function as a transcriptase on this full-length (−)RNP producing six capped and polyadenylated mRNAs containing the N, P, M (cyan crescent), F (blue arrow), H (green bullet) and L gene ORFs. Translation of these proteins allows assembly of virions at the plasma membrane. When virions are examined by electron cryotomography the M protein can be seen coating the RNP (0.8 nm thick slice from a tomogram, scale bar 100 nm). The herringbone structure of the (−)RNP is clearly visible within the virion, a schematic (−)RNP is overlaid for comparison (left panel). A fringe of spikes of the F and H fusion complex decorates the membrane of the virion; these are represented schematically at a higher magnification on the same tomograph. Recombinant MVs based on clinical isolates expressing fluorescent proteins from additional transcription units have been invaluable in illuminating viral pathogenesis. These viruses permit the microscopic imaging of virus infected cells with unprecedented levels of sensitivity, for example in epithelia (center panel). They also allow macroscopic imaging and targeted pathology to be performed at the time of necropsy, for example in the gut associated lymphoid tissue of a macaque (right panel).

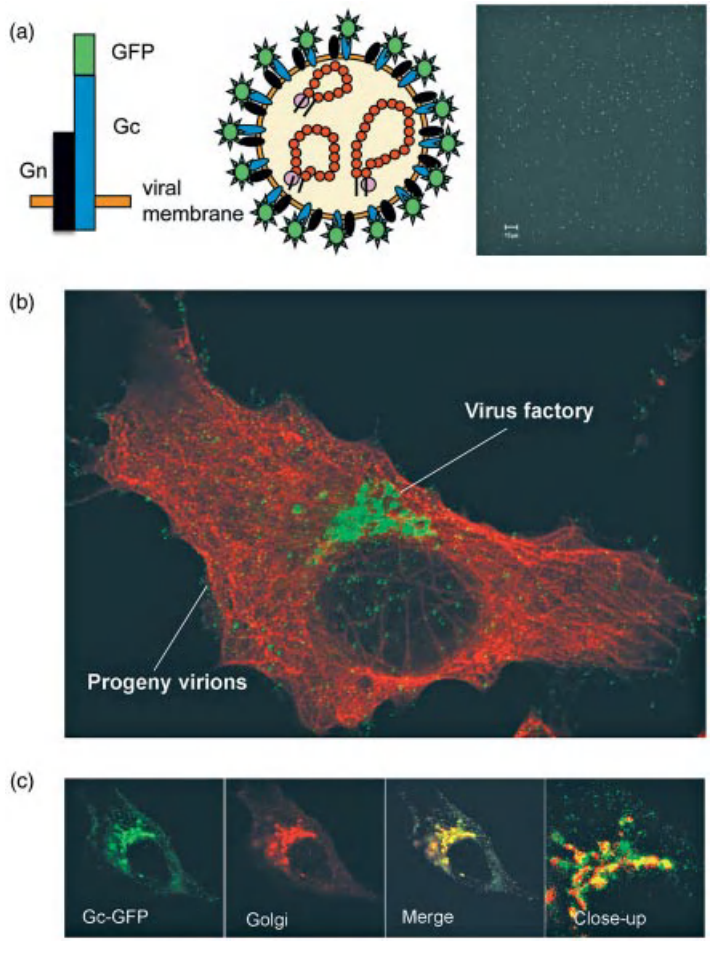

Plate 4 Recombinant Bunyamwera virus expressing GFP-Gc fusion protein. (a) Schematics of the chimeric glycoprotein and recombinant virus (rBUNGc-eGFP) are shown on the left. On the right is supernatant fluid from infected cells examined under UV light showing autofluoresent virus particles. (b) BSR-T7/5 cells were infected for 8 h with rBUNGc-eGFP at an MOI of 1 PFU/ cell, fixed and co-stained with anti-tubulin antibody (in red). The virus factory in the Golgi region of the cell and autofluorescent progeny virus particles are indicated. (c) Detail of virus budding at the Golgi. BSRT7/5 cells were infected with rBUNGc-eGFP and co-stained with antibodies to the Golgi marker GM130 (in red). Colocalization between Gc proteins and the Golgi protein are shown in yellow in the merged image, and the enlarged image shows budding virions. *Source*: Adapted from Shi *et al*., (2010). Copyright © American Society for Microbiology. *Journal of Virology,* Vol. 84, 2010, p. 8460–8469. doi:10.1128/JVI.00902-10.

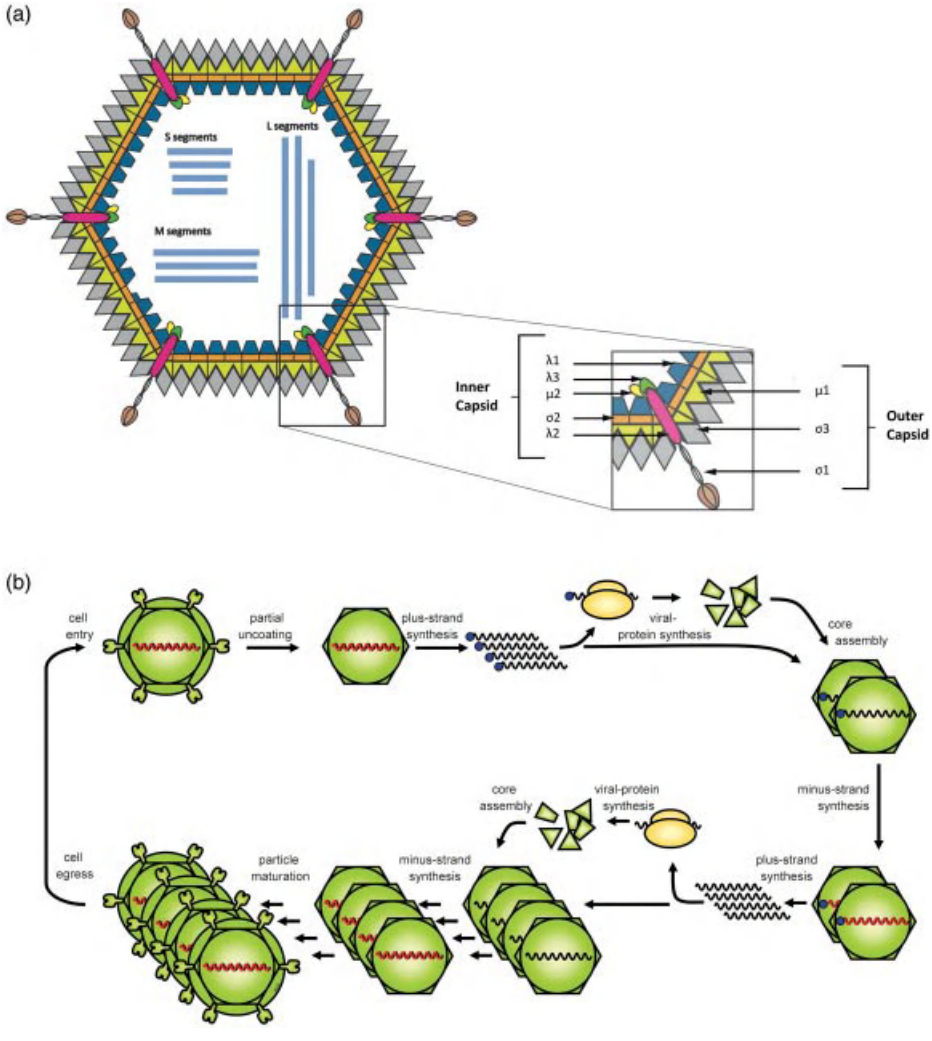

Plate 5 (a) Schematic representation of the mammalian orthoreovirus. The virus contains a non-enveloped icosahedral capsid, containing 10 dsRNA segments. These encode the structural proteins: five proteins comprise the inner capsid, and the three others form the outer capsid. The positions of the various capsid components are indicated. (b) Schematic representation of the reovirus' genome replication. After cell entry the viral particle is partially uncoated, and penetrates the endosomal membrane. In the cytoplasm the primary transcription process yields capped plus-strand RNA molecules, which are translated and can associate with the newly assembled cores. In the cores these transcripts serve as templates for minus-strand synthesis yielding double-stranded RNA. Subsequently the secondary transcription process yields uncapped transcripts which are translated and associate with the new core particles. Minus-strand synthesis proceeds to yield double-stranded RNA genome segments. The particles mature and egress from the cells. In the figure only one of the genome segments is drawn.

1

Introduction

Anne Bridgen
Croft Dhu, Newtonmore, Inverness-shire, Scotland

1.1 Background

Viruses with ribonucleic acid (RNA) genomes make up many of our current most serious human pathogens. For example, influenza A virus, poliovirus, rotaviruses, dengue virus, hepatitis C virus, West Nile fever virus, yellow fever virus and measles virus are all RNA viruses, and are between them responsible for millions of human deaths each year. Rotaviruses alone are responsible for around 350,000–600,000 infant deaths each year from diarrhoea (Parashar *et al*., 2003). One of the features of RNA viruses is that the viral polymerase responsible for their replication is not very accurate as there is no proof-reading capacity. This low accuracy means that, in the presence of antiviral drugs, viral escape mutants soon arise which no longer respond to the drug. There are thus very few effective antivirals directed against RNA viruses. In addition, many of the new emerging viruses which arise through viral mutation, genome segment reassortment or host switching to suddenly enter the human population are RNA viruses. These include the coronavirus severe acute respiratory syndrome (SARS) virus, Ebola and Marburg filoviruses, and avian and swine flu, and are the viruses that tend to cause the highest mortality rates. There is thus a high requirement to be able to analyse these viruses and to develop effective vaccines and antivirals.

RNA viruses possess several different types of RNA genomes. Some have a non-segmented genome, or the genome can be split into a number of different segments, for example, 2 for arenaviruses, 3 for bunyaviruses, 7–8 for influenza viruses and 10–12 for reoviruses. In addition, they can comprise positive sense, negative sense or ambisense RNA, and be either single- or double-stranded. Positive sense RNAs can be translated directly into protein, while a negative sense RNA has first to be transcribed by the viral proteins to form positive stranded RNA that can be translated. Ambisense RNAs are those which contain genes running in both orientations within the same genome or genome segment. There are also retroviruses and hepadnaviruses which go through both RNA and deoxyribonucleic acid (DNA) phases via reverse transcription of their RNA. These last named groups of viruses, which

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

include the human immunodeficiency viruses, will not be discussed in this volume despite their importance, as the amount of research in this area would easily require a separate volume.

In classical genetics, the specific genes in an organism were deduced from observations of the phenotype of the organism. Reverse genetics is a term coined to describe processes where information flows in the opposite direction, that is, the gene is determined or altered directly, and the resultant phenotype observed. In the context of virology, this then refers to changes introduced directly into the complementary DNA (cDNA) used to generate infectious RNA virus or virus-like particles, in order to study the function of specific gene sequences and proteins, and the term has come to be applied to the ability to go from a DNA copy of the viral genome to a new virus. Neumann and Kawaoka (2004) define reverse genetics as the generation of a virus entirely from cDNA. It is an incredibly powerful tool both for the generation of modified viruses, which can act as vaccines or vectors, and for the analysis of viral genes and non-coding sequences.

1.2 Reverse genetics for different classes of genome

One of the most definitive ways in which to study the roles of specific sequences in viral genomes is to modify them and to generate infectious virus, that is, to 'rescue' the virus, from these modified sequences. For DNA viruses this was relatively straightforward once molecular biological techniques became sufficiently sophisticated to allow this, as the DNA could be introduced directly into cells to generate infectious virus. Thus, infectious T2 bacteriophage was rescued from DNA as early as 1957 (Fraser *et al*., 1957). The first RNA virus to be rescued from its cDNA was the bacteriophage Qbeta rescued by Taniguchi *et al*. (1978), while the first mammalian plus stranded RNA virus to be rescued was poliovirus by Racaniello and Baltimore (1981). Researchers subsequently discovered that this process was more efficient if the RNA was transcribed *in vitro* and the nascent RNA transfected into cells (Boyer and Haenni, 1994); this process was then applied to many plus sense RNA viruses. Some difficulties were encountered with specific families of viruses, however, such as coronaviruses, as is discussed in Section 1.4.

Negative sense RNA viruses proved less amenable to such studies as the minimal infectious unit comprises the viral RNA encapsidated by the nucleocapsid and replication proteins to form a ribonucleoprotein (RNP) complex. It was not until 1994 that Schnell *et al*. (1994) succeeded in rescuing the first negative sense RNA virus, the rhabdovirus rabies virus, from cDNA. One of the main reasons for this breakthrough was the decision to transfect cells with cDNA plasmids encoding the viral antigenome rather than the genome. This meant that there was less negative sense RNA present in the cell which could hybridise to the positive sense viral mRNAs and thus induce host innate immune responses.

To add to the difficulties of rescuing negative sense RNA viruses from cDNA, many of them comprise segmented genomes, so, for their rescue, cells must be

transfected with constructs for each of the genome segments as well as for the replication proteins. Early rescue experiments for the eight-segmented genome virus influenza A virus involved modification of single RNA segments and use of helper viruses (Luytjes *et al*., 1989; Enami *et al*., 1990). However, this is not an efficient process as only a small proportion of the helper viruses acquires the novel segment. The first segmented, negative sense RNA virus to be rescued entirely from cDNA was the tri-segmented bunyavirus Bunyamwera virus (Bridgen and Elliott, 1996). This used the approach initiated for the non-segmented rabies virus in using positive sense, antigenomic constructs for rescue. Rescue of influenza A virus entirely from cDNA followed later (Fodor *et al*., 1999; Neumann *et al*., 1999), in a procedure involving transfection of cells with 12 different plasmids. This original technique has been modified extensively and now rescue can be achieved using far fewer plasmids.

Of the viruses described in this volume, the last to be rescued were the double-stranded RNA (dsRNA) genome viruses. Not only do these have genomes of dsRNA, a structure which does not naturally occur in cells and which therefore induces host innate immune responses, but also many have multiple genome segments, making the rescue more complex.

Thus, the practical way in which rescue is achieved is very different depending on the nature of the genome, as is summarised in Figure 1.1. In this volume we are showcasing the RNA viruses from each of these genomic groups, so that there are examples of what has been achieved and what the problems have been for each of these groups. To date, representatives of most of the human and animal virus families have been rescued from cDNA (see Table 1.1). There has also been

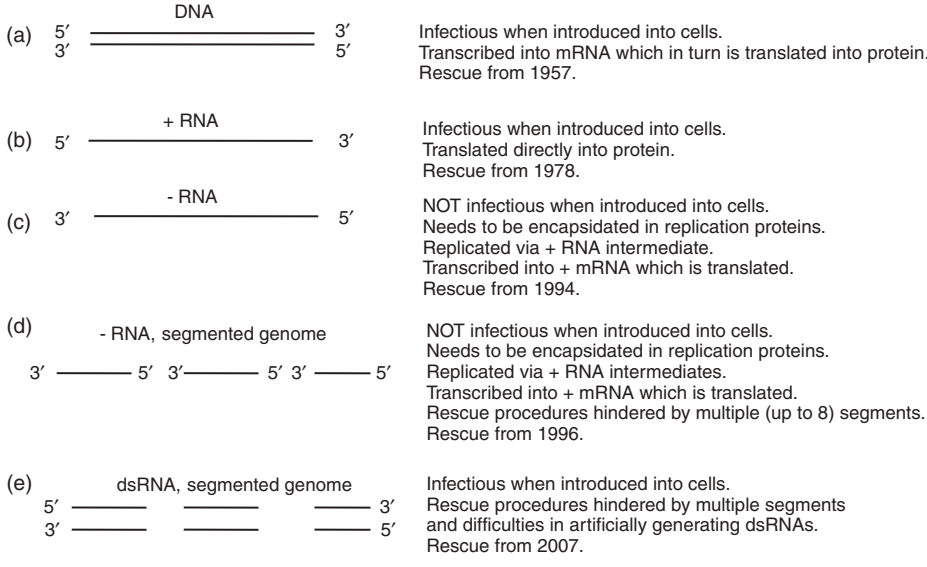

Figure 1.1 How rescue is achieved.

Table 1.1 Important dates in the history of human and animal virus reverse genetics.

Date	Event	Nature of virus	Reference
1981	Rescue of poliovirus from cDNA	+ve sense ss RNA	Racaniello and Baltimore, 1981
1981–1994	Summary of viruses rescued in this period from cDNA or *in vitro* transcribed RNA; includes members of alphavirus, picornavirus and flavivirus familes	+ve sense ss RNA	Boyer and Haenni, 1994
1994	Rescue of rhabdovirus rabies virus	-ve sense ss RNA	Schnell *et al*., 1994
1995	Rescue of paramyxovirus measles virus	-ve sense ss RNA	Radecke *et al*., 1995
1995	Rescue of rhabdovirus VSV	-ve sense ss RNA	Whelan *et al*., 1995
1995	Rescue of paramyxovirus RSV	-ve sense ss RNA	Collins *et al*., 1995
1995	Rescue of feline calicivirus from RNA transcripts	+ve sense ss RNA	Sosnovtsev and Green, 1995
1996	Rescue of trisegmented bunyavirus Bunyamwera virus	-ve sense ss RNA, 3 segments	Bridgen and Elliott, 1996
1996	Rescue of birnavirus infectious bursal disease virus from transcribed RNA	ds RNA 2 segments	Mundt and Vakharia, 1996
1997	Rescue of paramyxovirus SV5	-ve sense ss RNA	He *et al*., 1997
1997	Rescue of astrovirus from transfected RNA	+ve sense ss RNA	Geigenmuller *et al*., 1997
1997	Rescue of hepatitis C from RNA in chimpanzees	+ve sense ss RNA	Kolykhalov *et al*., 1997 Yanagi *et al*., 1997
1998	Rescue of arterivirus PRRSV	+ve sense ss RNA	Meulenberg *et al*., 1998
1999	Rescue of orthomyxovirus influenza A virus	-ve sense ss RNA, 8 segments	Neumann *et al*., 1999 Fodor *et al*., 1999
1999	Rescue of hepatitis C virus from cDNA in cell culture	+ve sense ss RNA	Kolykhalov *et al*., 1999 Lohmann *et al*., 1999
2000	Rescue of coronaviruses TGEV and HCV 229E	+ve sense ss RNA	Almazan *et al*., 2000; Thiel *et al*., 2001; Yount *et al*., 2000
2001	Rescue of filovirus Ebolavirus	-ve sense ss RNA,	Volchov *et al*., 2001 Neumann *et al*., 2002
2001	Rescue of picornavirus Aichi virus (genus Kobuvirus)	+ve sense ss RNA	Sasaki *et al*., 2001
2001	Rescue of orthomyxovirus Thogoto virus	-ve sense ss RNA, 6 segments	Wagner *et al*., 2001
2002	Rescue of orthomyxovirus influenza B virus	-ve sense ss RNA, 8 segments	Hoffmann *et al*., 2002 Jackson *et al*., 2002
2005	Rescue of bornavirus Borna disease virus	-ve sense ss RNA	Schneider *et al*., 2005
2006	Rescue of arenavirus LCMV	-ve sense ss RNA, 2 segments	Flatz *et al*., 2006; Sanchez, de la Torre, 2006
2007	Rescue of orthomyxovirus influenza C virus	-ve sense ss RNA, 7 segments	Crescenzo-Chaigne van der Werf, 2007 Muraki *et al*., 2007
2007	Rescue of reovirus	+ve sense ss RNA 10 segments	Kobayashi *et al*., 2007

Notes: Rescue was from transfected cDNA unless otherwise stated.
HCV Human coronavirus; VSV Vesicular stomatitis virus; RSV Respiratory syncytial virus; TGEV transmissible gastroenteritis virus; PRRSV porcine reproductive and respiratory syndrome virus; LCMV lymphocytic choriomeningitis virus.

an explosion of work in plant virology, which has seen a considerable number of plant pathogens rescued in the past decade. There are, however, many viruses of invertebrates and plants that have not yet been studied by this approach, and indeed many species within families in which the prototype virus has been well studied by reverse genetic technology but other members have not.

The book also discusses viral quasispecies and the implication of this theory on approaches to reverse genetics (see Chapter 11). The theory implies that viruses are not unique, but instead comprise a swarm of inseparable and related molecules, which therefore instantly creates a problem for researchers trying to produce a single synthetic virus molecule. The practical procedures used during rescue, such as the cell type or level of plaque purification, will impact on the level of heterogeneity of the virus (Section 11.2.3) so this theory has practical application to the way in which rescue experiments are conducted.

1.3 Methodology

In this chapter we are going to consider several facets of the methodology behind current reverse genetics techniques, specifically:

1. Stages in virus reverse genetics.
2. Use of different promoters.
3. Obtaining precise genome ends.
4. Increasing rescue efficiencies.
5. Combining material from different genetic segments.
6. Confirmation of the rescue phenotypes.

1.3.1 Stages in virus reverse genetics: minigenome replication; replication of virus-like particles (VLPs) and whole virus rescue

Many researchers start their rescue experiments by using a minigenome system comprising a reporter gene bounded by viral sequences which provide the signals for viral transcription and replication. These can be replicated and transcribed *in vitro* by the viral replicative genes supplied from appropriate plasmids. This is a useful first stage to ensure that the cloned polymerase is functional and the replication signals are correct before attempting full virus rescue. One commonly used reporter gene used is that encoding the jellyfish *Aequorea victoria* Green Fluorescent Protein (GFP), which can fluoresce in the presence of ultraviolet light, as well as its spectral variants including blue, red and yellow fluorescent proteins. Luciferase genes from the firefly *Photinus pyralis* or the sea pansy *Renilla reniformis* are

capable of bioluminescence in the presence of suitable substrates. Another reporter gene is that for chloramphenicol acetyl transferase (CAT). Choice of reporter depends on the cloning capacity (CAT and GFP are both quite small proteins), the application, and the predicted stability/toxicity (GFP features less well here). The reporter genes are cloned in the same sense as the viral genes. They therefore have to be transcribed into mRNA before they can be translated for negative-sense RNA viruses.

Use of the minigenome systems ensures everything is working well before full rescue is attempted. If a quantitative reporter gene such as luciferase or CAT is used, the ratios and amounts of plasmids encoding the replication proteins can be optimised. Variant constructs can be made with slightly different promoters to see if there is a higher level of replication with additional nucleotides in the promoter to enhance activity, or whether these are deleterious to replication. If a GFP reporter is used, this can be used to visualise the proportion of cells infected in different cell types.

In addition, minigenome experiments can also provide a substantial amount of information in their own right, for example, in determining the role of particular sequence elements and whether additional viral genes are required for replication (see, for instance, Fearns and Collins, 1999; Gauliard *et al*., 2006; Bergeron *et al*., 2010, for different examples of the use of minigenome systems).

A second step along the way is to also include viral glycoprotein constructs such that the minigenome can also be packaged into virus-like particles (VLPs) and used to infect further cells. This can then be used to investigate packaging requirements of the virus. Since the VLPs do not contain the genes for the replication proteins, they cannot be passaged further unless a helper virus is added or the infected cells are also transfected with genes for the replication proteins. This is therefore a useful approach for working with serious pathogens. Two examples of the use of VLP systems are given in Overby *et al*. (2006); Wenigenrath *et al*. (2010).

The final stage is to rescue the entire viral genome. These different stages are described excellently with examples given in Chapter 7 by Richard Elliott on bunyavirus rescue, so we refer you to Section 7.3 of this book rather than duplicating material here.

1.3.2 Use of different promoters

To generate viral RNAs and proteins from cDNA in cell culture the viral sequences need to be flanked by a suitable promoter, either one which is present within the cell or one which is added with the cDNA. The most widely used transcription system is that of phage T7 RNA polymerase, which allows cytoplasmic transcription of viral RNAs, thus mimicking what would happen in cells infected with viruses which replicate in the cytoplasm. The T7 RNA polymerase can be expressed from transfected DNA or from helper viruses that express the protein. These can include vaccinia virus (vTF7-3), the less pathogenic modified vaccinia virus *Ankara*

(MVA-T7), or *fowlpox* (FPT7) (Fuerst *et al*., 1986; Sutter *et al*., 1995; Britton *et al*., 1996). The vaccinia virus vTF7-3 system is very efficient, with T7 transcripts making up 30% of cytoplasmic mRNA within 24 hours of infection (Elroy-Stein *et al*., 1989), but is quite toxic to cells. This limits the time during which viruses can be rescued, though this may be minimised by the use of vaccinia virus inhibitors (Kato *et al*., 1996). The attenuated MVA virus replicates well in BHK cells and has a much reduced host range compared to vTF7-3 (Drexler *et al*., 1998). Recently use of MVA-T7 was enhanced by the generation of compatible destination vectors for the widely used Gateway cloning system to express the gene of interest under control of the T7 RNA polymerase following recombination with the MVA-T7 virus genome (Pradeau-Aubreton *et al*., 2010). The FP-T7 system was developed for reverse genetics experiments with avian viruses, but is used more widely for mammalian virus rescue as no infectious virus is produced on infection of mammalian cells with fowlpox (Britton *et al*., 1996).

In order to avoid the toxicity associated with helper viruses, several groups have generated cell lines permanently transfected with the T7 gene. Radecke *et al*. (1995) generated 293 cells that expressed T7 RNA polymerase as well as measles virus proteins. Permanently transfected BHK cell lines that express T7 RNA polymerase were generated by Buchholz *et al.,* (1999). Both BHK cells and the derived BSR -T7/5 cells are also deficient in RIG-I signalling so there is less interferon induction within these cells, which fortuitously benefits rescue procedures (Habjan *et al*., 2008). The BSR-T7/5 cell line has been used to rescue a wide range of viruses including the paramyxoviruses bovine respiratory syncytial virus and human respiratory syncytial virus (BRSV and HRSV; Buchholz *et al*., 1999; Kaur *et al*., 2008), the bunyaviruses Bunyamwera virus, La Crosse virus and Rift Valley fever virus (Lowen *et al*., 2004; Blakqori and Weber, 2005; Habjan *et al*., 2008) and the rhabdoviruses vesicular stomatitis virus and rabies virus (Harty *et al*., 2001; Wu and Rupprecht, 2008). Another set of permanently transfected T7 cell lines produced includes the swine kidney cell line SK6.T7 used to rescue the pestivirus classical swine fever virus (van Gennip *et al*., 1999). More recently, BHK cells have been transduced by retroviral gene transfer to express the T7 RNA polymerase and these cells were used to rescue foot and mouth disease virus (Zheng *et al*., 2009). Another possibility for producing the T7 RNA polymerase within cells is to clone the gene into a plasmid vector using an endogenously expressed promoter such as the CMV immediate early promoter or the chicken beta actin promoter of the pCAGGS vector, which is highly transcribed in both avian and mammalian cells (Jiang *et al*., 2009).

Use of endogenously expressed T7 RNA polymerase has been particularly pertinent for the rescue of viruses that are to be used as potential vaccines. Vaccine production has very specific requirements in that the procedure has to be consistent and reproducible, use only helper viruses that have had their origin and passage history checked extensively, and must not contain any contamination from infectious agents including prion proteins (Witko *et al*., 2006). These authors have therefore developed a technique that has been used for rescue of recombinant paramyxo- and

rhabdo- viruses from Vero and other cell lines based on efficient electroporation. In these experiments the T7 gene was cloned into the plasmid pCI-neo (Promega) 3 of the hCMV immediate-early promoter/enhancer region. In addition, use of a heat shock process and plasmids expressing glycoproteins increased efficiency, thus allowing more attenuated viruses to be rescued (Witko *et al*., 2006). The technique has since been further modified such that the entire procedure can be completed in only 15 minutes (Surman *et al*., 2007).

Alternatively, endogenous cellular promoters such as RNA polymerase I or II (pol I or pol II) may be used rather than T7 RNA polymerase. These are highly transcriptionally active in the nucleus, which works well for those viruses such as influenza A virus that normally replicate in the nucleus. For viruses that replicate in the cytoplasm (the majority), there may be problems due to splicing of the RNAs using pol II. The pol I system was developed for influenza A virus reverse genetics experiments (Zobel *et al*., 1993; Neumann *et al*., 1994) and uses endogenous RNA polymerase I, a cellular nucleolar protein that produces transcripts lacking 5′ caps and 3′ polyadenylated tails. Likewise the pol II system was also used in influenza A rescue to reduce the complexity of the virus rescue protocol (Hoffmann *et al*., 2000). RNA polymerase II is the main eukaryotic cellular transcriptase responsible for transcription of mRNAs and small nuclear RNAs. In an innovative approach Hoffmann *et al*. (2000) cloned the influenza A segments such that the vRNAs were expressed from pol I promoters and the mRNAs from pol II promoters in bidirectional transcription units. This reduced the number of plasmids necessary to be transfected from 12 to 8. Transcription by pol II has also been used to rescue other viruses such as the birnavirus infectious bursal disease virus (Qi *et al*., 2007).

1.3.3 Generating precise genome ends

A major issue for the generation of synthetic RNAs from cDNA is the ability to generate exact transcripts, since many viruses cannot replicate with additional residues on their genome or antigenome RNAs. This is generally more of a problem for negative sense RNA viruses. Most rescue constructs for negative sense RNA viruses involve inserting the cDNA immediately downstream of the promoter to generate appropriate 5′ ends, although efficient transcription from the T7 promoter requires up to 3 additional G residues, which then remain present on the nascent RNA. These additional residues do not appear to hinder viral replication for many viruses (Collins *et al*., 1991; Conzelmann, 2004). A self-cleaving ribozyme sequence is then positioned between the viral cDNA and the T7 termination signal to generate an exact 3′ end; this is essential as additional residues at this point prevent replication (Ghanem *et al*., 2012). The hepatitis delta virus antigenomic ribozyme was first described by Perrotta and Been (1991) and applied to virus rescue shortly thereafter by two groups to rescue VSV and nodavirus constructs, respectively (Pattnaik *et al*., 1992; Ball, 1992). Since then, some groups have used additional hammerhead ribozymes to generate an exact 5′ end (le Mercier *et al*., 2002; Martin *et al*.,

2006); these ribozymes are probably not essential, as the virus itself removes additional 5′ nucleotides, but do make rescue more efficient. The initial hepatitis delta virus ribozyme sequence has also been modified to increase its efficiency (Perrotta and Been, 1998; Perrotta *et al*., 1999). Use of both these modifications (that is, the modified hepatitis delta virus ribozyme at the 3′ end and hammerhead ribozymes at the 5′ end to remove non-templated G residues) has increased the speed of rabies virus rescue as well as the rescue efficiency by a factor of a hundred (Ghanem *et al*., 2012).

Another issue in addition to the requirement to generate exact genomic ends is the requirement for some viruses, notably the paramyxoviruses, to comprise a precise number of nucleotides. Difficulties in rescuing paramyxoviruses eventually led to the discovery, published by Calain and Roux (1993), that the genomes needed to consist of a multiple of 6 nucleotides, as each nucleocapsid protein encapsidates exactly 6 nucleotides. This became known as the 'Rule of six' and many paramyxoviruses including the respiroviruses (for example, Sendai virus) and the morbilliviruses (for example, measles virus) can only be deleted or extended by a multiple of 6 nucleotides, although the restriction seems less tight for the rubulaviruses (for example, mumps virus), and members of the pneumovirus genus do not seem to have this requirement at all (Kolakofsky *et al*., 1998). Current understanding of how this affects the phase of the RNA is discussed for the measles virus in Chapter 6. Similar findings have been obtained for Ebola virus, for which deletions or insertions have to be a multiple of 6, although for this virus the total number of nucleotides is not divisible by 6 (Weik *et al*., 2005).

1.3.4 Increasing rescue efficiencies

In order to generate infectious virus from cDNA, particularly if the aim is to create a mutant virus that replicates inefficiently, it is important to maximise rescue efficiencies. This is particularly important for segmented genome viruses for which multiple clones need to be transfected into the same cell. This can be achieved by use of better transfection reagents, use of more transfectable cells or both. The former has become easier with the commercial production of many highly efficient and non-toxic transfection agents that produce consistent levels of transfection and can be used to transfect a wide range of cell types. Several of them are compared in the context of virus rescue by Gonzales *et al*. (2007). Other researchers use high efficiency electroporation to obtain high yields of recombinant virus (Surman *et al*., 2007). A novel reagent Nucleofector™ allows DNA or RNA to be electroporated directly into the nucleus of cells including embryonic stem cells and primary cell cultures (www.lonza.com). As well as being efficient, this procedure can be conducted without the presence of possibly contaminated bovine material, a requirement for clinical vaccine production.

Some cell types such as the human embryonic kidney 293 cells can be transfected to very high efficiencies but clearly cell choice has to depend first and foremost on

the efficiency of the virus to replicate in these cell lines. If one is attempting to generate a mutant virus deficient in host antagonism genes, it is worth bearing in mind the immunocompetence of the host cell: many cell lines including Veros and BHKs are deficient in interferon production, while the modified BHK cell line BSR-T7 is even more disrupted. It seems they have a complete defect in the activation of IRF-3, the transcription factor required for IFN-β expression (see Chapter 5 in this volume). Cell lines can be modified before transfection by incorporating a viral gene that antagonises host innate immune responses, for example, the pestivirus NPro gene. NPro blocks IRF-3 binding to DNA as well as targeting IRF-3 for polyubiquitination and subsequent destruction by cellular proteasomes (Hilton *et al*., 2006). Alternatively, the V protein of parainfluenza virus *5* blocks IFN signalling by targeting STAT1 for proteasome-mediated degradation. A range of human cell lines were generated that express the V protein and can no longer respond to IFN. When these cells were used to rescue viruses, many of them formed bigger plaques and grew to titres 10- to 4,000-fold higher than in the IFN-responsive cells. This is particularly pertinent for the generation of disabled vaccine candidates as well as for other slow-growing viruses (Young *et al*., 2003).

Many other factors can contribute to the efficiency of virus rescue. One group found that the use of capped RNA transcripts increased rescue efficiency for caliciviruses, as this more accurately mimics the natural RNA (Yunus *et al*., 2010).

1.3.5 Combining material from different genetic segments

A very specialised way of improving rescue efficiency is to reduce the plasmid number to be transfected, since one of the challenges of virus rescue is the transfection of all of the cDNA plasmids or RNAs into the same cell. This is particularly the case for viruses with multiple genetic segments, but applies to all rescues where more than one cDNA plasmid is required. Any reduction in plasmid number thus improves the chance of all the genetic material being present in the same cell and hence the rescue efficiency. Some approaches to this are described in Chapter 8 on influenza A virus. Figure 8.2 on p. 230 shows the reduction in plasmid number from 12 (a plasmid for each of the 8 genome segments and 4 protein encoding plasmids) to 8 by the generation of bi-directional constructs. Elderfield, Hartgroves and Barclay in Chapter 8 do comment that the plasmid numbers have been reduced further but many labs are continuing with the 8 plasmid rescue.

Similarly, the number of plasmids used for reovirus rescue has been reduced from 10 to 4, considerably enhancing efficiency (Kobayashi *et al*., 2010). Interestingly these authors postulate a variety of reasons why this is more efficient:

1. Greater probability that all genomic cDNAs will enter a single cell.
2. On the basis that much DNA entering a cell is degraded, this increases the probability that all genomic cDNAs will remain in a single cell.

3. Increased likelihood of more essential protein-protein or protein-RNA interactions required for virus recovery resulting from closer proximity of cDNAs.

1.3.6 Confirmation of the rescue genotypes/phenotypes

In order to be confident that the wild type virus produced after rescue is in fact a rescued virus and not a stray contaminant, it is advisable to introduce silent mutations into the viral genome at the development stage as we did with the first bunyavirus rescue (Bridgen and Elliott, 1996). Clearly modified viruses containing non-silent mutations can be identified by sequence, protein profiles or other phenotypic characteristic.

1.4 Difficulties in establishing a reverse genetics system

Reverse genetics systems are not always the easiest to set up. Difficulties which may be encountered fall into several categories:

(a) viral sequence;

(b) clone generation and stability;

(c) transfection and rescue;

(d) complex virus genome;

(e) inability of the virus to replicate in cell culture.

There are often problems with the sequence of the virus: is the published sequence correct? Have the viral termini been sequenced fully? Does the sequence represent the viral genome or have mutations been introduced as a result of viral attenuation in cell culture or the development of viral quasispecies? Or even through errors introduced during reverse transcription? Many rescues have been made harder by incorrect published sequences. Section 3.3 describes the incorrect initial sequencing of hepatitis C virus by omission of one non-coding region. It also goes on to describe how the incredibly high sequence variation in this virus required cDNA clones to be made using consensus sequences. Another example of a rescue made harder by sequence issues is that for the arenavirus lymphocytic choriomeningitis virus (LCMV). There often seem to be problems associated with an 8 nucleotide-long sequence in the long (L) segment intergenic region, a region of stem loop structure located between the two open reading frames in this segment. This region shows sequence variability and also deletions in the stem loop region which could have

come in either at the cloning stage or be inherent to the virus (Sanchez and de la Torre, 2006).

These difficulties are now less common since the advent of effective long-range and high fidelity PCR enzymes, which can be used to amplify the complete genome of several viruses. These amplified genomes can then be sequenced to check for any sequence variation, or several full length clones can made from different PCR products and then tested to see which will yield rescued virus on transfection. Care has to be taken at this stage, as errors here will make the final results meaningless. It is essential to sequence the initial cDNA clone as well as the rescued virus in order to ascertain that the rescued product has the same sequence.

Then there are difficulties in cloning the cDNA into bacteria. Many cDNAs can be cloned successfully but there are others such as the genomic cDNAs of yellow fever virus, coronaviruses and nairoviruses that seem to be toxic when expressed in *E. coli*. For example, in 1989, Rice *et al*. rescued yellow fever virus from cDNA but, due to the instability of the full-length cDNA clone and its toxicity in *E. coli*, needed to do this by *in vitro* ligation and transcription followed by RNA transfection (Rice *et al*., 1989). Another alphavirus cDNA, that of Japanese encephalitis virus, was stabilised by intron insertion (Yamshchikov *et al*., 2001). These authors also summarise many different approaches used to circumvent cloning difficulties in *E. coli*. The same group also reduced the enhancer of CMV to reduce spurious transcription in *E. coli* to improve viral stability even further (Mishin *et al*., 2001). For coronaviruses, three different approaches were used to circumvent the toxicity of the polymerase gene for the initial rescues: one group used an *in vitro* ligation transcription strategy, no mean feat for a 29 kb virus, one group made a vaccinia virus recombinant while a third used a bacterial artificial chromosome for expression (Almazan *et al*., 2000; Yount *et al*., 2000; Thiel *et al*., 2001).

Among negative sense RNA viruses, nairovirus polymerase (L) genes cloned into *E. coli* acquire spontaneous deletions or point mutations, or contain regions that cannot be cloned (unpublished results). Only one nairovirus L gene has been cloned, that of Crimean Congo haemorrhagic fever virus (CCHFV), and this was not straightforward (Frias-Staheli *et al*., 2007; Bergeron 2010).

Difficulties in transfection and rescue can arise from a number of different sources. Cells should be in good condition, ideally low passage, free from contamination and sub-confluent for most cell lines. The quality of the DNA is generally less of an issue; we obtained good results with pooled miniprep DNA! The ratio of plasmids is crucial; for the first measles rescue 5 μg of the plasmid harbouring the measles virus antigenomic DNA was mixed with only 1-100 ng of the plasmid encoding the measles virus L polymerase mRNA (Radecke *et al*., 1995). These ratios are usually determined at the minigenome stage. Non-templated nucleotides derived from the promoter may or may not be crucial as some viruses can tolerate additional 5′ nucleotides while others cannot. Use of helper viruses may interfere with virus rescue through competition for cellular resources, through damage to Golgi apparatus or other means.

The double-stranded RNA viruses have been one of the last categories of virus to be rescued largely because of their complex genome, with up to 12 segments of RNA. The first group to rescue reovirus entirely from cDNA (Kobayashi *et al*., 2007), estimated that viable virus was only made in $1{:}10^5$–$1{:}10^6$ cells, though this has now been improved upon (Kobayashi *et al*., 2010).

A final difficulty arises for those viruses which do not grow well in cell culture, for example C virus, since it is not too practical or ethical to conduct rescue experiments on chimpanzees! See Sections 3.4 and 3.7 for further details of how this difficulty was overcome. Another group of viruses which does not replicate well in cell culture is the *Norovirus* genus of the *Caliciviridae* family (Yunus *et al*., 2010). For this reason most of the reverse genetics experiments have been performed with murine norovirus, which does replicate well in cell culture, rather than human norovirus (see Chapter 4.1).

1.5 Recent developments

Most of the virus rescue systems have been optimised and improved since the first rescues. Some of these improvements are shown in Table 1.2. This is by no means a comprehensive listing, but is meant to illustrate with examples many of the developments which have helped to improved the efficiencies of virus rescues over the past decade or so. Further details of these systems can be found in the indicated chapters of this volume.

1.6 Are there any boundaries for conducting reverse genetics?

Now we have the potential to conduct reverse genetics experiments on nearly every group of human and animal viruses, are there any constraints which can or should limit what experiments are done? There are clearly scientific limitations to virus rescue. One constraint is that lethal mutations or mutations that are so detrimental that they are genetically unstable cannot easily be examined. For example, it is very hard to rescue full-length clones with modified promoter sequences or mutations in the active sites of the viral replicase/transcriptase. Another major constraint is how viruses are packaged: many viruses with rigid capsids cannot contain genomes above a certain size. Yet others, such as rhabdoviruses, which have elongated capsids that can just be extended, can incorporate a substantial additional amount of genetic material. This topic is touched on in several chapters in this volume, for example, Section 10.2.5 for orthoreoviruses. In contrast, altering viral gene order has become common, and plays a vital role in altering the level of expression of viral and additional genes. The more we understand about the packaging signals

Table 1.2 Examples of improvements to reverse genetic procedures.

Original rescue	Modified procedure(s)	Major improvements
Coronavirus (Chapter 2) Almazan *et al*., 2000	Gonzalez *et al*., 2002	1. Insertion of an intron into the toxic region of the polymerase gene increased rescue efficiency via the BAC system
Hepatitis C virus (Chapter 3) Multiple, see Chapter 3.	Lohmann *et al*., 2001 Kato *et al*., 2003 Blight *et al*., 2002	1. Re-engineering of viral cDNA to remove hindering mutations 2. Use of cDNA from a fulminant virus 3. Modification of Huh cell lines to reduce interferon signalling
Norovirus (Chapter 4) Chaudhry *et al*., 2007	Yunus *et al*., 2010	1. Change from use of fowl pox T7 to *in vitro* transcription and RNA capping using ScriptCap post-transcriptional enzymatic capping system followed by electroporation increased yield 10 fold
Rabies virus (Chapter 5) Schnell *et al*., 1994	Ghanem *et al*., 2012	1. Substitution of the "core" 3′ HDV ribozyme by one with enhanced cleavage activity resulted in c. 10-fold more rescue events and faster initiation of an infectious cycle 2. Use of hammerhead ribozyme to generate an exact 5′-end 3. Replacing both 5′ and 3′ ribozymes increased rescue efficency c. 100 fold
Measles virus (Chapter 6) Radecke *et al*., 1995	Schneider *et al*., 1997	1. Switched from plasmid derived T7 to vaccinia virus MVA-T7
Bunyamwera virus (Chapter 7) Bridgen and Elliott, 1996	Lowen *et al*., 2004	1. Switched from vaccinia T7 to BSR-T7 cell line as source of T7: much less toxicity and no need to passage through mosquito cells 2. Reduction of plasmids from 6 to 3 ie removal of protein expression plasmids
Influenza A virus (Chapter 8) Neumann *et al*., 1999	Hoffmann *et al*., 2000 Neumann *et al*., 2005	1. Reduction in plasmid number from 12-17 to 8 2. Further reduction in plasmid number from 8 then 1 (though 3-4 work better)
Reovirus (Chapters 9, 10) Kobayashi *et al*., 2007	Kobayashi *et al*., 2010	1. Constructs combined on plasmids so reduction in plasmid number from 10 to 4 2. Switched from vaccinia T7 (rDIs-T7pol) to BSR-T7 cell lines as source of T7

for specific segments in segmented viruses, the more we can know what the options are for adding or removing specific segments, which is discussed further in Section 12.12.

A more contentious issue is which viruses *should* be rescued. A few years ago there was much debate on the advisability of regenerating the 1918 H1N1 influenza A virus from patient samples (see Section 12.3). Currently, the main issue under discussion is the generation of avian H5N1 influenza A virus with enhanced transmissibility between mammalian species: two groups have made mutant viruses which pass easily between ferrets. Staggeringly, the U.S. National Science Advisory Board for Biosecurity (NSABB) has recommended that this research be redacted in order to avoid misuse of the information (Faden and Karron, 2012). In addition, the scientists themselves have agreed to a 60-day moratorium on using these pathogenic viruses until international agreement is reached about the wisdom and extent of this research (Fouchier *et al*., 2012).

The above provides two specific examples of ways in which viruses could possess altered tropism or pathogenicity. In general, alteration of the viral glycoprotein gene is likely to alter the viral tropism; care should therefore be taken particularly if any replacement glycoprotein has a likely wider tropism than the original. These experiments should always be subject to local genetic manipulation rules and appropriate levels of containment used. This is particularly the case for any virus which is a potential bioterrorism weapon. Several viruses which can now be generated by reverse genetic means, or are likely to be shortly, are on the CDC list of bioterrorism agents. In fact their very pathogenicity drives research into them. For example, haemorrhagic fever viruses such as Rift Valley fever virus (RVFV), Ebola, Marburg and the arenaviruses Lassa fever virus and Machupo viruses are category A bioterrorism weapons. Viral encephalitis-causing viruses such as the alphaviruses Venezuelan equine encephalitis, Eastern and Western equine encephalitis viruses are class B weapons, while the paramyxovirus Nipah virus and hantaviruses are class C weapons (http://www.bt.cdc.gov/agent/agentlist-category.asp#a, accessed 13 Dec. 2011). For the majority of viruses, they are already present in laboratories and in the wild, so experimentation just makes them more available to a wider range of people. However, the examples cited in the previous paragraph are viruses which were generated in the laboratory, leading to the potential for misuse.

Reverse genetics is thus an area which impinges on ethics and requires wisdom in its usage. Bhutkar (2005) comments that synthetic biology in general raises issues of ethics, regulation and patentability and this is clearly relevant to virus reverse genetics.

References

Almazan, F., Gonzalez, J. M., Penzes, Z., Izeta, A., Calvo, E., Plana-Duran, J. and Enjuanes, L. 2000. Engineering the largest RNA virus genome as an infectious

bacterial artificial chromosome. *Proceedings of the National Academy of Sciences of the United States of America* **97**(10): 5516–5521.

Ball, L. A. 1992. Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. *Journal of Virology* **66**(4): 2335–2345.

Bergeron, E., Albarino, C. G., Khristova, M. L. and Nichol, S. T. 2010. Crimean-Congo hemorrhagic fever virus-encoded ovarian tumor protease activity is dispensable for virus RNA polymerase function. *Journal of Virology* **84**(1): 216–226.

Bhutkar, A. 2005. Synthetic biology: navigating the challenges ahead. *The Journal of Biolaw & Business* **8**(2): 19–29.

Blakqori, G. and Weber, F. 2005. Efficient cDNA-based rescue of La Crosse bunyaviruses expressing or lacking the nonstructural protein NSs. *Journal of Virology* **79**(16): 10420–10428.

Blight, K. J., McKeating, J. A. and Rice, C. M. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *Journal of Virology* **76**(24): 13001–13014.

Boyer, J. C. and Haenni, A. L. 1994. Infectious transcripts and cDNA clones of RNA viruses. *Virology* **198**(2): 415–426.

Bridgen, A. and Elliott, R. M. 1996. Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proceedings of the National Academy of Sciences of the United States of America* **93**(26): 15400–15404.

Britton, P., Green, P., Kottier, S., Mawditt, K. L., Penzes, Z., Cavanagh, D. and Skinner, M. A. 1996. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. *The Journal of General Virology* **77**(5): 963–967.

Buchholz, U. J., Finke, S. and Conzelmann, K. K. 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *Journal of Virology* **73**(1): 251–259.

Calain, P. and Roux, L. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *Journal of Virology* **67**(8): 4822–4830.

Chaudhry, Y., Skinner, M. A. and Goodfellow, I. G. 2007. Recovery of genetically defined murine norovirus in tissue culture by using a fowlpox virus expressing T7 RNA polymerase. *The Journal of General Virology* **88**(8): 2091–2100.

Collins, P. L., Hill, M. G., Camargo, E., Grosfeld, H., Chanock, R. M. and Murphy, B. R. 1995. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proceedings of the National Academy of Sciences of the United States of America* **92**(25): 11563–11567.

Collins, P. L., Mink, M. A. and Stec, D. S. 1991. Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on

the expression of a foreign reporter gene. *Proceedings of the National Academy of Sciences of the United States of America* **88**(21): 9663–9667.

Conzelmann, K. K. 2004. Reverse genetics of mononegavirales. *Current Topics in Microbiology and Immunology* **283**: 1–41.

Crescenzo-Chaigne, B. and van der Werf, S. 2007. Rescue of influenza C virus from recombinant DNA. *Journal of Virology* **81**(20): 11282–11289.

Drexler, I., Heller, K., Wahren, B., Erfle, V. and Sutter, G. 1998. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. *The Journal of General Virology* **79**(2): 347–352.

Elroy-Stein, O., Fuerst, T. R. and Moss, B. 1989. Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. *Proceedings of the National Academy of Sciences of the United States of America* **86**(16): 6126–6130.

Enami, M., Luytjes, W., Krystal, M. and Palese, P. 1990. Introduction of site-specific mutations into the genome of influenza virus. *Proceedings of the National Academy of Sciences of the United States of America* **87**(10): 3802–3805.

Faden, R. R. and Karron, R. A. 2012. The obligation to prevent the next dual-use controversy. *Science* **335**(6070): 802–804.

Fearns, R. and Collins, P. L. 1999. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. *Journal of Virology* **73**(7): 5852–5864.

Flatz, L., Bergthaler, A., de la Torre, J. C. and Pinschewer, D. D. 2006. Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA, *Proceedings of the National Academy of Sciences of the United States of America* **103**(12): 4663–4668.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. and Garcia-Sastre, A. 1999. Rescue of influenza A virus from recombinant DNA. *Journal of Virology* **73**(11): 9679–9682.

Fouchier, R. A. M., Garcia-Sastre, A., Kawaoka, Y. *et al*. 2012. Pause on avian flu transmission research. *Science* **335**(6067): 400–401.

Fraser, D., Mahler, H. R., Shug, A. L. and Thomas, C.A. 1957. The infection of sub-cellular Escherichia Coli, strain B, with a DNA preparation from T2 bacteriophage. *Proceedings of the National Academy of Sciences of the United States of America* **43**(11): 939–947.

Frias-Staheli, N., Giannakopoulos, N. V., Kikkert, M., Taylor, S. L., Bridgen, A., Paragas, J., Richt, J. A., *et al*. 2007. Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. *Cell Host and Microbe* **2**(6): 404–416.

Fuerst, T. R., Niles, E. G., Studier, F. W. and Moss, B. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **83**(21): 8122–8126.

Gauliard, N., Billecocq, A., Flick, R. and Bouloy, M. 2006. Rift Valley fever virus noncoding regions of L, M and S segments regulate RNA synthesis. *Virology* **351**(1): 170–179.

Geigenmuller, U., Ginzton, N. H. and Matsui, S. M. 1997. Construction of a genome-length cDNA clone for human astrovirus serotype 1 and synthesis of infectious RNA transcripts. *Journal of Virology* **71**(2): 1713–1717.

Ghanem, A., Kern, A. and Conzelmann, K. K. 2012. Significantly improved rescue of rabies virus from cDNA plasmids. *European Journal of Cell Biology*, **91**: 10–16.

Gonzalez, G., Pfannes, L., Brazas, R. and Striker, R. 2007. Selection of an optimal RNA transfection reagent and comparison to electroporation for the delivery of viral RNA. *Journal of Virological Methods* **145**(1): 14–21.

Gonzalez, J. M., Penzes, Z., Almazan, F., Calvo, E. and Enjuanes, L. 2002. Stabilization of a full-length infectious cDNA clone of transmissible gastroenteritis coronavirus by insertion of an intron. *Journal of Virology* **76**(9): 4655–4661.

Habjan, M., Penski, N., Spiegel, M. and Weber, F. 2008. T7 RNA polymerase-dependent and -independent systems for cDNA-based rescue of Rift Valley fever virus. *The Journal of General Virology* **89**(9): 2157–2166.

Harty, R. N., Brown, M. E., Hayes, F. P., Wright, N. T. and Schnell, M. J. 2001. Vaccinia virus-free recovery of vesicular stomatitis virus. *Journal of Molecular Microbiology and Biotechnology* **3**(4): 513–517.

He, B., Paterson, R. G., Ward, C. D. and Lamb, R. A. 1997. Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* **237**(2): 249–260.

Hilton, L., Moganeradj, K., Zhang, G., Chen, Y. H., Randall, R. E., McCauley, J. W. and Goodbourn, S. 2006. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. *Journal of Virology* **80**(23): 11723–11732.

Hoffmann, E., Mahmood, K., Yang, C. F., Webster, R. G., Greenberg, H. B. and Kemble, G. 2002. Rescue of influenza B virus from eight plasmids. *Proceedings of the National Academy of Sciences of the United States of America* **99**(17): 11411–11416.

Hoffmann, E., Neumann, G., Hobom, G., Webster, R. G. and Kawaoka, Y. 2000. 'Ambisense' approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. *Virology* **267**(2): 310–317.

Jackson, D., Cadman, A., Zurcher, T. and Barclay, W. S. 2002. A reverse genetics approach for recovery of recombinant influenza B viruses entirely from cDNA. *Journal of Virology* **76**(22): 11744–11747.

Jiang, Y., Liu, H., Liu, P. and Kong, X. 2009. Plasmids driven minigenome rescue system for Newcastle disease virus V4 strain. *Molecular Biology Reports* **36**(7): 1909–1914.

Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M. and Nagai, Y. 1996. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative

or positive sense. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms* **1**(6): 569–579.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., Nozaki, A. and Shimotohno, K. 2003. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. *Biochemical and Biophysical Research Communications* **306**(3): 756–766.

Kaur, J., Tang, R. S., Spaete, R. R. and Schickli, J. H. 2008. Optimization of plasmid-only rescue of highly attenuated and temperature-sensitive respiratory syncytial virus (RSV) vaccine candidates for human trials. *Journal of Virological Methods* **153**(2): 196–202.

Kobayashi, T., Antar, A. A., Boehme, K. W., Danthi, P., Eby, E. A., Guglielmi, K. M., Holm, G. H., *et al*. 2007. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. *Cell Host & Microbe* **1**(2): 147–157.

Kobayashi, T., Ooms, L. S., Ikizler, M., Chappell, J. D. and Dermody, T. S. 2010. An improved reverse genetics system for mammalian orthoreoviruses. *Virology* **398**(2): 194–200.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. and Roux, L. 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *Journal of Virology* **72**(2): 891–899.

Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M. and Rice, C. M. 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science (New York, N.Y.)* **277**(5325): 570–574.

Kolykhalov, A. A., Reed, K. E. and Rice, C. M. 1999 Cloning and assembly of complex libraries of full-length HCV cDNA clones. *Methods in Molecular Medicine* **19**: 289–301.

Le Mercier, P., Jacob, Y., Tanner, K. and Tordo, N. 2002. A novel expression cassette of lyssavirus shows that the distantly related Mokola virus can rescue a defective rabies virus genome. *Journal of Virology* **76**(4): 2024–2027.

Lohmann, V., Korner, F., Dobierzewska, A. and Bartenschlager, R. 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *Journal of Virology* **75**(3): 1437–1449.

Lowen, A. C., Noonan, C., McLees, A. and Elliott, R. M. 2004. Efficient bunyavirus rescue from cloned cDNA. *Virology* **330**(2): 493–500.

Luytjes, W., Krystal, M., Enami, M., Parvin, J. D. and Palese, P. 1989. Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**(6): 1107–1113.

Martin, A., Staeheli, P. and Schneider, U. 2006. RNA polymerase II-controlled expression of antigenomic RNA enhances the rescue efficacies of two different members of the Mononegavirales independently of the site of viral genome replication. *Journal of Virology* **80**(12): 5708–5715.

Meulenberg, J. J., Bos-de Ruijter, J. N., van de Graaf, R., Wensvoort, G. and Moormann, R. J. 1998. Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. *Journal of Virology* **72**(1): 380–387.

Mishin, V. P., Cominelli, F. and Yamshchikov, V. F. 2001. A 'minimal' approach in design of flavivirus infectious DNA. *Virus Research* **81**(1–2): 113–123.

Mundt, E. and Vakharia, V. N. 1996. Synthetic transcripts of double-stranded Birnavirus genome are infectious. *Proceedings of the National Academy of Sciences of the United States of America* **93**(20): 11131–11136.

Muraki, Y., Murata, T., Takashita, E., Matsuzaki, Y., Sugawara, K. and Hongo, S. 2007. A mutation on influenza C virus M1 protein affects virion morphology by altering the membrane affinity of the protein. *Journal of Virology* **81**(16): 8766–8773.

Neumann, G., Feldmann, H., Watanabe, S., Lukashevich, I. and Kawaoka, Y. 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. *Journal of Virology* **76**(1): 406–410.

Neumann, G., Fujii, K., Kino, Y. and Kawaoka, Y. 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. *Proceedings of the National Academy of Sciences of the United States of America* **102**(46): 16825–16829.

Neumann, G. and Kawaoka, Y. 2004. Reverse genetics systems for the generation of segmented negative-sense RNA viruses entirely from cloned cDNA. *Current Topics in Microbiology and Immunology* **283**: 43–60.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., *et al*. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proceedings of the National Academy of Sciences of the United States of America* **96**(16): 9345–9350.

Neumann, G., Zobel, A. and Hobom, G. 1994. RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* **202**(1): 477–479.

Overby, A. K., Popov, V., Neve, E. P. and Pettersson, R. F. 2006. Generation and analysis of infectious virus-like particles of uukuniemi virus (bunyaviridae): a useful system for studying bunyaviral packaging and budding. *Journal of Virology* **80**(21): 10428–10435.

Parashar, U. D., Hummelman, E. G., Bresee, J. S., Miller, M. A. and Glass, R. I. 2003. Global illness and deaths caused by rotavirus disease in children. *Emerging Infectious Diseases* **9**(5): 565–572.

Pattnaik, A. K., Ball, L. A., LeGrone, A. W. and Wertz, G. W. 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**(6): 1011–1020.

Perrotta, A. T. and Been, M. D. 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. *Nature* **350**(6317): 434–436.

Perrotta, A. T. and Been, M. D. 1998. A toggle duplex in hepatitis delta virus self-cleaving RNA that stabilizes an inactive and a salt-dependent pro-active ribozyme conformation. *Journal of Molecular Biology* **279**(2): 361–373.

Perrotta, A. T., Nikiforova, O. and Been, M. D. 1999. A conserved bulged adenosine in a peripheral duplex of the antigenomic HDV self-cleaving RNA

reduceskinetic trapping of inactive conformations. *Nucleic Acids Research* **27**(3): 795–802.

Pradeau-Aubreton, K., Ruff, M., Garnier, J. M., Schultz, P. and Drillien, R. 2010. Vectors for recombinational cloning and gene expression in mammalian cells using modified vaccinia virus Ankara. *Analytical Biochemistry* **404**(1): 103–105.

Qi, X., Gao, Y., Gao, H., Deng, X., Bu, Z., Wang, X., Fu, C. and Wang, X. 2007. An improved method for infectious bursal disease virus rescue using RNA polymerase II system. *Journal of Virological Methods* **142**(1–2): 81–88.

Racaniello, V. R. and Baltimore, D. 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science (New York, N.Y.)* **214**(4523): 916–919.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G. and Billeter, M. A. 1995. Rescue of measles viruses from cloned DNA. *The EMBO Journal* **14**(23): 5773–5784.

Rice, C. M., Grakoui, A., Galler, R. and Chambers, T. J. 1989. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *The New Biologist* **1**(3): 285–296.

Sanchez, A. B. and de la Torre, J. C. 2006. Rescue of the prototypic Arenavirus LCMV entirely from plasmid. *Virology* **350**(2): 370–380.

Sasaki, J., Kusuhara, Y., Maeno, Y., Kobayashi, N., Yamashita, T., Sakae, K., Takeda, N. and Taniguchi, K. 2001. Construction of an infectious cDNA clone of Aichi virus (a new member of the family Picornaviridae) and mutational analysis of a stem-loop structure at the 5′ end of the genome. *Journal of Virology* **75**(17): 8021–8030.

Schneider, H., Spielhofer, P., Kaelin, K., Dotsch, C., Radecke, F., Sutter, G. and Billeter, M. A. 1997. Rescue of measles virus using a replication-deficient vaccinia-T7 vector. *Journal of Virological Methods* **64**(1): 57–64.

Schneider, U., Schwemmle, M. and Staeheli, P. 2005. Genome trimming: a unique strategy for replication control employed by Borna disease virus. *Proceedings of the National Academy of Sciences of the United States of America* **102**(9): 3441–3446.

Schnell, M. J., Mebatsion, T. and Conzelmann, K. K. 1994. Infectious rabies viruses from cloned cDNA. *The EMBO Journal* **13**(18): 4195–4203.

Sosnovtsev, S. and Green, K. Y. 1995. RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VpG for infectivity. *Virology* **210**(2): 383–390.

Surman, S. R., Collins, P. L., Murphy, B. R. and Skiadopoulos, M. H. 2007. An improved method for the recovery of recombinant paramyxovirus vaccine candidates suitable for use in human clinical trials. *Journal of Virological Methods* **141**(1): 30–33.

Sutter, G., Ohlmann, M. and Erfle, V. 1995. Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Letters* **371**(1): 9–12.

Taniguchi, T., Palmieri, M. and Weissmann, C. 1978. A Qbeta DNA-containing hybrid plasmid giving rise to Qbeta phage formation in the bacterial host (proceedings). *Annales de Microbiologie* **129**: B(4): 535–536.

Thiel, V., Herold, J., Schelle, B. and Siddell, S. G. 2001. Infectious RNA transcribed in vitro from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. *The Journal of General Virology* **82**(6): 1273–1281.

van Gennip, H. G., van Rijn, P. A., Widjojoatmodjo, M. N. and Moormann, R. J. 1999. Recovery of infectious classical swine fever virus (CSFV) from full-length genomic cDNA clones by a swine kidney cell line expressing bacteriophage T7 RNA polymerase. *Journal of Virological Methods* **78**(1–2): 117–128.

Volchkov, V. E., Volchkova, V. A., Muhlberger, E., Kolesnikova, L. V., Weik, M., Dolnik, O. and Klenk, H. D. 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. *Science (New York, N.Y.)* **291**(5510): 1965–1969.

Wagner, E., Engelhardt, O. G., Gruber, S., Haller, O. and Kochs, G. 2001. Rescue of recombinant Thogoto virus from cloned cDNA. *Journal of Virology* 7**5**(19): 9282–9286.

Weik, M., Enterlein, S., Schlenz, K. and Muhlberger, E. 2005. The Ebola virus genomic replication promoter is bipartite and follows the rule of six. *Journal of Virology* **79**(16): 10660–10671.

Wenigenrath, J., Kolesnikova, L., Hoenen, T., Mittler, E. and Becker, S. 2010. Establishment and application of an infectious virus-like particle system for Marburg virus. *Journal of General Virology* **91**(5): 1325–1334.

Whelan, S. P., Ball, L. A., Barr, J. N. and Wertz, G. T. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proceedings of the National Academy of Sciences of the United States of America*, **92**(18): 8388–8392.

Witko, S. E., Kotash, C. S., Nowak, R. M., Johnson, J. E., Boutilier, L. A., Melville, K. J., Heron, S. G., *et al*. 2006. An efficient helper-virus-free method for rescue of recombinant paramyxoviruses and rhadoviruses from a cell line suitable for vaccine development. *Journal of Virological Methods* **135**(1): 91–101.

Wu, X. and Rupprecht, C. E. 2008. Glycoprotein gene relocation in rabies virus. *Virus Research* **131**(1): 95–99.

Yamshchikov, V., Mishin, V. and Cominelli, F. 2001. A new strategy in design of +RNA virus infectious clones enabling their stable propagation in E. coli. *Virology* **281**(2): 272–280.

Yanagi, M., Purcell, R. H., Emerson, S. U. and Bukh, J. 1997. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proceedings of the National Academy of Sciences of the United States of America* **94**(16): 8738–8743.

Young, D. F., Andrejeva, L., Livingstone, A., Goodbourn, S., Lamb, R. A., Collins, P. L., Elliott, R. M. and Randall, R. E. 2003. Virus replication in engineered human cells that do not respond to interferons. *Journal of Virology* **77**(3): 2174–2181.

Yount, B., Curtis, K. M. and Baric, R. S. 2000. Strategy for systematic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. *Journal of Virology* **74**(22): 10600–10611.

Yunus, M. A., Chung, L. M., Chaudhry, Y., Bailey, D. and Goodfellow, I. 2010. Development of an optimized RNA-based murine norovirus reverse genetics system. *Journal of Virological Methods* **169**(1): 112–118.

Zheng, H., Tian, H., Jin, Y., Wu, J., Shang, Y., Yin, S., Liu, X. and Xie, Q. 2009. Development of a hamster kidney cell line expressing stably T7 RNA polymerase using retroviral gene transfer technology for efficient rescue of infectious foot-and-mouth disease virus. *Journal of Virological Methods* **156**(1–2): 129–137.

Zobel, A., Neumann, G. and Hobom, G. 1993. RNA polymerase I catalysed transcription of insert viral cDNA. *Nucleic Acids Research* **21**(16): 3607–3614.

Part I

Positive sense RNA viruses

2

Coronavirus reverse genetics

Maria Armesto, Kirsten Bentley, Erica Bickerton, Sarah Keep and Paul Britton
Avian Viral Diseases, Institute for Animal Health, Compton Laboratory, Newbury, Berkshire

2.1 The *Coronavirinae*

The *Coronaviridae* form part of the order *Nidovirales*, which comprises two subfamilies, the *Coronavirinae* and *Torovirinae*. There are three genera of coronaviruses, *alpha-*, *beta-* and *gammacoronaviruses* (Carstens, 2010), which were so named for their visual resemblance to the corona of the sun in negatively stained preparations (Figure 2.1) (Tyrrell *et al*., 1968). Representative members of each of the coronavirus genera are shown in Table 2.1. Possibly the most publicised coronavirus of recent years has been the human coronavirus SARS-CoV, which emerged in China in 2002 causing the severe acute respiratory syndrome epidemic (Drosten *et al*., 2003; Ksiazek *et al*., 2003; Marra *et al*., 2003; Rota *et al*., 2003). Coronaviruses have, however, been isolated from many vertebrates and cause several economically important diseases in livestock species, including pigs, cows and chickens, and domestic species, such as dogs and cats.

Coronaviruses are enveloped viruses with a single-stranded positive-sense RNA genome of 26–32kb, the largest genomes of all RNA viruses currently known, that replicate in the cytoplasm of infected cells. The genome associates with the nucleoprotein (N), forming a helical nucleocapsid within the virus particles. Although common among negative-sense RNA viruses, coronaviruses are the only positive-sense RNA viruses to possess helical nucleocapsids, which are enclosed within lipid envelopes containing the spike (S) glycoprotein, membrane (M) protein and envelope (E) protein (Figure 2.1). For general reviews, see (Siddell, 1995; Lai and Cavanagh, 1997; Enjuanes, 2005; Siddell *et al*., 2005; Enjuanes *et al*., 2006; Gorbalenya *et al*., 2006; Masters, 2006; Thiel, 2007; Britton and Cavanagh, 2008; Perlman *et al*., 2008; Norkin, 2010).

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

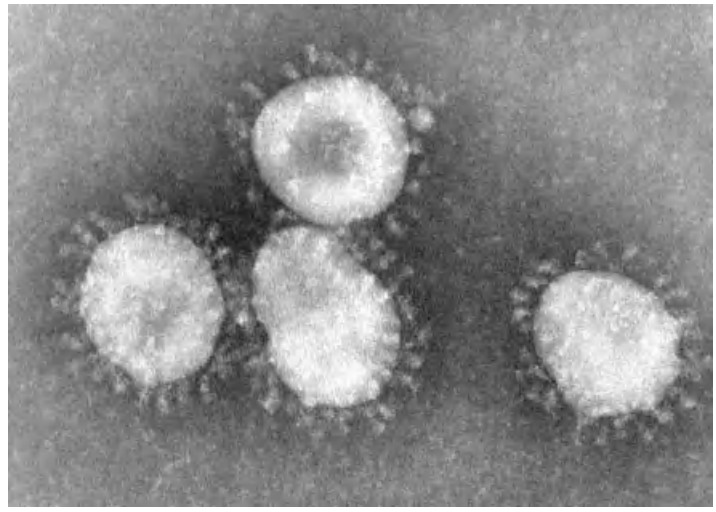

Figure 2.1 Coronavirus structure and electron micrograph of Coronavirus particles. All coronavirus particles contain three membrane proteins, the S glycoprotein, the M and the E proteins, which are embedded in the lipid membrane. The virus particle also contains the N protein, which interacts with the RNA genome to form a helical nucleocapsid. The large size of the S glycoprotein gives the coronavirus particle the distinctive corona.
Source: Micrograph obtained from the CDC Public Health Image Library, ID number 4814.

2.2 Infectious bronchitis

The avian coronavirus infectious bronchitis virus (IBV) is the aetiological agent of the disease infectious bronchitis (IB) that affects poultry. IBV replicates primarily in the respiratory tract, causing the highly contagious respiratory disease IB in chickens characterised by nasal discharge, snicking, tracheal ciliostasis and rales (Britton and Cavanagh, 2007). Replication also occurs in other epithelial surfaces including enteric surfaces, oviducts and kidneys (Ambali and Jones, 1990; Cavanagh, 2005; Cavanagh and Gelb, 2008; Jones, 2010). Following an IBV infection, egg production and quality are impaired in layers, and weight gain in broilers is reduced (Cook and Mockett, 1995). Infected birds are predisposed to secondary bacterial infections such as colibacillosis and mortality in young chicks is not uncommon. Faecal excretion of the virus is a consequence of replication in the intestinal tract; however, this does not normally result in clinical disease.

Infectious bronchitis was first described in the US in the 1930s (Schalk and Hawn, 1931; Beach and Schalm, 1936; Beaudette and Hudson, 1937) and is prevalent in poultry farming across the world due to the intensive nature of poultry production, estimated to involve the global production of 55 billion chickens (50 billion broilers and 5 billion layers) on an annual basis. In a report, commissioned by Defra in 2005 (Defra, 2005), IBV was indicated as a major cause of ill health among chickens and was implicated as being responsible for more economic loss in the UK

Table 2.1 Coronavirus genera and species.

Genus	Species	
alphacoronavirus	Canine coronavirus (CCoV)	
	Feline coronavirus (FCoV)	
	Feline infectious peritonitis virus (FIPV)	
	Human coronavirus 229E (HCoV-229E)	
	Porcine epidemic diarrhoea virus (PEDV)	
	Porcine transmissible gastroenteritis virus (TGEV)	
betacoronavirus	Bovine coronavirus (BCoV)	
	Human coronavirus HKU1 (HCoV-HKU1)	
	Human coronavirus OC43 (HCoV-OC43)	
	Human enteric coronavirus (HECoV)	
	Murine hepatitis virus (MHV)	
	Porcine haemagglutinating encephalomyelitis virus (HEV)	
	Rat coronavirus (RtCoV)	
	Severe acute respiratory syndrome coronavirus (SARS-CoV)	
gammacoronavirus	IBV-like avian	Infectious bronchitis virus (IBV)
		Turkey coronavirus (TCoV)
		Pheasant coronavirus (PhCoV)
	Non-IBV-like avian	Munia coronavirus (MunCoV)
		Bulbul coronavirus (BuCoV)
		Thrush coronavirus (ThCoV)
	Mammalian	Beluga whale coronavirus SW1 (BeCoV)
		Asian leopard cat coronavirus
	Others*	Goose coronavirus
		Pigeon coronavirus
		Duck coronavirus

Note: *The derivation of these species of *gammacoronavirus* has yet to be determined, according to the International Committee on Taxonomy of Viruses, Index of Viruses (Coronaviridae, 2008).

poultry industry than any other disease (Bennett, 2003; Bennett and Jpelaar, 2005); IBV was estimated to cost the UK economy nearly £19 million per year, mainly due to loss of egg production, with serious implications for animal welfare. The cost of control through vaccination is approximately £5 million per year in the UK.

2.3 Coronavirus genome organisation

The genomic RNA has a 5′ m7GpppN-cap and a 3′ poly(A) tail with untranslated regions (UTRs) at the 5′ and the 3′ ends that have been shown to be involved in replication and translation (Senanayake and Brian, 1999), reviewed in (Brian and Baric, 2005; Van den Born and Snijder, 2008). The same general genome organisation is shared within the genus: 5′ UTR – replicase gene – structural protein genes – UTR 3′ (Figure 2.2). The 3′-end of the genome (∼8kb) encodes the structural protein genes in the order S – E – M – N, with some *betacoronaviruses* also producing an haemagglutinin esterase (HE) protein, the gene of which is situated

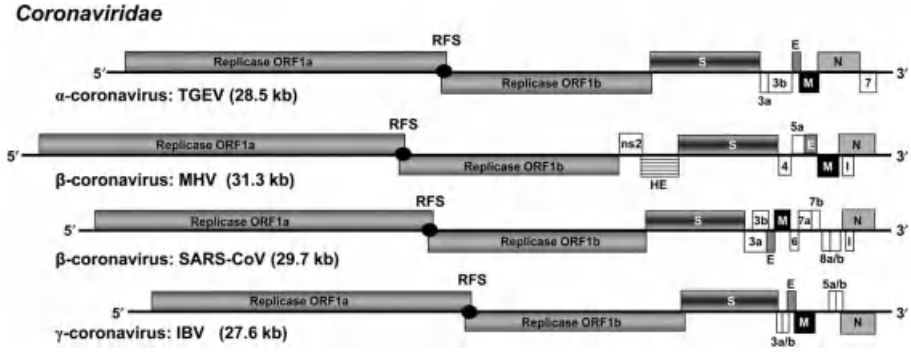

Figure 2.2 Comparison of the coronavirus genomic organisations of viruses belonging to the three genera. The replicase gene is comprised of ORFs 1a and 1b, which are located distal to the 5′ UTR and the leader sequence found at the 5′ end of the genome. ORFs 1a and 1b encode the proteins associated with RNA replication and are translated as two polyproteins, pp1a and pp1ab via a -1 ribosome frameshift site (RFS) between the two ORFs. The structural protein genes S, E, M and N, are located proximal to the 3′ UTR. Some *betacoronaviruses*, such as MHV, also encode an extra membrane associated structural protein, the HE protein, found 5′ of the S gene. Interspersed between the structural protein genes are the accessory genes encoding non-structural proteins, which are not essential for replication *in vitro*. TGEV encodes three accessory proteins, 3a, 3b and 7. MHV also encodes three accessory proteins, 2, 4 and 5a. SARS-CoV encodes seven accessory proteins, 3a, 3b, 6, 7a, 7b, 8a and 8b. IBV encodes four accessory proteins, 3a, 3b, 5a and 5b. Although genes encoding accessory proteins have the same location within the genome of a coronavirus, for example, 3a and 3b in TGEV, SARS-CoV and IBV, they are not homologous.

upstream of the S gene. The replicase gene (gene 1) encompasses the 5′ most two-thirds of the entire genome and consists of two large open reading frames, ORF1a and 1b (Boursnell *et al*., 1987), reviewed in (Britton and Cavanagh, 2008; Ziebuhr, 2008). ORFs 1a and 1b overlap and the 1b sequence is translated as a result of a -1 frameshift mechanism (Brierley *et al*., 1987), the signal for which, consisting of a pseudoknot structure and a slippery sequence, lies in the overlapping region between ORF1a and 1b. In addition to the replicase gene and structural protein genes, coronavirus genomes also have several polycistronic genes encoding non-structural or accessory proteins, often referred to as group-specific genes. For example, IBV encodes four accessory proteins, 3a, 3b, 5a and 5b encoded by two polycistronic genes, 3 and 5 (Figure 2.2), the functions of which are as yet unknown.

2.4 The coronavirus replication cycle

The coronavirus replication cycle occurs in the cell cytoplasm as outlined in Figure 2.3, in which the S glycoprotein mediates attachment to host cell receptors and fusion of the virion membrane to the host cell membrane (Koch *et al*., 1990; Luo and Weiss, 1998). Binding of the S glycoprotein, via the receptor binding domain on the S1 subunit, to the host cell receptor induces conformational changes in

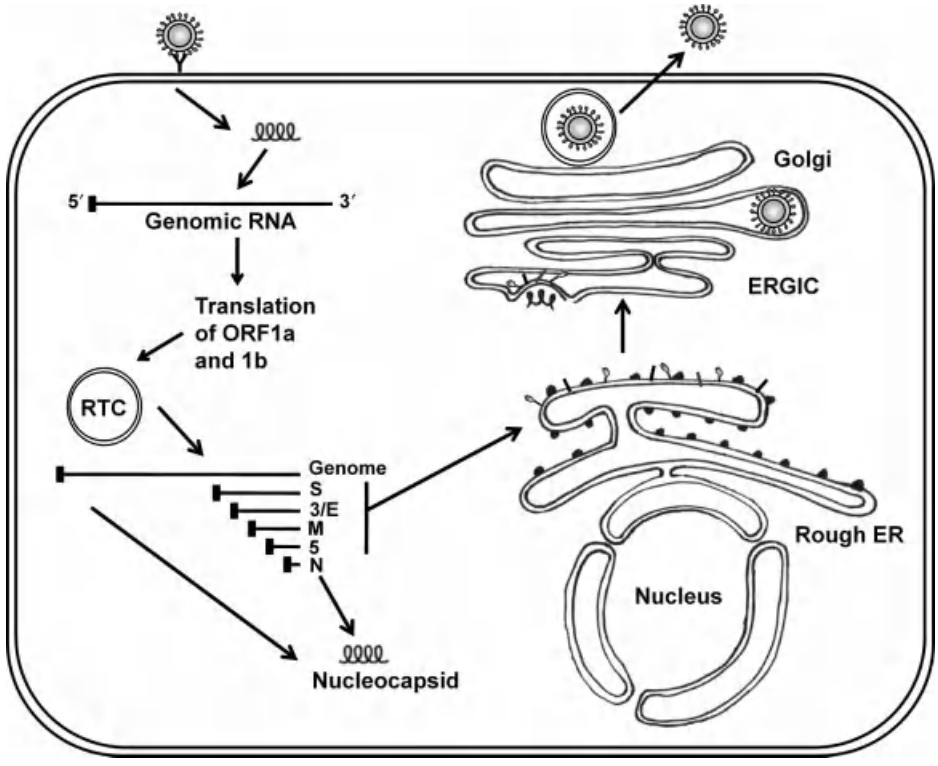

Figure 2.3 The replication cycle of a coronavirus following infection of a susceptible cell. The virus particle attaches to the host cell receptor and fuses with the cell membrane via the S glycoprotein. Genomic RNA is released and acts as a mRNA for the translation of the replicase proteins. Virus-encoded proteinases proteolytically cleave the replicase polyproteins generating 15–16 products, which assemble into replication-transcription complexes (RTCs) associated with virus-induced double membrane vesicles (DMVs). Sub-genomic mRNAs are produced from the genomic RNA for the expression of the structural and accessory proteins at the rough ER. The membrane associated structural proteins assemble into virus particles at the ERGIC. The N protein associates with the genomic RNA to form the nucleocapsid and is incorporated into virions at the ERGIC. Virus particles bud off from the Golgi apparatus and exit the cell by exocytosis.

the S glycoprotein (Zelus *et al*., 2003; Tripet *et al*., 2004; Guo *et al*., 2009; Shulla and Gallagher, 2009), leading to virus–cell fusion and release of the nucleocapsid into the cytoplasm. Some coronaviruses such as SARS-CoV (Inoue *et al*., 2007) and MHV (Eifart *et al*., 2007) have been shown to utilise the clathrin-dependent endocytosis pathway for entry before being transported to early endosomes. Conformational changes in the S glycoprotein leading to virus–cell fusion may be pH-dependent, as in the cases of IBV (Chu *et al*., 2006) and SARS-CoV (Yang *et al*., 2004), or may be activated by proteases, as in the cases of HCoV-229E (Kawase *et al*., 2009) and SARS-CoV (Simmons *et al*., 2005; Matsuyama *et al*., 2010).

Following virus entry and uncoating, gene 1 of genomic RNA is directly translated into the two large replicase polyproteins, pp1a and pp1ab. Both polyproteins are proteolytically cleaved by two or three virus-encoded proteinases (Ziebuhr *et al*., 2000; Ziebuhr, 2008) and form replication-transcription complexes (RTC) on virus-induced double membrane vesicles (DMVs); autoproteolytic processing of pp1a and pp1ab polyproteins produces the 15 (IBV) or 16 (other coronaviruses) replicase non-structural proteins (nsp). The RTCs are then responsible for the replication and transcription of genomic and subgenomic mRNAs.

A model for coronavirus transcription and subsequent translation (Figure 2.4) has been described by Sawicki and Sawicki (Sawicki and Sawicki, 1995, 1998, 2005; Sawicki *et al*., 2007), reviewed in (Pasternak *et al*., 2006; Van den Born and Snijder, 2008). Minus strand RNA templates are synthesised from the genomic RNA; genome-length RNA copies are then produced by continuous transcription whereas subgenome-length RNAs are produced by a discontinuous transcription mechanism. A transcription regulation sequence (TRS), CTTAACAA for IBV, found upstream of each gene on the genomic RNA and at the leader junction site at the 5′ end of the genome is responsible for the generation of coronavirus subgenomic mRNAs. The generation of coronavirus subgenomic mRNAs starts with the synthesis of a negative-strand copy that initiates from the 3′ end of the genomic RNA and continues until a TRS is reached on the genomic RNA. The RTC either pauses and then continues on to the next TRS or translocates to the TRS comprising the leader junction sequence at the 5′ end of the genome and results in the discontinuous addition of a negative-sense copy of the leader RNA sequence at the 3′ end. The overall process results in a series of negative-sense copies of the sub-genomic RNAs with an anti-leader sequence at the 3′ end. The negative sense genome-length and sub-genomic RNAs are used as templates for synthesis of genomic RNA and a nested set of sub-genomic mRNAs, in which each mRNA has the same 3′ terminus and short 5′ leader sequence, identical to the 5′ end of the genome (Lai *et al*., 1983). Most sub-genomic mRNAs are structurally polycistronic but functionally monocistronic in which only the ORF at the 5′ end is translated by a cap-dependent mechanism. However, some subgenomic mRNAs are functionally bi- or tricistronic and are subsequently translated via a leaky-scanning mechanism or by internal ribosome entry (Liu and Inglis, 1991, 1992; Le *et al*., 1994). Newly synthesised viral RNA is found associated with convoluted membranes and DMVs (Gosert *et al*., 2002), reviewed in (Baker and Denison, 2008), that are thought to originate from the endoplasmic reticulum (ER) (Knoops *et al*., 2008), although autophagy may also be involved (Prentice *et al*., 2004). These membranes may serve to protect viral RNA from degradation or provide an optimal environment for viral RNA synthesis (van Hemert *et al*., 2008).

The nucleocapsid associates with the M protein (Sturman *et al*., 1980) and structural proteins assemble at the ER-Golgi intermediate compartment (ERGIC) (Klumperman *et al*., 1994). Complete virus particles bud off from the Golgi apparatus and exit the cell by exocytosis (Tooze *et al*., 1987).

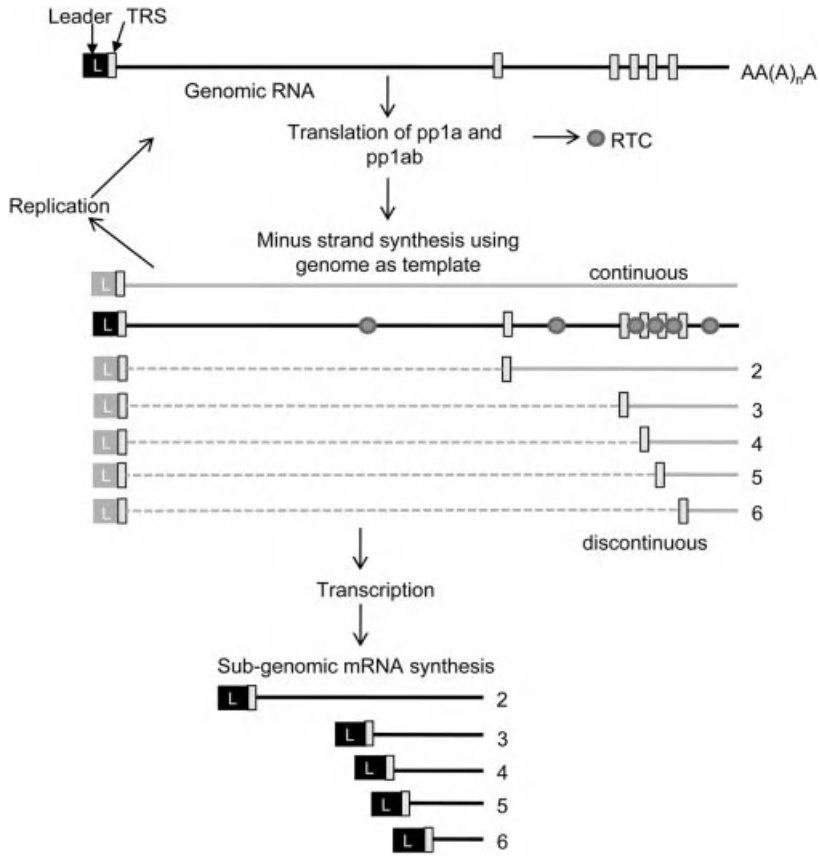

Figure 2.4 Coronavirus replication and transcription. Coronavirus-derived replicase proteins within the RTCs recognise *cis*-acting elements at the 5′ and 3′ ends of the genomic RNA and copy the genome into either a genome-length negative-strand template or generate sub-genomic negative-strand templates by a discontinuous process. Negative strands are shown in light grey and are used as templates for genomic and sub-genomic mRNA synthesis, generating a 3′-coterminal nested set of sub-genomic mRNAs. Anti-leader sequences are also shown in light grey. The RTCs age, releasing the minus strand templates for degradation.
Source: Adapted from (Sawicki *et al*., 2007).

2.5 Development of reverse genetics system for coronaviruses including IBV

Coronaviruses have a single-stranded, non-segmented positive sense RNA genome, requiring the generation of a cDNA that can function as a template for the generation of infectious RNA. The initial stage for a coronavirus-based reverse genetics system involves conversion of the RNA genome into an authentic cDNA that can be

manipulated using standard DNA technologies or utilising homologous recombination. The final stage of the process requires the generation of an infectious RNA from the modified cDNA utilising a DNA-dependent RNA polymerase. Viruses, including coronaviruses, with a positive-sense single-stranded RNA genome have the advantage that the infectious RNA derived from a cDNA copy, like the genomic RNA, can be recognised by a host cell's transcriptional machinery as an mRNA, resulting in the synthesis of the protein(s) required for replication of the RNA genome, in the case of coronaviruses, this involves 15–16 distinct proteins. Historically, the development of the first reverse genetics system for a single-stranded RNA virus recovered from a cDNA was for the bacteriophage Qβ (4.5kb) (Taniguchi *et al*., 1978). This early success was followed by the recovery of viruses from cDNAs generated from RNA viruses with increasing size as outlined by (Racaniello and Baltimore, 1981; Rice *et al*., 1987; Liljestrom *et al*., 1991).

The first reverse genetics system for coronaviruses was developed during the 1990s using targeted RNA recombination rather than recovery of a virus from a full-length cDNA of the virus genome, reviewed in (Masters, 1999; Masters and Rottier, 2005). This technology allowed the modification of a coronavirus genome utilising a recombination event between either a non-replicating or replicating RNA, generated from a modifiable cDNA, introduced into the same cell as the replicating coronavirus genome and a selective marker to differentiate recombinant viruses. Targeted RNA recombination was devised as a method of modifying a coronavirus genome as it was unclear at that time whether the construction of a full-length cDNA and subsequent generation of an infectious RNA were possible for an RNA virus with such a large genome size. The method was originally based on a temperature sensitive (*ts*) lesion within the N protein gene of MHV (Koetzner *et al*., 1992) and later utilised selection via retargeting a recombinant coronavirus, by the use of heterologous S glycoprotein, to different cell types (Kuo *et al*., 2000). For example, modifications were made to MHV by producing a virus, fMHV, which expressed the ectodomain of the S glycoprotein from FIPV allowing for selection on feline cells, modifications were made to the MHV genome with concomitant replacement of the FIPV S glycoprotein with the MHV S glycoprotein allowing selection of the recombinant MHV (rMHV) on murine cells (Kuo *et al*., 2000). The technology is still a useful tool for specifically modifying some coronavirus genomes; however, the main disadvantage is that it is difficult to modify the replicase region of the genome.

Following the use of targeted recombination to modify a coronavirus genome, cDNAs capable of generating infectious RNAs were being produced for increasingly larger RNA genomes ranging from 15 kb (arteriviruses) (van Dinten *et al*., 1997) to 20 kb (citrus tristeza virus of the genus *Closterovirus*) (Satyanarayana *et al*., 2001). However, despite these successes, it was found that the size of the RNA virus genome was not the main constraint on generating a successful reverse genetics system. The instability of some virus-derived cDNAs in bacteria was identified as a problem that required ingenious strategies for the assembly of full-length cDNAs for generating infectious RNAs. For example, *in vitro* ligation, without

assembly of the full-length cDNA in bacteria, was used to develop an infectious clone system for yellow fever virus (Rice *et al*., 1989); construction of full-length cDNAs in yeast (Polo *et al*., 1997), or the introduction of short introns to allow propagation of cDNAs in *Escherichia coli* (*E. coli*) (Yamshchikov *et al*., 2001) have proven successful strategies for the generation of full-length, stable genomic cDNAs of dengue and Japanese encephalitis viruses.

Several groups, including our own, discovered that certain regions of the coronavirus replicase gene proved to be highly unstable in *E. coli*, therefore preventing the assembly of a full-length coronavirus-derived cDNA. The breakthrough in the development of the first coronavirus reverse genetics system based on a full-length cDNA for generating infectious RNA was reported in 2000 for the porcine coronavirus TGEV (Almazán *et al*., 2000), reviewed in (Enjuanes *et al*., 2005; Deming and Baric, 2008). The TGEV full-length cDNA was assembled in a bacterial artificial chromosome (BAC), immediately downstream of a cytomegalovirus (CMV) RNA polymerase II promoter for subsequent generation of infectious RNA. The BAC system was chosen due to the presence of only a single copy per bacterial cell and because it allowed the introduction of the unstable region as a final step. Construction of the TGEV cDNA was initiated from a cDNA representing a defective RNA (D-RNA) that could be rescued by helper TGEV, thus indicating that all the RNA sequences required for replication were present on the D-RNA (Izeta *et al*., 1999). The authors then sequentially introduced the TGEV sequence absent from the D-RNA to create a full-length cDNA. During this process a sequence, corresponding to part of the replicase sequence, was found to be unstable in *E. coli* to such an extent that an intact cDNA could not be maintained in the bacteria. The Enjuanes group successfully produced the TGEV cDNA by initially generating a cDNA lacking the sequence that gave rise to instability in *E. coli*, under the control of the CMV promoter in a BAC. The unstable sequence was introduced into the cDNA as a final cloning step, resulting in a relatively stable full-length cDNA that could be amplified in *E. coli*. Transfection of the TGEV-BAC construct into susceptible cells resulted in the synthesis of infectious RNA in the nucleus by cellular RNA polymerase II and subsequent amplification in the cytoplasm by virus-encoded enzymes for the recovery of infectious recombinant virus, reviewed in (Enjuanes *et al*., 2005).

A second reverse genetics system was reported for TGEV involving the *in vitro* assembly of a full-length cDNA using a series of contiguous cDNAs containing engineered unique restriction sites, dispensing with the requirement for *E. coli* (Yount *et al*., 2000). Infectious RNA was produced *in vitro* using bacteriophage T7-RNA polymerase, utilising a T7-RNA polymerase promoter immediately upstream of the 5′ end of the TGEV cDNA, and electroporated into susceptible cells for the rescue of infectious virus. The authors found that this system required TGEV N protein for the recovery of infectious virus.

A third coronavirus reverse genetics system utilising vaccinia virus (VV) as the vector for the full-length cDNA was reported for the recovery of HCoV 229E (Thiel *et al*., 2001) and the avian coronavirus IBV (Casais *et al*., 2001). In both systems,

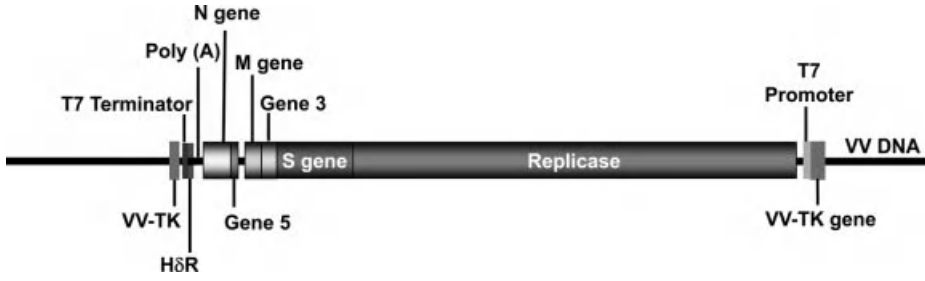

Figure 2.5 Schematic diagram of the IBV Beau-R full-length cDNA inserted into the vaccinia virus TK gene. The IBV cDNA, representing a full-length copy of the IBV Beau-R genomic RNA (Casais *et al*., 2001), is shown inserted within a *Not*I restriction site within the TK gene of VV vNotI/tk (Merchlinsky and Moss, 1992). The IBV cDNA is shown in a 3′–5′ direction as the VV DNA is in the 5′–3′ orientation. The IBV genes are indicated as are the positions of the T7 promoter, the HδR and T7 termination sequences in relation to the IBV cDNA.

sequential cDNA fragments corresponding to the two genomes were generated and systematically ligated together *in vitro* before direct cloning into the genome of VV vNotI/tk, via a NotI site introduced into the thymidine kinase (TK) gene of vNotI/tk (Merchlinsky and Moss, 1992). This resulted in a full-length cDNA under the control of a T7 RNA polymerase promoter with a hepatitis δ ribozyme (HδR) placed downstream of the coronavirus poly(A) tail followed by a T7 termination sequence (Figure 2.5), reviewed in (Thiel and Siddell, 2005). Infectious RNA can be generated *in vitro* from VV templates using T7 RNA polymerase and transfected into permissive cells for the recovery of infectious virus (Thiel *et al*., 2001). Alternatively, infectious RNA can be generated *in situ* in which VV DNA is transfected into cells infected with a recombinant fowlpox virus, rFPV-T7 expressing T7 RNA polymerase (Britton *et al*., 1996), for the recovery of infectious virus (Casais *et al*., 2001). We found that the IBV N protein is an absolute requirement for the recovery of IBV using primary chick kidney (CK) cells. The requirement of an N protein for the recovery of other coronaviruses is not an absolute requirement, however, recovery is significantly enhanced by the presence of the appropriate N protein (Yount *et al*., 2000; Yount *et al*., 2002; Yount *et al*., 2003; Almazan *et al*., 2004; Schelle *et al*., 2005; Schelle *et al*., 2006; Coley *et al*., 2005). A possible explanation for this observed enhancement comes from some recent studies in which an interaction between the MHV nsp3 replicase protein and the N protein was found to be critical for replication (Hurst *et al*., 2010).

Reverse genetics systems for several coronaviruses, belonging to all three genera, have been developed and successfully used to recover infectious viruses (Table 2.2). We will, therefore, describe in more detail how we have used our IBV reverse genetics system, as an example, to modify a coronavirus genome. The use of VV as a vector for a full-length coronavirus cDNA offers a highly stable system for producing and maintaining an authentic cDNA, dispensing with the need for repeated

Table 2.2 Reverse genetics systems for the recovery of infectious coronaviruses.

Virus	Genus	System	Reference
TGEV	*alphacoronavirus*	BAC	Almazán *et al*., 2000
TGEV	*alphacoronavirus*	*In vitro* ligation	Yount *et al*., 2000
HCoV 229E	*alphacoronavirus*	Vaccinia virus	Thiel *et al*., 2001
mFIPV	*alphacoronavirus*	Targeted recombination	Haijema *et al*., 2003
FCoV	*alphacoronavirus*	Vaccinia virus	Tekes *et al*., 2008
HCoV NL63	*alphacoronavirus*	*In vitro* ligation	Donaldson *et al*., 2008
MHV	*betacoronavirus*	Targeted recombination	Koetzner *et al*., 1992
fMHV	*betacoronavirus*	Targeted recombination	Kuo *et al*., 2000
MHV	*betacoronavirus*	*In vitro* ligation	Yount *et al*., 2002
MHV	*betacoronavirus*	Vaccinia virus	Coley *et al*., 2005
HCoV OC43	*betacoronavirus*	BAC	St-Jean *et al*., 2006
SARS-CoV	*betacoronavirus*	BAC	Almazan *et al*., 2006
SARS-CoV	*betacoronavirus*	*In vitro* ligation	Yount *et al*., 2003
Bat-SCoV*	*betacoronavirus*	*In vitro* ligation	Becker *et al*., 2008
IBV	*gammacoronavirus*	Vaccinia virus	Casais *et al*., 2001
IBV	*gammacoronavirus*	*In vitro* ligation	Youn *et al*., 2005, Fang *et al*., 2007

Note: *Bat-SCoV = bat SARS-like coronavirus in which the sequence was synthesised from a consensus Bat-SCoV genome where the Bat-SCoV S glycoprotein receptor binding domain (RBD) was replaced with the SARS-CoV RBD for rescue of infectious recombinant virus in Vero cells (Becker *et al*., 2008).

cloning of cDNA fragments. A major advantage of the VV-based system is that the coronavirus cDNA can be modified or replaced using homologous recombination; the transient dominant selection (TDS) system that we use (Britton *et al*., 2005) is described below. The resultant rIBVs, apart from the introduced modification, are isogenic as they are derived from the same cDNA sequence.

2.6 Reverse genetics system for IBV

A complete cDNA copy of the IBV Beaudette genome was assembled and inserted into the TK gene of VV (Casais *et al*., 2001). The IBV cDNA is under the control of a T7 promoter and has a HδR sequence placed downstream of the coronavirus poly(A) tail followed by a T7 termination sequence (Figure 2.5). IBV infectious RNA is generated from the T7 promoter immediately adjacent to the 5′ end of the IBV cDNA using T7 RNA polymerase and terminates at the T7 termination sequence downstream of the HδR sequence, which autocleaves itself and the T7-termination sequence at the end of the poly(A) sequence, resulting in an authentic IBV genomic RNA copy.

2.6.1 Transient dominant selection for modification of the IBV genome

The VV-based TDS recombination method (Falkner and Moss, 1990) we use to modify our IBV cDNA sequence (Britton *et al*., 2005; Armesto *et al*., 2008) is outlined in Figure 2.6. The TDS method for modifying sequences in VV and related poxviruses involves the use of a plasmid containing a selective marker gene, *E. coli* guanine phosphoribosyltransferase (GPT); (Mulligan and Berg, 1981; Falkner and Moss, 1988), as well as the IBV sequence to be modified, the donor sequence. A recombinant VV (rVV), containing either an IBV full-length cDNA or an IBV cDNA, in which part of the IBV genome has been deleted, is used as a receiver sequence for modification. As a result of a single-step homologous recombination event, between the donor IBV cDNA sequence in the GPT plasmid and the IBV receiver sequence in the VV genome, the complete plasmid sequence is inserted into the genome of the receiver VV. Resultant rVVs express the selective GPT protein and can be identified by plaque purification in the presence of selection medium containing mycophenolic acid (MPA), xanthine and hypoxanthine. MPA is an inhibitor of purine biosynthesis, therefore only those viruses carrying the GPT gene (Figure 2.7), which provides an alternative pathway for purine biosynthesis, are able to replicate in the presence of MPA and the alternative purine precursors xanthine and hypoxanthine (Falkner and Moss, 1988). Recombinant VVs that are phenotypically GPT^+ are then plaque purified in the absence of selection medium. The removal of the selection agent results in a second recombination event between repeat sequences in the VV genome causing the loss of the GPT gene (Figure 2.6). This second recombination step results in two possible outcomes; one event will result in the original (unmodified) IBV sequence and the other in the generation of an IBV cDNA containing the desired modification (Figure 2.6). Once the desired IBV cDNA sequence within the VV genome has been identified and confirmed, stocks of this virus are grown in BHK-21 cells for isolation of VV DNA.

2.6.2 Rescue of recombinant IBVs

The rescue of infectious rIBVs is carried out in primary CK cells using the VV DNA containing the modified IBV sequence and a helper rFPV-T7 (Britton *et al*., 1996) to generate the infectious IBV RNA. The rFPV-T7 infected CK cells are transfected with the VV DNA and a plasmid expressing the IBV N protein (Hiscox *et al*., 2001), under the control of both the CMV promoter and the T7 RNA promoter. As indicated in Section 2.6.1, we found that the presence of the IBV N protein with the IBV infectious RNA is essential for recovery of infectious virus (Casais *et al*., 2001). Cell supernatants are filtered to remove any rFPV-T7 (Evans *et al*., 2000) and potential rIBVs are passaged three times in CK cells to produce stocks of virus for sequence analysis to confirm the presence of the modified IBV sequence.

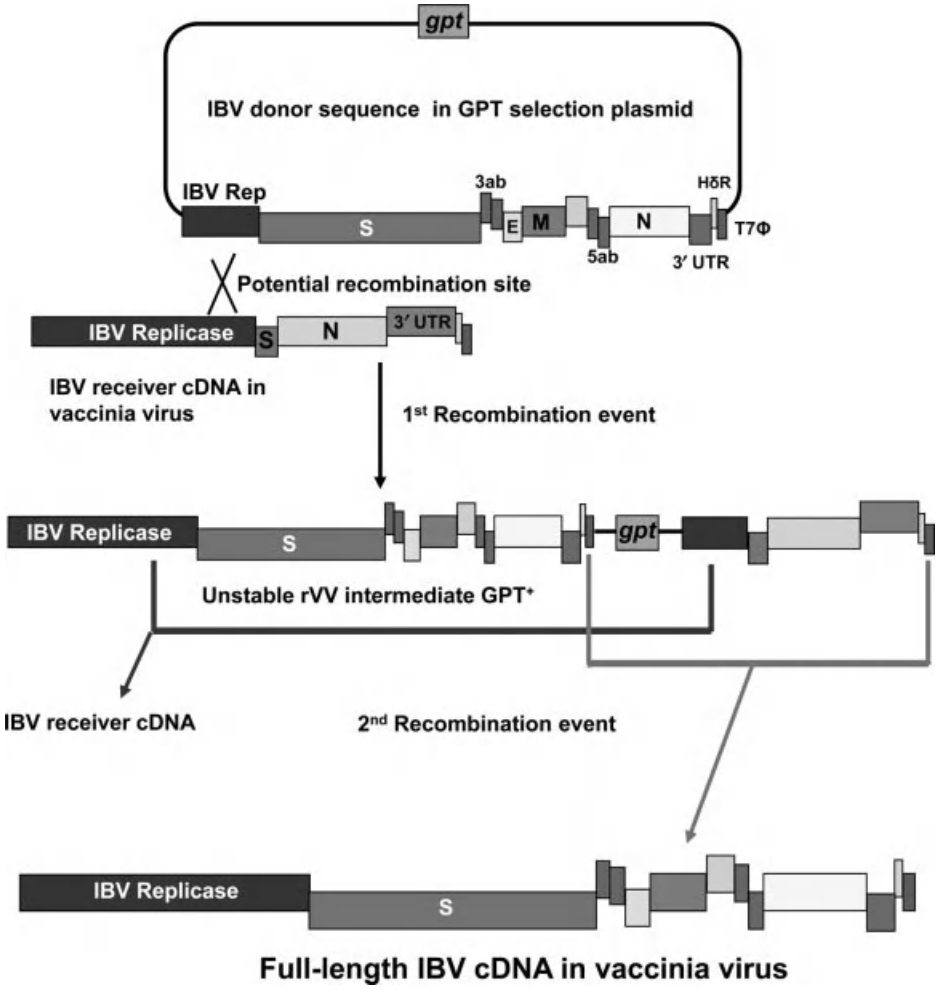

Figure 2.6 Schematic diagram of the transient dominant selection process for modifying Beau-R cDNA within a vaccinia virus genome. The figure shows the general TDS process for modifying IBV cDNA using a GPT selection plasmid with the modified IBV donor sequence; in this case the addition of IBV structural and accessory genes. The receiver IBV cDNA is within the VV genome and in this example shows an IBV cDNA lacking sequence from the start of the S gene to the end of the N gene. An intermediate rVV is generated, in which the complete donor plasmid DNA sequence is integrated into the truncated IBV cDNA, by a single-step homologous recombination event via a replicase sequence common to both sequences. The rVV has a GPT^+ phenotype allowing selection in the presence of MPA. Removal of MPA can result in two types of spontaneous intramolecular recombination events due to the presence of tandem repeat sequences of the IBV cDNA, resulting in either generation of rVVs with a truncated IBV cDNA (no modification) similar to the receiver sequence or a complete full-length IBV cDNA, the desired end product. Both recombination events result in the loss of the GPT gene. The IBV genes representing the structural and accessory genes are shown; a potential recombination event is indicated between the IBV replicase gene sequence common to both constructs.

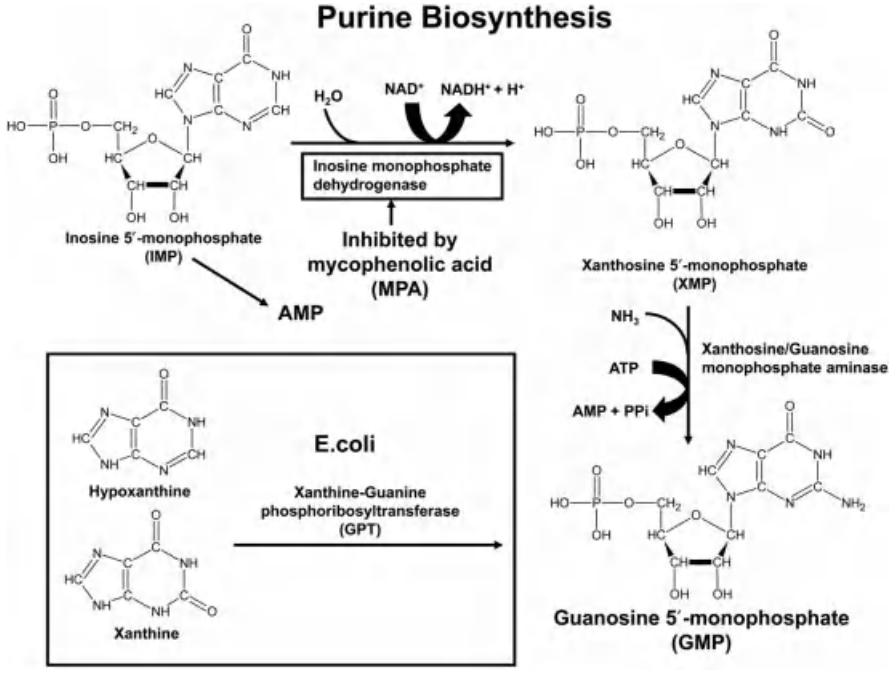

Figure 2.7 Schematic diagram showing the effect of MPA on purine metabolism. MPA inhibits the enzyme inosine monophosphate dehydrogenase, preventing the formation of xanthine monophosphate required for the generation of guanosine monophosphate, a precursor for nucleic acid biosynthesis, resulting in the intracellular depletion of purine nucleotides and inhibition of cell growth. The inhibition of the *de novo* synthesis of the purines by MPA can be overcome by alternative enzymes such as GPT, which is able to convert the substrates xanthine and hypoxanthine into guanosine monophosphate. Therefore, rVVs expressing GPT can grow in the presence of MPA.

Primary CK cells are refractory for growth of most IBV isolates; therefore, rIBVs expressing S glycoproteins from such isolates cannot be recovered using CK cells. In order to recover such rIBVs, the supernatants from the transfected CK cells are used to infect 10-day-old embryonated hen's eggs. Allantoic fluid is collected and any potential virus passed a further three times in 10-day-old embryos. RNA is extracted from the allantoic fluid of infected eggs and RT-PCR followed by sequencing is used to confirm the identity of the rIBV.

2.7 Reverse genetics systems for the modification of coronavirus genomes

This section describes how reverse genetics systems have been used to study the molecular biology of coronaviruses with respect to interactions and functions of the

replicase, structural and accessory proteins and to determine whether they play an essential role in the coronavirus replication cycle.

2.7.1 Modifications to coronavirus structural genes

The coronavirus M glycoprotein

The most abundant coronavirus structural protein is the 25 kDa M glycoprotein, which spans the viral envelope three times (Godeke *et al*., 2000; Hogue and Machamer, 2008). The first membrane-spanning domain targets the M protein to the *cis* Golgi (Machamer *et al*., 1990) and has been shown to be sufficient for membrane binding, retention in the Golgi and formation of multimers (Tseng *et al*., 2010). The M protein has a short amino-terminal ectodomain and a large carboxy-terminal cytoplasmic domain that interacts with the E protein (Corse and Machamer, 2003) and the nucleocapsid and is involved in assembly of virus particles (Narayanan *et al*., 2003; Hogue and Machamer, 2008). Modification of the MHV M protein gene was achieved using fMHV targeted RNA recombination to map the interactions of the M and N proteins of MHV (Kuo and Masters, 2002). Interaction of the M protein with the S glycoprotein retains the S glycoprotein in the ERGIC (McBride *et al*., 2007) and a single amino acid residue in the SARS-CoV M protein has been identified as being necessary for interaction of the S and M proteins and assembly of virions (McBride and Machamer, 2010). The *alpha*- and *gammacoronaviruses* have M proteins glycosylated with N-linked sugars whereas *betacoronaviruses* such as MHV have M proteins that are O-glycosylated. Targeted RNA recombination was used to modify the MHV M protein so that it was either N-glycosylated or not glycosylated (de Haan *et al*., 2003a). Glycosylation of the M protein is not required for virus assembly, indicating that glycosylation of the M protein is involved in a virus–host interaction. Infection of cells in culture showed that the glycosylation status of the MHV M protein did not influence the growth kinetics of the viruses. However, rMHVs with an N-glycosylated M protein induced type I interferon (IFN) to a higher level when compared to viruses expressing M proteins either lacking glycosylation or with O-linked sugars. *In vivo* studies showed that the rMHVs differed in their ability to replicate in the livers but not in the brains of infected mice (de Haan *et al*., 2003a).

The coronavirus N protein

The N protein is a 50 kDa phosphorylated, highly basic structural protein that forms a helical nucleocapsid when bound to the coronavirus RNA genome within virus particles (Hogue and Machamer, 2008). The carboxy-terminal domain interacts with the M protein (Hurst *et al*., 2005) and is packaged into viral particles by the M protein (Narayanan *et al*., 2003; Hogue and Machamer, 2008). Targeted RNA recombination using fMHV identified that the carboxy termini of the MHV N protein was involved in the interaction of the M and N proteins (Kuo and Masters, 2002).

The coronavirus E protein

The 10 kDa E protein is an integral membrane protein and a minor component of the virus envelope containing a single hydrophobic domain flanked by two hydrophilic domains. Two different topologies for the orientation of the E protein in the virus membrane have been proposed with either one or two transmembrane (TM) domains (Hogue and Machamer, 2008). Recent studies have shown that the SARS-CoV E protein has a topological conformation indicative of a single TM domain with the amino-terminus orientated towards the lumen of intracellular membranes and the carboxy-terminus facing the cell cytoplasm (Nieto-Torres *et al*., 2011). The cytoplasmic tail of the E protein contains Golgi targeting motifs (Corse and Machamer, 2003). It is thought that interaction of the E and M proteins may cause membrane curvature (Fischer *et al*., 1998), promoting budding of virus particles indicating that the E protein plays a fundamental role in the generation of virus particles (Lim and Liu, 2001). The E protein forms cation-selective ion channels in the lipid envelope, enhancing membrane permeability (Wilson *et al*., 2004). A rTGEV lacking the E gene has been shown to require helper E protein for recovery of infectious TGEV, indicating that the E protein is essential for TGEV replication (Ortego *et al*., 2002). In contrast, recovery of a rMHV with a deleted E gene showed that the E protein is not essential for MHV replication (Kuo and Masters, 2003); however, the rMHV grew less efficiently. The rMHV acquired variant M genes encoding M proteins with truncated endodomains, which enhanced virus growth and were incorporated into virions, indicating a role for E in mediating interactions between TM domains of M monomers (Kuo and Masters, 2010). The MHV E gene was replaced by heterologous E genes from other coronaviruses (Kuo *et al*., 2007). Despite extensive sequence variability of the coronavirus E proteins and possible structural differences, rMHVs expressing the BCoV, SARS-CoV or IBV E proteins functioned as wild-type viruses, suggesting that the role of the E protein is not dependent on sequence-specific interactions with an M protein. Substitution of the MHV E protein with that of TGEV, however, required compensatory mutations.

The coronavirus S glycoprotein

The 180 kDa S glycoprotein is a type I membrane protein projecting 20 nm from the virus surface (Figure 2.8) (Delmas and Laude, 1990; Cavanagh, 1995; Hogue and Machamer, 2008) and is composed of two subunits; the amino-terminal S1 subunit responsible for binding to host cell receptors (Koch *et al*., 1990) and the carboxyl-terminal S2 subunit responsible for cell–cell and virus–cell fusion (Luo and Weiss, 1998). The cell tropism of coronaviruses has been shown to be determined by the S glycoprotein (Kuo *et al*., 2000; Casais *et al*., 2003; Haijema *et al*., 2003).

Replacement of the MHV S glycoprotein ectodomain with that of FIPV resulted in a recombinant virus, fMHV, with the tropism of FIPV rather than MHV (Kuo *et al*., 2000). This virus has subsequently been used to select additional recombinant viruses that have regained the ability to grow in murine cells, confirming that

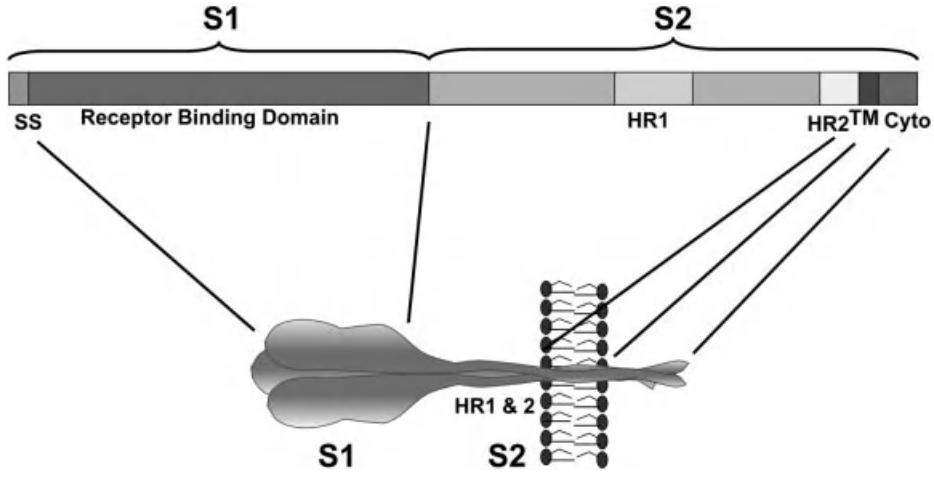

Figure 2.8 IBV S glycoprotein structure. The IBV S glycoprotein is a class I fusion membrane protein comprising the amino-terminal S1 subunit and the carboxy-terminal S2 subunit. A signal sequence (SS) located at the amino-terminal end of S1 is cleaved during synthesis; the IBV RBD is located within the S1 sequence. The S2 subunit contains two heptad repeats (HR1 and HR2), a TM domain and a cytoplasmic tail (Cyto). The IBV S glycoprotein is cleaved by host cell proteases at a highly basic region between the S1 and S2 subunits.

cell tropism is determined by the S glycoprotein. This approach was also utilised to create the reverse virus, mFIPV, with the S glycoprotein ectodomain of MHV expressed by FIPV, which gained the ability to grow on murine cells and lost the ability to grow on feline cells (Haijema *et al*., 2003). The FIPV reverse genetics system based on mFIPV gave rise to potential live attenuated vaccines against FIPV (Haijema *et al*., 2004).

We used our IBV reverse genetics system to investigate the role of the S glycoprotein, the tropism and growth characteristics of different IBV strains and to develop potential vaccines that can be propagated in cell lines rather than embryonated chicken eggs. Casais *et al*. (Casais *et al*., 2003) showed that the cellular tropism of IBV Beaudette is conferred by the S glycoprotein by replacing the ectodomain of the Beaudette S glycoprotein with that of M41 within the background of Beau-R, creating BeauR-M41(S). IBV Beaudette is not only able to replicate in primary CK cells but is also able to replicate in Vero and BHK-21 cell lines, an African green monkey kidney and baby hamster kidney cell line respectively; in contrast, the IBV M41 strain is only able to replicate in primary CK cells. BeauR-M41(S) exhibited the *in vitro* cellular tropism of M41, demonstrating that the S glycoprotein is a determinant of host range in IBV. The rIBV did not replicate as well as M41 in the trachea and nose of infected birds and was apathogenic like Beau-R (Hodgson *et al*., 2004). However, chickens vaccinated with BeauR-M41(S) were protected against M41 whereas vaccination with Beaudette did not induce protection (Hodgson *et al*., 2004).

Further rIBVs were created in the background of Beaudette with different S genes. Two recombinants, BeauR-4/91(S) and BeauR-B1648(S), express the S glycoprotein ectodomain from either the UK/4/91 strain or the BE/B1648/87 strain respectively, which are pathogenic strains belonging to different IBV serogroups to Beaudette and M41. As with BeauR-M41(S), the S gene of 4/91 did not confer pathogenicity to BeauR-4/91(S) although the resultant virus had the tropism and *in vitro* growth characteristics of the donor strain 4/91. Chickens vaccinated with BeauR-4/91(S) were protected from challenge with 4/91 (Armesto *et al*., 2011).

The potential for random recombination events within the IBV S glycoprotein was explored using the TDS system in which the S gene from the BE/B1648/87 nephropathogenic strain of IBV was recombined with the Beaudette S gene in the full-length IBV cDNA (Izadkhasti, 2006). Random recombination events occurred at different cross-over points between the two S genes. An rIBV possessing an S gene with the HR1 domain from BE/B1648/87 and the HR2 domain from Beaudette was recovered, whereas viruses containing chimaeric HR1 sequences were not viable, indicating the importance of the HR1 domain in the functionality of the spike. None of the resulting viruses were able to grow on CK or Vero cells, as with BE/B1648/87, although all possessed similar growth characteristics to the parental BE/B1648/87 in embryonated eggs. Viruses resulting from recombination within the S gene were shown to be viable, supporting the theory that IBV evolution is linked with recombination events (Cavanagh *et al*., 2007).

To investigate which subunit of the IBV S glycoprotein is involved in conferring the ability of Beaudette to grow on Vero cells, two rIBVs with the genomic background of Beaudette with either the S1 or S2 subunit from M41 were generated; BeauR-M41(S1) has the background of Beau-R with the S1 subunit from M41, BeauR-M41(S2) has the S2 subunit from M41. Both rIBVs replicated to similar titres as the parent viruses on CK cells. Growth of BeauR-M41(S2) on Vero cells was poor like M41, in contrast, BeauR-M41(S1) was found to replicate on Vero cells with similar growth kinetics as Beau-R albeit at a lower titre (Figure 2.9) (Bickerton, 2010). However, after seven passages on Vero cells, BeauR-M41(S1) replicated to a similar titre as observed for Beau-R (Figure 2.9). As the S1 subunit is responsible for receptor binding, it was anticipated that this subunit would be responsible for the ability of Beau-R to replicate in Vero and BHK-21 cells rather than the S2 subunit, which is involved in virus-to-cell and cell-to-cell fusion. The extended host range of a variant of MHV has previously been mapped to the S1 subunit (Schickli *et al*., 2004). Surprisingly, we found that the ability of IBV Beaudette to infect and replicate in Vero and BHK-21 cells was conferred by the S2 subunit, not the S1 subunit (Bickerton, 2010) that contains the receptor binding domain (RBD).

2.7.2 Modification of coronavirus accessory protein genes

All coronaviruses contain group-specific genes located in the 3′ end of the genome interspersed among the structural protein genes. These group-specific genes vary in

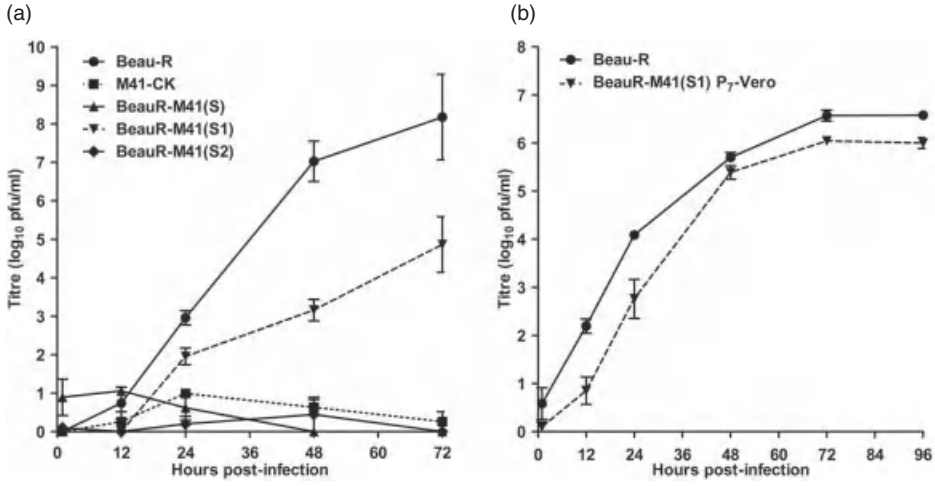

Figure 2.9 Growth kinetics of rIBVs on Vero cells. Vero cells were infected with (a) Beau-R, M41-CK, BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2); and (b) Beau-R and BeauR-M41(S1) P_7-Vero. Supernatants were harvested at 1, 12, 24, 48, 72 and 96 hours post-infection and titrated on CK cells. Three replicates of each growth curve were performed and the averages taken. Error bars indicate standard error of the mean.

number and location between different coronaviruses and express proteins with potentially diverse functions. Reverse genetics systems have enabled manipulation of these genes to elucidate their role(s) in replication. IBV contains two group-specific genes, 3 and 5, encoding accessory proteins 3a and 3b, and 5a and 5b, respectively (Figure 2.2). By generating a series of rIBVs, Hodgson *et al*. (Hodgson *et al*., 2006) were able to show that the 3a and 3b proteins were dispensable for replication of IBV. Through mutation of the translation initiation codons of one or both proteins, it was demonstrated that, despite the loss of expression of these proteins, the growth kinetics of the viruses remained similar to that of wild-type parental virus when grown in primary CK cells or 11-day-old embryonated eggs (Hodgson *et al*., 2006). A similar approach was taken by Casais *et al*. (Casais *et al*., 2005) to investigate the requirement of IBV gene 5. Recombinant IBVs were generated in which either the 5a or 5b, or both 5a and 5b, translation initiation codons were modified to prevent expression of the proteins. Further recombinants were generated in which either the first, or both, of the IBV Beaudette gene 5 TRSs were scrambled to prevent transcription of the gene 5 subgenomic mRNA. As with gene 3, each of the recombinant viruses showed growth kinetics similar to that of wild-type parental both *in vitro* in CK cells, *in ovo* in 11-day-old embryonated eggs, and also *ex vivo* in tracheal organ cultures (Casais *et al*., 2005). In an alternative approach Youn *et al*. (Youn *et al*., 2005) also demonstrated that the IBV 5a protein was not essential for replication by replacing the 5a sequence with that of enhanced green fluorescent protein (EGFP). A rIBV that expressed EGFP instead of the 5a protein was successfully rescued and grew on Vero cells, albeit to a 10-fold lower titre compared with the wild-type

virus. These results indicate that the proteins from IBV genes 3 and 5 are not essential for replication *in vitro* but may have a role *in vivo* and are therefore classified as accessory proteins.

MHV contains four group-specific genes, 2a and HE located between ORF 1b and the S gene, 4 and 5a located between the S and E genes (Figure 2.2). Not all of these genes are expressed in all strains of MHV. Targeted RNA recombination was used to generate a series rMHVs with group-specific gene deletions to investigate their requirements (de Haan *et al*., 2002). The rMHVs had either genes 2a and HE or genes 4 and 5a deleted or all four genes deleted. *In vitro* characterisation of the rMHVs showed they had similar growth kinetics to wild-type parental virus, although deletion of genes 4 and 5a alone, or in combination with the deletion of gene 2a and HE, resulted in approximately 10-fold lower titres than wild-type virus or the gene 2a/HE deletion rMHV. However, inoculation of mice with each of rMHVs identified that they were highly attenuated, concluding that the MHV group-specific genes, although not essential *in vitro*, have a role to play in virulence *in vivo*.

TGEV contains two group-specific genes, 3a/b and gene 7. Ortego *et al*. (Ortego *et al*., 2003) investigated whether gene 7 is dispensable for TGEV replication. The growth kinetics of the rTGEV, in which gene 7 had been deleted, were similar to those shown by the parental virus, viral titres were also similar at 24 hours post-infection indicating that TGEV gene 7 is not essential for replication in cell culture. When used *in vivo* to infect piglets, the rTGEV showed approximately 100-fold reduction in virus titres compared to parental virus with an increase in the survival of piglets. This again highlights the potential role for the group-specific genes in virus virulence within the natural host.

SARS-CoV encodes a large number of group-specific genes. As with other coronaviruses, these genes, 3a/b, 6, 7a/b, 8a/b, and 9b, are located among the structural genes and the functions of most are still unknown. Yount *et al*. (Yount *et al*., 2005) systematically deleted five of the eight group-specific genes and assessed the replication and gene expression of the resulting recombinants *in vitro* and *in vivo* in mice. Deletion of the SARS-CoV group-specific genes individually, or in combination, resulted in little effect on the replication of the virus *in vitro*, supporting the previous results that coronavirus group-specific genes are not essential for growth in cell culture. Freundt *et al*. (Freundt *et al*., 2010) showed that the deletion of gene 3a resulted in a reduction of cell death at 48 hours post-infection when compared to the wild-type virus. Gene 3a was also found to be necessary for the formation of intracellular vesicles, a prominent feature of cells from SARS-CoV-infected patients, and fragmentation of the Golgi apparatus. Again this implies that the group-specific proteins have a role to play *in vivo* that may be difficult to determine in cell culture alone.

Although the group-specific genes are not conserved across the different coronaviruses, it seems clear that they are dispensable for replication in cell culture but are often implicated in roles specific to the host, particularly with regards to virulence and pathogenesis. This non-essential nature of these genes has led to research

to investigate whether the deletion, or replacement, of these genes can result in attenuated vaccines or in the use of coronaviruses as gene delivery systems.

2.7.3 Modification of the coronavirus replicase gene

The replicase gene is over 20kb in size and the availability of coronavirus reverse genetics systems has allowed the modification of several replicase nsps to investigate their role in the virus life cycle, in pathogenicity, host–virus interactions and to identify sequence changes involved in attenuation.

Nsp1 of MHV and SARS-CoV has been implicated in reducing cellular gene expression as a result of mRNA degradation (Kamitani *et al*., 2006; Zust *et al*., 2007). Deletion of the carboxy-terminal part of MHV nsp1, K_{124}-L_{241}, was found to be dispensable for replication in cell culture but was required for efficient proteolytic cleavage of nsp1 and for optimal replication (Brockway and Denison, 2005). Further work showed that deletions proximal to residue K_{124} did not result in recovery of infectious virus, although, point mutagenesis in the amino-terminal region did result in infectious virus albeit with altered replication and RNA synthesis; indicating a potential role for nsp1 in RNA synthesis and virus replication. Zust *et al*. (Zust *et al*., 2007) deleted nucleotides 829–927 from the carboxy-terminal region of the MHV nsp1 and rescued a virus that had growth kinetics in cell culture that were indistinguishable to those showed by parental wild-type virus. The resulting recombinant, MHV-nsp1Δ99, was attenuated *in vivo*, however, replication and spread of the rMHV were restored in type I IFN receptor-deficient (IFNAR) mice when compared to wild-type mice; leading the authors to hypothesise that MHV nsp1 interferes with the type I IFN system.

Deletion of nsp2 in both MHV and SARS-CoV resulted in the recovery of infectious virus (Graham *et al*., 2005). The authors reported that RTCs in cells infected with the MHVΔnsp2 virus were morphologically identical to those infected with wild-type parental virus, even though they lacked nsp2 protein; nsp2 co-expressed using a retroviral system was recruited to the replication complexes in the MHVΔnsp2 infected cells. Overall the results showed that nsp2 is not required for replication in cell culture.

The nsp3 replicase protein is the largest replicase component and a multifunctional protein that encodes several enzymatically active domains, including two papain-like proteases and ADP-ribose-1”-monophosphatase (ADRP; previously known as the coronavirus X domain). Generation of an ADRP-deficient HCoV-229E virus showed that this conserved coronavirus enzyme is not required for replication in cell culture with no detectable differences in RNA synthesis or virus growth when compared to parental virus (Putics *et al*., 2005). A rMHV encoding an inactivated ADRP replicated, although with reduced titres, in the livers of infected mice but did not induce liver disease (Eriksson *et al*., 2008). The authors observed reduced IL-6 production in the spleens and livers of mice infected with the ADRP-deficient MHV and postulated that ADRP exacerbates MHV-induced liver

pathology through the induction of inflammatory cytokines. Recombinant HCoV-229E and SARS-CoV viruses expressing inactivated ADRPs have been shown to have an increased sensitivity to IFN-α when compared to the parental viruses indicating that ADRP may play a role in virus escape from host innate immune responses induced by a coronavirus infection (Kuri *et al*., 2011).

The nsp4 replicase protein is a membrane protein, with four predicted TM-spanning domains, involved in the assembly of RTCs on DMVs by anchoring the RTC to the membranes (Gorbalenya *et al*., 1989). Various deletions of the TM domains 1-3 from MHV nsp4 either did not result in recovery of infectious virus, resulted in viruses that were impaired for growth or those that demonstrated wild-type replication (Sparks *et al*., 2007). Indicating that nsp4 is required for MHV replication and that TM domains 1–3 are essential but TM4 and the carboxy-terminal amino acids K_{398}-T_{492} are dispensable for replication. Amino acid substitution N258T of MHV nsp4 resulted in a rMHV with a *ts* phenotype that caused a reduction in DMV formation and partial localisation of nsp3 and nsp4 to mitochondria at the non-permissive temperature, indicating a role of nsp4 in DMV assembly (Clementz *et al*., 2008).

The nsp12-16 replicase proteins, encoded by the ORF1b region of gene 1, have a variety of enzymatic activities associated with RNA synthesis and processing (Ziebuhr, 2008); in which nsp12 has the RNA-dependent-RNA polymerase activity, nsp13 helicase activity, nsp14 3′-to-5′ exoribonuclease (ExoN) activity, nsp15 nidoviral endoribonuclease (NendoU) (this enzyme is unique to and conserved in all nidoviruses) activity and nsp16 2′-*O*-ribose methyltransferase (MT) activity. A SARS-CoV-based replicon system was used to show that the RNA-processing enzymes ExoN, NendoU and 2′-*O*-MT are essential for RNA synthesis (Almazan *et al*., 2006). Inactivation of the 2′-*O*-MT domain in the MHV and HCoV-229E nsp16 replicase proteins resulted in recovery of infectious recombinant viruses that induced an increased expression of type I IFN in virus-infected cells and were highly sensitive to type I IFN (Zust *et al*., 2011). The authors reported that induction of type I IFN by the 2′-*O*-MT-deficient viruses was dependent on the cytoplasmic RNA sensor Mda5. They proposed, due to the link between Mda5-mediated sensing of viral RNA and 2′-*O*-methylation of viral mRNAs, that RNA modification by 2′-*O*-methylation provides a molecular signature allowing discrimination between self and non-self mRNA in the context of innate immune responses by the cell. Interestingly, the 2′-*O*-MT-deficient MHV was apathogenic in wild-type mice but both replication and virus spread were restored in IFNAR mice or in mice lacking Toll-like receptor 7 and Mda5 (Zust *et al*., 2011).

In order to study the pathogenicity determinants in IBV, the VV-based IBV reverse genetics system was used to replace the genes downstream of the replicase gene in the apathogenic IBV strain Beaudette with those from the pathogenic strain M41. A GPT plasmid containing the distal part of the Beau-R replicase gene fused to the M41 sequence from the S gene to the poly(A) tail was used as a donor sequence. A Beau-R-based cDNA consisting of the complete replicase gene, followed by part of the S gene fused to the N gene and 3′-UTR, was used as a receiver

sequence (Armesto *et al*., 2009). The resultant chimaeric virus, rBeauR-Rep-M41-Struct, was tested for pathogenicity in chickens. Observations of clinical signs, ciliary activity and analyses for viral RNA in the trachea of the infected chickens, showed that rBeauR-Rep-M41-Struct was not pathogenic, suggesting that determinants of pathogenicity reside in the replicase gene (Armesto *et al*., 2009). Therefore, to determine which region or regions of the Beaudette replicase gene are involved in loss of pathogenicity, the replicase gene of rBeauR-Rep-M41-Struct was sequentially replaced with the replicase gene sequence from M41 using the TDS system. Several rVVs, containing chimaeric Beau-R/M41 replicase sequences, were generated and viable rIBVs with the chimaeric replicase sequences are being used for virus pathogenicity studies.

2.8 Using coronavirus reverse genetics systems for gene delivery

The advent of reverse genetics systems for coronaviruses has also allowed the study of their potential to act as vectors for the expression and delivery of foreign genes. The large size of the genome means in principle the virus could accommodate large inserts while the specific tropism afforded by the S glycoproteins means that vectors could be targeted to the desired tissues. Due to the nature of coronavirus transcription, provided a TRS signal is also included, foreign genes can in principle be inserted into the coronavirus genome and expressed as a new sub-genomic mRNA. The non-essential phenotype of the group-specific genes means that targeted replacement of these genes may offer an alternative to insertion in order to express a foreign gene from TRS signals already available.

Although any of the coronavirus accessory genes could be replaced with a foreign gene, or new genes inserted at any point in the genome distal to gene 1, studies carried out have shown that the stability of such recombinant viruses may depend on a number of factors including the spatial position of the foreign gene and the virus backbone, as well as the intrinsic properties of the genes themselves. These aspects of stability were studied, using a combination of FIPV or MHV expressing the firefly or *Renilla* luciferase genes (de Haan *et al*., 2003b; de Haan *et al*., 2005). Expression of a foreign gene in MHV was found to be greater the closer to the 3′ end of the genome it was inserted (de Haan *et al*., 2003b) and that *Renilla* luciferase was maintained more stably than the firefly luciferase (de Haan *et al*., 2005). The stability of the firefly gene was also dependent on genome position with higher instability seen at more 3′ positions, indicating that not only the gene but the position in which it is inserted can affect the stability of the resulting viruses. The virus background can also make a difference; firefly luciferase expression was found to be more stable following replacement of gene 3abc in FIPV than when inserted between the E and M genes of MHV (de Haan *et al*., 2005), indicating that replacement of non-essential genes, rather than insertion between viral genes, may result in recombinants with greater stability.

IBV has been proposed as a vaccine vector to protect against IB and other viral infections of chickens based on the insertion of a foreign gene. We have investigated the possibility of such vectors through the generation of a number of rIBVs expressing the reporter genes EGFP or *Renilla* luciferase using the Beau-R molecular clone. Recombinants in which gene 5 has been replaced show stability of expression to at least passage 8 in primary CK cells, and this is increased to at least passage 10 if the foreign gene has been codon optimised to that of IBV (unpublished data). Shen *et al*. (Shen *et al*., 2009) also generated a number of rIBVs expressing EGFP or firefly luciferase from their P65 Vero cell-adapted Beaudette strain. The greatest genetic stability was identified following replacement of gene 3ab with firefly luciferase, which maintained high levels of expression up to passage 15. This supports previous work (de Haan *et al*., 2005) proposing that greater stability was found with the replacement of non-essential genes. Shen *et al*. (Shen *et al*., 2009) also demonstrated the ability of IBV to express a number of other viral and host proteins including the SARS-CoV gene 6, the dengue virus 1 core protein and the eukaryotic translation initiation factor eIF3f. All the genes were found to be expressed although the dengue virus core protein was found to be the least stable, only reaching passage 3 in Vero cells, reiterating the fact that the inserted gene itself can have an effect on the stability of the virus.

An rTGEV in which EGFP replaced the non-essential gene 3a was found to be highly stable; the virus continued to express similar levels of EGFP after 20 passages (Sola *et al*., 2003). When studied *in vivo*, in swine, the insertion of EGFP was found to have an effect on the behaviour of the virus; growth in enteric tissues was found to decrease 10 to 100-fold and the mean day of death observed in piglets was delayed by 1 or 1.5 days. The study of such recombinants on their ability to induce immunity is required in order to assess their usefulness as vaccine vectors. This study addressed the issue by immunising pregnant sows with the recombinant virus expressing EGFP and assessing TGEV- and EGFP-specific antibody levels in both sows and progeny. Significant levels of both TGEV- and EGFP-specific antibodies were found in the serum of piglets, demonstrating that this vector had elicited lactogenic immunity, thus establishing that vaccine vectors based on coronaviruses are viable options as they are able to illicit immune responses *in vivo*.

In addition to utilising viruses as vectors for delivering foreign antigens for potential vaccines, reverse genetics has also allowed the modification of viruses for virotherapy, that is, a process in which viruses can be modified to target and destroy cancer cells. The potential of coronaviruses to be utilised as virotherapy vectors has been investigated. MHV has been retargeted to a non-native receptor through the use of a soluble adapter protein to initiate an infection of cells normally non-permissive for MHV (Verheije *et al*., 2006). The adapter protein consisted of the N-terminal D1 domain of the MHV cellular receptor mCEACAM1a, which binds the S glycoprotein of MHV. The mCEACAM1a D1 domain was linked to a six-amino acid histidine (His) tag via three alanine residues and a myc tag. The His tag binds to a sFvHis receptor expressed on target cells resulting in infection of the target cell with MHV. Additional adapter proteins were designed with a hinge

region that would facilitate dimerisation of the adapter protein mimicking the natural mCEACAM1a receptor. The mechanism of cell entry by MHV when mediated by the adapter protein was shown to be via S-mediated membrane fusion as used by MHV when naturally infecting murine cells.

Utilising knowledge previously gained from the deletion of the MHV group-specific genes (de Haan *et al*., 2002), the adapter proteins were inserted into the MHV genome replacing genes 2a and HE, resulting in the generation of an rMHV that could express its own adapter protein for targeting, and therefore could be independently propagated on target cells. However, only viruses expressing adapter proteins containing the hinge region were able to infect target cells expressing the sFvHis receptor and establish a multi-cycle infection resulting in the killing of cells. By generating a new adapter protein, coupling the mCEACAM1a receptor to the epidermal growth factor (EGF) protein and inserting this into the MHV genome, it was possible to target MHV to EGF receptor-expressing human cells (Verheije *et al*., 2009). The EGF receptor is abundantly expressed on a majority of tumours and it was shown that targeting MHV to glioblastoma cells expressing this receptor resulted in efficient killing of the cancer cells. While further work is required in this area, these studies demonstrate the potential for exploiting coronaviruses in the treatment of cancers.

Acknowledgements

The authors would like to acknowledge the support of the following organisations: Department of the Environment, Food and Rural Affairs (Defra), Biotechnology and Biological Sciences Research Council (BBSRC), Pfizer Animal Health, Intervet Schering-Plough, British Egg Marketing Board Research (BEMB) and Education Trust, British Poultry Council (BPC) and the British Egg Industry Council (BEIC).

References

Almazan, F., Dediego, M. L., Galan, C., Escors, D., Alvarez, E., Ortego, J., Sola, I., Zuniga, S., Alonso, S., Moreno, J. L., Nogales, A., Capiscol, C. and Enjuanes, L. 2006. Construction of a severe acute respiratory syndrome coronavirus infectious cDNA clone and a replicon to study coronavirus RNA synthesis. *Journal of Virology* **80**: 10900–10906.

Almazan, F., Galan, C. and Enjuanes, L. 2004. The nucleoprotein is required for efficient coronavirus genome replication. *Journal of Virology* **78**: 12683–12688.

Almazán, F., González, J. M., Pénzes, Z., Izeta, A., Calvo, E., Plana-Durán, J. and Enjuanes, L. 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. *Proceedings of the National Academy of Science USA* **97**: 5516–5521.

Ambali, A. G. and Jones, R. C. 1990. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. *Avian Diseases* **34**: 809–817.

Armesto, M., Casais, R., Cavanagh, D. and Britton, P. 2008. Transient dominant selection for the modification and generation of recombinant infectious bronchitis coronaviruses. *Methods in Molecular Biology* **454**: 255–273.

Armesto, M., Cavanagh, D. and Britton, P. 2009. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. *PLoS ONE* **4**: e7384.

Armesto, M., Evans, S., Cavanagh, D., Abu-Median, A. B., Keep, S. and Britton, P. 2011. A recombinant avian infectious bronchitis virus expressing a heterologous spike gene belonging to the 4/91 serotype. *PLoS ONE* **6**(8): e24352. Epub. 30 Aug. 2011.

Baker, S. C. and Denison, M. R. 2008. Cell biology of nidovirus replication complexes. In Perlman, S., Gallagher, T. and Snijder, E. J. (eds) *Nidoviruses*. ASM Press: Washington, DC.

Beach, J. R. and Schalm, O. W. 1936. A filtrable virus distinct from that of laryngotracheitis: the cause of respiratory disease of chicks. *Poultry Science* **15**: 199–206.

Beaudette, F. R. and Hudson, C. B. 1937. Cultivation of the virus of infectious bronchitis. *Journal of the American Veterinary Medical Association* **90**: 51–60.

Becker, M. M., Graham, R. L., Donaldson, E. F., Rockx, B., Sims, A. C., Sheahan, T., Pickles, R. J., Corti, D., Johnston, R. E., Baric, R. S. and Denison, M. R. 2008. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proceedings of the National Academy of Science USA* **105**: 19944–19949.

Bennett, R. 2003. The direct costs of livestock disease: The development of a system of models for the analysis of 30 endemic livestock diseases in Great Britain. *Journal of Agricultural Economics* **54**: 55–71.

Bennett, R. and Jpelaar, J. I. 2005. Updated estimates of the costs associated with thirty-four endemic livestock diseases in Great Britain. *Journal of Agricultural Economics* **56**: 135–144.

Bickerton, E. J. 2010. Cellular tropism and cell-cell fusion properties of the infectious bronchitis virus spike glycoprotein. PhD, University of Warwick.

Boursnell, M. E. G., Brown, T. D. K., Foulds, I. J., Green, P. F., Tomley, F. M. and Binns, M. M. 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *Journal of General Virology* **68**: 57–77.

Brian, D. A. and Baric, R. S. 2005. Coronavirus genome structure and replication. *Current Topics in Microbiology and Immunology* **287**: 1–30.

Brierley, I., Boursnell, M. E. G., Binns, M. M., Bilimoria, B., Blok, V. C., Brown, T. D. K. and Inglis, S. C. 1987. An efficient ribosomal frame-shifting signal in the polymerase encoding region of the coronavirus IBV. *EMBO Journal* **6**: 3779–3785.

Britton, P. and Cavanagh, D. 2007. Avian coronavirus diseases and infectious bronchitis vaccine development. In Thiel, V. (ed.) *Coronaviruses: Molecular and Cellular Biology.* Caister Academic Press: Norfolk, UK.

Britton, P. and Cavanagh, D. 2008. Nidovirus genome organization and expression mechanisms. In Perlman, S., Gallagher, T. and Snijder, E. J. (eds) *Nidoviruses*. ASM Press: Washington, DC.

Britton, P., Evans, S., Dove, B., Davies, M., Casais, R. and Cavanagh, D. 2005. Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection. *Journal of Virological Methods* **123**: 203–211.

Britton, P., Green, P., Kottier, S., Mawditt, K. L., Pénzes, Z., Cavanagh, D. and Skinner, M. A. 1996. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. *Journal of General Virology* **77**: 963–967.

Brockway, S. M. and Denison, M. R. 2005. Mutagenesis of the murine hepatitis virus nsp1-coding region identifies residues important for protein processing, viral RNA synthesis, and viral replication. *Virology* **340**: 209–223.

Carstens, E. B. 2010. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses 2009. *Archives of Virology* **155**: 133–146.

Casais, R., Davies, M., Cavanagh, D. and Britton, P. 2005. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. *Journal of Virology* **79**: 8065–8078.

Casais, R., Dove, B., Cavanagh, D. and Britton, P. 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *Journal of Virology* **77**: 9084–9089.

Casais, R., Thiel, V., Siddell, S. G., Cavanagh, D. and Britton, P. 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. *Journal of Virology* **75**: 12359–12369.

Cavanagh, D. 1995. The coronavirus surface glycoprotein. In Siddell, S. G. (ed.) *The Coronaviridae*. Plenum Press: New York and London.

Cavanagh, D. 2005. Coronaviruses in poultry and other birds. *Avian Pathology* **34**: 439–448.

Cavanagh, D., Casais, R., Armesto, M., Hodgson, T., Izadkhasti, S., Davies, M., Lin, F., Tarpey, I. and Britton, P. 2007. Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins. *Vaccine* **25**: 5558–5562.

Cavanagh, D. and Gelb, J., JR. 2008. Infectious bronchitis. In Saif, Y. M. (ed.) *Diseases of Poultry*. Twelfth edition. Blackwell Publishing: Iowa.

Chu, V. C., Mcelroy, L. J., Chu, V., Bauman, B. E. and Whittaker, G. R. 2006. The avian coronavirus infectious bronchitis virus undergoes direct low-pH-dependent fusion activation during entry into host cells. *Journal of Virology* **80**: 3180–3188.

Clementz, M. A., Kanjanahaluethai, A., O'brien, T. E. and Baker, S. C. 2008. Mutation in murine coronavirus replication protein nsp4 alters assembly of double membrane vesicles. *Virology* **375**: 118–129.

Coley, S. E., Lavi, E., Sawicki, S. G., Fu, L., Schelle, B., Karl, N., Siddell, S. G. and Thiel, V. 2005. Recombinant mouse hepatitis virus strain A59 from cloned, full-length cDNA replicates to high titers in vitro and is fully pathogenic in vivo. *Journal of Virology* **79**: 3097–3106.

Cook, J. K. A. and Mockett, A. P. A. 1995. Epidemiology of infectious bronchitis virus. In Siddell, S. G. (ed.) *The Coronaviridae*. Plenum Press: New York and London.

Corse, E. and Machamer, C. E. 2003. The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. *Virology* **312**: 25–34.

Defra 2005. *Defra Report on the Economic Assessment of Livestock Diseases in the United Kingdom (UK)*. ZZ0102. Available at: http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&ProjectID=9781&FromSearch=Y&Publisher=1&SearchText=ZZ0102&SortString=ProjectCode&SortOrder=Asc&Paging=10"\l.

De Haan, C. A., De Wit, M., Kuo, L., Montalto-Morrison, C., Haagmans, B. L., Weiss, S. R., Masters, P. S. and Rottier, P. J. 2003a. The glycosylation status of the murine hepatitis coronavirus M protein affects the interferogenic capacity of the virus in vitro and its ability to replicate in the liver but not the brain. *Virology* **312**: 395–406.

De Haan, C. A., Haijema, B. J., Boss, D., Heuts, F. W. and Rottier, P. J. 2005. Coronaviruses as vectors: stability of foreign gene expression. *Journal of Virology* **79**: 12742–12751.

De Haan, C. A., Masters, P. S., Shen, X., Weiss, S. and Rottier, P. J. 2002. The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. *Virology* **296**: 177–189.

De Haan, C. A., Van Genne, L., Stoop, J. N., Volders, H. and Rottier, P. J. 2003b. Coronaviruses as vectors: position dependence of foreign gene expression. *Journal of Virology* **77**: 11312–11323.

Delmas, B. and Laude, H. 1990. Assembly of coronavirus spike protein into trimers and its role in epitope expression. *Journal of Virology* **64**: 5367–5375.

Deming, D. J. and Baric, R. S. 2008. Genetics and Reverse Genetics of Nidoviruses. In Perlman, S., Gallagher, T. and Snijder, E. J. (eds) *Nidoviruses*. ASM Press: Washington, DC.

Donaldson, E. F., Yount, B., Sims, A. C., Burkett, S., Pickles, R. J. and Baric, R. S. 2008. Systematic assembly of a full-length infectious clone of human coronavirus NL63. *Journal of Virology* **82**: 11948–11957.

Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H. R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R. A., Berger, A., Burguiere, A. M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J. C., Muller, S., Rickerts, V., Sturmer, M., Vieth, S., Klenk, H. D., Osterhaus, A. D., Schmitz, H. and Doerr, H. W. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *New England Journal of Medicine* **348**: 1967–1976.

Eifart, P., Ludwig, K., Bottcher, C., De Haan, C. A., Rottier, P. J., Korte, T. and Herrmann, A. 2007. Role of endocytosis and low pH in murine hepatitis virus strain A59 cell entry. *Journal of Virology* **81**: 10758–10768.

Enjuanes, L. 2005. *Coronavirus Replication and Reverse Genetics*. Springer Verlag: Berlin.

Enjuanes, L., Almazan, F., Sola, I. and Zuniga, S. 2006. Biochemical aspects of coronavirus replication and virus-host interaction. *Annual Reviews in Microbiology* **60**: 211–230.

Enjuanes, L., Sola, I., Alonso, S., Escors, D. and Zuniga, S. 2005. Coronavirus reverse genetics and development of vectors for gene expression. *Current Topics in Microbiology and Immunology* **287**: 161–197.

Eriksson, K. K., Cervantes-Barragan, L., Ludewig, B. and Thiel, V. 2008. Mouse hepatitis virus liver pathology is dependent on ADP-ribose-1″-phosphatase, a viral function conserved in the alpha-like supergroup. *Journal of Virology* **82**: 12325–12334.

Evans, S., Cavanagh, D. and Britton, P. 2000. Utilizing fowlpox virus recombinants to generate defective RNAs of the coronavirus infectious bronchitis virus. *Journal of General Virology* **81**: 2855–2865.

Falkner, F. G. and Moss, B. 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. *Journal of Virology* **62**: 1849–1854.

Falkner, F. G. and Moss, B. 1990. Transient dominant selection of recombinant vaccinia viruses. *Journal of Virology* **64**: 3108–3111.

Fang, S., Chen, B., Tay, F. P., Ng, B. S. and Liu, D. X. 2007. An arginine-to-proline mutation in a domain with undefined functions within the helicase protein (Nsp13) is lethal to the coronavirus infectious bronchitis virus in cultured cells. *Virology* **358**: 136–147.

Fischer, F., Stegen, C. F., Masters, P. S. and Samsonoff, W. A. 1998. Analysis of constructed E gene mutants of mouse hepatitis virus confirms a pivotal role for E protein in coronavirus assembly. *Journal of Virology* **72**: 7885–7894.

Freundt, E. C., Yu, L., Goldsmith, C. S., Welsh, S., Cheng, A., Yount, B., Liu, W., Frieman, M. B., Buchholz, U. J., Screaton, G. R., Lippincott-Schwartz, J., Zaki, S. R., Xu, X. N., Baric, R. S., Subbarao, K. and Lenardo, M. J. 2010. The open reading frame 3a protein of severe acute respiratory syndrome-associated coronavirus promotes membrane rearrangement and cell death. *Journal of Virology* **84**: 1097–1109.

Godeke, G. J., De Haan, C. A., Rossen, J. W., Vennema, H. and Rottier, P. J. 2000. Assembly of spikes into coronavirus particles is mediated by the carboxy-terminal domain of the spike protein. *Journal of Virology* **74**: 1566–1571.

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J. and Snijder, E. J. 2006. Nidovirales: evolving the largest RNA virus genome. *Virus Research* **117**: 17–37.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. and Blinov, V. M. 1989. Coronavirus genome: prediction of putative functional domains in the non-structural polyprotein by comparative amino acid analysis. *Nucleic Acids Research* **17**: 4847–4861.

Gosert, R., Kanjanahaluethai, A., Egger, D., Bienz, K. and Baker, S. C. 2002. RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. *Journal of Virology* **76**: 3697–3708.

Graham, R. L., Sims, A. C., Brockway, S. M., Baric, R. S. and Denison, M. R. 2005. The nsp2 replicase proteins of murine hepatitis virus and severe acute respiratory syndrome coronavirus are dispensable for viral replication. *Journal of Virology* **79**: 13399–13411.

Guo, Y., Tisoncik, J., Mcreynolds, S., Farzan, M., Prabhakar, B. S., Gallagher, T., Rong, L. and Caffrey, M. 2009. Identification of a new region of SARS-CoV S protein critical for viral entry. *Journal of Molecular Biology* **394**: 600–605.

Haijema, B. J., Volders, H. and Rottier, P. J. 2004. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. *Journal of Virology* **78**: 3863–3871.

Haijema, B. J., Volders, H. and Rottier, P. J. M. 2003. Switching species tropism: an effective way to manipulate the feline coronavirus genome. *Journal of Virology* **77**: 4528–4538.

Hiscox, J. A., Wurm, T., Wilson, L., Britton, P., Cavanagh, D. and Brooks, G. 2001. The coronavirus infectious bronchitis virus nucleoprotein localizes to the nucleolus. *Journal of Virology* **75**: 506–512.

Hodgson, T., Britton, P. and Cavanagh, D. 2006. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. *Journal of Virology* **80**: 296–305.

Hodgson, T., Casais, R., Dove, B., Britton, P. and Cavanagh, D. 2004. Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. *Journal of Virology* **78**: 13804–13811.

Hogue, B. G. and Machamer, C. E. 2008. Coronavirus structural proteins and virus assembly. In Perlman, S., Gallagher, T. and Snijder, E. J. (eds) *Nidoviruses*. ASM Press: Washington, DC.

Hurst, K. R., Kuo, L., Koetzner, C. A., Ye, R., Hsue, B. and Masters, P. S. 2005. A major determinant for membrane protein interaction localizes to the carboxy-terminal domain of the mouse coronavirus nucleocapsid protein. *Journal of Virology* **79**: 13285–13297.

Hurst, K. R., Ye, R., Goebel, S. J., Jayaraman, P. and Masters, P. S. 2010. An interaction between the nucleocapsid protein and a component of the replicase-transcriptase complex is crucial for the infectivity of coronavirus genomic RNA. *Journal of Virology* **84**: 10276–10288.

Inoue, Y., Tanaka, N., Tanaka, Y., Inoue, S., Morita, K., Zhuang, M., Hattori, T. and Sugamura, K. 2007. Clathrin-dependent entry of severe acute respiratory syndrome coronavirus into target cells expressing ACE2 with the cytoplasmic tail deleted. *Journal of Virology* **81**: 8722–8729.

Izadkhasti, S. 2006. Generation of recombinant infectious bronchitis viruses with chimaeric spike proteins. PhD, University of London.

Izeta, A., Smerdou, C., Alonso, S., Penzes, Z., Mendez, A., Plana-Durán, J. and Enjuanes, L. 1999. Replication and packaging of transmissible gastroenteritis coronavirus-derived synthetic minigenomes. *Journal of Virology* **73**: 1535–1545.

Jones, R. C. 2010. Viral respiratory diseases (ILT, aMPV infections, IB): are they ever under control? *British Poultry Science* **51**: 1–11.

Kamitani, W., Narayanan, K., Huang, C., Lokugamage, K., Ikegami, T., Ito, N., Kubo, H. and Makino, S. 2006. Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. *Proceedings of the National Academy of Science USA* **103**: 12885–12890.

Kawase, M., Shirato, K., Matsuyama, S. and Taguchi, F. 2009. Protease-mediated entry via the endosome of human coronavirus 229E. *Journal of Virology* **83**: 712–721.

Klumperman, J., Locker, J. K., Meijer, A., Horzinek, M. C., Geuze, H. J. and Rottier, P. J. M. 1994. Coronavirus M proteins accumulate in the Golgi complex beyond the site of virion budding. *Journal of Virology* **68**: 6523–6534.

Knoops, K., Kikkert, M., Worm, S. H., Zevenhoven-Dobbe, J. C., Van Der Meer, Y., Koster, A. J., Mommaas, A. M. and Snijder, E. J. 2008. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biology* **6**: e226.

Koch, G., Hartog, L., Kant, A. and Van Roozelaar, D. J. 1990. Antigenic domains of the peplomer protein of avian infectious bronchitis virus: correlation with biological function. *Journal of General Virology* **71**: 1929–1935.

Koetzner, C. A., Parker, M. M., Ricard, C. S., Sturman, L. S. and Masters, P. S. 1992. Repair and mutagenesis of the genome of a deletion mutant of the coronavirus mouse hepatitis virus by targeted RNA recombination. *Journal of Virology* **66**: 1841–1848.

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., ZakI, S. R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J. A., Lim, W., Rollin, P. E., Dowell, S. F., Ling, A. E., Humphrey, C. D., Shieh, W. J., Guarner, J., Paddock, C. D., Rota, P., Fields, B., Derisi, J., Yang, J. Y., Cox, N., Hughes, J. M., Leduc, J. W., Bellini, W. J. and Anderson, L. J. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *New England Journal of Medicine* **348**: 1953–1966.

Kuo, L., Godeke, G. J., Raamsman, M. J., Masters, P. S. and Rottier, P. J. 2000. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. *Journal of Virology* **74**: 1393–1406.

Kuo, L., Hurst, K. R. and Masters, P. S. 2007. Exceptional flexibility in the sequence requirements for coronavirus small envelope protein function. *Journal of Virology* **81**: 2249–2262.

Kuo, L. and Masters, P. S. 2002. Genetic evidence for a structural interaction between the carboxy termini of the membrane and nucleocapsid proteins of mouse hepatitis virus. *Journal of Virology* **76**: 4987–4999.

Kuo, L. and Masters, P. S. 2003. The small envelope protein E is not essential for murine coronavirus replication. *Journal of Virology* **77**: 4597–4608.

Kuo, L. and Masters, P. S. 2010. Evolved variants of the membrane protein can partially replace the envelope protein in murine coronavirus assembly. *Journal of Virology* **84**: 12872–12885.

Kuri, T., Eriksson, K. K., Putics, A., Züst, R., Snijder, E. J., Davidson, A. D., Siddell, S. G., Thiel, V., Ziebuhr, J. and Weber, F. 2011. The ADP-ribose-1″-monophosphatase domains of SARS-coronavirus and Human coronavirus 229E mediate resistance to antiviral interferon responses. *Journal of General Virology* **92**: 1899–1905.

Lai, M. M. and Cavanagh, D. 1997. The molecular biology of coronaviruses. *Advances in Virus Research* **48**: 1–100.

Lai, M. M. C., Patton, C. D., Baric, R. S. and Stohlman, S. A. 1983. Presence of leader sequences in the mRNA of mouse hepatitis virus. *Journal of Virology* **46**: 1027–1033.

Le, S. Y., Sonenberg, N. and Maizel Jr., J. V. 1994. Distinct structural elements and internal entry of ribosomes in mRNA3 encoded by infectious bronchitis virus. *Virology* **198**: 405–411.

Liljestrom, P., Lusa, S., Huylebroeck, D. and Garoff, H. 1991. *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *Journal of Virology* **65**: 4107–4113.

Lim, K. P. and Liu, D. X. 2001. The missing link in coronavirus assembly: retention of the avian coronavirus infectious bronchitis virus envelope protein in the pre-Golgi compartments and physical interaction between the envelope and membrane proteins. *Journal of Biological Chemistry* **276**: 17515–17523.

Liu, D. X. and Inglis, S. C. 1991. Association of the infectious bronchitis virus 3c protein with the virion envelope. *Virology* **185**: 911–917.

Liu, D. X. and Inglis, S. C. 1992. Internal entry of ribosomes on a tricistronic mRNA encoded by infectious bronchitis virus. *Journal of Virology* **66**: 6143–6154.

Luo, Z. L. and Weiss, S. R. 1998. Roles in cell-to-cell fusion of two conserved hydrophobic regions in the murine coronavirus spike protein. *Virology* **244**: 483–494.

Machamer, C. E., Mentone, S. A., Rose, J. K. and Farquhar, M. G. 1990. The E1 glycoprotein of an avian coronavirus is targeted to the cis Golgi complex. *Proceedings of the National Academy of Science USA* **87**: 6944–6948.

Marra, M. A., Jones, S. J., Astell, C. R., Holt, R. A., Brooks-Wilson, A., Butterfield, Y. S., Khattra, J., Asano, J. K., Barber, S. A., Chan, S. Y., Cloutier, A., Coughlin, S. M., Freeman, D., Girn, N., Griffith, O. L., Leach, S. R., Mayo, M., McDonald, H., Montgomery, S. B., Pandoh, P. K., Petrescu, A. S., Robertson, A. G., Schein, J. E., Siddiqui, A., Smailus, D. E., Stott, J. M., Yang, G. S., Plummer, F., Andonov, A., Artsob, H., Bastien, N., bernard, K., Booth, T. F., Bowness, D., Czub, M., Drebot, M., Fernando, L., Flick, R., Garbutt, M., Gray, M., Grolla, A., Jones, S., Feldmann, H., Meyers, A., Kabani, A., Li, Y., Normand, S., Stroher, U., Tipples, G. A., Tyler, S., Vogrig, R., Ward, D., Watson, B., Brunham, R. C., Krajden, M., Petric, M., Skowronski, D. M., Upton, C. and Roper, R. L. 2003. The genome sequence of the SARS-associated coronavirus. *Science* **300**: 1399–1404.

Masters, P. S. 1999. Reverse genetics of the largest RNA viruses. *Advances in Virus Research* **53**: 245–264.

Masters, P. S. 2006. The molecular biology of coronaviruses. *Advances in Virus Research* **66**: 193–292.

Masters, P. S. and Rottier, P. J. 2005. Coronavirus reverse genetics by targeted RNA recombination. *Current Topics in Microbiology and Immunology* **287**: 133–159.

Matsuyama, S., Nagata, N., Shirato, K., Kawase, M., Takeda, M. and Taguchi, F. 2010. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *Journal of Virology* **84**: 12658–12664.

Mcbride, C. E., Li, J. and Machamer, C. E. 2007. The cytoplasmic tail of the severe acute respiratory syndrome coronavirus spike protein contains a novel endoplasmic reticulum retrieval signal that binds COPI and promotes interaction with membrane protein. *Journal of Virology* **81**: 2418–2428.

Mcbride, C. E. and Machamer, C. E. 2010. A single tyrosine in the severe acute respiratory syndrome coronavirus membrane protein cytoplasmic tail is important for efficient interaction with spike protein. *Journal of Virology* **84**: 1891–1901.

Merchlinsky, M. and Moss, B. 1992. Introduction of foreign DNA into the vaccinia virus genome by in vitro ligation: recombination-independent selectable cloning vectors. *Virology* **190**: 522–526.

Mulligan, R. and Berg, P. 1981. Selection for animal cells that express the E. coli gene coding for xanthine-guanine phosphoribosyl transferase. *Proceedings of the National Academy of Science USA* **78**: 2072–2076.

Narayanan, K., Chen, C. J., Maeda, J. and Makino, S. 2003. Nucleocapsid-independent specific viral RNA packaging via viral envelope protein and viral RNA signal. *Journal of Virology* **77**: 2922–2927.

Nieto-Torres, J. L., Dediego, M. L., Álvarez, E., Jiménez-Guardeño, J. M., Regla-Nava, J. A., Llorente, M., Kremer, L., Shuo, S. and Enjuanes, L. 2011. Subcellular location and topology of severe acute respiratory syndrome coronavirus envelope protein. *Virology* **415**: 69–82.

Norkin, L. C. 2010. Coronaviruses. In *Virology: Molecular Biology and Pathogenesis*. ASM Press: Washington, DC.

Ortego, J., Escors, D., Laude, H. and Enjuanes, L. 2002. Generation of a replication-competent, propagation-deficient virus vector based on the transmissible gastroenteritis coronavirus genome. *Journal of Virology* **76**: 11518–11529.

Ortego, J., Sola, I., Almazan, F., Ceriani, J. E., Riquelme, C., Balasch, M., Plana, J. and Enjuanes, L. 2003. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. *Virology* **308**: 13–22.

Pasternak, A. O., Spaan, W. J. and Snijder, E. J. 2006. Nidovirus transcription: how to make sense. . .? *Journal of General Virology* **87**: 1403–1421.

Perlman, S., Gallagher, T. and Snijder, E. J. 2008. *Nidoviruses*. ASM Press: Washington, DC.

Polo, S., Ketner, G., Levis, R. and Falgout, B. 1997. Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. *Journal of Virololgy* **71**: 5366–5374.

Prentice, E., Jerome, W. G., Yoshimori, T., Mizushima, N. and Denison, M. R. 2004. Coronavirus replication complex formation utilizes components of cellular autophagy. *Journal of Biological Chemistry* **279**: 10136–10141.

Putics, A., Filipowicz, W., Hall, J., Gorbalenya, A. E. and Ziebuhr, J. 2005. ADP-ribose-1″-monophosphatase: a conserved coronavirus enzyme that is dispensable for viral replication in tissue culture. *Journal of Virology* **79**: 12721–12731.

Racaniello, V. R. and Baltimore, D. 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**: 916–919.

Rice, C. M., Grakoui, A., Galler, R. and Chambers, T. J. 1989. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *New Biology* **1**: 285–296.

Rice, C. M., Levis, R., Strauss, J. H. and Huang, H. V. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *Journal of Virology* **61**: 3809–3819.

Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., Penaranda, S., Bankamp, B., Maher, K., Chen, M. H., Tong, S., Tamin, A., Lowe, L., Frace, M., Derisi, J. L., Chen, Q., Wang, D., Erdman, D. D., Peret, T. C., Burns, C., Ksiazek, T. G., Rollin, P. E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A. D., Drosten, C., Pallansch, M. A., Anderson, L. J. and Bellini, W. J. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* **300**: 1394–1399.

Satyanarayana, T., Bar-Joseph, M., Mawassi, M., Albiach-Marti, M. R., Ayllon, M. A., Gowda, S., Hilf, M. E., Moreno, P., Garnsey, S. M. and Dawson, W. O. 2001. Amplification of Citrus tristeza virus from a cDNA clone and infection of citrus trees. *Virology* **280**: 87–96.

Sawicki, S. G. and Sawicki, D. L. 1995. Coronaviruses use discontinuous extension for synthesis of subgenome-length negative strands. *Advances in Experimental Medicine and Biology* **380**: 499–506.

Sawicki, S. G. and Sawicki, D. L. 1998. A new model for coronavirus transcription. *Advances in Experimental Medicine and Biology* **440**: 215–219.

Sawicki, S. G. and Sawicki, D. L. 2005. Coronavirus transcription: a perspective. *Current Topics in Microbiology and Immunology* **287**: 31–55.

Sawicki, S. G., Sawicki, D. L. and Siddell, S. G. 2007. A contemporary view of coronavirus transcription. *Journal of Virology* **81**: 20–29.

Schalk, A. F. and Hawn, M. C. 1931. An apparently new respiratory disease of baby chicks. *Journal of the American Veterinary Association* **78**: 413–422.

Schelle, B., Karl, N., Ludewig, B., Siddell, S. G. and Thiel, V. 2005. Selective replication of coronavirus genomes that express nucleocapsid protein. *Journal of Virology* **79**: 6620–6630.

Schelle, B., Karl, N., Ludewig, B., Siddell, S. G. and Thiel, V. 2006. Nucleocapsid protein expression facilitates coronavirus replication. *Advances in Experimental Medicine and Biology* **581**: 43–48.

Schickli, J. H., Thackray, L. B., Sawicki, S. G. and Holmes, K. V. 2004. The N-terminal region of the murine coronavirus spike glycoprotein is associated with the extended host range of viruses from persistently infected murine cells. *Journal of Virology* **78**: 9073–9083.

Senanayake, S. D. and Brian, D. A. 1999. Translation from the 5′ untranslated region (UTR) of mRNA 1 is repressed, but that from the 5′ UTR of mRNA 7 is stimulated in coronavirus-infected cells. *Journal of Virology* **73**: 8003–8009.

Shen, H., Fang, S. G., Chen, B., Chen, G., Tay, F. P. and Liu, D. X. 2009. Towards construction of viral vectors based on avian coronavirus infectious bronchitis virus for gene delivery and vaccine development. *Journal of Virological Methods* **160**: 48–56.

Shulla, A. and Gallagher, T. 2009. Role of spike protein endodomains in regulating coronavirus entry. *Journal of Biological Chemistry* **284**: 32725–32734.

Siddell, S., Ziebuhr, J. and Snijder, E. J. 2005. Coronaviruses, toroviruses and arteriviruses. In Mahy, B. W. J. and Ter Meulen, V. (eds) *Topley and Wilson's Microbiology and Microbial Infections, Virology*. Hodder Arnold: London.

Siddell, S. G. (ed.) 1995. *The Coronaviridae*. New York: Plenum.

Simmons, G., Gosalia, D. N., Rennekamp, A. J., Reeves, J. D., Diamond, S. L. and Bates, P. 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proceedings of the National Academy of Science USA* **102**: 11876–11881.

Sola, I., Alonso, S., Zuniga, S., Balasch, M., Plana-Duran, J. and Enjuanes, L. 2003. Engineering the transmissible gastroenteritis virus genome as an expression vector inducing lactogenic immunity. *Journal of Virology* **77**: 4357–4369.

Sparks, J. S., Lu, X. and Denison, M. R. 2007. Genetic analysis of murine hepatitis virus nsp4 in virus replication. *Journal of Virology* 8B1: 12554–12563.

St-Jean, J. R., Desforges, M., Almazan, F., Jacomy, H., Enjuanes, L. and Talbot, P. J. 2006. Recovery of a neurovirulent human coronavirus OC43 from an infectious cDNA clone. *Journal of Virology* **80**: 3670–3674.

Sturman, L. S., Holmes, K. V. and Behnke, J. 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *Journal of Virology* **33**: 449–462.

Taniguchi, T., Palmieri, M. and Weissmann, C. 1978. QB DNA-containing hybrid plasmids giving rise to QB phage formation in the bacterial host. *Nature* **274**: 223–228.

Tekes, G., Hofmann-Lehmann, R., Stallkamp, I., Thiel, V. and Thiel, H. J. 2008. Genome organization and reverse genetic analysis of a type I feline coronavirus. *Journal of Virology* **82**: 1851–1859.

Thiel, V. 2007. *Coronaviruses Molecular and Cellular Biology*. Caister Academic Press: Norfolk, UK.

Thiel, V., Herold, J., Schelle, B. and Siddell, S. G. 2001. Infectious RNA transcribed *in vitro* from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. *Journal of General Virology* **82**: 1273–1281.

Thiel, V. and Siddell, S. G. 2005. Reverse genetics of coronaviruses using vaccinia virus vectors. *Current Topics in Microbiology and Immunology* **287**: 199–227.

Tooze, J., Tooze, S. A. and Fuller, S. D. 1987. Sorting of progeny coronavirus from condensed secretory proteins at the exit from the trans-golgi network of att20 cells. *Journal of Cell Biology* **105**: 1215–1226.

Tripet, B., Howard, M. W., Jobling, M., Holmes, R. K., Holmes, K. V. and Hodges, R. S. 2004. Structural characterization of the SARS-coronavirus spike S fusion protein core. *Journal of Biological Chemistry* **279**: 20836–20849.

Tseng, Y. T., Wang, S. M., Huang, K. J., Lee, A. I., Chiang, C. C. and Wang, C. T. 2010. Self-assembly of severe acute respiratory syndrome coronavirus membrane protein. *Journal of Biological Chemistry* **285**: 12862–12872.

Tyrrell, D. A. J., Almeida, J. D., Berry, D. M., Cunningham, C. H., Hamre, D., Hofstad, M. S., Mallucci, L. and Mcintosh, K. 1968. Coronaviruses. *Nature* **220**: 650.

Van Den Born, E. and Snijder, E. J. 2008. RNA signals Regulating Nidovirus RNA synthesis. In Perlman, S., Gallagher, T. and Snijder, E. J. (eds) *Nidoviruses*. ASM Press: Washington, DC.

Van Dinten, L. C., Den Boon, J. A., Wassenaar, A. L. M., Spaan, W. J. M. and Snijder, E. J. 1997. An infectious arterivirus cDNA clone: identification of a replicase point mutation that abolishes discontinuous mRNA transcription. *Proceedings of the National Academy of Science USA* **94**: 991–996.

Van Hemert, M. J., Van Den Worm, S. H. E., Knoops, K., Mommaas, A. M., Gorbalenya, A. E. and Snijder, E. J. 2008. SARS-coronavirus replication/transcription complexes are membrane-protected and need a host factor for activity *in vitro*. *PLoS Pathogens* **4**: e1000054.

Verheije, M. H., Lamfers, M. L., Wurdinger, T., Grinwis, G. C., Gerritsen, W. R., Van Beusechem, V. W. and Rottier, P. J. 2009. Coronavirus genetically redirected to the epidermal growth factor receptor exhibits effective antitumor activity against a malignant glioblastoma. *Journal of Virology* **83**: 7507–7516.

Verheije, M. H., Wurdinger, T., Van Beusechem, V. W., De Haan, C. A., Gerritsen, W. R. and Rottier, P. J. 2006. Redirecting coronavirus to a nonnative receptor through a virus-encoded targeting adapter. *Journal of Virology* **80**: 1250–1260.

Wilson, L., Mckinlay, C., Gage, P. and Ewart, G. 2004. SARS coronavirus E protein forms cation-selective ion channels. *Virology* **330**: 322–331.

Yamshchikov, V., Mishin, V. and Cominelli, F. 2001. A new strategy in design of (+)RNA virus infectious clones enabling their stable propagation in *E.coli*. *Virology* **281**: 272–280.

Yang, Z. Y., Huang, Y., Ganesh, L., Leung, K., Kong, W. P., Schwartz, O., Subbarao, K. and Nabel, G. J. 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced

by dendritic cell transfer through DC-SIGN. *Journal of Virology* **78**: 5642–5650.

Youn, S., Leibowitz, J. L. and Collisson, E. W. 2005. *In vitro* assembled, recombinant infectious bronchitis viruses demonstrate that the 5a open reading frame is not essential for replication. *Virology* **332**: 206–215.

Yount, B., Curtis, K. M. and Baric, R. S. 2000. Strategy for systematic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. *Journal of Virology* **74**: 10600–10611.

Yount, B., Curtis, K. M., Fritz, E. A., Hensley, L. E., Jahrling, P. B., Prentice, E., Denison, M. R., Geisbert, T. W. and Baric, R. S. 2003. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. *Proceedings of the National Academy of Science USA* **100**: 12995–13000.

Yount, B., Denison, M. R., Weiss, S. R. and Baric, R. S. 2002. Systematic assembly of a full-length infectious cDNA of mouse hepatitis virus strain A59. *Journal of Virology* **76**: 11065–11078.

Yount, B., Roberts, R. S., Sims, A. C., Deming, D., Frieman, M. B., Sparks, J., Denison, M. R., Davis, N. and Baric, R. S. 2005. Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. *Journal of Virology* **79**: 14909–14922.

Zelus, B. D., Schickli, J. H., Blau, D. M., Weiss, S. R. and Holmes, K. V. 2003. Conformational changes in the spike glycoprotein of murine coronavirus are induced at 37 degrees C either by soluble murine CEACAM1 receptors or by pH 8. *Journal of Virology* **77**: 830–840.

Ziebuhr, J. 2008. Coronavirus replicative proteins. In Perlman, S., Gallagher, T. and Snijder, E. J. (eds) *Nidoviruses*. ASM Press: Washington, DC.

Ziebuhr, J., Snijder, E. J. and Gorbalenya, A. E. 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. *Journal of General Virology* **81**: 853–879.

Zust, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B. W., Ziebuhr, J., Szretter, K. J., Baker, S. C., Barchet, W., Diamond, M. S., Siddell, S. G., Ludewig, B. and Thiel, V. 2011. Ribose 2′-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nature Immunology* **12**: 137–143.

Zust, R., Cervantes-Barragan, L., Kuri, T., Blakqori, G., Weber, F., Ludewig, B. and Thiel, V. 2007. Coronavirus non-structural protein 1 is a major pathogenicity factor: implications for the rational design of coronavirus vaccines. *PLoS Pathogens* **3**: e109.

3

Reverse genetic tools to study hepatitis C virus

Alexander Ploss
Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Diseases, The Rockefeller University, New York

3.1 Introduction: hepatitis C

Infectious hepatitis was already recognised as a major emerging medical problem in the 1950s (Ward *et al*., 1958). With the advent of reliable serological tests in the mid-1970s, many clinical cases could be attributed to hepatitis A and B viruses, however, a substantial fraction of cases remained of unknown cause, and were termed Non-A, Non-B hepatitis (NANBH). It was demonstrated that the NANBH agent is sensitive to organic solvents and could pass through 80 μm filters, suggesting that it to be a small-enveloped virus. Despite substantial efforts it was not until 1989 that Michael Houghton and his team discovered hepatitis C virus (HCV) as the major etiologic agent of NANBH (Choo *et al*., 1989). Development of diagnostic assays and testing of patients and blood supplies highlighted the prevalence and severity of HCV disease. Screening test of blood products drastically reduced the frequency of new infections due to exposure to contaminated materials.

Nonetheless, HCV remains a major medical problem with at least 130 million chronic carriers – approximately 2% of the world's population. Today, HCV is primarily transmitted by intravenous drug use and, in developing countries, unclean medical practices remain a risk factor. Acute infection with HCV is usually clinically asymptomatic. About 20–30% of exposed individuals will spontaneously clear the infection, while the majority go on to develop persistent disease. Chronic hepatitis C results in progressive liver disease leading within 10–30 years to fibrosis, cirrhosis and liver cancer. In fact, HCV is associated with more than half of the newly diagnosed hepatocellular carcinomas in the United States (Alter *et al*., 1999; Montalto *et al*., 2002) and is currently a leading cause of liver transplants worldwide (Brown Jr., 2005). Disease progression is accelerated in those individuals who are co-infected with human immuno-deficiency virus (HIV) or alcoholics.

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

Development of an HCV vaccine has been complicated by the genetic and antigenic diversity of the virus: seven genotypes, differing by 30–35% at the nucleotide level, as well as multiple subtypes are recognised. Treatment options for HCV patients are also limited. The current standard of care, consisting of pegylated interferon (IFN) alpha, ribavirin and one of two protease inhibitors (telaprevir or boceprevir), has significant side effects and is not effective in all individuals (Jacobson *et al*., 2011; Poordad *et al*., 2011). IFN/ribavirin therapy is even less effective in several patient groups, including those bearing certain IFN-λ gene polymorphisms (Suppiah *et al*., 2009, Tanaka *et al*., 2009; Rauch *et al*. 2010), patients with HIV-coinfection or advanced liver disease, and those who have undergone liver transplantation (Fried *et al*., 2002; Manns *et al*., 2001). Liver transplantation is merely a palliative procedure due to universal re-infection of the graft, often resulting in rapid fibrosis progression and subsequent graft failure (Brown Jr, 2005). A broader spectrum of more effective directly acting antivirals (DAAs) is likely to ameliorate the problem of chronic infections in the future. Due to the remarkable replicative capacity of the virus and its error-prone polymerase, however, resistance may be a major problem and early clinical data highlight the dangers of mono-therapy (De Francesco and Migliaccio, 2005). Thus, the current goal for HCV therapy is to achieve sustained virologic response in most patients with an efficient combination of DAAs not leading to the emergence of escape mutations.

3.2 Hepatitis C virus

HCV is a small, enveloped virus that belongs to the family of Flaviviridae, genus *Hepacivirus*. HCV uptake into its primary target cell – the human hepatocyte – requires numerous host proteins including glycosaminoglycans (GAGs) (Barth *et al*., 2003; Koutsoudakis *et al*., 2006), the low density lipoprotein receptor (LDL-R) (Agnello *et al*., 1999; Monazahian *et al*., 1999; Molina *et al*., 2007; Owen *et al*., 2009), the high density lipoprotein receptor scavenger receptor class B type 1 (SCARB1) (Scarselli *et al*., 2002), tetraspanin CD81 (Pileri *et al*., 1998), and two tight junction (TJ) proteins, claudin-1 (CLDN1) (Evans *et al*., 2007) and occludin (OCLN) (Liu *et al*., 2009; Ploss *et al*., 2009). In addition, two receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphRA2) appear to modulate the interaction between CD81 and CLDN1 (Lupberger *et al*., 2011). The exact mechanism of HCV entry remains incompletely defined (reviewed in Zeisel *et al*., 2011). HCV enters the hepatocyte via clathrin-dependent receptor mediated endocytosis. The virion is primed for membrane fusion in the acidified endosome eventually leading to uncoating and release of the viral genome in the cytoplasm (Figure 3.1).

The positive-sense single-stranded RNA genome is approximately 9600 nucleotides in length and encodes a single large open reading frame (ORF) (Figure 3.2). The HCV ORF is flanked by 5′ and 3′ non-translated regions (NTR) containing RNA elements that are important for viral replication and translation (reviewed

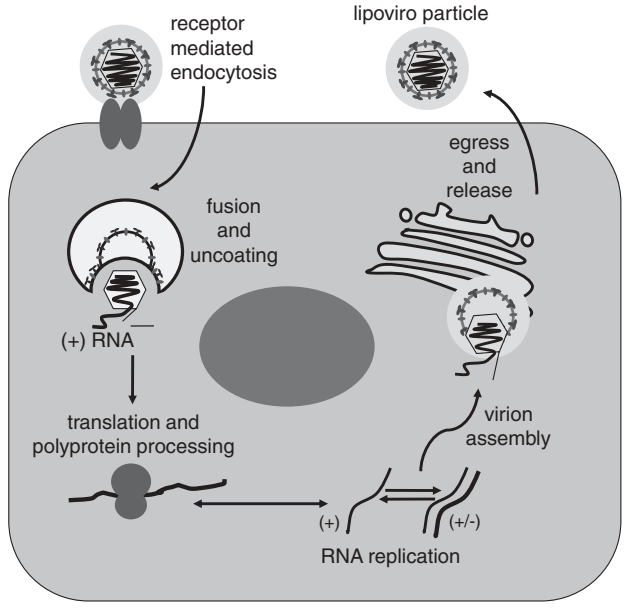

Figure 3.1 The HCV life-cycle: HCV engages numerous cellular entry factors during the entry process. After fusion and coating the + sense single stranded RNA genome is translated and the nascent polyprotein processed. The RNA replication complex, which forms within HCV-induced modified cytoplasmic membrane structures, replicates the positive sense RNA genome through a negative strand intermediate. Interactions between viral proteins and cellular lipid components are essential for a number of viral life-cycle events, including replication, virion assembly, and host cell entry.

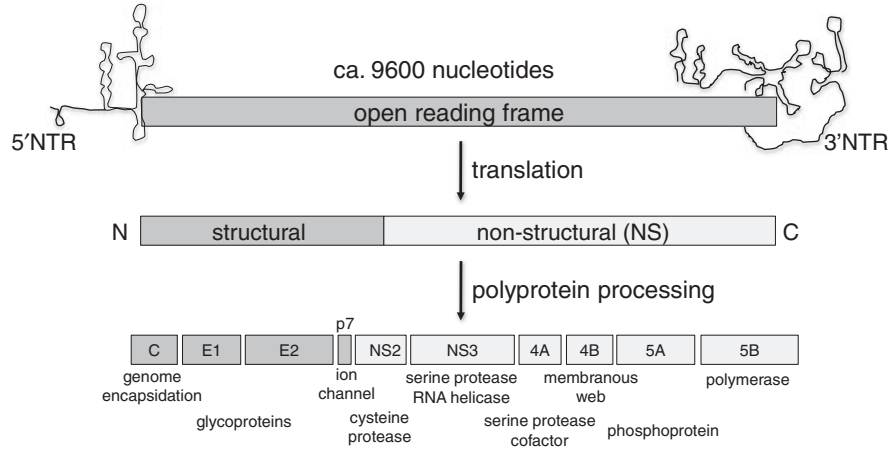

Figure 3.2 HCV genome and polyprotein organisation. The RNA structures in the 5′ and 3′ non-translated regions (NTR) of the genome are depicted. Those include an internal ribosome entry site in the 5′ NTR and a variable region, poly-uridine tract, and 3′-X tail in the 3′ NTR. RNA structures in the NS5B coding region form a kissing-loop interaction with the 3′-X tail. The HCV proteins, which are encoded by the open reading frame and their functions are indicated.

in Tellinghuisen *et al*., 2007). Recently, interactions between the liver-specific microRNA, miR-122, with two sites in the HCV 5′NTR have been shown to be essential for efficient HCV RNA replication in cultured cells (Jopling *et al*., 2005; Jopling *et al*., 2008) and in HCV-infected chimpanzees(Lanford *et al*., 2010). The 5′ NTR encompasses an internal ribosomal entry site (IRES), which drives translation of the viral proteins in a single continuous polyprotein, NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Polyprotein processing occurs by multiple mechanisms. Endogenous peptidases, signal peptidase (Santolini *et al*., 1994) and signal peptide peptidase (McLauchlan *et al*., 2002; Pene *et al*., 2009) mediate the cleavage of the structural proteins, core, E1, E2 and the p7/NS2 junction. Processing of nonstructural proteins (NS) is affected by *cis*- and *trans*-cleaving autoproteases. The present model suggests that dimerised NS2, together with the N-terminal domain of NS3, mediates autoproteolytic cleavage of NS2 cleavage from NS3. NS3 in cis cleaves NS4A from itself and NS4B, after which NS4A associates with the N-terminus of NS3 to form the NS3/4A protease complex. NS3-4A is responsible for cleavage at the NS4B/NS5A and NS5A/NS5B junctions in *trans*. HCV also produces an additional protein, known as ARFP, F, or core+1. This protein is encoded by an ORF that overlaps the core gene in the +1 frame. In vitro, this protein is produced by a ribosomal frameshift mechanism (Walewski *et al*., 2001). ARFP is dispensable for the HCV life-cycle (McMullan *et al*., 2007; Vassilaki *et al*., 2008) and it is unclear whether it contributes to some aspect of viral pathogenesis.

The structural proteins build the viral particle: C forms the nucleocapsid, and the glycoproteins E1 and E2 decorate the host cell-derived virion envelope. E1 and E2 in conjunction with virion-associated lipoproteins mediate the viral entry process, including interactions with cellular receptor molecules and catalysis of fusion between the virion and cellular membranes. After translation, the HCV proteins are associated with membranes derived from the endoplasmic reticulum (ER). Here, they exert their respective functions, including also the inactivation of host molecules by the NS3-4A protease which are critical for anti-viral signaling (reviewed in Horner and Gale, 2009). NS4B, possibly in combination with NS5A, induces the formation of the so-called membranous web, an accumulation of membranous replication vesicles, which contains ER-derived membranes and lipid droplets (LDs). Here, the RNA-dependent RNA polymerase (RdRp) NS5B in combination with presumably most, if not all, other non-structural proteins and critical host factors such as cyclophilin A amplify viral RNA (reviewed in Bartenschlager *et al*., 2010). The RNA replication complex replicates the positive sense RNA genome through a negative strand intermediate. Nascent RNA genomes are translated to produce new viral proteins, serve as new/additional RNA templates for further RNA replication and are assembled to infectious virions. Interactions between viral proteins and cellular lipid components are essential for a number of viral life-cycle events, including replication, virion assembly, and host cell entry. Infectious particles are formed in close association with LD, a subcellular organelle where the HCV core protein was shown to accumulate (reviewed in Bartenschlager *et al*., 2011). The exact mechanism of HCV assembly is still incompletely understood but the process is tightly linked to the very low-density lipoprotein (VLDL) pathway. HCV hijacks several

enzymes, such as microsomal triglyceride transfer protein (MTP), but also several apolipoproteins (apo), in particular, apoE and possibly apoB for its assembly. This close association of virions with apolipoproteins results in so-called lipoviroparticles, which have a VLDL-like but variable composition (Merz *et al*., 2011).

HCV has a narrow species tropism, which is limited to humans and chimpanzees. The basis for this species barrier is incompletely understood. Expression of the human orthologs of CD81 and OCLN can render mouse and hamster cells permissive to viral uptake (Ploss *et al*., 2009). HCV can replicate in murine cells, albeit at very low levels (Zhu *et al*., 2003; Uprichard *et al*., 2006). Later stages in the HCV life-cycle are likely to be supported in mice, as it was recently reported that infectious particles can assemble and be released from murine hepatoma cell lines (Long *et al*., 2011b).

3.3 Construction of infectious clones for hepatitis C virus

In order to study HCV genetically it was necessary to construct an infectious clone. An infectious clone is a complementary cDNA that can produce infectious virus when its corresponding *in vitro* transcript is introduced in suitable cell lines or susceptible animal models. The first published sequence of HCV lacked of the genomic 3′NTR, which are critical for viral replication. Subsequent studies revealed that this region of the genome contains a variable region, a poly-pyrimidine tract and a conserved 3′ terminal stem-loop structure (Tanaka *et al*., 1995; Kolykhalov *et al*., 1996; Blight and Rice, 1997). However, intrahepatic injection of the first full-length clones into chimpanzees did not result in productive infection. Given the remarkable sequence diversity of HCV within patients as well as possible PCR and sequencing errors, it was speculated that incompatible mutations rendered these first genomes non-infectious. Consensus genomes were therefore created. These incorporated the most prominent nucleotide in each position, reasoning that deleterious mutations would be less common. Intrahepatic inoculation of *in vitro* transcribed consensus RNA into chimpanzees resulted in sustained HCV infection that clinically and virologically resembled infection by the clinical material from which the infectious clone was derived (Kolykhalov *et al*., 1997; Yanagi *et al*., 1997). Currently chimpanzee infectious clones for genotypes 1a (Hong *et al*., 1999; Lanford *et al*., 2001; Sakai *et al*., 2007), 1b (Yanagi *et al*., 1998; Beard *et al*., 1999; Thomson *et al*., 2001; Bukh *et al*., 2002), 2a (Yanagi *et al*., 1999; Wakita *et al*., 2005), 3a (Gottwein *et al*., 2010) and 4a (Gottwein *et al*., 2010) have been constructed but are still lacking for the other genotypes.

3.4 Study of HCV RNA replication in cell culture systems

Although chimpanzee-infectious clones were available, detailed analysis of the HCV life-cycle *in vitro* was still precluded by the lack of a tractable cell culture

system. Initial attempts to infect primary hepatocytes (Carloni *et al*., 1993; Iacovacci *et al*., 1993; Iacovacci *et al*., 1997; Ito *et al*., 1996) or human hepatoma cells (Ikeda *et al*., 1997) with sera from HCV-infected individuals or experimentally infected chimpanzees resulted in low-level replication or inconclusive results. Identification of clinical HCV isolates that robustly replicate in cell culture and conditions conducive to infection remain a priority to this day.

The generation of a recombinant cDNA clone of HCV proven to be infectious *in vivo* was a major step forward and suggested that it might be feasible to initiate a HCV tissue culture system. However, HCV RNA failed to amplify when RNA was transfected directly into cultured cells (Lohmann *et al*., 1999; Blight *et al*., 2000). These early setbacks suggested that the specific HCV RNA genomes were not compatible with the cellular environment of human liver cancer cell lines, which are arguably quite different from human hepatocytes *in vivo*. In 1999, Lohmann *et al*. generated genotype 1b (strain Con1) bicistronic genomes that expressed the antibiotic-selectable marker neomycin phosphotransferase (neo) (Lohmann *et al*., 1999). The 5′ NTR drives expression of the first 12 codons of C fused to the neo coding sequence, while a heterologous encephalomyocarditis virus (EMCV) IRES drives expression of the HCV non-structural proteins NS3 through NS5B; the genome ends with the native HCV 3′ NTR. Upon transfection of this selectable genome, termed a replicon, into human hepatoma cells, drug-resistant colonies containing HCV RNA and non-structural proteins emerged (Figure 3.3).These results became the basis of the first cell culture platform to study HCV RNA replication (Lohmann *et al*., 1999; Pietschmann *et al*., 2001).

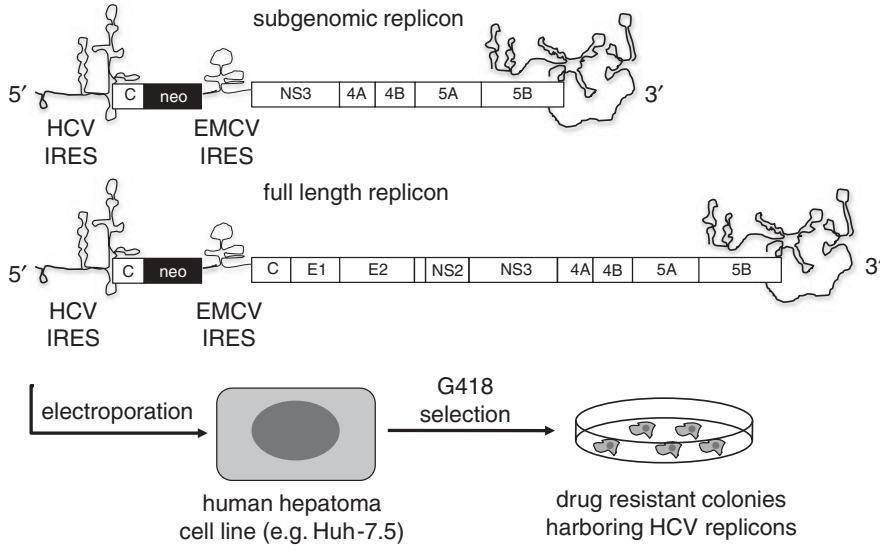

Figure 3.3 HCV replicons. Schematic representation of subgenomic and full-length HCV replicons. After transfection of *in vitro* transcribed RNA, drug selectable genomes replicate in permissive human hepatoma cell lines.

While the development of HCV replicons clearly marked a milestone in HCV research, the efficiency of establishing productive RNA replication remained very low, leading to drug resistance in only a small fraction of transfected cells. Sequence analysis of HCV replicon clones revealed mutations in NS3, NS5A and NS5B which, when re-engineered in the parental genome, resulted in considerably higher levels of RNA replication (Blight *et al*., 2000; Blight *et al*., 2002; Lohmann *et al*., 2001; Lohmann *et al*., 2003). It was demonstrated that these replication-enhancing mutations also increased the replicative fitness of other genotype 1b replicons, such as AH1 (Mori *et al*., 2008), HCV-O (Kato *et al*., 2003a; Ikeda *et al*., 2005; Abe *et al*., 2007), HCV-BK (Grobler *et al*., 2003) and J4 (Maekawa *et al*., 2004) but not other genotypes (Blight *et al*., 2000; Gu *et al*., 2003; Lanford *et al*., 2003; Liang *et al*., 2005; Yi and Lemon, 2004). Subsequently, different sets of adaptive mutations, which also primarily mapped to NS3, NS4B, NS5A and NS5B were shown to enhance replication of genotype 1a replicons (Blight *et al*., 2003; Grobler *et al*., 2003; Yi and Lemon, 2004). Replicons for all other genotypes, except one derived from a genotype 2a isolate (Kato *et al*., 2003b), are not yet available.

3.5 Use of HCV replicons to study viral replication

The development of the HCV replicon system opened unprecedented opportunities to dissect the viral and cellular components influencing HCV RNA replication. It became possible to map the functional determinants of HCV proteins involved in viral replication. Furthermore, genetic interactions between HCV gene products could now be analysed by introducing mutations or deleting parts of the viral genome, which alter replication, and subsequently selecting for compensatory mutations. Identification of flexible regions within non-structural proteins, mutation of which did not impair or only minimally impacted HCV RNA replication, allowed the insertion of reporter genes. This provided tools for studying multiple aspects of viral replication, for example, the formation and turnover of HCV replication complexes in living cells (Moradpour *et al*., 2004; McCormick *et al*., 2006; Jones *et al*., 2007). Inclusion of luciferase with a dominant selectable marker in bicistronic HCV replicons was also shown to be functional (Vrolijk *et al*., 2003; Ikeda *et al*., 2005). This configuration yielded a useful tool for the study of HCV replication in high throughput. Indeed, targeted and genome-wide siRNA screens using luciferase expressing replicons helped to identify several candidate host molecules which affect HCV replication (Wang *et al*., 2005; Watashi *et al*., 2005; Ng *et al*., 2007; Randall *et al*., 2007; Supekova *et al*., 2008; Tai *et al*., 2009; Vaillancourt *et al*., 2009; Reiss *et al*., 2011).

The development of the replicon system also enabled studies to explore the ability of HCV to replicate in cell lines of non-hepatic or even non-human origin. It was indeed possible to select for HCV replicon containing human non-hepatic epithelial cells (Zhu *et al*., 2003), mouse hepatoma cells (Zhu *et al*., 2003; Uprichard *et al*., 2006) and even murine embryonic fibroblasts (Chang *et al*., 2006; Lin *et al*.,

2010). These data suggest that all the essential host factors required for HCV RNA replication may be present in non-hepatic cells, even those derived from rodents.

3.6 Utility of replicons for drug screening

HCV replicons provided the first cell-based platform to functionally assess the efficacy of lead anti-viral compounds in drug development. The replicon became essential for assaying the activity of enzymatic targets, such as NS3-4A protease or NS5B polymerase, in the context of authentic viral replication.

HCV replicons also enabled the discovery of new classes of inhibitors that target viral proteins without apparent enzymatic activity. For example, initial leads for the non-nucleoside NS5B inhibitor GS-9190 (Vliegen *et al*., 2009), the NS4A antagonist ACH-806 (Yang *et al*., 2008), and the NS5A inhibitor BMS-790052 (Gao *et al*., 2010) were discovered using replicon-based screens. Similarly, the synthetic non-immunosuppressive cyclosporine analog, DEBIO-025 (also known as Alisporovir), which interferes with the essential HCV replication host factor, cyclophilin A, was shown to effectively inhibit viral replication in HCV replion assays (Paeshuyse *et al*., 2006).

HCV replicons also allowed for a reverse genetic analysis of mutations that accumulated, for example, in patient cohorts treated with DAA compounds. Analyses of the sequence variants allowed correlation between resistant variants emerging *in vivo* and those observed in the *in vitro* replicon system; resistance profile and replicative ability (i.e., fitness) of the variants could also be studied (Sarrazin and Zeuzem, 2010; Fridell *et al*., 2011).

The lack of a full panel of replicons representing all HCV genotypes has hindered the rational selection of compounds with pan-genotype activity. While the first approved DAAs are active against HCV genotypes 1a and 1b, and to some extent genotype 2, there is a considerable need for effective drugs for all genotypes. One example is genotype 4, which is dominant in Egypt – a country with one of the highest HCV prevalence worldwide. To overcome this gap, chimeras expressing HCV enzymes of heterologous genotypes, for example, the protease (genotypes 1-4) (Binder *et al*., 2011) or polymerase (genotype 1-6) (Herlihy *et al*., 2008) domains, have been created and exhibit varying susceptibilities to inhibitors. NS3/4A or NS5B genes, respectively were cloned from the plasma of HCV-infected individuals and inserted into HCV genotype 2a (protease chimeras) or 1b (polymerase chimeras) replicons, replacing the native sequences.

3.7 Development of the infectious cell culture systems for HCV

In 2001, an HCV sequence from a Japanese patient who developed fulminant hepatitis, an extremely rare clinical complication during the acute phase of the infection

was reported (Kato *et al*., 2001). It became quickly clear that Japanese fulminant hepatitis (JFH1), a genotype 2a strain, had unique properties. Unlike all previously constructed HCV replicons, transfection of JFH1 RNA into human hepatoma cells resulted in efficient RNA replication without the need for adaptive mutations (Kato *et al*., 2003b). Furthermore, transfection of Huh7 hepatoma cells or derivatives thereof with full-length *in vitro* transcribed JFH1 RNA resulted in detectable levels of infectious virions in the cell culture supernatant (Wakita *et al*., 2005; Zhong *et al*., 2005). Cell culture-derived HCV (HCVcc) uptake was glycoprotein dependent and could be blocked by antibodies directed against E2 or the HCV entry factor CD81. Infection with JFH1 HCVcc was also sensitive to type I IFN and prototype HCV polymerase and protease inhibitors (Lindenbach *et al*., 2005).

3.8 Construction of intergenotypic viral chimeras

Maximal titers of full-length JFH1 transfections reached approximately 50,000 infectious units per milliliter within 3 weeks of culture (Zhong *et al*., 2005). Chimeric genomes consisting of the C through NS2 protein of HCV genotype 2a isolate J6 and the non-structural proteins NS3 through NS5B of JFH1(Figure 3.4), however, reached the same titers within two days following transfection (Lindenbach *et al*., 2005). This first intergenotypic chimera provided a more robust system to study the entire HCV life-cycle *in vitro*. The original breakpoint between J6 and JFH1 was originally not empirically determined. It was later demonstrated that shifting the original breakpoint between J6 and JFH1 from the end of NS2 to a site located right after the first putative transmembrane domain of NS2, designated Jc1, yielded infectious titers 100- to 1,000-fold higher than the parental JFH1 isolate (Pietschmann *et al*., 2006). These observations suggested that determinants within the structural proteins govern the kinetics and efficiency of virus assembly and release. Subsequently, iterative passage of J6/JFH1 derived genomes was found to yield adaptive mutations that further improved the yield of infectious virus and allowed for more efficient viral spread in a cell culture (Walters *et al*., 2009; Pokrovskii *et al*., 2011).

Following these seminal reports, intergenotypic chimeras consisting of the C-NS2 regions of representative genomes of all seven HCV genotypes were constructed (Pietschmann *et al*., 2006; Gottwein *et al*., 2007; Yi *et al*., 2007; Jensen *et al*., 2008; Scheel *et al*., 2008; Gottwein *et al*., 2009; Pietschmann *et al*., 2009). These tools have become indispensable for *in vitro* and *in vivo* assays of HCV entry and neutralisation. The utility of such chimeras has recently been extended to test 5′ NTR-based treatment approaches, such as antagonists of the liver-specific microRNA miR-122 122 (Jopling *et al*., 2005) using JFH1-based recombinants. 5′ NTR-NS2 recombinants of HCV genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, and 6a paired with NS3-NS5B from JFH1 were generated, and replicate and produce infectious virus in cell culture (Imhof and Simmonds, 2010; Li *et al*., 2011). In order to facilitate genotype-specific studies of HCV RNA replication inhibitors and viral resistance, J6/JFH1-based recombinants with genotypes 1-7 NS3 serine protease domain (NS3P) and NS4A (Gottwein *et al*., 2011b) or NS5A

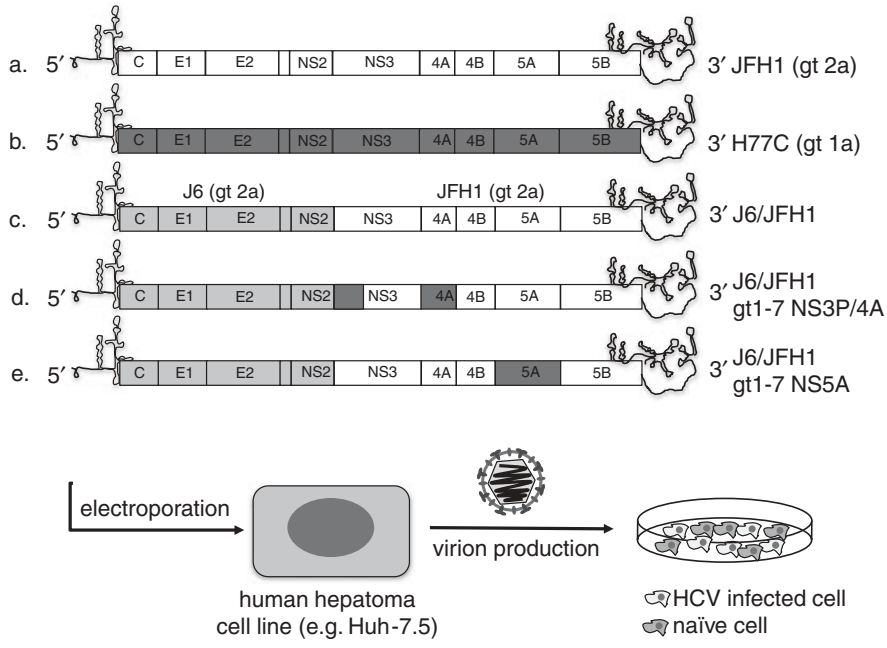

Figure 3.4 HCV cell culture systems. Schematic representation of HCV full length genomes which can replicate and produce infectious virions in human hepatoma cells. Japanese fulminant hepatitis (JFH1) genotype 2a genome (a.), and the cell culture adapted Hutchinson strain (H77C) (b.). c. J6/JFH1 is an intergenotypic chimera composed of the C-NS2 of isolate J6 paired with the non-structural proteins NS3-NS5B of JFH1. Examples of J6/JFH1 chimeras harboring the NS3 protease domain and NS4A or NS5A of diverse HCV genotypes.

(Scheel *et al*., 2011) were constructed. Interestingly, recombinant HCV variants harboring non-structural proteins of genotypes 1–7 exhibited genotype-specific susceptibility to certain classes of compounds, highlighting the utility of these platforms for pan-genotype drug development. Almost all intergenotypic chimeras required adaptive mutations, which increase replicative fitness and/or production of infectious particles.

The advent of a cell culture system that supports the entire HCV life-cycle opened unparalleled opportunities for genetic analysis of the role of specific host and viral proteins, not only those involved in HCV RNA replication but also virion assembly. Introduction of specific mutations that impair replication and/or virus production, and subsequent selection for compensatory mutations, allowed genetic interactions between HCV gene products to be identified (Pietschmann *et al*., 2006; Yi *et al*., 2007; Murray *et al*., 2007). Reporter virus genomes have been engineered to facilitate rapid quantification of HCV replication. Reporters have been expressed using bicistronic genomes or inserted, in-frame, in the HCV polyprotein often as fusions with the HCV NS5A protein (Koutsoudakis *et al*., 2006; Schaller *et al*., 2007; Gottwein *et al*., 2011a).

3.9 Non-JFH1 derived genomes

Despite considerable effort, it has proven difficult to identify additional genomes that, like JFH1, robustly replicate in cell culture. Based on the cDNA clones of the Hutchinson strain, which generated RNA transcripts shown to be infectious *in vivo*, a genotype 1a infection system (H77S) has been generated. The H77S genome contains five cell culture-adaptive mutations that are distributed throughout the nonstructural protein-coding region, resulting in robust RNA replication in human hepatoma cells and moderate titers of cell culture-infectious virus (although significantly less efficient than even parental JFH1) (Yi *et al*., 2006). Identification of additional HCV genomes covering all genotypes that can complete the entire viral life-cycle in cell culture remains a high priority.

3.10 Cell lines that support HCV replication

Hepatic and hepatoma cell lines that have been used for many years to study HCV replication and production of cell culture-derived virus. Most cell lines efficiently supporting HCV replication have been derived from the human hepatoma cell line Huh-7. The Huh-7 cell line was originally derived from a 57-year-old Japanese male patient with a well-differentiated hepatocellular carcinoma (Nakabayashi *et al*., 1982). It was observed that elimination of self-replicating HCV RNA from Huh-7 clones, for example. by prolonged treatment with alpha interferon would sometimes result in cured cells that could more efficiently support subgenomic and full-length HCV replication. Examples of such a cured cell lines are Huh-7.5 (Blight *et al*., 2002), Huh-7-lunet (Friebe *et al*., 2005) and Huh-7.5.1 (Zhong *et al*., 2005) cells. All these cell lines were later shown to also support efficient replication and particle production of JFH1-based genomes. It has been suggested that the marked increase in HCV permissiveness of Huh-7.5 cells correlates with their reduced ability to mount type I interferon mediated antiviral responses. This failure has been attributed to a mutation in the helicase domain of the RIG-I protein in Huh-7.5, which severely impairs binding to viral RNA (Sumpter *et al*., 2005). However, other studies demonstrated that reconstitution of RIG-I signaling in Huh-7.5 did not have any impact on HCV replication (Binder *et al*., 2007). Thus, the reason for the higher permissiveness of Huh-7 derivatives remains a mystery.

A few other cell lines support HCV RNA replication to some extent, including LH86 cells. LH86 cells were derived from resected well-differentiated hepatocellular carcinoma tissue (Zhu *et al*., 2007). JFH1 infection of LH86 cells triggers innate antiviral defense mechanisms that culminate in apoptotic cell death. HCV is generally thought to be a non-cytopathic virus and thus these exacerbated responses probably do not reflect the cellular response to infection *in vivo*. Expression of mir-122 renders two human hepatoma cell lines, Hep3B and HepG2 supplemented with CD81, permissive to HCV infection (Kambara *et al*., 2011; Narbus *et al*., 2011).

Hepatoma lines have certainly been instrumental for studies of the HCV lifecycle, but abnormal proliferation and deregulated gene expression of continuously growing cancer cell lines may not reflect *in vivo* hepatocytes. Thus, it would desirable to dissect HCV infection in better hepatocyte-like cell culture models.

3.11 Study of HCV in physiologically more relevant cell culture systems

Primary hepatocyte cultures have proven to be valuable tools in the study of liver physiology, viral hepatitis, and liver regeneration. Although one of the first studies describing HCV infection in primary human hepatocytes was reported almost 20 years ago (Iacovacci *et al*., 1993), the use of primary hepatocytes in HCV research has not found widespread use, likely due to the difficulties associated with culturing these cells, maintaining their differentiated phenotype and achieving reliable HCV infection. Despite such challenges, study of HCV in primary human hepatocytes has provided valuable insights in the development of HCV quasispecies (Rumin *et al*., 1999), the role of HCV entry factors (in particular, LDLR and CD81) in HCV infection (Molina *et al*., 2007, Molina *et al*., 2008), IFN inhibition of HCV replication (Castet *et al*., 2002) and the biophysical properties of infectious virions (Podevin *et al*., 2010). However, analysis of other phenomena, such as the response of the virus to drug regimens, which are metabolised by sets of enzymes expressed in hepatocytes or growth of patient-derived HCV genomes, which may only ramp up slowly, requires long-term stability of the hepatic phenotype.

It has been observed that a number of liver-specific functions are progressively lost when hepatocytes are isolated and cultivated (Bhatia *et al*., 1999). These phenotypic changes are mainly the result of diminished transcription of relevant liver-specific genes, and can be interpreted as a dedifferentiation of the isolated hepatocytes. Ischemia-reperfusion induced stress during the isolation process, disruption of the normal tissue architecture, and adaptation to the *in vitro* environment are underlying factors. Different approaches, mainly mimicking the *in vivo* hepatocyte environment, have been successfully used to prevent or slow the dedifferentiation process. Co-culture of primary hepatocytes with non-parenchymal cells has long been known to stabilise their cellular functions (Khetani *et al*., 2008). Advances in microfabrication techniques allowed for plating both cell populations in experimentally defined patterns, resulting in so-called micropatterned co-cultures (MPCCs), and yields hepatocytes that can maintain differentiated functions over several weeks (Khetani and Bhatia, 2008). Human hepatocytes in MPCCs support the entire HCV life-cycle although sensitive reporter strategies are typically employed to assess HCV replication (Ploss *et al*., 2010). Similar observations have also been made using fetal liver-derived hepatocyte cultures (Lazaro *et al*., 2003; Lazaro *et al*., 2007) and blunting of type I interferon signaling substantially increases HCV permissiveness and viral spread (Andrus *et al*., 2011; Marukian *et al*., 2011). Recently,

induced pluripotent stem cells (iPSCs) have been shown to differentiate into cells that share many features of mature hepatocytes, termed hepatocyte-like cells (HLCs) (reviewed in Behbahan *et al*., 2011). Using HLCs it may become possible to analyse patient specific phenotypes in cell culture. At this point, however, convincing evidence that HLCs can support HCV infection is still missing.

Use of advanced primary hepatocyte cell culture systems provides a new platform with which to study cellular responses to HCV infection in a physiologically more relevant context. It is conceivable that other patient-derived HCV genomes and recombinant derivatives thereof will be able to replicate more efficiently in this more native cellular environment as opposed to cell culture adapted liver cancer cell lines.

3.12 Animal models for HCV infection

The chimpanzee model played a pivotal role in the discovery of HCV and the validation of the first infectious clones (Kolykhalov *et al*., 1997). However, experiments in great apes are expensive, logistically challenging and have been under scrutiny because of ethical concerns. The reasons for limited species tropism of HCV remain poorly understood. At the level of viral entry, the virus requires CD81, SCARB1, CLDN1 and OCLN to infect rodent cells but only CD81 and OCLN have to be of human origin (Ploss *et al*., 2009; Dorner *et al*., 2011). On the basis of these observations, human CD81 and OCLN were adenovirally expressed in a reporter mouse strain. Such genetically humanised mice support uptake of HCV particles and viral RNA translation (Dorner *et al*., 2011); other parts of the viral life-cycle were not recapitulated in this model. HCV entry of multiple genotypes was observed using bi-cistronic intergenotypic HCV chimeras expressing CRE recombinase. Following entry into cells expressing suitable combinations of HCV entry factors, CRE activates a cellular reporter in cells, which could be quantified by bioluminescent imaging.

In principal, HCV genomes can replicate in murine hepatic cell lines, albeit at very low frequency (Zhu *et al*., 2003). Thus, the cellular factors essential for HCV replication are present in mouse cells, but the virally encoded replication machinery might not mesh efficiently with the murine factors. Species-specific differences in innate antiviral responses and the inability of HCV proteins to overcome them may also impair replication in mouse cells. In fact, targeted disruption of the PKR, a cytosolic sensor for viral RNA, or interferon response factor 3 (IRF3) in mouse embryonic fibroblasts yields higher levels of HCV replication (Chang *et al*., 2006; Lin *et al*., 2010). Remarkably, HCV virion assembly and release do take place in mouse hepatoma cells provided that essential host factors like apoE are provided (Long *et al*., 2011a). However, it yet remains to be demonstrated that the entire HCV life-cycle can be recapitulated in an inbred mouse model.

Alternatively, mice can be humanised by transplantation of human hepatocytes into suitable xenorecipients. A high degree of chimerism (up to 99%) can be

achieved by transplanting human hepatocytes into immunodeficient mice where liver injury has been induced to ablate the endogenous murine hepatocytes. The best-characterised model to date is the immunodeficient urokinase-type plasminogen activator (uPA) mouse, in which an albumin (Alb) promoter directs high-level toxic expression of uPA (Heckel *et al*., 1990). The hepatotoxicity creates a permissive environment for the expansion of functional, transgene-free human hepatocytes. Human hepatocytes can also be successfully engrafted into fumaryl acetoacetate hydrolase (FAH)–deficient mice on a $Rag2^{-/-}$ $IL2\gamma_c{}^{null}$ immunodeficient background (FRG mice) (Azuma *et al*., 2007; Bissig *et al*., 2007). FAH is the last enzyme in the tyrosine breakdown pathway and its deficiency leads to lethal type 1 hypertyrosinemia in man and liver failure in mice. Treatment with 2-(2-nitro-4-trifluoromethylbenzyol)-cyclohexan-1,3-dione (NTBC) prevents the accumulation of toxic metabolites and hepatotoxicity allowing liver injury to be induced by withdrawal of the drug. A recently generated transgenic mouse expressing caspase 8 fused to the FK506 binding protein on an immunodecient background (AFC8) was shown not only to support engraftment of human fetal hepatocytes but also components of a human immune system (Washburn *et al*., 2011).

These liver chimeric mouse models are susceptible to hepatotropic human pathogens, including HBV (Dandri *et al*., 2001; Bissig *et al*., 2010), HCV (Mercer *et al*., 2001; Bissig *et al*., 2010; Washburn *et al*., 2011), and human malaria parasites (Sacci *et al*., 2006) and have been used in studies ranging from viral evolution to preclinical testing of antiviral compounds. In contrast to almost every human cell culture system, highly engrafted human liver chimeric mice are susceptible not only to cell culture-derived viruses (Lindenbach *et al*., 2006; Bissig *et al*., 2010) but also patient-derived isolates (Mercer *et al*., 2001; Bissig *et al*., 2010). Importantly, in contrast to cell culture-produced virus, animal passaged virus has a buoyant density profile that resembles more the low density profiles which have been ascribed to patient-derived viruses.

3.13 Reverse genetics of clinically relevant HCV genotypes *in vivo*

Human liver chimeric mice have the potential to enable reverse genetic studies on JFH1-based and non-JFH1-derived viruses *in vivo*. It has been demonstrated that injection of *in vitro* transcribed HCV RNA, including sequences cloned from patients with severe acute hepatitis has resulted in productive infection, yielding measurable and transmissible viremia in mice highly engrafted with human hepatocytes (Hiraga *et al*., 2007; Kimura *et al*., 2008). The impact of host genetics and viral amino acid substitutions associated with altered responses to IFN-based therapy were analysed in human liver chimeric mice (Hiraga *et al*., 2011). These studies establish precedence for the utility of human liver chimeric mice to study the mechanisms underlying the variable resistance of HCV genotypes and patient-specific variants to therapy.

3.14 Conclusion

Hepatitis C remains a major medical problem. Analysis of HCV infection and development of more effective therapeutics have been hampered by the lack of adequate cell culture systems and animal models. The development of the HCV replicon and infectious cell culture system has provided platforms to apply reverse genetic approaches to gain insights into HCV biology and to screen drug candidates. Despite these advances, more tools are needed, including a broader spectrum of HCV isolates which replicate efficiently in cell culture, more physiological cellular environments that reflect the hepatic environment, and more tractable animal models to dissect viral pathogenesis. More effective therapeutics have begun to emerge but, given the high global burden of HCV, it will remain a major challenge to conquer the infection world-wide.

Acknowledgments

I would like to thank Drs. Charles Rice, Catherine Murray, Troels Scheel for useful discussions and critically reading the manuscript. I also thank Ms. Bridget Donovan for technical assistance.

References

Abe, K., Ikeda, M., Dansako, H., Naka, K. and Kato, N. 2007. Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA. *Virus Research* **125**: 88–97.

Agnello, V., Abel, G., Elfahal, M., Knight, G. B. and Zhang, Q.-X. 1999. Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. *Proceedings of National Academy of Sciences of the USA* **96**: 12766–12771.

Alter, M. J., Kruszon-Moran, D., Nainan, O. V., McQuillan, G. M., Gao, F., Moyer, L. A., Kaslow, R. A. and Margolis, H. S. 1999. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *New England Journal of Medicine* **341**: 556–562.

Andrus, L., Marukian, S., Jones, C. T., Catanese, M. T., Sheahan, T. P., Schoggins, J. W., Barry, W. T., *et al*. 2011. Expression of paramyxovirus V proteins promotes replication and spread of hepatitis C virus in cultures of primary human fetal liver cells. *Hepatology*, in press.

Azuma, H., Paulk, N., Ranade, A., Dorrell, C., Al-Dhalimy, M., Ellis, E., Strom, S., Kay, M. A., *et al*. 2007. Robust expansion of human hepatocytes in Fah-/-/Rag2-/-/Il2rg-/- mice. *Nature Biotechnology* **25**: 903–910.

Bartenschlager, R., Cosset, F. L. and Lohmann, V. 2010. Hepatitis C virus replication cycle. *Journal of Hepatology* **53**: 583–585.

Bartenschlager, R., Penin, F., Lohmann, V. and Andre, P. 2011. Assembly of infectious hepatitis C virus particles. *Trends in Microbiology* **19**: 95–103.

Barth, H., Schafer, C., Adah, M. I., Zhang, F., Linhardt, R. J., Toyoda, H., Kinoshita-Toyoda, A., *et al*. 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *Journal of Biological Chemistry* **278**: 41003–41012.

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., *et al*. 1999. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30**: 316–324.

Behbahan, I. S., Duan, Y., Lam, A., Khoobyari, S., Ma, X., Ahuja, T. P. and Zern, M. A. 2011. New approaches in the differentiation of human embryonic stem cells and induced pluripotent stem cells toward hepatocytes. *Stem Cell Review* **7**: 748–759.

Bhatia, S. N., Balis, U. J., Yarmush, M. L. and Toner, M. 1999. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB Journal* **13**: 1883–1900.

Binder, J., Tetangco, S., Weinshank, M., Maegley, K., Lingardo, L., Diehl, W., *et al*. 2011. Development of hepatitis C virus chimeric replicons for identifying broad spectrum NS3 protease inhibitors. *Antiviral Research* **91**: 102–111.

Binder, M., Kochs, G., Bartenschlager, R. and Lohmann, V. 2007. Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. *Hepatology* **46**: 1365–74.

Bissig, K. D., Le, T. T., Woods, N. B. and Verma, I. M. 2007. Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model. *Proceedings of the National Academy of Sciences of the USA* **104**: 20507–205011.

Bissig, K. D., Wieland, S. F., Tran, P., Isogawa, M., Le, T. T., Chisari, F. V. and Verma, I. M. 2010. Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *Journal of Clinical Invesigation* **120**: 924–930.

Blight, K. J., Kolykhalov, A. A. and Rice, C. M. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**: 1972–1974.

Blight, K. J., McKeating, J. A., Marcotrigiano, J. and Rice, C. M. 2003. Efficient RNA replication of hepatitis C virus genotype 1a in cell culture. *Journal of Virology* **77**: 3181–3190.

Blight, K. J., McKeating, J. A. and Rice, C. M. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *Journal of Virology* **76**: 13001–13014.

Blight, K. J. and Rice, C. M. 1997. Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA. *Journal of Virology* **71**: 7345–7352.

Brown Jr, R. S. 2005. Hepatitis C and liver transplantation. *Nature* **436**: 973–978.

Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R. E., Govindarajan, S., *et al*. 2002. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proceedings of the National Academy of Sciences of the USA* **99**: 14416–14421.

Carloni, G., Iacovacci, S., Sargiacomo, M., Ravagnan, G., Ponzetto, A., Peschle, C. and Battaglia, M. 1993. Susceptibility of human liver cell cultures to hepatitis C virus infection. *Archives of Virology* **Suppl. 8**: 31–39.

Castet, V., Fournier, C., Soulier, A., Brillet, R., Coste, J., Larrey, D., Dhumeaux, D., *et al*. 2002. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected *in vitro*. *Journal of Virology* **76**: 8189–8199.

Chang, K. S., Cai, Z., Zhang, C., Sen, G. C., Williams, B. R. and Luo, G. 2006. Replication of hepatitis C virus (HCV) RNA in mouse embryonic fibroblasts: protein kinase R (PKR)-dependent and PKR-independent mechanisms for controlling HCV RNA replication and mediating interferon activities. *Journal of Virology* **80**: 7364–7374.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. and Houghton, M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**: 359–362.

Dandri, M., Burda, M. R., Torok, E., Pollok, J. M., Iwanska, A., Sommer, G., Rogiers, X., *et al*. 2001. Repopulation of mouse liver with human hepatocytes and *in vivo* infection with hepatitis B virus. *Hepatology* **33**: 981–988.

De Francesco, R. and Migliaccio, G. 2005. Challenges and successes in developing new therapies for hepatitis C. *Nature* **436**: 953–960.

Dorner, M., Horwitz, J. A., Robbins, J. B., Barry, W. T., Feng, Q., Mu, K., Jones, C. T., *et al*. 2011. A genetically humanized mouse model for hepatitis C virus infection. *Nature* **474**: 208–211.

Evans, M. J., Von Hahn, T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., *et al*. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**: 801–805.

Fridell, R. A., Wang, C., Sun, J. H., O'Boyle, D. R., 2nd, Nower, P., Valera, L., Qiu, D., *et al*. 2011. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: *in vitro* and *in vivo* correlations. *Hepatology,* forthcoming.

Friebe, P., Boudet, J., Simorre, J. P. and Bartenschlager, R. 2005. Kissing-loop interaction in the 3′ end of the hepatitis C virus genome essential for RNA replication. *Journal of Virology* **79**: 380–392.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Jr., Haussinger, D., *et al*. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *New England Journal of Medicine* **347**: 975–982.

Gao, M., Nettles, R. E., Belema, M., Snyder, L. B., Nguyen, V. N., Fridell, R. A., Serrano-Wu, M. H., *et al*. 2010. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* **465**: 96–100.

Gottwein, J. M., Jensen, T. B., Mathiesen, C. K., Meuleman, P., Serre, S. B., Lademann, J. B., *et al*. 2011a. Development and application of hepatitis C reporter viruses with genotype 1 to 7 core-nonstructural protein 2 (NS2) expressing fluorescent proteins or luciferase in modified JFH1 NS5A. *Journal of Virology* **85**: 8913–8928.

Gottwein, J. M., Scheel, T. K., Callendret, B., Li, Y. P., Eccleston, H. B., Engle, R. E., Govindarajan, S., *et al*. 2010. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and *in vivo* pathogenesis studies. *Journal of Virology* **84**: 5277–5293.

Gottwein, J. M., Scheel, T. K., Hoegh, A. M., Lademann, J. B., Eugen-Olsen, J., Lisby, G. and Bukh, J. 2007. Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. *Gastroenterology* **133**: 1614–1626.

Gottwein, J. M., Scheel, T. K., Jensen, T. B., Ghanem, L. and Bukh, J. 2011b. Differential efficacy of protease inhibitors against HCV genotypes 2a, 3a, 5a, and 6a NS3/4A protease recombinant viruses. *Gastroenterology* **141**: 1067–1079.

Gottwein, J. M., Scheel, T. K., Jensen, T. B., Lademann, J. B., Prentoe, J. C., Knudsen, M. L., *et al*. 2009. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* **49**: 364–377.

Grobler, J. A., Markel, E. J., Fay, J. F., Graham, D. J., Simcoe, A. L., Ludmerer, S. W., Murray, E. M., *et al*. 2003. Identification of a key determinant of hepatitis C virus cell culture adaptation in domain II of NS3 helicase. *Journal of Biological Chemistry* **278**: 16741–16746.

Gu, B., Gates, A. T., Isken, O., Behrens, S. E. and Sarisky, R. T. 2003. Replication studies using genotype 1a subgenomic hepatitis C virus replicons. *Journal of Virology* **77**: 5352–5359.

Heckel, J. L., Sandgren, E. P., Degen, J. L., Palmiter, R. D. and Brinster, R. L. 1990. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* **62**: 447–456.

Herlihy, K. J., Graham, J. P., Kumpf, R., Patick, A. K., Duggal, R. and Shi, S. T. 2008. Development of intergenotypic chimeric replicons to determine the broad-spectrum antiviral activities of hepatitis C virus polymerase inhibitors. *Antimicrobial Agents and Chemotherapy* **52**: 3523–3531.

Hiraga, N., Abe, H., Imamura, M., Tsuge, M., Takahashi, S., Hayes, C. N., Ochi, H., *et al*. 2011. Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology,* forthcoming.

Hiraga, N., Imamura, M., Tsuge, M., Noguchi, C., Takahashi, S., Iwao, E., Fujimoto, Y., *et al*. 2007. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Letters* **581**: 1983–1987.

Hong, Z., Beaudet-Miller, M., Lanford, R. E., Guerra, B., Wright-Minogue, J., Skelton, A., Baroudy, B. M., *et al*. 1999. Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. *Virology* **256**: 36–44.

Horner, S. M. and Gale, M., JR. 2009. Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. *Journal of Interferon Cytokine Research* **29**: 489–498.

Iacovacci, S., Manzin, A., Barca, S., Sargiacomo, M., Serafino, A., Valli, M. B., Macioce, G., *et al*. 1997. Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected *in vitro*. *Hepatology* **26**: 1328–1337.

Iacovacci, S., Sargiacomo, M., Parolini, I., Ponzetto, A., Peschle, C. and Carloni, G. 1993. Replication and multiplication of hepatitis C virus genome in human foetal liver cells. *Research in Virology* **144**: 275–279.

Ikeda, M., Abe, K., Dansako, H., Nakamura, T., Naka, K. and Kato, N. 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochemical and Biophysical Research Communications* **329**: 1350–1359.

Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Shimotohno, K. and Kato, N. 1997. Hepatitis G virus replication in human cultured cells displaying susceptibility to hepatitis C virus infection. *Biochemical and Biophysical Research Communications* **235**: 505–508.

Imhof, I. and Simmonds, P. 2010. Development of an intergenotypic hepatitis C virus (HCV) cell culture method to assess antiviral susceptibilities and resistance development of HCV NS3 protease genes from HCV genotypes 1 to 6. *Journal of Virology* **84**: 4597–4610.

Ito, T., Mukaigawa, J., Zuo, J., Hirabayashi, Y., Mitamura, K. and Yasui, K. 1996. Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus. *Journal of General Virology* **77**(Pt 5): 1043–1054.

Jacobson, I. M., McHutchison, J. G., Dusheiko, G., Di Bisceglie, A. M., Reddy, K. R., Bzowej, N. H., Marcellin, P., *et al*. 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. *New England Journal of Medicine* **364**: 2405–2416.

Jensen, T. B., Gottwein, J. M., Scheel, T. K., Hoegh, A. M., Eugen-Olsen, J. and Bukh, J. 2008. Highly efficient JFH1-based cell-culture system for hepatitis C virus genotype 5a: failure of homologous neutralizing-antibody treatment to control infection. *Journal of Infectious Diseases* **198**: 1756–1765.

Jones, D. M., Gretton, S. N., Mclauchlan, J. and Targett-Adams, P. 2007. Mobility analysis of an NS5A-GFP fusion protein in cells actively replicating hepatitis C virus subgenomic RNA. *Journal of General Virology* **88**: 470–475.

Jopling, C. L., Schutz, S. and Sarnow, P. 2008. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host Microbe* **4**: 77–85.

Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. and Sarnow, P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* **309**: 1577–1581.

Kambara, H., Fukuhara, T., Shiokawa, M., Ono, C., Ohara, Y., Kamitani, W. and Matsuura, Y. 2011. Establishment of a novel permissive cell line for propagation of hepatitis C virus by the expression of microRNA122. *Journal of Virology,* forthcoming.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., *et al*. 2003a. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected *in vitro*. *Biochemical and Biophysical Research Communications* **306**: 756–766.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M. and Wakita, T. 2003b. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**: 1808–1817.

Kato, T., Furusaka, A., Miyamoto, M., Date, T., Yasui, K., Hiramoto, J., Nagayama, K., *et al*. 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *Journal of Medical Virology* **64**: 334–339.

Khetani, S. R. and Bhatia, S. N. 2008. Microscale culture of human liver cells for drug development. *Nature Biotechnology* **26**: 120–126.

Khetani, S. R., Chen, A. A., Ranscht, B. and Bhatia, S. N. 2008. T-cadherin modulates hepatocyte functions *in vitro*. *The FASEB Journal* **22**(11): 3768–3775.

Kimura, T., Imamura, M., Hiraga, N., Hatakeyama, T., Miki, D., Noguchi, C., Mori, N., *et al*. 2008. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. *Journal of General Virology* **89**: 2108–2113.

Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M. and Rice, C. M. 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* **277**: 570–574.

Kolykhalov, A. A., Feinstone, S. M. and Rice, C. M. 1996. Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. *Journal of Virology* **70**: 3363–3371.

Koutsoudakis, G., Kaul, A., Steinmann, E., Kallis, S., Lohmann, V., Pietschmann, T. and Bartenschlager, R. 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *Journal of Virology* **80**: 5308–5320.

Lanford, R. E., Guerra, B., Lee, H., Averett, D. R., Pfeiffer, B., Chavez, D., Notvall, L. and Bigger, C. 2003. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(i)-poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. *Journal of Virology* **77**: 1092–1104.

Lanford, R. E., Hildebrandt-Eriksen, E. S., Petri, A., Persson, R., Lindow, M., Munk, M. E., *et al*. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **327**: 198–201.

Lanford, R. E., Lee, H., Chavez, D., Guerra, B. and Brasky, K. M. 2001. Infectious cDNA clone of the hepatitis C virus genotype 1 prototype sequence. *Journal of General Virology* **82**: 1291–1297.

Lazaro, C. A., Chang, M., Tang, W., Campbell, J., Sullivan, D. G., Gretch, D. R., Corey, L., *et al*. 2007. Hepatitis C virus replication in transfected and serum-infected cultured human fetal hepatocytes. *American Journal of Pathology* **170**: 478–489.

Lazaro, C. A., Croager, E. J., Mitchell, C., Campbell, J. S., Yu, C., Foraker, J., Rhim, J. A., *et al*. 2003. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. *Hepatology* **38**: 1095–1106.

Li, Y. P., Gottwein, J. M., Scheel, T. K., Jensen, T. B. and Bukh, J. 2011. MicroRNA-122 antagonism against hepatitis C virus genotypes 1-6 and reduced efficacy by host RNA insertion or mutations in the HCV 5′ UTR. *Proceedings of the National Academy of Sciences of the USA* **108**: 4991–4996.

Liang, C., Rieder, E., Hahm, B., Jang, S. K., Paul, A. and Wimmer, E. 2005. Replication of a novel subgenomic HCV genotype 1a replicon expressing a puromycin resistance gene in Huh-7 cells. *Virology* **333**: 41–53.

Lin, L. T., Noyce, R. S., Pham, T. N., Wilson, J. A., Sisson, G. R., Michalak, T. I., Mossman, K. L. and Richardson, C. D. 2010. Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. *Journal of Virology* **84**: 9170–9180.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., *et al*. 2005. Complete replication of hepatitis C virus in cell culture. *Science* **309**: 623–626.

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., McKeating, J. A., Lanford, R. E., *et al*. 2006. Cell culture-grown hepatitis C virus is infectious *in vivo* and can be recultured *in vitro*. *Proceedings of the National Academy of Sciences of the USA* **103**: 3805–3809.

Liu, S., Yang, W., Shen, L., Turner, J. R., Coyne, C. B. and Wang, T. 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *Journal of Virology* **83**: 2011–2014.

Lohmann, V., Hoffmann, S., Herian, U., F, F. P. and Bartenschlager, R. 2003. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *Journal of Virology* **77**: 3007–3019.

Lohmann, V., Korner, F., Dobierzewska, A. and Bartenschlager, R. 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *Journal of Virology* **75**: 1437–1449.

Lohmann, V., Korner, F., Koch, J. O., Herian, U., Theilmann, L. and Bartenschlager, R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**: 110–113.

Long, G., Hiet, M. S., Windisch, M. P., Lee, J. Y., Lohmann, V. and Bartenschlager, R. 2011a. Mouse hepatic cells support assembly of infectious hepatitis C virus particles. *Gastroenterology,* forthcoming.

Long, G., Hiet, M. S., Windisch, M. P., Lee, J. Y., Lohmann, V. and Bartenschlager, R. 2011b. Mouse hepatic cells support assembly of infectious hepatitis C virus particles. *Gastroenterology* **141**: 1057–1066.

Lupberger, J., Zeisel, M. B., Xiao, F., Thumann, C., Fofana, I., Zona, L., Davis, C, *et al*. 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nature Medicine* **17**: 589–595.

Maekawa, S., Enomoto, N., Sakamoto, N., Kurosaki, M., Ueda, E., Kohashi, T., Watanabe, H., *et al*. 2004. Introduction of NS5A mutations enables subgenomic HCV replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells. *Journal of Viral Hepatology* **11**: 394–403.

Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K., Shiffman, M., Reindollar, R., Goodman, Z. D., *et al*. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**: 958–965.

Marukian, S., Andrus, L., Sheahan, T. P., Jones, C. T., Charles, E. D., Ploss, A., Rice, C. M. and Dustin, L. B. 2011. Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. *Hepatology*.

McCormick, C. J., Maucourant, S., Griffin, S., Rowlands, D. J. and Harris, M. 2006. Tagging of NS5A expressed from a functional hepatitis C virus replicon. *Journal of General Virology* **87**: 635–640.

McLauchlan, J., Lemberg, M. K., Hope, G. and Martoglio, B. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO Journal* **21**: 3980–3988.

McMullan, L. K., Grakoui, A., Evans, M. J., Mihalik, K., Puig, M., Branch, A. D., Feinstone, S. M. and Rice, C. M. 2007. Evidence for a functional RNA element in the hepatitis C virus core gene. *Proceedings of the National Academy of Sciences of the USA* **104**: 2879–2884.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., *et al*. 2001. Hepatitis C virus replication in mice with chimeric human livers. *Nature Medicine* **7**: 927–933.

Merz, A., Long, G., Hiet, M. S., Brugger, B., Chlanda, P., Andre, P., Wieland, F., *et al*. 2011. Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome. *Journal of Biological Chemistry* **286**: 3018–3032.

Molina, S., Castet, V., Fournier-Wirth, C., Pichard-Garcia, L., Avner, R., Harats, D., Roitelman, J., *et al*. 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *Journal of Hepatology* **46**: 411–419.

Molina, S., Castet, V., Pichard-Garcia, L., Wychowski, C., Meurs, E., Pascussi, J. M., Sureau, C., *et al*. 2008. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent. *Journal of Virology* **82**: 569–574.

Monazahian, M., Bohme, I., Bonk, S., Koch, A., Scholz, C., Grethe, S. and Thomssen, R. 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *Journal of Medical Virology* **57**: 223–229.

Montalto, G., Cervello, M., Giannitrapani, L., Dantona, F., Terranova, A. and Castagnetta, L. A. 2002. Epidemiology, risk factors, and natural history of hepatocellular carcinoma. *Annals of the N Y Academy of Sciences* **963**: 13–20.

Moradpour, D., Evans, M. J., Gosert, R., Yuan, Z., Blum, H. E., Goff, S. P., Lindenbach, B. D. and Rice, C. M. 2004. Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. *Journal of Virology* **78**: 7400–7409.

Mori, K., Abe, K., Dansako, H., Ariumi, Y., Ikeda, M. and Kato, N. 2008. New efficient replication system with hepatitis C virus genome derived from a patient

with acute hepatitis C. *Biochemical and Biophysical Research Communications* **371**: 104–109.

Murray, C. L., Jones, C. T., Tassello, J. and Rice, C. M. 2007. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. *Journal of Virology* **81**: 10220–10231.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. and Sato, J. 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Research* **42**: 3858–3863.

Narbus, C. M., Israelow, B., Sourisseau, M., Michta, M. L., Hopcraft, S. E., Zeiner, G. M. and Evans, M. J. 2011. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *Journal of Virology* **85**: 12087–120892.

Ng, T. I., Mo, H., Pilot-Matias, T., He, Y., Koev, G., Krishnan, P., Mondal, R., *et al*. 2007. Identification of host genes involved in hepatitis C virus replication by small interfering RNA technology. *Hepatology* **45**: 1413–1421.

Owen, D. M., Huang, H., Ye, J. and Gale, M., JR. 2009. Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. *Virology* **394**: 99–108.

Paeshuyse, J., Kaul, A., De Clercq, E., Rosenwirth, B., Dumont, J. M., Scalfaro, P., Bartenschlager, R. and Neyts, J. 2006. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication *in vitro*. *Hepatology* **43**: 761–770.

Pene, V., Hernandez, C., Vauloup-Fellous, C., Garaud-Aunis, J. and Rosenberg, A. R. 2009. Sequential processing of hepatitis C virus core protein by host cell signal peptidase and signal peptide peptidase: a reassessment. *Journal of Viral Hepatology* **16**: 705–715.

Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., Steinmann, E., Abid, K., *et al*. 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proceedings of the National Academy of Sciences of the USA* **103**: 7408–7413.

Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K. and Bartenschlager, R. 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *Journal of Virology* **75**: 1252–1264.

Pietschmann, T., Zayas, M., Meuleman, P., Long, G., Appel, N., Koutsoudakis, G., Kallis, S., *et al*. 2009. Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. *PLoS Pathogens* **5**: e1000475.

Pileri, P., Uematsu, Y., Compagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., *et al*. 998. Binding of hepatitis C virus to CD81. *Science* **282**: 938–941.

Ploss, A., Evans, M. J., Gaysinskaya, V. A., Panis, M., You, H., De Jong, Y. P. and Rice, C. M. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* **457**: 882–886.

Ploss, A., Khetani, S. R., Jones, C. T., Syder, A. J., Trehan, K., Gaysinkaya, V. A., Mu, K., *et al*. 2010. Persistent hepatitis C virus infection in microscale primary

human hepatocyte cultures. *Proceedings of the National Academy of Sciences of the USA*, in press.

Podevin, P., Carpentier, A., Pene, V., Aoudjehane, L., Carriere, M., Zaidi, S., Hernandez, C., *et al*. 2010. Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. *Gastroenterology* **139**(4): 1355–1364. Epub 2010 July 1.

Pokrovskii, M. V., Bush, C. O., Beran, R. K., Robinson, M. F., Cheng, G., Tirunagari, N., Fenaux, M., *et al*. 2011. Novel mutations in a tissue culture-adapted hepatitis C virus strain improve infectious-virus stability and markedly enhance infection kinetics. *Journal of Virology* **85**: 3978–3985.

Poordad, F., McCone, J., Jr., Bacon, B. R., Bruno, S., Manns, M. P., Sulkowski, M. S., Jacobson, I. M., *et al*. 2011. Boceprevir for untreated chronic HCV genotype 1 infection. *New England Journal of Medicine* **364**: 1195–1206.

Randall, G., Panis, M., Cooper, J. D., Tellinghuisen, T. L., Sukhodolets, K. E., Pfeffer, S., *et al*. 2007. Cellular cofactors affecting hepatitis C virus infection and replication. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 12884–12889.

Rauch, A., Kutalik, Z., Descombes, P., Cai, T., Di Iulio, J., Mueller, T., Bochud, M., *et al*. 2010. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* **138**: 1338–1345, e1–7.

Reiss, S., Rebhan, I., Backes, P., Romero-Brey, I., Erfle, H., Matula, P., Kaderali, L., *et al*. 2011. Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. *Cell Host Microbe* **9**: 32–45.

Rumin, S., Berthillon, P., Tanaka, E., Kiyosawa, K., Trabaud, M.-A., Bizollon, T., Gouillat, C., *et al*. 1999. Dynamic analysis of hepatitis C virus replication and quasispecies selection in long-term cultures of adult human hepatocytes infected *in vitro*. *Journal of General Virology* **80**: 3007–3018.

Sacci, J. B., Jr., Alam, U., Douglas, D., Lewis, J., Tyrrell, D. L., Azad, A. F. and Kneteman, N. M. 2006. Plasmodium falciparum infection and exoerythrocytic development in mice with chimeric human livers. *Int J Parasitol* **36**, 353–60.

Sakai, A., Takikawa, S., Thimme, R., Meunier, J. C., Spangenberg, H. C., Govindarajan, S., Farci, P., *et al*. 2007. In vivo study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone (pHC-TN) are infectious in chimpanzees but not in Huh7.5 cells. *Journal of Virology* **81**: 7208–7219.

Santolini, E., Migliaccio, G. and La Monica, N. 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. *Journal of Virology* **68**: 3631–3641.

Sarrazin, C. and Zeuzem, S. 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* **138**: 447–462.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., *et al*. 2002. The human scavenger receptor class B type I is

a novel candidate receptor for the hepatitis C virus. *EMBO Journal* **21**: 5017–5025.

Schaller, T., Appel, N., Koutsoudakis, G., Kallis, S., Lohmann, V., Pietschmann, T. and Bartenschlager, R. 2007. Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes. *Journal of Virology* **81**: 4591–4603.

Scheel, T. K., Gottwein, J. M., Jensen, T. B., Prentoe, J. C., Hoegh, A. M., Alter, H. J., Eugen-Olsen, J. and Bukh, J. 2008. Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. *Proceedings of the National Academy of Sciences of the USA* **105**: 997–1002.

Scheel, T. K., Gottwein, J. M., Mikkelsen, L. S., Jensen, T. B. and Bukh, J. 2011. Recombinant HCV variants with NS5A from genotypes 1-7 have different sensitivities to an NS5A inhibitor but not interferon-alpha. *Gastroenterology* **140**: 1032–1042.

Sumpter, R., Jr., Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M. and Gale, M., JR. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *Journal of Virology* **79**: 2689–2699.

Supekova, L., Supek, F., Lee, J., Chen, S., Gray, N., Pezacki, J. P., Schlapbach, A. and Schultz, P. G. 2008. Identification of human kinases involved in hepatitis C virus replication by small interference RNA library screening. *Journal of Biological Chemistry* **283**: 29–36.

Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M. L., Bassendine, M. *et al*. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nature Genetics* **41**: 1100–1104.

Tai, A. W., Benita, Y., Peng, L. F., Kim, S. S., Sakamoto, N., Xavier, R. J. and Chung, R. T. 2009. A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. *Cell Host Microbe* **5**: 298–307.

Tanaka, T., Kato, N., Cho, M.-J. and Shimotohno, K. 1995. A novel sequence found at the 3′ terminus of hepatitis C virus genome. *Biochemical and Biophysical Research Communications* **215**: 744–749.

Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., Nakagawa, M., *et al*. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nature Genetics* **41**: 1105–1109.

Tellinghuisen, T. L., Evans, M. J., Von Hahn, T., You, S. and Rice, C. M. 2007. Studying hepatitis C virus: making the best of a bad virus. *Journal of Virology* **81**: 8853–8867.

Thomson, M., Nascimbeni, M., Gonzales, S., Murthy, K. K., Rehermann, B. and Liang, T. J. 2001. Emergence of a distinct pattern of viral mutations in chimpanzees infected with a homogeneous inoculum of hepatitis C virus. *Gastroenterology* **121**: 1226–1233.

Uprichard, S. L., Chung, J., Chisari, F. V. and Wakita, T. 2006. Replication of a hepatitis C virus replicon clone in mouse cells. *Virology Journal* **3**: 89.

Vaillancourt, F. H., Pilote, L., Cartier, M., Lippens, J., Liuzzi, M., Bethell, R. C., Cordingley, M. G. and Kukolj, G. 2009. Identification of a lipid kinase as a host factor involved in hepatitis C virus RNA replication. *Virology* **387**: 5–10.

Vassilaki, N., Friebe, P., Meuleman, P., Kallis, S., Kaul, A., Paranhos-Baccala, G., Leroux-Roels, G., *et al*. 2008. Role of the hepatitis C virus core+1 open reading frame and core cis-acting RNA elements in viral RNA translation and replication. *Journal of Virology* **82**: 11503–11515.

Vliegen, I., Paeshuyse, J., De Burghgraeve, T., Lehman, L. S., Paulson, M., Shih, I. H., Mabery, E., *et al*. 2009. Substituted imidazopyridines as potent inhibitors of HCV replication. *Journal of Hepatology* **50**: 999–1009.

Vrolijk, J. M., Kaul, A., Hansen, B. E., Lohmann, V., Haagmans, B. L., Schalm, S. W. and Bartenschlager, R. 2003. A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C. *Journal of Virological Methods* **110**: 201–209.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K. *et al*. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Medicine* **11**: 791–796.

Walewski, J. L., Keller, T. R., Stump, D. D. and Branch, A. D. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* **7**: 710–721.

Walters, K. A., Syder, A. J., Lederer, S. L., Diamond, D. L., Paeper, B., Rice, C. M. and Katze, M. G. 2009. Genomic analysis reveals a potential role for cell cycle perturbation in HCV-mediated apoptosis of cultured hepatocytes. *PLoS Pathogens* 5, e1000269.

Wang, C., Gale, M., Jr., Keller, B. C., Huang, H., Brown, M. S., Goldstein, J. L. and Ye, J. 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Molecular Cell* **18**: 425–434.

Ward, R., Krugman, S., Giles, J. P., Jacobs, A. M. and Bodansky, O. 1958. Infectious hepatitis; studies of its natural history and prevention. *New England Journal of Medicine* **258**: 407–416.

Washburn, M. L., Bility, M. T., Zhang, L., Kovalev, G. I., Buntzman, A., Frelinger, J. A., Barry, W., *et al*. 2011. A humanized mouse model to study hepatitis C virus infection, immune response, and liver disease. *Gastroenterology*.

Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y. and Shimotohno, K. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Molecular Cell* **19**: 111–122.

Yanagi, M., Purcell, R. H., Emerson, S. U. and Bukh, J. 1997. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proceedings of the National Academy of Sciences of the USA* **94**: 8738–8743.

Yanagi, M., Purcell, R. H., Emerson, S. U. and Bukh, J. 1999. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* **262**: 250–263.

Yanagi, M., St. Claire, M., Shapiro, M., Emerson, S. U., Purcell, R. H. and Bukh, J. 1998. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious *in vivo*. *Virology* **244**: 161–172.

Yang, W., Zhao, Y., Fabrycki, J., Hou, X., Nie, X., Sanchez, A., Phadke, A., *et al*. 2008. Selection of replicon variants resistant to ACH-806, a novel hepatitis C virus inhibitor with no cross-resistance to NS3 protease and NS5B polymerase inhibitors. *Antimicrobial Agents and Chemotherapy* **52**: 2043–2052.

Yi, M. and Lemon, S. M. 2004. Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. *Journal of Virology* **78**: 7904–7915.

Yi, M., Ma, Y., Yates, J. and Lemon, S. M. 2007. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. *Journal of Virology* **81**: 629–638.

Yi, M., Villanueva, R. A., Thomas, D. L., Wakita, T. and Lemon, S. M. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proceedings of the National Academy of Sciences of the USA* **103**: 2310–2315.

Zeisel, M. B., Fofana, I., Fafi-Kremer, S. and Baumert, T. F. 2011. Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets for antiviral therapies. *Journal of Hepatology* **54**: 566–576.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., *et al*. 2005. Robust hepatitis C virus infection *in vitro*. *Proceedings of the National Academy of Sciences of the USA* **102**: 9294–9299.

Zhu, H., Dong, H., Eksioglu, E., Hemming, A., Cao, M., Crawford, J. M., Nelson, D. R. and Liu, C. 2007. Hepatitis C virus triggers apoptosis of a newly developed hepatoma cell line through antiviral defense system. *Gastroenterology* **133**: 1649–1659.

Zhu, Q., Guo, J. T. and Seeger, C. 2003. Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. *Journal of Virology* **77**: 9204–9210.

4

Calicivirus reverse genetics

Ian Goodfellow
Section of Virology, Department of Medicine, Imperial College, London

4.1 Introduction

The use of reverse genetics has greatly facilitated the understanding of many aspects of RNA virus biology. Despite many advances in other fields, knowledge of calicivirus biology has lagged significantly behind that of many other RNA viruses, primarily due to the inability to grow human noroviruses in cell culture. As a result, animal caliciviruses have often been used as model systems with which to dissect aspects of the calicivirus life-cycle (Vashist *et al*., 2009).

Members of the *Caliciviridae* family of small positive stranded RNA viruses cause a wide range of diseases in both man and animals (Vashist *et al*., 2009). In man, human noroviruses are now recognised as the major cause of food-borne gastroenteritis in the developed world (Scallan *et al*., 2011). There are currently five recognised genera: *Norovirus, Sapovirus, Vesivirus, Lagovirus* and the recently approved *Nebovirus* (Figure 4.1). Calicivirus genomes are typically ~7.5kb in length, with a 3′ poly-A tail and a 13–15kDa virus-encoded protein (VPg) linked to the 5′ end of the viral RNA. Caliciviruses share a very common genome structure (Figure 4.1) (Clarke and Lambden, 2000) which varies largely only by whether or not the open reading frame (ORF) encoding the major capsid protein (VP1) is in the same frame and therefore contiguous with ORF1. In all cases, however, expression of the VP1 protein is also thought to occur from a subgenomic RNA (sgRNA) produced during virus replication.

Open reading frame one is a large polyprotein that is post-translationally cleaved by the virus encoded protease (3C, NS6) into six or seven non-structural proteins (depending on the virus, Figure 4.1). The individual functions of each of the non-structural proteins have yet to be fully elucidated, however, three main enzymatic activities are present: NS3 encodes a protein with NTPase and helicase motifs; NS6 encodes a chymotrypsin-like protease which is responsible for the production of the mature non-structural proteins from the ORF1 polyprotein; and NS7 encodes the RNA-dependent RNA polymerase responsible for viral RNA synthesis.

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

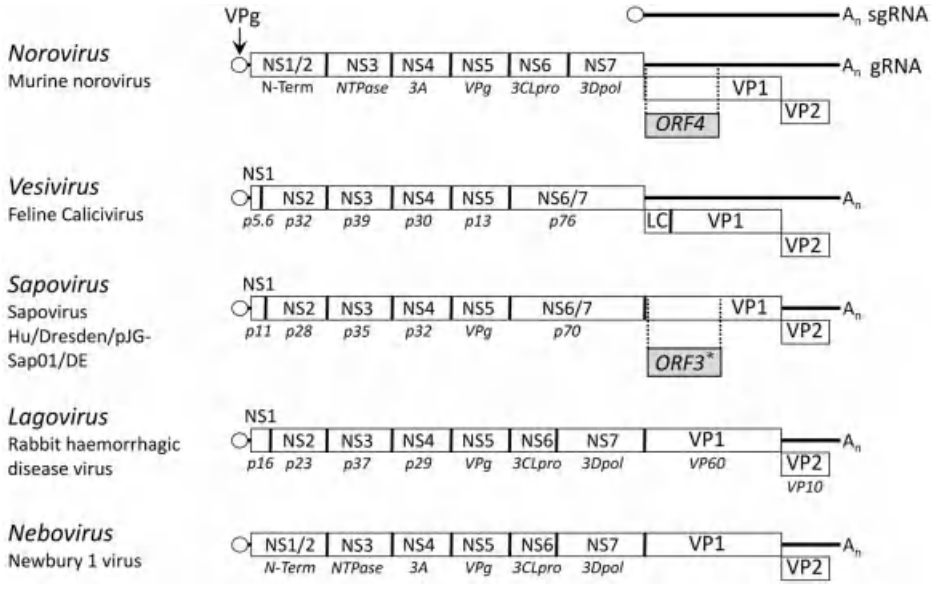

Figure 4.1 Schematic representation of calicivirus genomes. The genome layout of representative members of the five currently recognised *Caliciviridae* genera is highlighted. The NS1-7 nomenclature is used to distinguish the various non-structural proteins, with other synonyms widely used in the literature are also provided below each protein product in italics. Members of the *Norovirus* and *Vesivirus* genera typically contain three open reading frames (ORFs), although a fourth ORF is present in murine norovirus (ORF4). The *Lagovirus, Sapovirus* and *Nebovirus* genera members typically possess two open reading frames, although it is worth noting that the major capsid protein VP1 can also be translated as a separate ORF from the viral subgenomic RNA (sgRNA) produced during virus replication. Some members of the *Sapovirus* genus also possess a third ORF (highlighted with an asterisk, ORF3), thought to be expressed from the sgRNA. The positions of the major and minor capsid proteins VP1 and VP2 are also shown, along with the Vesivirus specific leader of the capsid protein (LC). Note that the figure is not to scale.

The unique mechanism that caliciviruses use for protein synthesis is likely to be one of the largest hurdles for the development of efficient calicivirus reverse genetics systems. Unlike other RNA viruses that rely on cap-dependent or internal ribosome entry site (IRES)-dependent mechanisms (Belsham and Sonenberg, 2000;Yanguez and Nieto, 2011), caliciviruses rely on the use of a virus-encoded, 13–15kDa protein known as VPg or NS5 covalently linked to the 5′ end of the viral genome (Figure 4.1). We and others have previously demonstrated that the calicivirus VPg protein functions as a proteinaceous cap substitute, recruiting cellular translation initiation factors to the 5′ end of the viral RNA to allow subsequent ribosomal recruitment and viral protein synthesis (Daughenbaugh *et al*., 2003; Goodfellow *et al*., 2005; Chaudhry *et al*., 2006). In addition, the 5′ untranslated regions of the calicivirus genomic and subgenomic RNAs are typically short (4 and 5 nucleotides for MNV respectively) (Karst *et al*., 2003). Therefore it is highly likely that efficient calicivirus translation initiation, as required for optimal virus recovery,

can really only occur when VPg is covalently linked to the viral RNA. To date, however, no method is readily available to allow the site-specific linkage of recombinant forms of calicivirus VPg proteins to *in vitro* transcribed RNA. Despite this, and as described in more detail below, it is now clear that recent advances in the field have resulted in the generation of reverse genetics systems for many members of the *Caliciviridae*. In some cases, the reverse genetics systems have been applied to study significant aspects of the virus life-cycle and pathogenesis. This has allowed a substantial increase in our understanding of this important group of pathogens and has provided the tools for future study. In a few cases, however, reverse genetics has only recently been developed, therefore the full potential of the systems has yet to be explored. For completeness, all the calicivirus reverse genetics systems developed to date are discussed, along with their applications where appropriate.

4.2 Feline calicivirus

4.2.1 Development of feline calicivirus reverse genetics

Early studies on feline calicivirus (FCV) clearly indicated that viral RNA either purified from infected cells or from virions was infectious when transfected into permissive cells (Love, 1973). However, it was not until 1995 that the first FCV reverse genetics system was developed by Dr Stanislav Sosnovtsev and Dr Kim Green at the National Institutes for Health, Bethesda, USA (Sosnovtsev and Green, 1995). This represented not only a major step forward for the study of FCV, but as this was the first reverse genetics system for any member of the *Caliciviridae*, it was a major breakthrough in the field. The FCV system as originally described (Sosnovtsev and Green, 1995) relied on the transfection of *in vitro* transcribed viral RNA derived from a full-length cDNA clone of FCV strain Urbana into permissive feline kidney cells (Figure 4.2). Cloning individual regions of the viral genome was initially achieved by generating a cDNA library from RNA isolated from infected cells. The genome was then assembled from a number of clones using unique enzymes naturally present in the viral genome. The full-length cDNA clone was first linearised and then *in vitro* transcribed using bacteriophage T7 RNA polymerase in the presence of high concentrations of the cap analogue m7G(5′)ppp(5′)G. As the cDNA construct was generated with a truncated T7 RNA polymerase promoter at the 5′ end, the resulting capped transcripts did not contain any non-viral sequences at the 5′ end of transcripts. RNA produced in this manner is infectious when transfected into almost any mammalian cell type, however, only feline cells express the feline Jam-1 receptor molecule required for efficient infection, allowing multiple rounds of virus replication and amplification (Makino *et al*., 2006). Crandell-Rees feline cells are typically the cell line of choice for virus recovery (Sosnovtsev and Green, 1995).

This system was further embellished by the use of a helper virus expressing T7 RNA polymerase which facilitates the recovery of virus from DNA bypassing the additional step of *in vitro* transcription (Figure 4.2) (Mitra *et al*., 2004). The

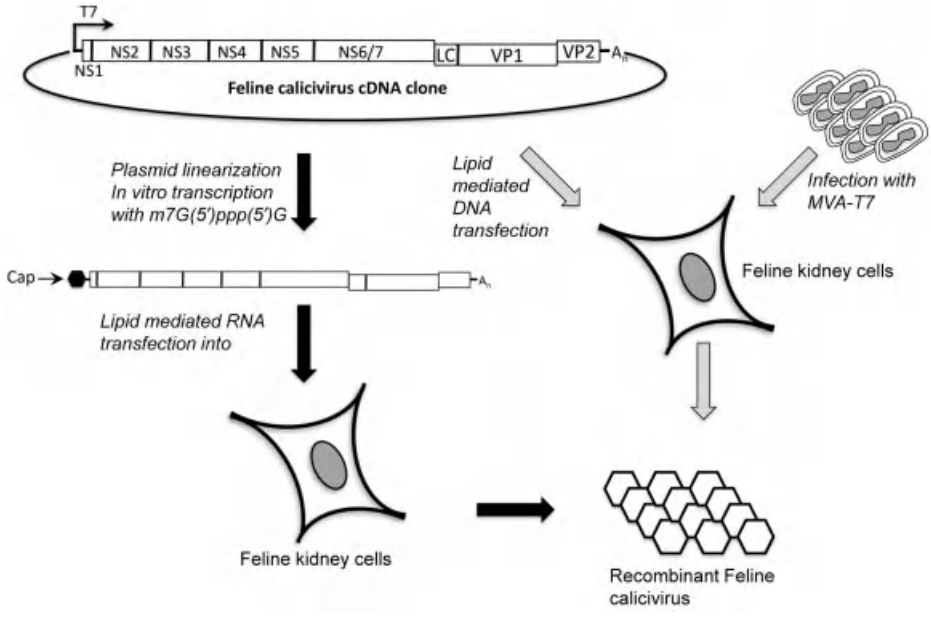

Figure 4.2 Feline calicivirus reverse genetics systems. Diagrammatic representations of the two available reverse genetics systems for the recovery of recombinant feline calicivirus (FCV) in cell culture. Black filled arrows illustrate the RNA mediated recovery system whereas the grey filled arrows illustrate the more recently described Vaccinia virus helper virus-based system. The RNA recovery system relies on the transfection of *in vitro* transcribed RNA derived from a full-length cDNA clone of the FCV genome. The more rapid DNA-based recovery system relies on use of a helper virus expressing T7 RNA polymerase (MVA-T7) to drive RNA synthesis in cells transfected with the cDNA clone of FCV. Both systems result in the production of recombinant FCV.

modified system relies on the transfection of the full-length cDNA clone of FCV into cells previously infected with a modified Vaccinia virus expressing T7 RNA polymerase (MVA-T7) as the helper virus. The T7 RNA polymerase produced by the helper virus results in high levels of transcription of the FCV RNA within the cell, and due to the capping activity of the helper virus-encoded enzymes (Fuerst and Moss, 1989), this is subsequently translated into the viral proteins and replication takes place. This minor modification effectively bypasses the need to produce *in vitro* transcribed capped RNA and can provide a more rapid approach to screening of viral mutants.

4.2.2 Applications of feline calicivirus reverse genetics

The FCV reverse genetics system has been used to address a number of fundamental questions relating to FCV biology, primarily viral polyprotein processing, capsid antigenicity and the function of viral RNA structures. The first such application was

the confirmation that the FCV capsid protein, which is synthesised as a precursor linked to the so-called Leader of the Capsid protein (LC) (Figure 4.1), is cleaved by the virus-encoded protease-polymerase fusion protein NS6-7, also known as p76 (Sosnovtsev *et al*., 1998). The FCV reverse genetics system has also been employed for the generation of chimeric viruses bearing the antigenic regions from various isolates (Neill *et al*., 2000), confirming that the hypervariable region E of the capsid protein plays an important role in the formation of the antigenic structure of the virion. This study provides a potential application for calicivirus reverse genetics in the generation of vaccine strains derived from cloned cDNA. In another study, the polyprotein cleavage sites, which lie between each of the non-structural proteins, were also shown to be essential for virus viability, whereas the potential protease cleavage site between the protease and polymerase domains of the NS6-7 protein (p76) was not required for virus viability (Sosnovtsev *et al*., 2002). This latter observation is in stark contrast to that described below where cleavage between the MNV NS6-7 proteins was essential for virus viability (Ward *et al*., 2007). These data highlight that for some members of the *Caliciviridae* the active form of the viral polymerase required during virus replication is in fact the protease–polymerase precursor and in the case of FCV, this is the only form of the viral polymerase detected during virus replication.

The generation of FCV variants bearing the fluorescent marker proteins GFP or dsRED2 has also been facilitated by the FCV reverse genetics system (Abente *et al*., 2010). Transposon-based mutagenesis followed by subsequent removal of the inserted transposon was initially used to identify positions within the FCV genome that could tolerate small (5 amino acid) insertions. Screening of a number of recovered viruses resulted in the identification of insertions in the LC (ntd 5577) and VP2 (ntd 7322) coding regions of the viral genome. These positions were then used to introduce a variety of epitope tags (HA, FLAG or tetra-cysteine) into the FCV genome and their effect on virus recovery was examined using the MVA-T7-based reverse genetics system. Only the HA tag was tolerated within VP2, however, the insertion of any of the three epitope tags was tolerated within the LC. The subsequent insertion of either GFP or dsRED2 into LC resulted in the generation of the first fluorescently labelled calicivirus (Abente *et al*., 2010), again representing a major breakthrough in the field. These viruses not only provide useful tools for the visualisation of the FCV replication complex in real time (an example is discussed below), but also provide proof of the principle that FCV may be engineered to express foreign proteins, facilitating the use of FCV for gene delivery.

The FCV reverse genetics system has also been used to examine the role of host cell factors in the viral life-cycle (Karakasiliotis *et al*., 2006; Karakasiliotis *et al*., 2010). Polypyrimidine tract binding protein (PTB) is a host cell nucleic acid binding protein initially identified as being involved in RNA splicing but is now known to play a role in polyadenylation (Castelo-Branco *et al*., 2004), RNA stability and translation initiation (Sawicka *et al*., 2008). PTB is a well-characterised trans-acting factor involved in RNA virus translation and replication and is primarily thought to act as an RNA chaperone to promote or alter the conformation of viral RNA

structures (Zuniga *et al*., 2009). PTB was initially described as binding to the 5′ end of the FCV genome functioning in a temperature-dependent manner in Crandell-Rees feline kidney cells (Karakasiliotis *et al*., 2006). Subsequent analysis identified two PTB binding sites in the first 250 nucleotide of the FCV genome, both of which lay within the coding region of the viral polyprotein (ORF1) (Karakasiliotis *et al*., 2010). Mutational analyses indicated that only by mutating both binding sites was PTB binding reduced *in vitro*, although it was not ablated entirely due to the need to maintain the coding capacity of that region of the viral RNA. Recombinant viruses that had a reduced capacity to bind PTB at the 5′ end showed altered growth kinetics in cells and this growth defect was particularly evident in AK-D feline lung cells, which displayed a very heterogeneous expression profile of PTB. To determine the potential role of PTB in the virus life-cycle, confocal microscopy was initially used to demonstrate that as the viral infection progresses, the levels of cytoplasmic PTB increase, and that cytoplasmic PTB can be found associated with viral replication complexes. In this particular case, the recombinant FCV expressing dsRED2 (Abente *et al*., 2010) provided an invaluable tool with which to visualise this process in real time. PTB is a nuclear-cytoplasmic shuttling protein, and previous reports indicated that phosphorylation of PTB might play a role in regulating localisation (Xie *et al*., 2003). Studies using dsRED2 fused to a nuclear localisation sequence demonstrated that the redistribution of PTB occurred prior to any global effect of infection on nuclear-cytoplasmic shuttling and is likely therefore to represent a specific effect on PTB. The role of cytoplasmic PTB in the FCV life-cycle appears to be to regulate the translation of viral RNA as recombinant forms of PTB inhibited FCV translation *in vitro* (Karakasiliotis *et al*., 2010). In addition, replication incompetent viral sgRNA, generated by inactivating the viral genomic RNA using oligonucleotide directed RNAse-H mediated digestion, was translated to a higher degree when PTB levels were reduced by siRNAs. These data suggest that PTB may contribute to the mechanism FCV uses to control viral genome translation and replication as, due to the polarity of the two processes, they are incompatible, that is, translation occurs by the translocation of the ribosome in a 5′–3′ direction, whereas replication occurs via movement of the polymerase in a 3′–5 direction on the template RNA. Together these data allowed the generation of a model of PTB function in the calicivirus life-cycle: (1) after virus binding and uncoating, the incoming viral RNA is delivered to the cytoplasm where the initial stage of viral translation, and possibly several primary rounds of viral RNA replication occur; (2) as the production of viral proteins increases, nuclear-cytoplasmic shuttling of PTB is altered by an as yet uncharacterised mechanism; (3) cytoplasmic levels of PTB increase and PTB binds to the 5′ end of the FCV genome, clearing viral RNA of ribosomes, stimulating viral RNA replication; and (4) newly synthesised viral RNA is then packaged into new infectious particles. It is worth noting that this model does not propose that all viral RNA within an individual cell is either translating or replicating at any particular stage in the viral life-cycle; instead it is expected that within any individual replication complex in an infected cell, the viral template RNA is either undergoing translation or replication/packaging. The switch between

translation and replication is a problem faced by all positive-stranded RNA viruses and has been the focus of a number of detailed studies. In poliovirus it is proposed that poly-C-binding protein 2 (PCBP2) interacts with the 5′ cloverleaf structure in combination with the viral protease-polymerase precursor (3CD) (Gamarnik and Andino, 1998). This interaction effectively sequesters PCBP2 from a binding site found within the viral internal ribosome entry site, effectively reducing viral translation and clearing the viral RNA of ribosomes. The effect is to stimulate RNA synthesis, promoting the production of new infectious particles.

4.3 Murine norovirus

Murine norovirus (MNV) was first reported in 2003 as a virus that caused a lethal infection in immunocompromised mice (Karst *et al*., 2003). Since then, MNV has become the model of choice for many researchers in the calicivirus field, as it remains the only norovirus for which a full infectious tissue culture system exists (Wobus *et al*., 2004). This, combined with the ready availability of genetically modified mouse strains and the ability to genetically manipulate the virus (described below), has provided an unprecedented ability to identify viral and host factors that contribute to norovirus pathogenesis and the immune response to infection. MNV displays a tropism for both macrophage and dendritic cells *in vitro* and *in vivo*, replicating to high titres in the RAW 264.7 and the BV-2 microglial cell lines in cell culture.

4.3.1 Development of MNV reverse genetics

Since the discovery of MNV in 2003, a number of reverse genetics systems and refinements have been developed. A system based on the use of a helper virus expressing T7 RNA polymerase was developed in 2007 (Chaudhry *et al*., 2007). Initial attempts to recover MNV from *in vitro* transcribed RNA prepared by the co-transcriptional incorporation of the cap analogue m7G(5′)ppp(5′)G, as proved successful for FCV (described above), failed to produce an infectious virus (Chaudhry *et al*., 2007). Initial conclusions were that the failure to recover a virus was due to the inefficient translation of the capped MNV RNA in cells. However, as will be described in more detail below, the presence of uncapped RNA within RNA preparations due to inefficient capping may have had an inhibitory effect on virus recovery via the induction of the innate immune response.

The use of Vaccinia virus expressing T7 RNA polymerase (MVA-T7) to drive RNA synthesis in cells previously transfected with a full-length MNV cDNA clone, an approach that had proved effective for other members of the *Caliciviridae* family, was also ineffective in recovering infectious MNV. Detailed analyses indicated that this was largely due to the observed inhibitory effect of Vaccinia virus replication on norovirus translation/replication (Chaudhry *et al*., 2007). Vaccinia virus replication

takes place in the cytoplasm of the infected cell and can have dramatic effects on the cellular architecture. Indeed, recent studies would indicate that many of the host cell translation initiation factors that are possibly involved in norovirus translation are actively recruited to the cytoplasmic replication factories produced during Vaccinia virus replication (Katsafanas and Moss, 2007) and this may have contributed to the observed inhibition of norovirus replication. In contrast, however, recovery of infectious MNV was achieved by transfection of a full-length cDNA clone into cells previously infected with a modified fowlpox virus expressing T7 RNA polymerase (FPV-T7, Figure 4.3a) (Chaudhry *et al*., 2007). Fowlpox is a member of the *Avipox* genus of the *Poxviridae* family and unlike Vaccinia virus, replicates only in avian cells. In mammalian cells, replication is abortive although cytopathic effect is sometimes observed at late stages post infection. The strain of fowlpox virus used to generate FPV-T7 (Britton *et al*., 1996), namely the attenuated FP9 strain, was derived from a plaque-purified isolate (HP438) of a virulent fowlpox isolate (HP1) via passage in chick embryo fibroblast cells (Mockett *et al*., 1992). FPV-T7 has since proved useful for the recovery of a number of viruses including infectious bursal disease virus (Boot *et al*., 1999), Newcastle disease virus (Peeters *et al*., 1999),

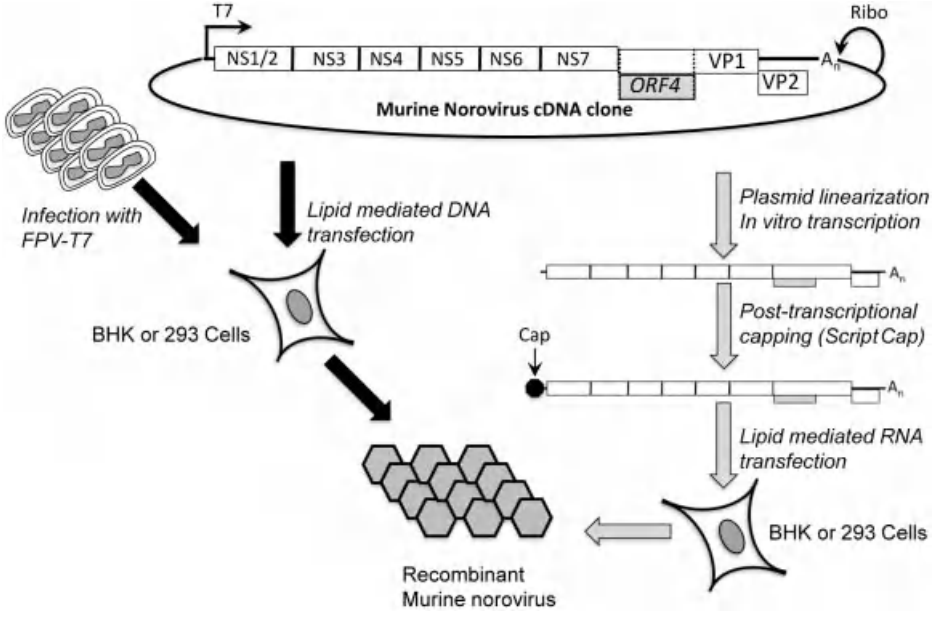

Figure 4.3a Murine Norovirus reverse genetics systems. Schematic illustration of the fowlpox helper virus-based (black filled arrows) and RNA-based (grey filled arrows) murine norovirus (MNV) recovery systems. The positions of the bacteriophage T7 RNA polymerase promoter (T7) and hepatitis δ virus ribozyme (Ribo) are highlighted. The RNA-based recovery system relies on the use of the ScriptCap post-transcriptional RNA capping system available from Epicentre to covert the 5′ end of *in vitro* transcribed RNA into a cap structure.

Rinderpest virus (Das *et al*., 2000) and the avian coronavirus infectious bronchitis virus (Casais *et al*., 2001). Studies with MNV indicated that typically >50,000 infectious units could be recovered per 35mm dish of cells and that a wide range of mammalian cell types were suitable (Chaudhry *et al*., 2007). Attempts to recover MNV using the FPV-T7 system from cells fully permissive for MNV failed, presumably due to poor transfection rates and inefficient FPV-T7 infection in the RAW264.7 cell line. Therefore, the system as initially described represents a single cycle of replication only, which allows the virus yield in this system to be used as a measure of viral fitness, which is particularly useful when examining the effect of mutations on viral growth. These initial studies also highlighted the importance of using the correct viral sequence for efficient recovery as a cDNA clone with a single incorrect nucleotide immediately upstream of the poly A tail rendered the clone non-infectious (Chaudhry *et al*., 2007).

A pol II-based MNV recovery system was also described in 2007 (Ward *et al*., 2007), although the reported yields of virus (∼200–5000 pfu/ml) were typically >10 fold lower than the FPV-T7 system described above (Chaudhry *et al*., 2007). The pol II system was first generated as a two-component baculovirus-based system that relied on the generation of recombinant baculoviruses containing the MNV cDNA under the control of a tetracycline inducible promoter and a baculovirus expressing a tetracycline activator. Transduction of cells with both baculoviruses resulted in the activation of RNA synthesis from the baculovirus genome containing the MNV cDNA clone (Figure 4.3b). Direct transfection of both the transfer vectors, containing the MNV cDNA and the tetracycline responsive transcriptional activator used to generate the recombinant baculoviruses, into a range of mammalian cells (HEK293T, HepG2, BHK21 and Cos7) resulted in the production of infectious virus (Figure 4.3b). Transfection of the MNV cDNA containing transfer vector alone into the HEK293T cell line also allowed recovery of infectious virus allowing the development of a single plasmid-based recovery system.

Recently a system that relies on the direct transfection of *in vitro* transcribed RNA into permissive cells has been developed and represents the most efficient calicivirus reverse genetics system described to date (Yunus *et al*., 2010). Previous attempts to recover infectious MNV from *in vitro* synthesised capped RNA had failed, largely due to the inability to efficiently transfect the MNV permissive cell line RAW264.7 with RNA, as well as the poor translation observed from MNV genomic RNA produced via co-transcriptional capping (Chaudhry *et al*., 2007). RNA is capped in this manner by the incorporation of the cap analogue m7 G(5′)pppG into RNA during the transcription reaction. This is achieved by modifying the components of the transcription reaction so that the cap analogue is typically present at a 10:1 or 4:1 ratio with GTP. RNA produced in this manner usually contains a variety of species resulting in 60–80% of RNA being capped, but up to 50% of this may have incorporated the cap analogues in the reverse orientation (Meis and Meis, 2007). The remaining uncapped RNA possesses a 5′ triphosphate group and as a result may be a potent stimulator of the innate immune response (Hornung *et al*.,

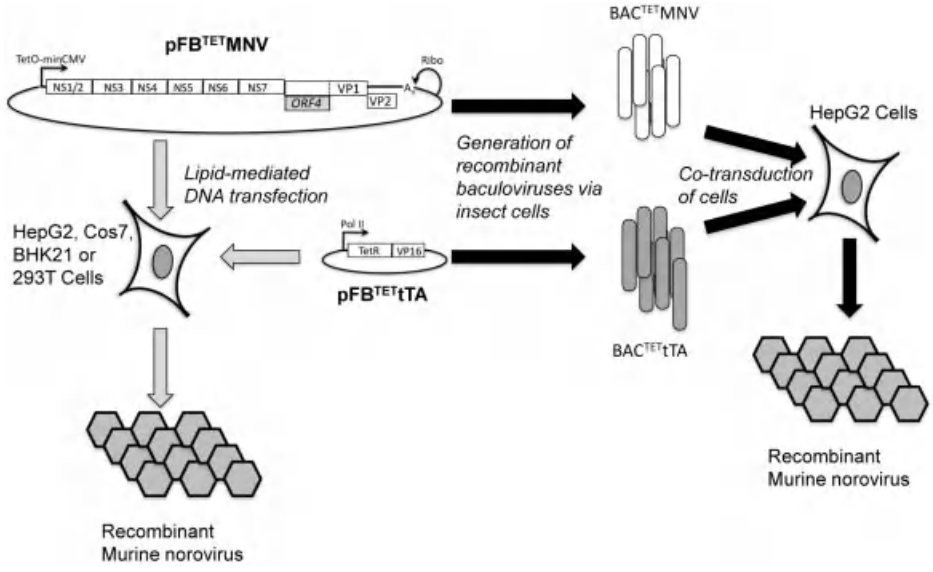

Figure 4.3b Illustration of the Pol-II based MNV recovery systems. Black filled arrows illustrate the use of a baculovirus transduction system used to derive recombinant MNV. This system relies on the prior generation of two recombinant baculoviruses; BACTETMNV to express the MNV genome with a ribozyme at the 3′ end (Ribo) under the control of a tetracycline regulatable a minimal CMV promoter (TetO-minCMV) and BACTETtTA to express a tetracycline repressor-VP16 transactivator fusion protein (TetR, VP16). Co-transduction of the hepatocellular carcinoma cell line HepG2 with both recombinant baculoviruses, results in RNA expression and virus replication. Grey filled arrows are used to illustrate the plasmid based recovery systems whereby a variety of mammalian cells can be transfected with the baculovirus transfer vectors pFBTETMNV and pFBTETtTA to drive MNV RNA expression and virus recovery.

2006; Pichlmair *et al*., 2006; Nallagatla *et al*., 2007). The consequences of this induction would be to stimulate many aspects of the antiviral response in cells that would inevitably reduce virus translation, replication and the subsequent production of infectious particles. Given the already described sensitivity of MNV to the interferon response (Karst *et al*., 2003; Mumphrey *et al*., 2007; Changotra *et al*., 2009), it is therefore possible that MNV recovery would be particularly sensitive to any induction of the innate immune response. Recently, however, systems have been established to allow the post-transcriptional capping of *in vitro* transcribed RNA using purified capping enzyme from Vaccinia virus (Meis and Meis, 2007). This approach is based on the tri-functional guanyltransferase enzyme from Vaccinia virus that has the ability to convert the 5′ triphosphorylated end of RNA into a cap 0 structure. RNA produced in this manner is typically capped to efficiencies approaching 100%, allowing efficient translation and minimising the chances of inducing the innate immune response. MNV genomic RNA produced in this manner was shown to yield high titres of infectious MNV upon transfection into a variety

of mammalian cells (Figure 4.3a) (Yunus *et al*., 2010). Typical yields from a single cycle of replication were $> 5 \times 10^5$ infectious units per 35mm dish.

4.3.2 Applications of MNV reverse genetics

The application of the pol-II-based MNV recovery system has to date been somewhat limited in comparison to the FPV-T7-based approach. The initial report describing the generation of the pol-II recovery system demonstrated that proteolytic cleavage between the protease and polymerase proteins of ORF1 (NS6/7) was essential for MNV replication (Ward *et al*., 2007). This is in stark contrast to the results described for FCV whereby the cleavage between NS6/7 was not essential for virus replication (Sosnovtsev *et al*., 2002). The only other reported use of the pol-II-based MNV reverse genetics system to date was the generation of the first human norovirus-murine norovirus chimeric virus, containing seven amino acids from human norovirus (Taube *et al*., 2010). The authors replaced a Fab2 binding site recognised by the neutralising monoclonal antibody A6.2 at position 377 within the major capsid protein VP1 (SVTAAAS), with the corresponding structurally equivalent sequence from the Norwalk virus (NGIGSGN). The resulting recombinant virus was viable, did not introduce any compensatory mutations with VP1 and was no longer neutralised by the A6.2 monoclonal antibody (Taube *et al*., 2010). This allowed the confirmation of neutralising monoclonal antibody site visualised by structural analysis of a protruding or 'P' domain of the MNV capsid.

The FPV-T7-based recovery system has been used to undertake a number of significant studies on MNV biology. Initial studies focused on trying to understand the genetic basis of the tissue culture adaptation and attenuation observed upon repeated passage of MNV1 in the RAW 264.7 cell line (Wobus *et al*., 2004). Wild-type MNV1 isolated from the brain of infected STAT1-/- mice displayed 100% lethality when reintroduced into STAT1-/- mice, even when using a low dose of around 300 plaque forming units (pfu) per animal. In contrast after three repeated passes through RAW 264.7 cells, even at a 10,000-fold increased dose, a >90% reduction in lethality was observed, indicating attenuation had occurred. Sequence analysis of the viral population during passage in a cell culture identified two coding changes: G2151A resulting in a valine to isoleucine change in NS4, and A5941G resulting in a lysine to glutamic acid change in the major capsid protein VP1. By building each mutation into a cDNA clone-based on the attenuated (passage 3) virus, it was then possible to determine that only the VP1 mutation contributed to virulence in STAT1-/- mice (Bailey *et al*., 2008). Analysis of viral replication kinetics in the RAW 264.7 cell line clearly indicated that the introduction of the 'virulent' mutation at position 5941 within VP1 reduced virus replication. While the NS4 tissue culture adaptive mutation (G2151A) did not contribute to viral virulence in mice, it did appear to stimulate replication in cell culture. The function of the NS4 protein has yet to be fully elucidated, however, it is possible that such adaptive mutations may simply alter the ability of NS4 to interact with

an as yet unknown important cellular factor required for norovirus replication in cell culture. The identification of such factors and similar adaptive mutations may contribute to the establishment of an efficient cell culture system for the study of human noroviruses.

The identification of RNA structures required for norovirus replication and pathogenesis has been facilitated using MNV reverse genetics (Simmonds *et al*., 2008; Bailey *et al*., 2010). Initially bioinformatic analysis was applied to the entire *Caliciviridae* family to identify regions within the viral genome that possess the ability to fold into defined RNA structures that are evolutionarily conserved between numerous isolates. The MNV reverse genetics system was then used to introduce mutations into a number of these RNA structures that disrupted the ability of the RNA to fold into a defined structure, but which left the protein coded by that region of the genome unaltered. By examining the ability of mutant cDNA clones to generate infectious virus, RNA structures at the 5′ and 3′ ends of the MNV genome were demonstrated to contribute to virus replication in cell culture. In some cases, e.g. the 5′ end, mutations were debilitating rather than lethal and viruses with reduced growth characteristics were generated. However, in some cases, for example, the 3′ untranslated region (UTR), significant disruptions of the RNA structure were in fact lethal (Simmonds *et al*., 2008). A small RNA structure was also identified immediately upstream of the predicted subgenomic RNA start site on the positive sense of the genomic RNA. This structure appeared to be more stable on the antisense genomic RNA, placing it immediately downstream of the subgenomic RNA start site, and it was hypothesised that it may at least in part contribute to the synthesis of the subgenomic RNA.

The modification of RNA structures that play a role in norovirus virulence has also been a benefit of the MNV reverse genetics system (Bailey *et al*., 2010). Sequence analysis of the 3′ UTR from a panel of MNV isolates highlighted that a polypyrimidine tract (U/C) was present in the single-stranded region of the terminal loop. The sequence varied in length and composition but was always present in some form. The FPV-T7 reverse genetics system was used to engineer viruses whereby the U/C rich sequence was replaced with a heterologous sequence. Viruses recovered in this manner appeared to display identical growth kinetics to that of the wild-type virus, yet competition analysis where wild-type and mutant viruses were mixed together indicated that the mutant virus displayed a fitness cost. The U/C rich sequence was found to be a binding site for polypyrimidine tract binding protein and poly rC binding protein *in vitro*. A mutant virus lacking the U/C rich tract was partially attenuated in the STAT1-/- mouse model, confirming that this sequence contributes in some manner to viral virulence (Bailey *et al*., 2010). As highlighted above (Section 4.2) we previously identified PTB as playing a role in FCV translational control by binding to the 5′ end of the viral genome. It is therefore possible that PTB plays a similar role in the norovirus life-cycle and that the deregulation of this control by reducing PTB binding to the 3′ end, as mutants lacking the U/C rich sequence retained an ability to bind PTB to reduced levels, resulted in attenuated *in vivo*. This ability to modify viral virulence via the alteration

of RNA-protein interactions, while well established for other RNA viruses (for example, Haller *et al*., 1996; Men *et al*., 1996; Rodriguez Pulido *et al*., 2009), was until recently now proven for any member of the *Caliciviridae*. The use of reverse genetics has now provided an additional approach to the generation of rationally attenuated calicivirus vaccines.

4.4 Porcine enteric calicivirus

Porcine enteric calicivirus (PEC), a member of the *Sapovirus* genus, was the first enteric calicivirus to be successfully cultured in cell culture (Flynn and Saif, 1988). PEC was first isolated from piglets with signs of diarrhoeal disease and was subsequently shown to infect pigs of all ages but causes diarrhoea only in piglets. In contrast, porcine noroviruses are detected in adult pigs without any clinical signs (Wang *et al*., 2007).

Replication of PEC in cell culture requires the addition of intestinal contents from gnotobiotic piglets to the cell culture media (Flynn and Saif, 1988) which was found to be dependent on cyclic AMP-mediated signalling events occurring as a result of intestinal contents (Chang *et al*., 2002). This was further determined to be a direct result of bile acids on the down regulation of the interferon response in the cultured porcine kidney cells (Chang *et al*., 2004). Again, this data, in addition to data on MNV (Karst *et al*., 2003; Mumphrey *et al*., 2007; Changotra *et al*., 2009) and that described below for human norovirus (Chang and George, 2007), highlights that the innate immune response appears to play a critical role in regulating calicivirus replication in cell culture. Attempts to grow human norovirus in cell culture in the presence of intestinal contents have, however, failed (Duizer *et al*., 2004).

A reverse genetics system for PEC was initially described in 2005 (Chang *et al*., 2005) and used a similar approach to that previously described for FCV (Sosnovtsev and Green, 1995) (Figure 4.2) whereby *in vitro* transcribed and co-transcriptionally capped RNA is transfected into permissive cells. In the case of PEC, the LLC-PK immortalised porcine kidney cell line was the cell line of choice and virus recovery was only observed when transfections were performed in the presence of bile acids. The genetically defined recombinant PEC generated by reverse genetics was found to retain an ability to infect piglets using the oral route, however, it displayed an attenuated phenotype similar to that of other tissue culture adapted isolates of PEC (Guo *et al*., 2001; Chang *et al*., 2005). Sequence analysis of the tissue culture adapted and virulent isolates suggested that attenuating mutations may map to the major capsid protein VP1 as also demonstrated for MNV (Bailey *et al*., 2008). The PEC reverse genetics system has yet to be used extensively, however, PEC holds great promise as a model for human norovirus infection given the diarrhoeal disease and the ready availability of a reverse genetics system. The specialised containment facilities required to generate and maintain gnotobiotic piglets may have contributed to limited use of PEC as a model, but future studies with this experimental system would be of great benefit to the field.

4.5 Rabbit haemorrhagic disease virus

Rabbit haemorrhagic disease virus (RHDV) causes a lethal and highly contagious disease. It was initially reported in China but has subsequently spread to many parts of the world including several European countries (Cooke, 2002). The first RHDV reverse genetics system was described in 2006, in which *in vitro* transcribed RNA was shown to be infectious not only when transfected directly into rabbit RK13 cells, but also when directly injected into the liver of rabbits (Liu *et al*., 2006). Surprisingly, uncapped RHDV RNA was found to be infectious, which is in stark contrast to all other calicivirus reverse genetics systems described to date (described in detail above). This data might suggest that the absolute requirement of VPg for viral protein synthesis, as reported for other caliciviruses is not as strict in RHDV. One might therefore predict that the RHDV genome may contain translational enhancer sequences that contribute to this observed infectivity of uncapped RNAs, however, further studies would be required to validate this. This particular system has subsequently been used to examine the role of the poly A tail in viral infectivity. Surprisingly, deletion of the entire poly A tail did not affect RHDV infectivity and in fact virus recovered after transfection of uncapped non-poly adenylated RNA into cells had restored the poly A tail (Liu *et al*., 2008b). Therefore, as has been demonstrated in a number of other viral systems, RHDV has the ability to generate a poly A tail *de novo*, either via the viral RNA polymerase or an as yet uncharacterised cellular mechanism. Further developments of the reverse genetics system led to the generation of a DNA-based recovery method whereby the RHDV genome was placed under the control of a pol-II cellular promoter (Liu *et al*., 2008a). Transfection of a full-length cDNA clone of RHDV under the control of the CMV immediate early (pol-II) promoter resulted in robust levels of viral protein production and the generation of infectious particles. This system was used to illustrate that unlike FCV (Sosnovtsev *et al*., 2005), the RHDV VP2 protein is not required for virus replication in cell culture as viruses lacking VP2 were readily generated (Liu *et al*., 2008a). Studies with FCV had indicated that while cis-acting RNA structures present in the VP2 coding region are essential for RNA replication, VP2 is only required for virion production (Sosnovtsev *et al*., 2005). Again this contrast between FCV and RHDV has highlighted that considerable variation in genome replication strategies exist between these closely related members of the *Caliciviridae* family and therefore comparative studies are well justified.

4.6 Human norovirus

As described above, the development of a full infectious culture system for human norovirus has been the focus of intensive research over many years, yet despite valiant efforts no robust system currently exists (Duizer *et al*., 2004). A highly differentiated cell culture system was reported as supporting human norovirus replication (Straub *et al*., 2007), however, this has not been validated in other

laboratories despite numerous efforts. Despite these setbacks, there has been substantial progress in the norovirus field over the past few years and while a fully infectious reverse genetics system remains to be generated, primarily due to the inability to confirm the production of infectious virus particles, a number of systems do exist. These will be described below along with their applications:

4.6.1 Norovirus replicons and their application

The first system to be developed that allowed some aspects of the norovirus replication cycle to be studied was replicon-bearing cell lines (Chang *et al*., 2006). This system relies on the generation of cell lines that stably maintain the Norwalk virus genome, selected by virtue of the insertion of an antibiotic resistance gene into the VP1 capsid sequence of the Norwalk virus. Transfection of *in vitro* transcribed capped Norwalk replicon RNA into baby hamster kidney cells (BHK), followed by several weeks of antibiotic selection resulted in the generation of antibiotic resistant clones at low frequency. Surprisingly, RNA extracted from these clones was then able to replicate in the human hepatoceullar carcinoma cell line Huh-7, generating the first human cell line stably replicating norovirus RNA (Chang *et al*., 2006). The efficiency with which this system is generated excludes the ability to examine the effect of mutations on replication but it has proved useful in examining the effect of norovirus replication on the cell and the identification of inhibitors. This system was used to demonstrate an antiviral effect of ribavirin and interferon on norovirus replication in cell culture (Chang and George, 2007; Kim *et al*., 2011), identifying the first possible therapeutic treatment for human noroviruses. Microarray analysis of replicon bearing cells identified a number of transcripts that were modified because of human norovirus replication. Subsequent detailed analysis indicated that genes involved in cholesterol biosynthesis were altered and that reduced cholesterol levels favoured norovirus replication in cells (Chang, 2009). This was further traced to an effect of low-density lipoprotein receptor (LDLR) receptor expression in that over-expression of the LDLR stimulated norovirus replication in cell culture. Sequestration of cholesterol via the use of statins also resulted in increased virus replication (Chang, 2009). This last observation is particularly significant given the recent observation that statin use exacerbates norovirus disease and can lead to increased mortality rates in norovirus-infected patients (Rondy *et al*., 2011). This has also highlighted how basic studies performed in cell culture can provide novel insights into risk factors associated with excessive clinical disease.

4.6.2 Human norovirus recovery systems

As discussed above, a full replication cycle of human norovirus in cell culture has yet to be achieved. Immortalised cell lines appear to be at least partially competent for norovirus replication as Norwalk virus RNA isolated from faecal samples can

undergo a single round of replication when transfected into cells (Guix *et al*., 2007). These studies would suggest that the major block to achieving a full infectious norovirus cell culture system is due to the inability of the virus to spread from cell to cell. This could be due to a lack of a suitable receptor, co-receptor or entry factor but may also be due to the effect of antiviral signalling events limiting virus spread. In the latter case, this is unlikely to be due to RIG-I mediated signalling as cells possessing a RIG-I mutation (Huh-7.5 cells) are also not able to support the full human norovirus replicative cycle (Guix *et al*., 2007).

Two human norovirus recovery systems have been developed in the past six years. The first was built on the prototype genogroup I human norovirus, namely Norwalk virus, and relied on the use of a Vaccinia virus-based helper virus expressing T7 RNA polymerase (MVA-T7) to drive expression of the viral RNA (Asanaka *et al*., 2005). In this system, similar to the MVA-T7 recovery system described for FCV (Figure 4.2), cDNA clones of the genomic and subgenomic regions of the viral genome were cloned downstream of a T7 RNA polymerase promoter, with a hepatitis delta virus ribozyme and T7 transcriptional promoter placed at the 3′ end after the poly A tail. Transfection of the subgenomic RNA alone into cells (human embryonic kidney, 293T cells) previously infected with the helper virus MVA-T7 resulted in the production of empty viral particles only, which lacked any associated viral RNA. However, transfection of the viral genomic RNA led to the production of viral replicase components, subgenomic RNA and virions with a sedimentation similar to that reported for Norwalk virus isolated from faecal samples. One fundamental observation made as a result of these studies was that, while transfection of the viral subgenomic RNA resulted in robust capsid production, these capsids did not in fact contain any RNA. This provided at least circumstantial evidence that any packaging signal within the norovirus genome, if one should exist, must lie within the region outside of the viral subgenomic RNA. Although quite inefficient and lacking the ability to spread from cell to cell, this system has again provided another tool for the analysis of aspects of the norovirus life-cycle in cell culture. A more or less identical system was developed for a genogroup II norovirus in 2006 with similar results (Katayama *et al*., 2006). Viral negative strand RNA was also identified in this system as additional proof that the viral RNA containing capsids observed were the likely result of authentic norovirus replication.

4.7 Conclusion

As illustrated above, recent progress in the calicivirus field has resulted in the generation of several reverse genetics systems for members of this poorly characterised family of RNA viruses. While many of the systems have yet to be exploited to their full potential, progress in recent years has been rapid and wide-reaching, providing novel insights into calicivirus–host interactions and calicivirus pathogenesis. While there still remain many unknowns, the future of calicivirus reverse genetics holds great potential. The development of a full infectious human norovirus cell culture

system remains the focus of much research, but until such a time this becomes available, studies with other members of the *Caliciviridae* as described above continue to increase our understanding of this significant group of pathogens.

Acknowledgements

Work in the lab of the author is supported by the Wellcome Trust, BBSRC and European Union. The author is a Wellcome Senior Fellow.

References

Abente, E. J., Sosnovtsev, S. V., Bok, K. and Green, K. Y. 2010. Visualization of feline calicivirus replication in real-time with recombinant viruses engineered to express fluorescent reporter proteins. *Virology* **400**: 18–31.

Asanaka, M., Atmar, R. L., Ruvolo, V., Crawford, S. E., Neill, F. H. and Estes, M. K. 2005. Replication and packaging of Norwalk virus RNA in cultured mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 10327–10332.

Bailey, D., Karakasiliotis, I., Vashist, S., Chung, L. M., Rees, J., McFadden, N., Benson, A., *et al*. 2010. Functional analysis of RNA structures present at the 3′ extremity of the murine norovirus genome: the variable polypyrimidine tract plays a role in viral virulence. *Journal of Virology* **84**: 2859–2870.

Bailey, D., Thackray, L. B. and Goodfellow, I. G. 2008. A single amino acid substitution in the murine norovirus capsid protein is sufficient for attenuation in vivo. *Journal of Virology* **82**: 7725–7728.

Belsham, G. J. and Sonenberg, N. 2000. Picornavirus RNA translation: roles for cellular proteins. *Trends in Microbiology* **8**: 330–335.

Boot, H. J., Ter Huurne, A. A., Peeters, B. P. and Gielkens, A. L. 1999. Efficient rescue of infectious bursal disease virus from cloned cDNA: evidence for involvement of the 3′-terminal sequence in genome replication. *Virology* **265**: 330–341.

Britton, P., Green, P., Kottier, S., Mawditt, K. L., Penzes, Z., Cavanagh, D. and Skinner, M. A. 1996. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. *Journal of General Virology* **77**: 963–967.

Casais, R., Thiel, V., Siddell, S. G., Cavanagh, D. and Britton, P. 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. *Journal of Virology* **75**: 12359–12369.

Castelo-Branco, P., Furger, A., Wollerton, M., Smith, C., Moreira, A. and Proudfoot, N. 2004. Polypyrimidine tract binding protein modulates efficiency of polyadenylation. *Molecular Cell Biology* **24**: 4174–4183.

Chang, K. 2009. Role of cholesterol pathways in norovirus replication. *Journal of Virology* **83**: 8587–8595.

Chang, K. and George, D. W. 2007. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. *Journal of Virology* **81**: 12111–12118.

Chang, K., Kim, Y., Green, K. Y. and Saif, L. J. 2002. Cell-culture propagation of porcine enteric calicivirus mediated by intestinal contents is dependent on the cyclic AMP signaling pathway. *Virology* **304**: 302–310.

Chang, K., Sosnovtsev, S. V., Belliot, G., Kim, Y., Saif, L. J. and Green, K. Y. 2004. Bile acids are essential for porcine enteric calicivirus replication in association with down-regulation of signal transducer and activator of transcription 1. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 8733–8738.

Chang, K., Sosnovtsev, S. V., Belliot, G., King, A. D. and Green, K. Y. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. *Virology* **353**: 463–473.

Chang, K. O., Sosnovtsev, S. S., Belliot, G., Wang, Q., Saif, L. J. and Green, K. Y. 2005. Reverse genetics system for porcine enteric calicivirus, a prototype sapovirus in the Caliciviridae. *Journal of Virology* **79**: 1409–1416.

Changotra, H., Jia, Y., Moore, T. N., Liu, G., Kahan, S. M., Sosnovtsev, S. V. and Karst, S. M. 2009. Type I and type II interferons inhibit the translation of murine norovirus proteins. *Journal of Virology* **83**: 5683–5692.

Chaudhry, Y., Nayak, A., Bordeleau, M. E., Tanaka, J., Pelletier, J., Belsham, G. J., Roberts, L. O. and Goodfellow, I. G. 2006. Caliciviruses differ in their functional requirements for eIF4F components. *Journal of Biological Chemistry* **281**: 25315–25325.

Chaudhry, Y., Skinner, M. A. and Goodfellow, I. G. 2007. Recovery of genetically defined murine norovirus in tissue culture by using a fowlpox virus expressing T7 RNA polymerase. *Journal of General Virology* **88**: 2091–2100.

Clarke, I. N. and Lambden, P. R. 2000. Organization and expression of calicivirus genes. *Journal of Infectious Diseases* **181**(Suppl. 2): S309–S316.

Cooke, B. D. 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. *Rev Sci Tech* **21**: 347–358.

Cox, C., Cao, S. and Lu, Y. 2009. Enhanced detection and study of murine norovirus-1 using a more efficient microglial cell line. *Virology Journal* **6**: 196.

Das, S. C., Baron, M. D., Skinner, M. A. and Barrett, T. 2000. Improved technique for transient expression and negative strand virus rescue using fowlpox T7 recombinant virus in mammalian cells. *Journal of Virological Methods* **89**: 119–127.

Daughenbaugh, K. F., Fraser, C. S., Hershey, J. W. and Hardy, M. E. 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. *EMBO Journal*, **22**: 2852–2859.

Duizer, E., Schwab, K. J., Neill, F. H., Atmar, R. L., Koopmans, M. P. G. and Estes, M. K. 2004. Laboratory efforts to cultivate noroviruses. *Journal of General Virology* **85**: 79–87.

Flynn, W. T. and Saif, L. J. 1988. Serial propagation of porcine enteric calicivirus-like virus in primary porcine kidney cell cultures. *Journal of Clinical Microbiology* **26**: 206–212.

Fuerst, T. R. and Moss, B. 1989. Structure and stability of mRNA synthesized by vaccinia virus- encoded bacteriophage T7 RNA polymerase in mammalian cells. Importance of the 5′ untranslated leader. *Journal of Molecular Biology* **206**: 333–348.

Gamarnik, A. V. and Andino, R. 1998. Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes & Development* **12**: 2293–304.

Goodfellow, I., Chaudhry, Y., Gioldasi, I., Gerondopoulos, A., Natoni, A., Labrie, L., Laliberte, J. F. and Roberts, L. 2005. Calicivirus translation initiation requires an interaction between VPg and eIF 4 E. *EMBO Report* **6**: 968–972.

Guix, S., Asanaka, M., Katayama, K., Crawford, S. E., Neill, F. H., Atmar, R. L. and Estes, M. K. 2007. Norwalk virus RNA is infectious in mammalian cells. *Journal of Virology* **81**: 12238–12248.

Guo, M., Hayes, J., Cho, K. O., Parwani, A. V., Lucas, L. M. and Saif, L. J. 2001. Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhoea by intravenous inoculation of wild-type PEC. *Journal of Virology* **75**: 9239–9251.

Haller, A. A., Stewart, S. R. and Semler, B. L. 1996. Attenuation stem-loop lesions in the 5′-noncoding region of poliovirus RNA: neuronal cell-specific translation defects. *Journal of Virology* **70**: 1467–1474.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., *et al*. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science*, **314**, 994–7.

Karakasiliotis, I., Chaudhry, Y., Roberts, L. O. and Goodfellow, I. G. 2006. Feline calicivirus replication: requirement for polypyrimidine tract-binding protein is temperature-dependent. *Journal of General Virology* **87**: 3339–3347.

Karakasiliotis, I., Vashist, S., Bailey, D., Abente, E. J., Green, K. Y., Roberts, L. O., Sosnovtsev, S. V. and Goodfellow, I. G. 2010. Polypyrimidine tract binding protein functions as a negative regulator of feline calicivirus translation. *PLoS One*, **5**: e9562.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J. and Virgin, H. W. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. *Science*, **299**: 1575–1578.

Katayama, K., Hansman, G. S., Oka, T., Ogawa, S. and Takeda, N. 2006. Investigation of norovirus replication in a human cell line. *Archives of Virology* **151**: 1291.

Katsafanas, G. C. and Moss, B. 2007. Colocalization of transcription and translation within cytoplasmic poxvirus factories coordinates viral expression and subjugates host functions. *Cell Host Microbe* **2**: 221–228.

Kim, Y., Thapa, M., Hua, D. H. and Chang, K. O. 2011. Biodegradable nanogels for oral delivery of interferon for norovirus infection. *Antiviral Research* **89**: 165–173.

Liu, G., Ni, Z., Yun, T., Yu, B., Chen, L., Zhao, W., Hua, J. and Chen, J. 2008a. A DNA-launched reverse genetics system for rabbit hemorrhagic disease virus reveals that the VP2 protein is not essential for virus infectivity. *Journal of General Virology* **89**: 3080–3085.

Liu, G. Q., Ni, Z., Yun, T., Yu, B., Zhu, J. M., Hua, J. G. and Chen, J. P. 2008b. Rabbit hemorrhagic disease virus poly(A) tail is not essential for the infectivity of the virus and can be restored in vivo. *Archives of Virology* **153**: 939–944.

Liu, G., Zhang, Y., Ni, Z., Yun, T., Sheng, Z., Liang, H., Hua, J., Li, S., *et al*. 2006. Recovery of infectious rabbit hemorrhagic disease virus from rabbits after direct inoculation with in vitro-transcribed RNA. *Journal of Virology* **80**: 6597–6602.

Love, D. N. 1973. The effect of DEAE-dextran on the infectivity of a feline calicivirus and its RNA. *Archiven Gesamte Virusforschungen*, **41**: 52–58.

Makino, A., Shimojima, M., Miyazawa, T., Kato, K., Tohya, Y. and Akashi, H. 2006. Junctional adhesion molecule 1 is a functional receptor for feline calicivirus. *Journal of Virology* **80**: 4482–4490.

Meis, J. E. and Meis, M. 2007. The new mScript^{TM} mRNA Production System: efficient mRNA transcription, capping and tailing for the highest yields of active protein. *EPICENTRE Biotechnologies Forum*.

Men, R. H., Bray, M., Clark, D., Chanock, R. M. and Lai, C. J. 1996. Dengue type 4 virus mutants containing deletions in the 3′ noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. *Journal of Virology* **70**: 3930–3937.

Mitra, T., Sosnovtsev, S. V. and Green, K. Y. 2004. Mutagenesis of tyrosine 24 in the VPg protein is lethal for feline calicivirus. *Journal of Virology* **78**: 4931–4935.

Mockett, B., Binns, M. M., Boursnell, M. E. and Skinner, M. A. 1992. Comparison of the locations of homologous fowlpox and vaccinia virus genes reveals major genome reorganization. *Journal of General Virology* **73**(10): 2661–2268.

Mumphrey, S. M., Changotra, H., Moore, T. N., Heimann-Nichols, E. R., Wobus, C. E., Reilly, M. J., Moghadamfalahi, M., *et al*. 2007. Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. *Journal of Virology* **81**: 3251–3263.

Nallagatla, S. R., Hwang, J., Toroney, R., Zheng, X., Cameron, C. E. and Bevilacqua, P. C. 2007. 5′-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science*, **318**: 1455–1458.

Neill, J. D., Sosnovtsev, S. V. and Green, K. Y. 2000. Recovery and altered neutralization specificities of chimeric viruses containing capsid protein domain exchanges from antigenically distinct strains of feline calicivirus. *Journal of Virology* **74**: 1079–1084.

Peeters, B. P., De Leeuw, O. S., Koch, G. and Gielkens, A. L. 1999. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *Journal of Virology* **73**: 5001–5009.

Pichlmair, A., Schulz, O., Tan, C. P., Naslund, T. I., Liljestrom, P., Weber, F. and Reis E Sousa, C. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science*, **314**: 997–1001.

Rodriguez Pulido, M., Sobrino, F., Borrego, B. and Saiz, M. 2009. Attenuated foot-and-mouth disease virus RNA carrying a deletion in the 3′ noncoding region can elicit immunity in swine. *Journal of Virology* **83**: 3475–3485.

Rondy, M., Koopmans, M., Rotsaert, C., Van Loon, T., Beljaars, B., Van Dijk, G., Siebenga, J., *et al*. 2011. Norovirus disease associated with excess mortality and use of statins: a retrospective cohort study of an outbreak following a pilgrimage to Lourdes. *Epidemiology and Infection*, **139**: 453–463.

Sawicka, K., Bushell, M., Spriggs, K. A. and Willis, A. E. 2008. Polypyrimidine-tract-binding protein: a multifunctional RNA-binding protein. *Biochemical Society Transactions* **36**: 641–647.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L. and Griffin, P. M. 2011. Foodborne illness acquired in the United States: major pathogens. *Emerging Infectious Diseases* **17**: 7–15.

Simmonds, P., Karakasiliotis, I., Bailey, D., Chaudhry, Y., Evans, D. J. and Goodfellow, I. G. 2008. Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses. *Nucleic Acids Research* **36**: 2530–2546.

Sosnovtsev, S. V., Belliot, G., Chang, K., Onwudiwe, O. and Green, K. Y. 2005. Feline calicivirus VP2 is essential for the production of infectious virions. *Journal of Virology* **79**: 4012–4024.

Sosnovtsev, S. V., Garfield, M. and Green, K. Y. 2002. Processing map and essential cleavage sites of the nonstructural polyprotein encoded by ORF1 of the feline calicivirus genome. *Journal of Virology* **76**: 7060–7072.

Sosnovtsev, S. V. and Green, K. Y. 1995. RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VPg for infectivity. *Virology*, **210**: 383–390.

Sosnovtsev, S. V., Sosnovtseva, S. A. and Green, K. Y. 1998. Cleavage of the feline calicivirus capsid precursor is mediated by a virus-encoded proteinase. *Journal of Virology* **72**: 3051–3059.

Straub, T. M., Bentrup, K. H. Z., Orosz-Coghlan, P., Dohnalkova, A., Mayer, B. K., Bartholomew, R. A. O., *et al*. 2007. In vitro cell culture infectivity assay for human noroviruses. *Emerging Infectious Diseases*, **13**: 396–403.

Taube, S., Rubin, J. R., Katpally, U., Smith, T. J., Kendall, A., Stuckey, J. A. and Wobus, C. E. 2010. High-resolution X-ray structure and functional analysis of the murine norovirus 1 capsid protein protruding domain. *Journal of Virology* **84**: 5695–5705.

Vashist, S., Bailey, D., Putics, A. and Goodfellow, I. 2009. Model systems for the study of human norovirus biology. *Future Virology* **4**: 353–367.

Wang, Q. H., Costantini, V. and Saif, L. J. 2007. Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. *Vaccine* **25**: 5453–5466.

Ward, V. K., McCormick, C. J., Clarke, I. N., Salim, O., Wobus, C. E., Thackray, L. B., Virgin, H. W. and Lambden, P. R. 2007. Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11050–11055.

Wobus, C. E., Karst, S. M., Thackray, L. B., Chang, K., Sosnovtsev, S. V., Belliot, G., Krug, A., *et al*. 2004. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biology* **2**, e432.

Xie, J., Lee, J. A., Kress, T. L., Mowry, K. L. and Black, D. L. 2003. Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 8776–8781.

Yanguez, E. and Nieto, A. 2011. So similar, yet so different: selective translation of capped and polyadenylated viral mRNAs in the influenza virus infected cell. *Virus Research* **156**: 1–12.

Yunus, M. A., Chung, L. M., Chaudhry, Y., Bailey, D. and Goodfellow, I. 2010. Development of an optimized RNA-based murine norovirus reverse genetics system. *Journal of Virological Methods* **169**: 112–118.

Zuniga, S., Sola, I., Cruz, J. L. and Enjuanes, L. 2009. Role of RNA chaperones in virus replication. *Virus Research* **139**: 253–266.

Part II

Negative sense RNA viruses

5

Reverse genetics of rhabdoviruses

Alexander Ghanem and Karl-Klaus Conzelmann
Max von Pettenkofer Institute and Gene Center, Ludwig-Maximilians-Universität München, Germany

5.1 Introduction: the *Rhabdoviridae* family

5.1.1 A diversity of similar viruses

The order *Mononegavirales*, or non-segmented negative strand RNA viruses (NNSV), which comprises the families of *Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae*, and *Bornaviridae*, was the first virus order to be established. These viruses originated from a common NNSV ancestor, as indicated by the similar organisation of genomes and a common ingenious mode of gene expression. The RNA of *Mononegavirales* is always present in a highly stable, helical ribonucleocapsid (NC) and the information encoded is expressed by sequential and polar transcription of discrete subgenomic mRNAs from the NCs (for review, see Whelan *et al*., 2004). Of the NNSV (and maybe of all RNA viruses), members of the *Rhabdoviridae* family have the widest host range, including insects, plants, fish, aquatic and terrestrial vertebrates, illustrating the versatility of the NNSV blueprint for conquering space in at least two different kingdoms of life (for reviews, see Pringle, 1997; Fu, 2005).

Approximately 170 *Rhabdoviridae* species have been described, most of which are characterised by a typical rod- or bullet-shaped morphology of the enveloped virions, but only a few have been assigned to the six genera established so far. Members of the *Lyssavirus* genus include the neurotropic rabies virus (RABV) and rabies-related viruses from bats and other animals. Human rabies is a zoonosis and causes more than 50,000 deaths per year, though effective vaccines and post-exposure treatments are available. Most of the human cases occur in India and other parts of South-Asia and Africa where rabies is mainly transmitted by dogs. The *Vesiculovirus* genus includes animal viruses like the prototypic vesicular stomatitis virus (VSV), which can induce severe disease in cattle and pigs causing enormous economic loss. A closely related vesiculovirus, Chandipura virus, has recently

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

emerged in India as serious human pathogen associated with severe acute encephalitis (for review, see Basak *et al*., 2007). Members of the *Ephemerovirus* genus such as bovine ephemeral fever virus (BEFV) are important pathogens of cattle. Notably, in contrast to RABV and other lyssaviruses, vesiculoviruses and ephemeroviruses are typically transmitted by insect vectors. The *Novirhabdovirus* genus includes typical rhabdoviruses from fish, such as viral hemorrhagic septicemia virus (VHSV) which infects numerous marine and freshwater fish species (Skall *et al*., 2005). Finally, rhabdoviruses that infect plants are separated into the genera *Cytorhabdovirus* and *Nucleorhabdovirus*, based on their sites of replication and morphogenesis (Jackson *et al*., 2005). They include important plant pathogens such as lettuce necrotic yellows virus (LNYV), or sonchus yellow net virus (SYNV). Plant rhabdoviruses are also transmitted by insect vectors. This has led to the suggestion that the family evolved from an ancestral insect virus and that the natural host range is largely determined by the insect host (Fu, 2005).

5.1.2 Rhabdovirus genomes

Some rhabdoviruses of mammals, like RABV and VSV have the simplest and smallest (only 11 kb) genomes of all *Mononegavirales*, comprising only five genes in the order 3′-N-P-M-G-L-5′. These encode the nucleoprotein (N), which encapsidates the viral RNA to form the NC, and the phosphoprotein (P), which is an essential cofactor for RNA synthesis and a chaperone for specific encapsidation of viral RNA by N. The matrix protein (M) provides a structural lattice between the NC and the virus envelope, and thus has a critical role in virus assembly. The transmembrane spike glycoprotein (G) is responsible for attachment to target cells and membrane fusion, and the 'large' protein (L), is the catalytic subunit of the viral RNA polymerase. These five genes represent the minimal set of genes in all *Mononegavirales*. The most highly conserved N and L proteins of rhabdoviruses still share common sequence blocks with those from other *Mononegavirales* families. Moreover, the order of the genes is highly conserved. In contrast, though also critically involved in RNA synthesis, the rhabdovirus P proteins show a relatively high variability, even within the genera.

The coding capacity of the minimal rhabdovirus genomes can be easily enlarged by expression of multiple products from a single RNA, and/or by the addition of extra genes, as the helical nature of the NCs allows for elongation (Figure 5.1). Lyssaviruses like RABV can express shorter variants of their P proteins by ribosomal leaky-scanning and translation initiation at downstream in-frame AUG codons (Chenik *et al*., 1995). In the P gene of vesiculoviruses a second, overlapping open reading frame is present, which can encode a non-essential C protein with unknown function. Ephemeroviruses may carry up to six extra genes located between the G and L genes and at least one of them is a second glycoprotein, G_{NS} (Walker, 2005). At the same position, the fish novirhabdoviruses have a typical small 'non-virion' (NV) protein-coding gene. In addition to the analogues of the five rhabdovirus core genes N, P, M, G, and L, plant rhabdoviruses may carry multiple extra genes, at

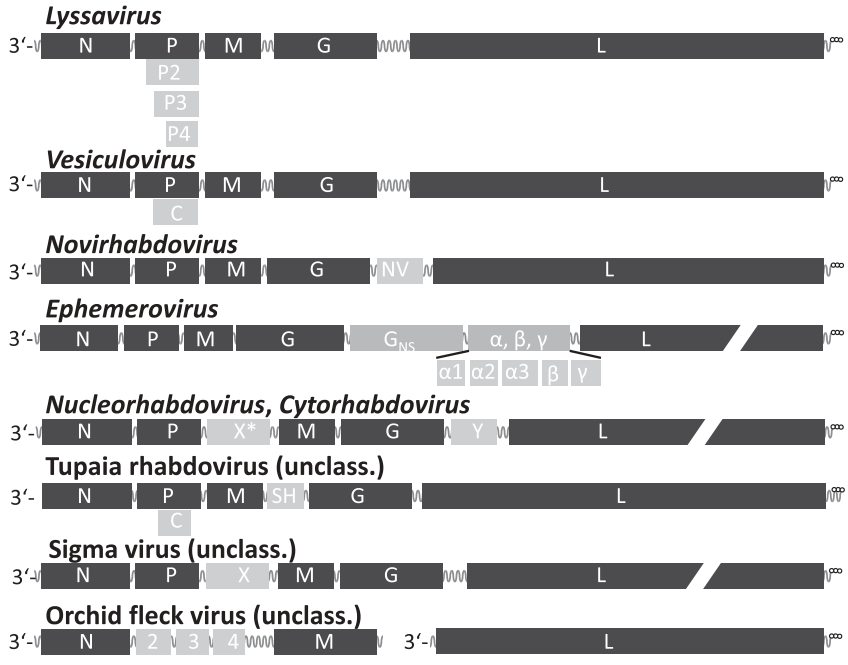

Figure 5.1 Diagrammatic representation of the 3′ to 5′ arrangement of transcription units of selected rhabdoviruses. Abbreviations: N nucleo(capsid)protein, P phosphoprotein, M matrix protein, G glycoprotein, L (large) polymerase, NV non-virion, SH small hydrophobic X undefined, Note that the order 3′-N-P-M-G-L-5′ is conserved throughout the rhabdoviruses, but additional transcription units may be present at different positions. Differently sized P proteins are expressed in the *Lyssavirus* genus by ribosomal leaky scanning from in frame AUG codons. The C protein of vesiculoviruses and Tupaia rhabdovirus is encoded in a second reading frame of the P gene. The plant virus genera *Nucleorhabdovirus* and *Cytorhabdovirus* comprise viruses which can have up to 4 extra genes (X*) downstream of P , and a Y gene downstream of G. A highly atypical example is Orchid fleck virus, which has a bipartite genome.

different positions. Some of the products show similarity with the 30K superfamily of plant virus movement proteins. The N, P, M and L proteins of nucleorhabdoviruses contain nuclear localisation signals consistent with virus replication and morphogenesis of these viruses taking place in the nucleus (Redinbaugh and Hogenhout, 2005). Sigma virus of *Drosophila* has an extra gene inserted downstream of the P gene (Longdon *et al*., 2010). Most remarkably, the plant rhabdovirus Orchid fleck virus has a bipartite genome, but shares significant structural and sequence similarities with members of the genus *Nucleorhabdovirus* (Kondo *et al*., 2006).

5.1.3 Rhabdovirus nucleocapsids and rhabdovirus RNAs

The typical helical NCs of rhabdoviruses are extremely tight and stable (Naeve *et al*., 1980). Within the host cells, they are present in the form of relaxed NCs,

which represent the synthetically active forms. In the rod-shaped virions, a condensed NC superhelix is present, probably held together by M protein (Mebatsion *et al*., 1999; Ge *et al*., 2010). Despite little sequence homology in the N proteins of VSV and RABV, N folding, RNA binding, and assembly are highly conserved in VSV and RABV, as resolved by X-ray analyses (Albertini *et al*., 2006; Green *et al*., 2006; Luo *et al*., 2007). The viral RNA is firmly sequestered in a cavity at the interface between amino- and carboxyterminal lobes of the N protein, which appear to clamp down on to the bound RNA. The complete occlusion of the RNA explains the excellent protection of the viral RNA against high salt concentration, attack by RNases, or silencing by siRNAs (Bitko and Barik, 2001). For RNA synthesis, which requires access of the viral polymerase to the RNA, conformational changes in the N protein by the polymerase and/or P protein must be postulated. Access appears to be achieved easiest (or exclusively) at the ends of the NCs, which are known as promoters.

The NCs are templates for two distinct modes of RNA synthesis by the L-P polymerase complex: transcription of subgenomic mRNAs and replication of full-length NCs (for a comprehensive review, see Whelan *et al*., 2004). There is evidence in favour of the presence of two distinct forms of polymerase complexes, a 'transcriptase', which produces the subgenomic mRNAs, and a 'replicase', which is responsible for amplification of full-length NCs. The two forms are not only distinguished by association with N (see below) but also by differential association with cellular proteins (Qanungo *et al*., 2004). However, there is still little known about the regulation of these two processes.

Transcription is obligatory sequential and starts off at the NC 3′ end with the synthesis of a short, approximately 50 nucleotide-long 5′-triphosphate leader RNA (Abraham and Banerjee, 1976; Colonno and Banerjee, 1976, 1978). The 3′ end of the leader RNA is determined in the genome template by a conserved transcriptional start sequence preceding the first (N) gene. At this junction, the leader RNA is released and transcription of the first mRNA (N) is initiated, which involves the addition of a 5′-methylguanosine cap structure (Both *et al*., 1975) by the L protein (Li *et al*., 2006; Ogino and Banerjee, 2007). Transcription proceeds until the polymerase runs into a stretch of U residues on which it stutters back and forth to produce an approximately 50–150 nucleotide long poly(A) tail, which is followed by discharge of the mRNA. A nearby downstream start signal directs the resident polymerase to re-engage in transcription of the next gene. The transcriptional stop/polyadenylation and restart signals are typically separated by a few nucleotides, known as the 'intergenic region', whose length and sequence composition influences the degree of reinitiation (Finke *et al*., 2000a). The stop signal of the most downstream gene (L) lacks a restart sequence, indicating that the terminal sequence is not transcribed. The exclusive entry of the polymerase at the 3′ end and eventual dissociation at the gene junctions result in the typical transcript gradient of *Mononegavirales* in which upstream (3′-proximal) RNAs are produced much more abundantly than downstream mRNAs. This is a simple and very efficient mechanism to direct adequate expression levels of gene products.

For replication to occur, N-P protein complexes must be present as a substrate for the L-P polymerase since the product of synthesis is not a free RNA but an N-RNA complex (NC). Replication again starts at the 3′ end of the genome N-RNA, but any transcription signal is ignored, resulting in the synthesis of a full-length encapsidated antigenome (positive strand) N-RNA. In the presence of an insufficient N-P concentration, replication is thought to be aborted predominantly at the junction of the leader region and first gene (usually leader region/N gene). Indeed, a considerable portion of the leader RNA is found encapsidated by N protein (Blumberg and Kolakofsky, 1981), which might suggest that initiation of all RNA synthesis is achieved by a single form of the polymerase, namely, the replicase. This hypothesis is supported by the recent observation that the N protein of Sendai virus, a paramyxovirus, is critically required not only for replication but also for transcription (Wiegand *et al*., 2007). Whether the resident replicase can switch to a transcriptase form of the polymerase at the leader-N junction, or whether the polymerase is able to transcribe the leader template or to scan the leader template without transcribing, or if the transcriptase form can enter the template at the leader-N junction, are undecided issues.

The 3′ end of the antigenome NC serves as template for replicative synthesis only. The production of a leader RNA equivalent, the trailer RNA, suggests a similar mechanism for aborting replication in unfavourable conditions. Since the 3′-terminal sequences of the antigenome RNA usually acts as a very strong promoter compared to the genome promoter, a huge excess of genome over antigenome NCs is usually produced. In the case of RABV- and VSV-infected cells, an approximately 50- and 10-fold excess of genome RNA is observed, respectively (Finke and Conzelmann, 1997b; Finke and Conzelmann, 1999;Whelan *et al*., 2004).

The coordinated expression of N, P, and L proteins, which are directly involved as enzymes or substrates for RNA synthesis, their mutual interplay, and the association with cellular proteins are therefore critically involved in rhabdovirus RNA synthesis and its regulation. In addition, other viral proteins may shape RNA synthesis. The RABV M protein, for example, has been shown to stimulate replication and suppress transcription. This is independent of its function in virus assembly, where it mediates condensation and transcriptional ‘freezing’ of NCs for envelopment and budding (Finke and Conzelmann, 2003; Finke *et al*., 2003).

5.1.4 Rhabdovirus envelopes

The M protein of rhabdoviruses is a key player in virus assembly (Figure 5.2). It is responsible for recruiting viral NCs to membranes, their condensation into the superhelical form thus specifying the shape of the virus particles, and inducing membrane curvature for budding of virions from the membrane. M forms a lattice underneath the viral envelope and thereby bridges the NC and the membrane (Ge *et al*., 2010). In RABV assembly, the M protein interacts with the C-terminal cytoplasmic tail (C-tail) of the G protein and thus is involved in recruiting G into RABV

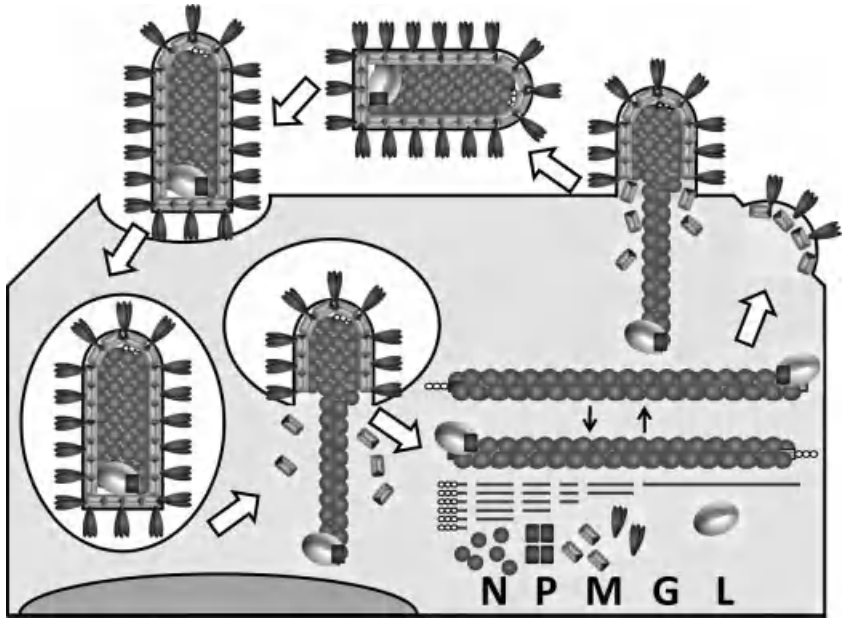

Figure 5.2 Replication cycle of a prototypical cytoplasmic rhabdovirus. Attachment of the virus to the cell surface proteins (upper left) is followed by receptor-mediated endocytosis, pH-dependent membrane fusion in late endosomes, disassembly of matrix protein and release of a relaxed ribonucleopcapsid (NC) into the cytoplasm. Primary transcription yields subgenomic capped and polyadenylated mRNAs, and upon translation and accumulation of viral proteins replication can ensue. NovelNCs are condensed at cellular membranes by matrix protein and progeny virus is released.

particles, which favours budding (Mebatsion *et al*., 1999). In VSV, M–G interactions are less stringent, but both M and G seem to have intrinsic budding activities (Rolls *et al*., 1994; Justice *et al*., 1995), such that budding of virions is most effective in G- and M-rich membrane areas.

The molecular mechanisms involved in M- (and G-) driven budding and release of viruses, however, are not clearly defined, and even the preferred sites of budding of related rhabdoviruses may differ considerably. For example, the two lyssaviruses, European bat lyssavirus type 1 (EBLV-1) and RABV, appear to preferentially bud at the plasma membrane and at intracellular membranes, respectively (Finke *et al*., 2010), this preference being dependent on the respective sequences of the M proteins.

Many other enveloped viruses like retroviruses rely on the cellular endosomal sorting complex for transport (ESCRT) machineries for pinching off particles during virus budding; these, however, appear to play only a minor role in rhabdovirus budding.

The trimeric transmembrane spikes of G on the newly generated virions are required for cell attachment and entry into new target cells by pH-dependent fusion. Notably, VSV G can fuse liposomes in the absence of any protein components,

indicating that specific fusion receptors are not needed for membrane fusion (Libersou *et al*., 2010). The proteins described as receptors for various rhabdoviruses are therefore only attachment proteins, the binding of which increases the probability of endocytosis and ending up in an endo-lysosome where low pH-mediated membrane fusion can occur. Hence, VSV G is being used for pseudotyping of retrovirus gene delivery vectors, in order to broaden their host range. *In vitro*, RABV can infect virtually every cell type, but *in vivo* it is highly neurotropic, probably involving better attachment to cells expressing neuronal attachment proteins (Lafon, 2005).

5.2 Rhabdovirus reverse genetics

In contrast to the vRNA of positive strand viruses, which can be directly translated at the ribosome to produce viral structural proteins and viral polymerase to initiate an infectious cycle, the RNAs of negative strand RNA viruses must first be assembled into an NC-like structure, and the L-P polymerase must be provided to allow transcription of translatable mRNAs. Indeed, this is accomplished within cells expressing the individual components from transfected cDNAs. In the past two decades 'reverse genetics' systems relying on such intracellular assembly of NCs have been developed and improved. Viruses from all *Mononegavirales* families can be generated *de novo* from cDNA, including several members of the mammalian and fish *Rhabdoviridae*. The availability of engineered viruses greatly facilitates the analysis of the molecular biology of these viruses and of virus–host interplay and allows for the design of valuable tracers, vectors, vaccines, and other biomedical tools.

5.2.1 Intracellular assembly of rhabdovirus NCs: DIs and minigenomes

In view of the natural co-transcriptional N-encapsidation of RNA by the viral polymerase on a natural NC template, the mission to package pre-made, free RNA into an NC-like structure appeared to be demanding. Initial encouraging success was obtained with the influenza virus and Sendai paramyxovirus by transfection of an *in vitro* packaged genome segment (Luytjes *et al*., 1989), or an *in vitro* transcribed minigenome RNA into virus-infected cells (Park *et al*., 1991), which could express the encoded reporter genes. Although much later this was achieved for some animal rhabdoviruses (Biacchesi *et al*., 2000b; Le Mercier *et al*., 2002b), a major breakthrough in this virus group was the establishment of helper-virus free systems, in which RNA and virus proteins were simultaneously expressed within cells from transfected circular plasmids. This was utilised first by the Gail Wertz group for VSV defective interfering (DI) particles (Pattnaik *et al*., 1992). VSV DI RNAs mostly contain only terminal sequences derived from the 5′ end of viral genomes (copyback DIs), or from 3′ and 5′ ends (internal deletion DI), but may lack any

protein-encoding genes. An expression system was used which involves infection of cells with a recombinant vaccinia virus (vv), (vTF7-3), encoding bacteriophage T7 RNA polymerase (T7 RNA pol) and transfection of the same cells with the protein expression plasmids driven by a T7 promoter (Fuerst *et al*., 1986). The vv/T7 system has the advantage of tremendously high expression levels combined with mostly cytoplasmic production of RNAs.

Initial experiments revealed that plasmid-expressed VSV N, P, and L proteins are sufficient to replicate a natural (encapsidated) copyback DI NC (Pattnaik and Wertz, 1990), and further expression of M and G proteins resulted in assembly and budding of complete VSV DI particles (Pattnaik and Wertz, 1991). This system also allowed the intracellular T7 RNA polymerase-driven transcription of high levels of RNAs from transfected circular plasmids containing a T7 terminator sequence. The generation of correct 3′ termini of transcripts was achieved by ribozyme sequences, as first successfully shown for intracellular generation of functional nodavirus RNA (Ball, 1992). Specifically, the antigenome ribozyme of hepatitis delta virus (HDVagRz) (Sharmeen *et al*., 1988; Perrotta and Been, 1990; Shih and Been, 2002), which requires only sequences downstream of the cleavage site for autocatalytic activity, was successfully employed. This set-up resulted in efficient encapsidation and replication of an RNA analogue of the VSV DI-T by the simultaneously expressed 'support' plasmids N, P, and L (Pattnaik *et al*., 1992).

As for other *Mononegavirales*, the crucial role of a precise 3′ end for rhabdovirus RNA rescue was confirmed by these authors. Constructs with short heterologous 3′ extensions or deletions were encapsidated by N protein, but not replicated. In contrast, extra non-viral residues at the 5′ end did not prevent 'rescue', and were removed rapidly during NC replication. Complete artificial VSV DI particles were assembled in cells co-expressing all five VSV proteins, thus allowing for analysis of both *cis*-acting sequences and *trans*-acting factors (Pattnaik *et al*., 1992). The vv/T7 protein and RNA expression system was highly effective and paved the way for similar progress with DI-like RNAs or artificial minigenomes not only of rhabdoviruses, but of all families of the *Mononegavirales* (for a comprehensive review, see Conzelmann, 2004; Theriault *et al*., 2005; Bukreyev *et al*., 2006; Biacchesi, 2011).

Typically, the minigenomes used in reverse genetics elements contain only the terminal non-coding 3′ and 5′ sequences, comprising the genomic promoter active in transcription and replication, and specifying the antigenomic promoter for replication, respectively. The coding sequences are often replaced by reporter genes such as CAT, GFP, or luciferase, to easily monitor successful rescue into NCs and gene expression. However, it has to be noted that *Mononegavirales* 'holo' promoters are not necessarily always confined to the non-translated terminal sequences. Although in all cases analysed so far, these sequences are sufficient for RNA synthesis, including replication and transcription, additional sequences enhancing the activity of the terminal 'core' promoters have been identified in the coding regions of Paramyxovirus N or L proteins (Whelan *et al*., 2004; Barr and Fearns, 2010).

By using bicistronic minigenomes in which reporter genes are separated by transcriptional stop/polyadenylation-restart signals, *cis*- and *trans*-acting factors of transcription can be studied. Though it is possible that minigenome RNAs may not reflect any aspect of viral RNA synthesis regulation and virus assembly faithfully, they are valuable tools for initial high throughput analysis of *cis*-acting sequences, virus protein mutants, and cellular proteins co-acting with viral functions, or screening of small molecule inhibitors (Whelan *et al*., 2004). Moreover, they are particularly valuable in working with highly pathogenic viruses as the experiments can be done at standard biosafety levels (Theriault *et al*., 2005).

5.2.2 Virus rescue systems

The vaccinia virus/T7 system

In spite of the excellent performance of the above reverse model genome systems, successful recovery of infectious virus lagged behind. The traditional approach, which involved co-expression of genomic virus RNA (i.e. minus strand RNA) and positive strand mRNAs for N, P, and L support proteins, consistently failed. The use of viral antigenomic RNA of RABV, however, resulted in reliable recovery of the first recombinant infectious negative strand RNA virus entirely from cDNA (Schnell *et al*., 1994). Once within a NC, the positive strand can be replicated by the plasmid-encoded support proteins to form full-length minus strand NCs as nascent RNA chains and thus are immune to hybrid formation with positive strand mRNAs. From functional genomic NCs, an infectious cycle is then initiated (Figure 5.3). This 'positive' approach turned out to be successful for many rhabdoviruses, including VSV, and other *Mononegavirales* families, indicating that hybridisation of genomic RNA and mRNAs had been the major obstacle for virus rescue in the traditional approach. The major direct adverse effects of dsRNA formation are probably the inhibition of efficient encapsidation of viral RNA and limiting the amounts of translatable mRNAs. In addition, dsRNA is a major pathogen-associated molecular pattern (PAMP), leading to the induction of type I interferon (IFN), and is activating the antiviral PKR which inhibits protein translation (for review, see Randall and Goodbourn, 2008; Rieder and Conzelmann, 2009). However, as the system employed vaccinia virus, which is able to antagonise these cellular responses, the direct effects are more critical.

The vv/T7 expression system is very robust and for virus rescue works well in a broad range of cell lines and conditions (Table 5.1). This is illustrated by successful rescue not only of mammalian rhabdoviruses like RABV or VSV in hamster cell lines (BSR, BHK) (Ito *et al*., 2001) but also fish rhabdoviruses such as snakehead rhabdovirus (SHRV) (Johnson *et al*., 2000), infectious hematopoietic necrosis virus (IHNV), and VHSV (Biacchesi *et al*., 2000a; Biacchesi *et al*., 2002) in *Epithelioma papulosum cyprinid* (EPC) cells from carp. This cell line can be grown for several hours at 37°C, allowing vv/T7 to express sufficient T7 RNA pol for virus rescue. Further cultivation at low temperature (14°C) supports replication of

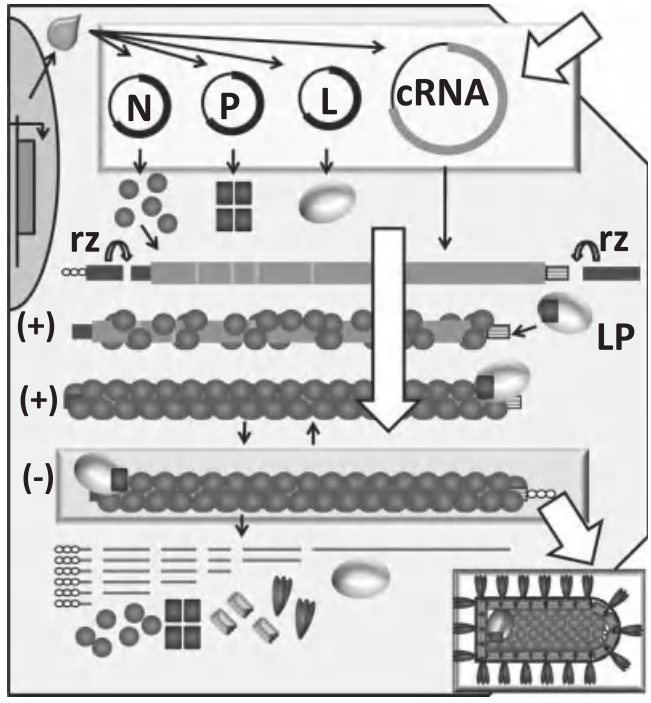

Figure 5.3 Rescue of rhabdoviruses from cDNA. After transfection of usually 4 plasmids the 'helper' proteins N, P and L are expressed and an antigenome-like (+) RNA is transcribed by T7 RNA polymerase or a cellular polymerase. Processing of the cRNA ends is achieved by ribozymes (rz) flanking the viral sequences. If the (+) RNA is encapsidated appropriately by N-P complexes, the plasmid-expressed polymerase (L-P) can synthesize a (-)NC. Initial transcription by the plasmid-expressed polymerase leads to the production of viral proteins which initiate an infectious cycle.

fish rhabdoviruses but not of vv, therefore active elimination of vv is not required (reviewed in Biacchesi, 2011). Elimination of vv after virus rescue in mammalian cells is easily achieved by filtration (Schnell *et al*., 1994; Lawson *et al*., 1995), using inhibitors of DNA replication such as AraC and rifampicin (Whelan *et al*., 1995), or transfer of the rescued viruses to cells not permissive for vv replication, such as mosquito cells (Bridgen and Elliott, 1996).

In addition to vTF7-3, a host range-restricted and highly attenuated vv strain expressing T7 RNA pol, based on a Modified vaccinia virus Ankara (MVA), MVA-T7, has been made available (Sutter *et al*., 1995; Wyatt *et al*., 1995). This virus is unable to multiply in human and most other mammalian cell lines, with the amazing exception of baby hamster kidney (BHK) cells (Drexler *et al*., 1998). Since viral gene expression is unimpaired in non-permissive cells, MVA-T7 is an efficient as well as safe vector. In variations of the system, other viruses have been used as a source of T7 RNA polymerase, like fowlpox virus, adenovirus, or Semliki forest virus for successful rescue of various paramyxoviruses (Conzelmann, 2004).

Table 5.1 Comparison of different rescue systems for members of the *Rhabdoviridae*.

Rescue system	Cell line	Virus	Strain	Remarks	Reference
vv encoded T7 RNA pol	BSR cells (BHK-clone)	*Lyssavirus* RABV	SAD B19		Schnell *et al.* 1994
			minigenome		Conzelmann 1994
	BHK-21		RC-HL		Ito *et al.* 2001
			minigenome	HHRz at 5′-end, superinfection with heterologous lyssaviruses	Le Mercier *et al.* 2002
		Vesiculovirus VSV	Serotype Indiana, San Juan strain		Lawson *et al.* 1995
			Serotype Indiana, San Juan strain	G from strain Orsay	Whelan *et al.* 1995
			minigenome	HDVagRz at 3′-end of cRNAs	Pattnaik *et al.* 1992
	EPC	*Novirhabdovirus* SHRV		warm water fish virus	Johnson *et al.* 2000
		Novirhabdovirus IHNV	European 32/87 strain	w/o N- or NV-"helper" plasmid	Biacchesi *et al.* 2000b
			minigenome	"helper" virus superinfection	Biacchesi *et al.* 2000a
		Novirhabdovirus VHSV	07/71 strain	heterologous combinations	Biacchesi *et al.* 2002
cell encoded T7 RNA pol	**BSR T7/5**	*Lyssavirus* RABV	SAD B19		Finke & Conzelman 1999
			HEP-Flury		Inoue *et al.* 2003
			SHBRV-18 strain, SN strain	chimeric genomes between both strains	Faber *et al.* 2004
		Vesiculovirus VSV	Serotype Indiana, San Juan strain		Harty *et al.* 2001
		Lyssavirus RABV	SAD B19	HHRz and HDVagRz(SC)	Ghanem *et al.* 2011
	BHK/T7-9		RC-HL		Ito *et al.* 2003
	EPC-T7	*Novirhabdovirus* SHRV		warm water fish virus	Alonso *et al.* 2004
plasmid encoded T7 RNA pol	BHK-21	*Lyssavirus* RABV	ERA		Wu and Rupprecht 2008
	Vero, HEK293T	*Vesiculovirus* VSV	Serotype Indiana, San Juan strain	5 "helper" plasmids: N,P,M,G;L	Witko *et al.* 2006
RNA pol II	BHK-21, HEK293T, NA	*Lyssavirus* RABV	HEP-Flury		Inoue *et al.* 2003
	BHK-21		CTN		Huang *et al.* 2010
	HEK293T	*Lyssavirus* EBLV-1	wt EBLV-1 from E. serotinus		Orbanz and Finke, 2010
	EPC	*Novirhabdovirus* IHNV	220-90 strain of IHNV		Ammayappan *et al.* 2011
		Novirhabdovirus VHSV	Great Lakes MI03 strain		Ammayappan *et al.* 2010

Non-viral expression of T7 RNA polymerase

T7 RNA pol can be expressed in transgenic cell lines and from transfected plasmids, thus avoiding handling of heterologous viruses like vv as a source for the polymerase (Lieber, Kiessling, and Strauss, 1989; Elroy-Stein and Moss, 1990). However, T7 transcripts have 5′-triphosphate ends and lack a poly(A) tail. While this is ideal for generating virus-like RNAs, translation of proteins is poor in the absence of the vv capping and polyadenylation enzymes. This can be partially compensated for by introducing an encephalomyocarditis virus (EMCV) IRES element into the protein-encoding plasmids, to mediate cap-independent translation (Elroy-Stein and Moss, 1990). Of note, 5′-triphosphate RNAs are ligands for the pattern receptor RIG-I (Hornung *et al*., 2006; Cui *et al*., 2008) and strongly induce expression of IFN, and expression of T7 RNA polymerase in cells always gives rise to 5′-triphosphate transcripts, probably initiated at genome sequences with similarity to the T7 promoter (5′-pTAATACGACTCACTATAGGG-3′; sense orientation) (Hornung *et al*., 2006). Cell clones expressing high amounts of T7 RNA pol may therefore be counter-selected through IFN-mediated apoptosis. A stable T7 RNA pol-expressing cell line successfully used in the past for rescue of numerous positive- and negative-strand RNA viruses is BSR T7/5, which is derived from BHK cells (Buchholz, Finke, and Conzelmann, 1999). Intriguingly, these cells have a complete defect in the activation of IRF3 (unpublished data), the transcription factor required for IFN-β expression. This IFN-negative cell line has been used for the rescue of various sorts of engineered RABVs (Faber *et al*., 2004), including ambisense viruses (Finke and Conzelmann, 1999), VSV (Harty *et al*., 2001), and other members of *Mononegavirales* (Conzelmann, 2004). One particular advantage of BSR T7/5 in virus rescue is that completely interferon-sensitive viruses, such as RABVs with mutations in the major viral IFN antagonist, can be rescued and propagated easily. BHK/T7-9 is another cell line derived from BHK cells which has been used to rescue RABV (Ito *et al*., 2003), and for the rescue of novirhabdoviruses, the carp cell line EPC-T7 was established (Alonso *et al*., 2004).

In addition, expression plasmids encoding T7 RNA pol, such as pSC6-T7-neo (Radecke *et al*., 1995), pNLS-T7 (Wu and Rupprecht, 2008), pCAGGS-T7 (Paterson *et al*., 2000), or pT7 (Witko *et al*., 2006) can be co-transfected with the viral plasmids into cells for direct rescue. This approach is limited only by the transfection efficiency and has the advantage that cells optimal for growth of the specific virus, or dictated by regulations for vaccine production or other manufacturing, can be used for both rescue and propagation of the viruses.

Efficiency of rhabdovirus rescue: critical effect of 3′ and 5′ ends

The efficiency of rescue of cDNA or RNA of *Mononegavirales* is several orders of magnitude lower than that of positive strand RNA viruses, which is due to the low efficiency of ‘illegitimate’ encapsidation of long RNA. This makes it all the more worthwhile to optimise the rescue system for every virus species,

including the selection of the right cell line for transfection and propagation of virus, and the expression of appropriate ratios of RNA, N, P, and L protein. Though exact ratios of N:P:L are not critically required for rescue of rhabdovirus RNAs as long as high amounts of N and P are expressed, there are examples from other *Mononegavirales,* in which only a very tight range of P concentrations is allowed (Schneider *et al*., 2003).

A critical factor for probably all viruses, however, is the availability of exact ends of the transcribed RNA. Although for minigenome and viral RNA rescue extra residues at the 5′ end do not preclude rescue, the efficiency is higher with exact ends. This is indicated by approaches in which hammerhead ribozymes (HHRz) (Blount and Uhlenbeck, 2002) were used to generate the 5′ ends. In the case of RABV, the traditional approach involved a transcript possessing three extra 5′-G residues in order to favour T7 transcription initiation. Introduction of an HHRz after the G residues, which should result in a transcript starting with the authentic 5′-A residue, increased the rescue efficiency 10-fold. In addition, we observed that the cleavage efficiency of the HDVagRz used so far is low in transfected cells, cleaving less than 10% of the T7 transcript, that is, only 10% of the RNA have the right 3′ end available for packaging and/or replication initiation. Exchange with a longer and much more effective HDV ribozyme (SC) cleaving 90% of intracellular transcripts again increased rescue efficiency more than 10-fold. Moreover, the combination of HHRz and HDVagRz SC in full-length clones of the RABV SAD L16 (pSAD HH-L16-SC) had a synergistic effect and improved rescue by 100-fold and yielded the most efficiently rescueable RABV construct so far (Ghanem *et al*., 2011).

Cellular polymerase systems for rhabdovirus rescue

The use of bacteriophage polymerases like T7 RNA pol has the advantage of allowing cytoplasmic transcription of transfected plasmids and is very well suited for the generation of viruses with a cytoplasmic replication cycle. Transcripts generated in the nucleus of cells must first be exported into the cytoplasm, which may involve processing of precursors by nucleases, addition of 5′-terminal cap structures and poly (A) tails or RNA splicing. Although nuclear transcription of rhabdovirus RNAs and the use of the autocatalytic HHRz and/or HDVagRz for rescue appear to be counter-intuitive, RNA polymerase II-based rescue systems have been used for the recovery of rhabdoviruses. CMV promoter-driven transcripts gave rise to individual RABVs, HEP-Flury (Inoue *et al*., 2003; Tao *et al*., 2010), CTN181 (Huang *et al*., 2010), and HN10 (Ming *et al*., 2009), and also the first rabies-related lyssavirus, EBLV-1 (Orbanz and Finke, 2010). In contrast, rescue of CMV promoter-driven full-length RNA of the RABV SAD L16 was not reported so far. However, recent reports show that the fish rhabdoviruses IHNV and VHSV can be rescued in Pol II systems (Ammayappan *et al*., 2011; Ammayappan, Lapatra, and Vakharia, 2010; Biacchesi, 2011). Therefore, in these approaches, the presence of virus-specific sequences representing cryptical RNA splice sites, which might affect the integrity of

full-length RNA and/or the efficient removal of the Pol II-derived poly(A) tails and 5′-caps, which may prevent nuclear export, may be decisive for success, and has to be determined empirically.

While recovery of vertebrate rhabdoviruses is straightforward (Table 5.1), the establishment of plant virus reverse genetics systems is obviously more challenging. This is likely due to technical problems with plant tissue or cell culture, hampering adequate gene transfer and expression of viral proteins (for review, see Jackson *et al*., 2005). Improvement of methods will certainly lead to the establishment of reverse genetics systems for plant rhabdoviruses, as well as for insect rhabdoviruses.

5.2.3 Design of recombinant rhabdoviruses

The possibilities of genetic manipulation of rhabdoviruses are enormous. This stems particularly from their modular genome organisation, their simple and efficient gene expression strategy, and the high stability of their genetic information. The latter is at least in part due to the characteristic structure of the rhabdovirus NC, which encumbers recombination and elimination of non-essential sequences. In addition, the rhabdovirus genome in the NC is one-dimensional and sophisticated RNA tertiary structures critical for replication, as observed in many positive strand RNA viruses, are not known.

Gene deletion, gene insertion

Apart from single or multiple mutations within genes, which may affect any aspect of the multifunctional virus proteins, the deletion of entire genes is particularly useful to initially address their roles within the virus life-cycle. As the genome is a succession of individual cistrons defined by transcriptional start and stop sequences, removal of an entire cistron is easily performed, which is most simply done by cutting in the usually spacious 3′ untranslated regions (UTR) of the target gene and the preceding gene (Figure 5.4). It has to be noted, however, that in this setting the upstream gene of the new construct has a hybrid 3′ UTR, and the lack of a cistron places the downstream genes in a more promoter proximal position such that they are transcribed more abundantly. It is therefore advantageous to exactly replace the viral ORF by that of a reporter gene, or an unrelated sequence. Obviously, gene deletion can be used to single out non-essential genes, like the NV of fish novirhabdoviruses, from the essential ones, like N, P, M, G, and L. Viruses lacking one or more essential genes can be produced in cells expressing the missing protein. Studying M gene-deficient RABVs, for example, not only verified the key role of M in virus budding (Mebatsion, Weiland, and Conzelmann, 1999) but also identified an important role of this protein in regulating the mode of RNA synthesis (Finke *et al*., 2000b; Finke *et al*., 2003). The deletion of the NV gene of the novirhabdoviruses SHRV (Alonso *et al*., 2004), IHRV, or VHSV (Biacchesi *et al*., 2000a; Biacchesi *et al*., 2000b; Thoulouze *et al*., 2004) did not prevent viral propagation *in vitro*, but

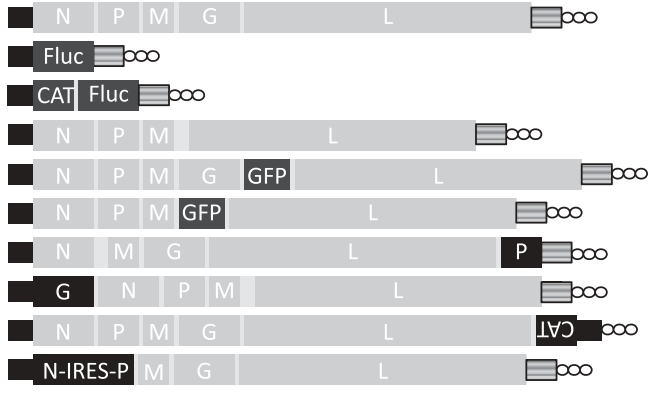

Figure 5.4 Design of recombinant virus genomes. Popular designs include (a) RABV wild-type organisation; (b) monocistronic or (c) bicistronic minigenomes comprising the genome ends and reporter gene transcription units like firefly luciferase (FLuc) and CAT; (d) deletion of genes; (e) insertion of additional genes; (f) gene replacement; (g, h) gene shifting to promoter distal (g) or proximal (h) positions to reduce or enhance gene expression, respectively; (i) represents an ambisense gene expression virus where antigenome RNAs comprise a copy of the genome promoter; (j) delineates an approach in which rhabdovirus transcription signals are replaced by internal translation initiation signals (IRES elements) such that P expression depends on translational activity of the IRES.

led to severe attenuation of IHRV and VHSH *in vivo*, confirming the NV gene as an important 'accessory' gene required for virus survival in natural conditions.

The introduction of an extra gene for vector or vaccines purposes is as easily achieved by introduction of a complete extra cistron, i.e., an ORF encoding protein of interest flanked by viral transcription start/stop signals. With RABV, in which the individual gene borders are distinct from each other, usually the N/P gene border sequence has been used, since this appears to support restart of the polymerase best. As is obvious from the sequential transcription mode and eventual dissociation of the polymerase at gene borders, the position of the extra cistron in principle determines the level of its transcripts and thus protein expression, and should reduce the levels of the downstream viral genes. However, the degree of the effects may depend on the virus used. While in VSV a steady decline of transcripts along the genome is suggested (see below), the transcript levels for the N, P, M, and G genes of RABV SAD L16 are rather similar and a steep step is only observed at the G/L border, which is characterised by a particularly long intergenic region. Using the classical and popular insertion site for RABV immediately downstream of the G gene (Schnell *et al*., 1994) still yields high levels of transcripts and reduces only the levels of the polymerase L, which is needed in catalytic amounts. Indeed, the addition of one or two extra cistrons to the RABV genome at this position did not greatly affect virus propagation.

No strict upper size limits for accommodation of additional foreign sequences are expected because of the helical nature of the rhabdovirus genomes. In view of

natural rhabdovirus genome sizes ranging from 11 to almost 20 kb, at least the small animal rhabdoviruses should have a capacity for approximately 10 kb of foreign sequences. More critical than size appears to be the number of individual cistrons, as the gene borders do not support reinitiation of all incoming polymerases, such that the loss of the polymerase may limit the expression of the most downstream genes. Bigger genomes obviously require more time to be replicated. Though this may not be a fatal problem in terms of replication *per se*, it is possible that proteins critically required for counteracting powerful cellular antiviral responses cannot be accumulated at sufficient speed. RNA viruses with big genomes (coronaviruses have up to 30 kb of RNA) also suffer from accumulation of detrimental mutations because of the relatively high error rates of RNA-dependent RNA polymerases.

Ambisense virus vectors

As outlined above, the loss of the polymerase at downstream genes and changes in the relative virus protein expression are probably the most serious consequences in adding extra cistrons to the typical rhabdovirus genome. An alternative strategy avoiding these problems was used in our laboratory for RABV. This involved an 'ambisense' strategy, which in nature is utilised by some members of the *Arenaviridae* and *Bunyaviridae* families, which possess segmented genomes (Bishop, 1986). In this case, both genome and antigenome NCs may serve as a template for transcription of subgenomic mRNAs (see Chapters 1 and 7 in this volume). To make a rhabdovirus an ambisense virus, the promoter of the antigenome has to be replaced with a copy of the genome promoter, including the leader and first gene's transcription start sequence, resulting in a 'copy-back' genome (Figure 5.4). In SAD-Ambi, a nearby transcriptional stop sequence prevents transcription into the L gene, in order to avoid the formation of long L dsRNAs. SAD Ambi-CAT contains in addition the ORF of chloramphenicol-acetyltransferase (CAT) reporter protein (Finke and Conzelmann, 1997).

The exchange of the promoter resulted not only in transcription and expression of the reporter gene from the antigenome NC, but also had effects on replication. While in standard virus a 50-fold excess of genome RNAs was present, the ambisense viruses directed the synthesis of equal amounts of genome and antigenome NCs (1:1) without a drastic loss of total replicative synthesis (twofold). Moreover, 50% of virions contained antigenomic NCs, as revealed by hybridisation experiments, revealing the lack of specific signals for incorporation into virus particles. The final infectious titres (that is, viruses with genome NCs) of the ambisense viruses were only 10–15-fold below those of wild-type (wt) virus. Hence, such RABVs appear to represent suitable vectors, particularly for expression of multiple foreign genes without disturbing the canonical gene order and expression levels of viral proteins. Whether or how this strategy is applicable to other viruses has to be determined empirically. For example, the VSV genome 5′ end appears to rely on specific sequences of the genomic 5′ end for incorporation into virions, as revealed by minigenome experiments (Whelan and Wertz, 1999). Ambisense Sendai viruses

reflect the findings with RABV in terms of gene expression (Le Mercier *et al*., 2002a), but are unstable in cell culture, indicating an important role of the viral trailer RNA in preventing apoptosis (Iseni *et al*., 2002) or induction of IFN by read-through dsRNAs (Le Mercier *et al*., 2002a).

Shifting viral genes

As the relative position of a gene to the 3′ terminal promoter determines its level of transcription, changing the gene order leads to changes in the relative protein expression and to viruses with altered phenotypes. In fact, gene-shifting to downstream positions is a valuable approach to study the functions of essential genes, complementing gene knock-out experiments. For example, stepwise downstream translocation of the N gene of VSV resulted in gradual attenuation of the virus *in vitro*, illustrating the important role of high N protein abundance for replication (Wertz *et al*., 1998). This holds true *in vivo*, revealing a correlation of replication and virulence (Flanagan *et al*., 2001; Flanagan *et al*., 2003). The role of the RABV M protein in the regulation of replication was illustrated by viruses expressing very little M protein from the 5′ terminal position downstream of the L protein (SAD-LM) (Finke and Conzelmann, 2003; Finke *et al*., 2003). Similarly, a corresponding construct in which the RABV P gene was expressed from the most downstream position (SAD ΔPLP) revealed the crucial roles of the RABV P protein as an IFN antagonist and yielded viruses strongly stimulating host cell innate immunity (Brzózka *et al*., 2005, 2006; Rieder and Conzelmann, 2009; Rieder *et al*., 2011). Gene shifting can also be exploited to increase the expression of major protective antigens, such as moving the VSV G gene more upstream (Flanagan, Ball, and Wertz, 2000).

Tuning transcription and translation

Expression levels of virus-encoded proteins can be further tuned by modified transcription signals that differ in their ability to direct restart or read through. In contrast to VSV, the four gene borders of RABV have intergenic regions of different length, namely 2, 5, 5 and 24–29 nucleotides, which alter reinitiation efficiencies. When the individual intergenic regions were introduced into the RABV N/P gene border, an attenuation of downstream transcription to 78% (P/M), 81% (M/G) or 11% (G/L) was observed. In a recombinant RABV, the replacement of the complete G/L gene border with a copy of the optimal N/P border resulted in over-expression of L mRNA, and a general increase in viral RNA synthesis (Finke *et al*., 2000b). Such enhancement of L expression could be helpful in compensating for poor L expression caused by multiple extra upstream cistrons.

A completely different approach to modulate the expression levels of viral proteins involves the removal of the rhabdovirus gene borders, and thus the transcription signals, and their replacement with RNA sequences which are active in translation, namely, internal ribosome entry site (IRES) sequences, as found, for

example, in picornaviruses. Such replacement of the N/P gene border in RABV, for example, leads to the transcription of a bicistronic N-P mRNA. The upstream N is translated in the typical 5′-cap-dependent manner while translation of the downstream P is internally mediated by the IRES element. As picornavirus IRES elements differ in their initiation activity, the expression levels of the downstream encoded protein can be fine-tuned by selecting the appropriate IRES. Translational attenuation of the RABV P by the poliovirus or human rhinovirus IRES accordingly attenuated the neurovirulence of recombinant RABV (Marschalek *et al*., 2009). In case of RABV, the P protein is not only essential for RNA encapsidation and polymerase function but is also a critical antagonist of IFN induction and IFN signalling. Reducing P levels therefore attenuates both virus replication and its ability to escape from antiviral host response (Brzózka *et al*., 2005; Marschalek *et al*., 2012).

As IRES elements direct internal entry of ribosomes, it was not surprising to see that rescue of the above recombinant viruses was possible without providing P protein from a separate expression plasmid (unpublished data). It can be envisaged that in the future full-length cDNA clones can be developed in which N, P, and L can be expressed from the full-length antigenome transcript (before it is encapsidated). Such single plasmid rescue systems may be particularly valuable in transgenic animal approaches for studying individual aspects of RABV biology and pathogenesis, or for *in vivo* tracing experiments.

5.3 Applications and examples

Rhabdoviruses of vertebrates, invertebrates and plants as well as other *Mononegavirales* represent a great source of tools for basic research and biomedical applications. Particularly, numerous vaccine and vector approaches have been initiated with the availability of reverse genetics systems, aimed at generating immunity against homologous and heterologous pathogens, or for oncolytic virotherapy. For comprehensive reviews, see Finke and Conzelmann, 2005a; Bukreyev *et al*., 2006; Lichty *et al*., 2004; Lech and Russell, 2010). Here, only the principal strategies will be outlined and a few important or exciting examples will be provided.

5.3.1 Vaccines and vectors

Approaches to attenuate live virus

The amenability of a virus to genetic manipulation immediately offers possibilities for generating attenuated live vaccines. Most mutations and alterations of the genomes attenuating the replication of rhabdoviruses *in vitro* should also affect their replication *in vivo*, and likely their virulence, as illustrated, for example, by the above-mentioned VSV N gene shift mutants (Flanagan *et al*., 2001). However, stimulation of immune responses is often inversely correlated with replication capacity and poor growth of live vaccines *in vitro* is a serious cost issue in commercial

vaccine production. The identification and elimination of virulence markers which attenuate virulence but do not affect replication and immunogenicity are desirable (Bukreyev *et al*., 2006; Lichty *et al*., 2004).

With the more recent elucidation of innate immune response pathways and their great impact on the development of adaptive immunity, viral antagonists of these pathways, in particular of type I IFN and NF-κB, have emerged as virulence factors and promising targets for attenuation of rhabdoviruses, and viruses in general. IFN antagonists of *Mononegavirales* are often non-essential so-called 'accessory' proteins, which are encoded on separate genes, like RSV NS1 and NS2, or from the P gene, such as the V and C proteins of paramyxoviruses, or the NV genes of novirhabdoviruses, and the expression of which can be abolished (for review, see Randall and Goodbourn, 2008; Goodbourn and Randall, 2009; Biacchesi, 2011).

Notably, the mechanisms and proteins involved in IFN escape of the seemingly closely related RABV and VSV differ dramatically, reflecting their specific biology (for review, see Rieder and Conzelmann, 2009). In RABV, the P protein is a specific and powerful inhibitor of IFN induction and of the IFN signalling-mediated induction of antiviral and immune-stimulatory proteins. In VSV, the M protein is cytopathic and prevents transcriptionally regulated host cell responses in general, by interfering with host mRNA transcription and nuclear export (Ferran and Lucas-Lenard, 1997; Ahmed *et al*., 2003). RABV P and VSV M are essential proteins, which cannot be deleted without abrogating virus propagation. However, mutagenesis experiments were used to identify proteins specifically lacking these IFN escape functions. For RABV, P mutants lacking a few amino acids (176–181) were unable to prevent activation of the IFN transcription factor IRF3. Recombinant RABVs carrying the mutation replicated like wt virus *in vitro* but induced high levels of IFN and were completely attenuated even after intracerebral application at a high dose (Rieder *et al*., 2011). Similarly, residue M51 in the M protein of VSV was found to be critical for host cell shutdown, and mutant M51 VSV viruses strongly activate the host innate immune response and are attenuated *in vivo* (Ahmed *et al*., 2009; Ahmed, Brzoza, and Hiltbold, 2006). Such attenuated but immune-stimulating viruses are being used in numerous approaches *in vivo*, involving oncolytic gene therapy (Wu *et al*., 2008). In summary, rhabdoviruses with targeted deletions in IFN antagonist genes are excellent candidates for live virus vaccines and oncolytic virotherapy, because they can be grown to high titres in tissue culture and are highly attenuated in the host organism, due to a robust IFN and immune response.

Another approach aimed at stimulating the host immune response rather than attenuating the virus is expression of recombinant cytokines like IFN (Obuchi *et al*., 2003; Faul *et al*., 2008) and other cytokines and chemokines (Fernandez *et al*., 2002; Niu *et al*., 2011; Wen *et al*., 2011). However, very careful selection of immune-stimulatory molecules is required to avoid adverse effects (Zhao *et al*., 2009) .

The acute or residual inherent neurotropism of rhabdoviruses is of concern in any live vector application. Recent progress in the growing field of small RNAs, particularly micro-RNAs (miRNAs) has enabled an elegant strategy to restrict virus

replication in neuronal tissue. This is based on the finding that in neurones specific miRNAs are overexpressed. Engineering the target sequences of these miRNAs into 3′-UTRs of rhabdovirus transcripts results in specific attenuation of virus neuropathogenicity (Kelly *et al*., 2010). Though mostly applied in the design of oncolytic vectors, this approach is generally applicable.

The major virulence factor of RABV, which determines its high neurotropism, is the G protein (Finke and Conzelmann, 2005b; Dietzschold *et al*., 2008; Lafon, 2008), which is also the antigen for the induction of neutralising antibodies (Cox, Dietzschold, and Schneider, 1977). The identification of mutations affecting the functions of G in binding to neuronal receptors and entry into neurones, but leaving the major antigenic sites intact is a classical objective in the design of live RABV vaccines. Indeed, mutations of the arginine residue 333 render many RABV strains incapable of reaching the central nervous system (CNS) (Dietzschold *et al*., 1983; Seif *et al*., 1985; Coulon *et al*., 1989), making them safe vaccines, see, for example (Mebatsion, 2001). Reverse genetics allows vaccines to be further improved, for example, by forced expression of the G protein to better stimulate the formation of antibodies and simultaneously attenuate the virus. This is achieved in viruses which contain two or three copies of the authentic or combinations of authentic and mutated G gene (Faber *et al*., 2002; Faber *et al*., 2007; Faber *et al*., 2009). The high attenuation of these viruses is multifactorial: the extra cistrons attenuate transcription and replication, overexpression of G induces apoptosis (Faber *et al*., 2002; Prehaud *et al*., 2003), thereby destroying the integrity of neuronal connections required for RABV to reach the CNS (see below for details), and the R333 mutation in G prevents efficient entry into neurones. The high propensity of rhabdoviruses to accept genetic modifications which allows the combination of multiple attenuating strategies, should lead to the development of highly safe and effective live vaccines in the future.

Vectors

Mammalian rhabdoviruses like RABV and VSV are well known to induce strong humoral and cellular immune responses and are therefore regarded as promising carriers for heterologous antigens as well. VSV grows rapidly and to high titres and has therefore been used as a vector for numerous antigens, since the advent of reverse genetics. A major focus in the field of VSV- and RABV-based vaccines is immunisation against HIV-1, which mostly involves expression of the HIV-1 Env and/or Gag proteins from recombinant RABV (Gomme *et al*., 2011) or VSV (Johnson *et al*., 2009). In addition, antigens from many other major pathogens threatening human health have been expressed from rhabdovirus vectors, just to mention a few recent examples: the Ebola and Marburg filoviruses (Geisbert *et al*., 2009; Falzarano, Geisbert, and Feldmann, 2011), influenza A virus (Schwartz *et al*., 2010), SARS coronavirus (Kapadia *et al*., 2005; Kapadia *et al*., 2008), hepatitis C virus (Majid *et al*., 2011), hepatitis B virus (Cobleigh *et al*., 2010), norovirus (Ma and Li, 2011), vaccinia virus

(Chattopadhyay *et al*., 2008; Braxton *et al*., 2010), or against bacteria or toxins (Smith *et al*., 2006; Chattopadhyay *et al*., 2008). In many of these cases, the non-cytopathic VSV mutant M51R is being used for improved immune response.

The heterologous antigens, which are mostly viral surface glycoproteins, can be expressed as soluble protein antigens, or can be designed to form virus-like particles, as in the case of noroviruses (Ma and Li, 2011) to mimic infection with the heterologous virus. Preferably, individual proteins are displayed on the surface of rhabdovirus particles to mimic the densely packed para-crystalline arrays of the rhabdovirus G spikes in natural viruses. Such multimeric identical antigens are characteristic of some viruses and are able to activate B cells directly (Zinkernagel, 1997; Fehr *et al*., 1998). This can be achieved by fusing the antigen with the G protein, or by incorporation of the entire protein into the viral membrane, either in addition or instead of the vector glycoprotein.

Envelope switching

Though contributing to virus budding, the G proteins of RABV and VSV are not required for this process (Mebatsion, Konig, and Conzelmann, 1996; Schnell *et al*., 1998; Mebatsion, Weiland, and Conzelmann, 1999) and can be replaced entirely by foreign type I transmembrane proteins (Mebatsion *et al*., 1995; Mebatsion and Conzelmann, 1996; Schnell *et al*., 1996; Mebatsion *et al*., 1997; Schnell *et al*., 1997). If these proteins can replace G functions in terms of virus entry, viruses with an altered tropism or host range are generated. VSV particles seem to be more promiscuous than RABV and accept many envelope glycoproteins such as the human CD4 (Schubert *et al*., 1992), though a certain length of the C-tail seems to favour incorporation (Schnell *et al*., 1998). In contrast, RABV requires a C-tail sequence and structure similar to that of RABV G (Mebatsion and Conzelmann, 1996; Mebatsion *et al*., 1997). As opposed to RABV, the expression levels of VSV G proteins in VSV-infected cells are extremely high, such that a specific enrichment in particles may not be necessary, explaining the observed differences.

Pseudotype rhabdoviruses, in which the lack of their own G genes is complemented in cells expressing the heterologous protein, or surrogate rhabdoviruses, in which the G gene is replaced with the foreign gene, are valuable tools for basic and applied research. As opposed to the latter, pseudotype viruses can perform only a single round of infection and can therefore be used to study the functions of the glycoproteins of highly hazardous viruses, like HIV-1, filoviruses or arenaviruses (see references in Finke and Conzelmann, 2005a). In addition, retargeting of viruses by pseudotyping to cells of interest is a valuable approach in directing vaccine viruses to immune cells like DCs, or in oncolytic virotherapy. Recent promising approaches include, for example, the use of oncolytic VSV carrying the envelope of LCMV which show enhanced infectivity for glioma cells (Muik *et al*., 2011). Surrogate viruses may serve the same objectives. Their tropism and immunogenicity are also directed by the foreign protein but they are replication- and spread-competent viruses, which require appropriate precautions.

(Mono)synaptic tracing

While for many biomedical approaches the extreme neurotropism of RABV is counterproductive, it is extremely valuable when it comes to the study of the nervous system, where RABV has a home field advantage over any other virus. In fact, *in vivo* RABV is completely adapted to and dependent on neurones, and on the integrity of the neuronal network, in order to reach the CNS where it replicates and amplifies best, and where it causes behavioural symptoms that guarantee transmission.

The unique feature of RABV is an exclusive transsynaptic transmission (Astic *et al*., 1993; Coulon *et al*., 1989; Ugolini, 1995, 2010). At the infection site, RABV is endocytosed by neurones and is retrogradely transported in axonal transport vesicles towards the cell body (Klingen, Conzelmann, and Finke, 2008). Klingen *et al*. made use of dual-labelled RABV particles allowing the distinction of enveloped virus from non-enveloped NCs. Upon membrane fusion and release of the NC into the cytoplasm, replication takes place. Then, new virions are assembled, and only at synapses are they transferred to the presynaptic membrane of connected neurones, endocytosed, and further transported in the direction of the CNS. While G gene-deficient RABV pseudotyped with RABV G can infect neurones after injection into the brain, transsynaptic transmission from these initially infected neurones to connected presynaptic neurones is not possible (Etessami *et al*., 2000). Injections with such single round RABV equipped with a GFP gene leads to brilliant staining of neurones and neurites, such that neurones with projections to the injection site can be identified and their morphology studied in great detail (Wickersham *et al*., 2007a).

The connectivity of neuronal circuits is a central topic in the neurosciences since detailed knowledge of neuronal wiring is a prerequisite for understanding how the brain works. Direct synaptic connections of individual neurones, however, cannot be easily determined with conventional non-viral or viral tracers, including replication-competent RABV. Fortunately, exploiting the exclusive synaptic transmission of RABV and the possibility of envelope switching and re-targeting of single round G-deficient RABV have recently led to the establishment of the first so-called ‘monosynaptic’ tracer system (Wickersham *et al*., 2007b).

The system involves targeted infection of a defined (postsynaptic) neuron which expresses G protein and therefore supports transsynaptic transfer of the virus to connected (presynaptic) neurones. As no G is expressed there, the virus is not spread further. Specific targeting of the first neurone is achieved, for example, by expression of the avian ‘tumor virus a’ protein (TVA) in the neurones, and infection with viruses pseudotyped with EnvA, the envelope protein of an avian retrovirus which uses the receptor TVA for entry. Expression of different fluorophors in the postsynaptic neurone and from the virus genome allows easy discrimination of post- and presynaptic cells (Plates 1 and 2). This system is being increasingly used to dissect sensory and motor circuits in the nervous system (Stepien *et al*., 2010; Wall *et al*., 2010; Weible *et al*., 2010; Miyamichi *et al*., 2011; Yonehara *et al*., 2011).

Future progress in virus construction will in addition greatly facilitate readout and allow modulation of neurone activity, for example, by including genes encoding light-activated ion channels or calcium-indicators.

5.4 Conclusion

The ability to manipulate the genomes of rhabdoviruses at will, and to recover new viruses not existing in nature is a powerful tool to study all aspects of the viral life-cycle, including host anti-viral, and viral anti-host mechanisms and to understand pathogenicity. This is a prerequisite for safe and efficient prophylaxis and treatment of present and emerging viruses. Moreover, the particular skills of rhabdoviruses can be exploited to design valuable tools for biomedical research, including vectors and tracers revealing the intricacies of biological systems like the brain.

Acknowledgements

We thank A. Stepien, S. Arber, and M. Schwarz for providing images, and Anika Kern for critical comments on the manuscript. Work in the authors' laboratory was supported by the Deutsche Forschungsgemeinschaft through GraKo 1202, SFB 455, SFB 870, SPP1175, and BMBF grant no. 01KI1016A.

References

Abraham, G. and Banerjee, A. K. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America* **73**(5): 1504–1508.

Ahmed, M., Brzoza, K. L. and Hiltbold, E. M. 2006. Matrix protein mutant of vesicular stomatitis virus stimulates maturation of myeloid dendritic cells. *Journal of Virology* **80**(5): 2194–2205.

Ahmed, M., McKenzie, M. O., Puckett, S., Hojnacki, M., Poliquin, L. and Lyles, D. S. 2003. Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. *Journal of Virology* **77**(8): 4646–4645.

Ahmed, M., Mitchell, L. M., Puckett, S., Brzoza-Lewis, K. L., Lyles, D. S. and Hiltbold, E. M. 2009. Vesicular stomatitis virus M protein mutant stimulates maturation of Toll-like receptor 7 (TLR7)-positive dendritic cells through TLR-dependent and -independent mechanisms. *The Journal of Virology* **83**(7): 2962–2975.

Albertini, A. A., Wernimont, A. K., Muziol, T., Ravelli, R. B., Clapier, C. R., Schoehn, G., Weissenhorn, W. and Ruigrok, R. W. 2006. Crystal structure of the rabies virus nucleoprotein-RNA complex. *Science* **313**(5785): 360–363.

Alonso, M., Kim, C. H., Johnson, M. C., Pressley, M. and Leong, J. A. 2004. The NV gene of snakehead rhabdovirus (SHRV) is not required for pathogenesis, and a heterologous glycoprotein can be incorporated into the SHRV envelope. *The Journal of Virology* **78**(11): 5875–5882.

Ammayappan, A., Kurath, G., Thompson, T. M. and Vakharia, V. N. 2011. A reverse genetics system for the Great Lakes strain of viral hemorrhagic septicemia virus: the NV gene is required for pathogenicity. *Marine Biotechnology (NY)*. **13**(4): 672–683.

Ammayappan, A., Lapatra, S. E. and Vakharia, V. N. 2010. A vaccinia-virus-free reverse genetics system for infectious hematopoietic necrosis virus. *Journal of Virological Methods* **167**(2): 132–139.

Astic, L., Saucier, D., Coulon, P., Lafay, F. and Flamand, A. 1993. The CVS strain of rabies virus as transneuronal tracer in the olfactory system of mice. *Brain Research* **619**(1–2): 146–156.

Ball, L. A. 1992. Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. *The Journal of Virology* **66**(4): 2335–2345.

Barr, J. N. and Fearns, R. 2010. How RNA viruses maintain their genome integrity. *Journal of General Virology* **91**(Pt 6): 1373–1387.

Basak, S., Mondal, A., Polley, S., Mukhopadhyay, S. and Chattopadhyay, D. 2007. Reviewing Chandipura: a vesiculovirus in human epidemics. *Bioscience Report* **27**(4–5): 275–298.

Biacchesi, S. 2011. The reverse genetics applied to fish RNA viruses. *Veterinary Research* **42**(1): 12.

Biacchesi, S., Bearzotti, M., Bouguyon, E. and Bremont, M. 2002. Heterologous exchanges of the glycoprotein and the matrix protein in a Novirhabdovirus. *Journal of Virology* **76**(6): 2881–2889.

Biacchesi, S., Thoulouze, M. I., Bearzotti, M., Yu, Y. X. and Bremont, M. 2000a. Recovery of NV knockout infectious hematopoietic necrosis virus expressing foreign genes. *Journal of Virology* **74**(23): 11247–11253.

Biacchesi, S., Yu, Y. X., Bearzotti, M., Tafalla, C., Fernandez-Alonso, M. and Bremont, M. 2000b. Rescue of synthetic salmonid rhabdovirus minigenomes. *Journal of General Virology* **81**(Pt 8): 1941–1945.

Bishop, D. H. 1986. Ambisense RNA genomes of arenaviruses and phleboviruses. *Advances in Virus Research* **31**: 1–51.

Bitko, V. and Barik, S. 2001. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiology* **1**: 34.

Blount, K. F. and Uhlenbeck, O. C. 2002. The hammerhead ribozyme. *Biochemical Society Transactions* **30**(Pt 6): 1119–1122.

Blumberg, B. M. and Kolakofsky, D. 1981. Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. *Journal of Virology* **40**(2): 568–576.

Both, G. W., Banerjee, A. K. and Shatkin, A. J. 1975. Methylation-dependent translation of viral messenger RNAs in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **72**(3): 1189–1193.

Braxton, C. L., Puckett, S. H., Mizel, S. B. and Lyles, D. S. 2010. Protection against lethal vaccinia virus challenge by using an attenuated matrix protein mutant vesicular stomatitis virus vaccine vector expressing poxvirus antigens. *Journal of Virology* **84**(7): 3552–3561.

Bridgen, A. and Elliott, R. M. 1996. Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proceedings of the National Academy of Sciences of the United States of America* **93**(26): 15400–15404.

Brzózka, K., Finke, S. and Conzelmann, K. K. 2005. Identification of the rabies virus alpha/beta interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. *Journal of Virology* **79**(12): 7673–7681.

Brzózka, K., Finke, S. and Conzelmann, K. K. 2006. Inhibition of interferon signaling by rabies virus phosphoprotein P: activation-dependent binding of STAT1 and STAT2. *Journal of Virology* **80**(6): 2675–2683.

Buchholz, U. J., Finke, S. and Conzelmann, K. K. 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *Journal of Virology* **73**(1): 251–259.

Bukreyev, A., Skiadopoulos, M. H., Murphy, B. R. and Collins, P. L. 2006. Nonsegmented negative-strand viruses as vaccine vectors. *The Journal of Virology* **80**(21): 10293–10306.

Chattopadhyay, A., Park, S., Delmas, G., Suresh, R., Senina, S., Perlin, D. S. and Rose, J. K. 2008. Single-dose, virus-vectored vaccine protection against Yersinia pestis challenge: CD4+ cells are required at the time of challenge for optimal protection. *Vaccine* **26**(50): 6329–6337.

Chenik, M., Chebli, K. and Blondel, D. 1995. Translation initiation at alternate in-frame AUG codons in the rabies virus phosphoprotein mRNA is mediated by a ribosomal leaky scanning mechanism. *Journal of Virology* **69**(2): 707–712.

Cobleigh, M. A., Buonocore, L., Uprichard, S. L., Rose, J. K. and Robek, M. D. 2010. A vesicular stomatitis virus-based hepatitis B virus vaccine vector provides protection against challenge in a single dose. *The Journal of Virology* **84**(15): 7513–7522.

Colonno, R. J. and Banerjee, A. K. 1976. A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription in vitro. *Cell* **8**(2): 197–204.

Colonno, R. J. and Banerjee, A. K. 1978. Complete nucleotide sequence of the leader RNA synthesized in vitro by vesicular stomatitis virus. *Cell* **15**(1): 93–101.

Conzelmann, K. K. 2004. Reverse genetics of mononegavirales. *Current Topics in Microbiology and Immunology* **283**: 1–41.

Coulon, P., Derbin, C., Kucera, P., Lafay, F., Prehaud, C. and Flamand, A. 1989. Invasion of the peripheral nervous systems of adult mice by the CVS strain of rabies virus and its avirulent derivative AvO1. *The Journal of Virology* **63**(8): 3550–3554.

Cox, J. H., Dietzschold, B. and Schneider, L. G. 1977. Rabies virus glycoprotein. II. Biological and serological characterization. *Infection and Immunity* **16**(3): 754–759.

Cui, S., Eisenacher, K., Kirchhofer, A., Brzózka, K., Lammens, A., Lammens, K., Fujita, T., *et al*. 2008. The C-terminal regulatory domain is the RNA 5′-triphosphate sensor of RIG-I. *Molecular Cell* **29**(2): 169–179.

Dietzschold, B., Li, J., Faber, M. and Schnell, M. 2008. Concepts in the pathogenesis of rabies. *Future Virology* **3**(5): 481–490.

Dietzschold, B., Wunner, W. H., Wiktor, T. J., Lopes, A. D., Lafon, M., Smith, C. L. and Koprowski, H. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proceedings of the National Academy of Sciences of the United States of America* **80**(1): 70–74.

Drexler, I., Heller, K., Wahren, B., Erfle, V. and Sutter, G. 1998. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. *Journal of General Virology* **79**(Pt 2): 347–352.

Elroy-Stein, O. and Moss, B. 1990. Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **87**(17): 6743–6747.

Etessami, R., Conzelmann, K. K., Fadai-Ghotbi, B., Natelson, B., Tsiang, H. and Ceccaldi, P. E. 2000. Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: an in vitro and in vivo study. *Journal of General Virology*. **81**(Pt 9): 2147–2153.

Faber, M., Faber, M. L., Li, J., Preuss, M. A., Schnell, M. J. and Dietzschold, B. 2007. Dominance of a nonpathogenic glycoprotein gene over a pathogenic glycoprotein gene in rabies virus. *The Journal of Virology* **81**(13): 7041–7047.

Faber, M., Li, J., Kean, R. B., Hooper, D. C., Alugupalli, K. R. and Dietzschold, B. 2009. Effective preexposure and postexposure prophylaxis of rabies with a highly attenuated recombinant rabies virus. *Proceedings of the National Academy of Sciences of the United States of America* **106**(27): 11300–11305.

Faber, M., Pulmanausahakul, R., Hodawadekar, S. S., Spitsin, S., McGettigan, J. P., Schnell, M. J. and Dietzschold, B. 2002. Overexpression of the rabies virus glycoprotein results in enhancement of apoptosis and antiviral immune response. *Journal of Virology* **76**(7): 3374–3381.

Faber, M., Pulmanausahakul, R., Nagao, K., Prosniak, M., Rice, A. B., Koprowski, H., Schnell, M. J. and Dietzschold, B. 2004. Identification of viral genomic elements responsible for rabies virus neuroinvasiveness. *Proceedings of the National Academy of Sciences of the United States of America* **101**(46): 16328–16332.

Falzarano, D., Geisbert, T. W. and Feldmann, H. 2011. Progress in filovirus vaccine development: evaluating the potential for clinical use. *Expert Review of Vaccines* **10**(1): 63–77.

Faul, E. J., Wanjalla, C. N., McGettigan, J. P. and Schnell, M. J. 2008. Interferon-beta expressed by a rabies virus-based HIV-1 vaccine vector serves as a molecular adjuvant and decreases pathogenicity. *Virology* **382**(2): 226–238.

Fehr, T., Naim, H. Y., Bachmann, M. F., Ochsenbein, A. F., Spielhofer, P., Bucher, E., Hengartner, H., *et al*. 1998. T-cell independent IgM and enduring protective IgG antibodies induced by chimeric measles viruses. *Nature Medicine* **4**(8): 945–948.

Fernandez, M., Porosnicu, M., Markovic, D. and Barber, G. N. 2002. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. *The Journal of Virology* **76**(2): 895–904.

Ferran, M. C. and Lucas-Lenard, J. M. 1997. The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. *Journal of Virology* **71**(1): 371–377.

Finke, S. and Conzelmann, K. K. 1997. Ambisense gene expression from recombinant rabies virus: random packaging of positive- and negative-strand ribonucleoprotein complexes into rabies virions. *Journal of Virology* **71**(10): 7281–7288. WH

Finke, S. and Conzelmann, K. K. 1999. Virus promoters determine interference by defective RNAs: selective amplification of mini-RNA vectors and rescue from cDNA by a 3′ copy-back ambisense rabies virus. *Journal of Virology* **73**(5): 3818–3825.

Finke, S. and Conzelmann, K. K. 2003. Dissociation of rabies virus matrix protein functions in regulation of viral RNA synthesis and virus assembly. *Journal of Virology* **77**(22): 12074–12082.

Finke, S. and Conzelmann, K. K. 2005a. Recombinant rhabdoviruses: vectors for vaccine development and gene therapy. *Current Topics in Microbiology and Immunology* **292**: 165–200.

Finke, S. and Conzelmann, K. K. 2005b. Replication strategies of rabies virus. *Virus Research* **111**(2): 120–131.

Finke, S., Cox, J. H. and Conzelmann, K. K. 2000a. Differential transcription attenuation of rabies virus genes by intergenic regions: generation of recombinant viruses overexpressing the polymerase gene. *Journal of Virology* **74**(16): 7261–7269.

Finke, S., Cox, J. H. and Conzelmann, K. K. 2000b. Differential transcription attenuation of rabies virus genes by intergenic regions: generation of recombinant viruses overexpressing the polymerase gene. *Journal of Virology* **74**(16): 7261–7269.

Finke, S., Granzow, H., Hurst, J., Pollin, R. and Mettenleiter, T. C. 2010. Intergenotypic replacement of lyssavirus matrix proteins demonstrates the role of lyssavirus M proteins in intracellular virus accumulation. *The Journal of Virology* **84**(4): 1816–1827.

Finke, S., Mueller-Waldeck, R. and Conzelmann, K. K. 2003. Rabies virus matrix protein regulates the balance of virus transcription and replication. *Journal of General Virology* **84**(Pt 6): 1613–1621.

Flanagan, E. B., Ball, L. A. and Wertz, G. W. 2000. Moving the glycoprotein gene of vesicular stomatitis virus to promoter-proximal positions accelerates and enhances the protective immune response. *Journal of Virology* **74**(17): 7895–7902.

Flanagan, E. B., Schoeb, T. R. and Wertz, G. W. 2003. Vesicular stomatitis viruses with rearranged genomes have altered invasiveness and neuropathogenesis in mice. *Journal of Virology* **77**(10): 5740–5748.

Flanagan, E. B., Zamparo, J. M., Ball, L. A., Rodriguez, L. L. and Wertz, G. W. 2001. Rearrangement of the genes of vesicular stomatitis virus eliminates clinical disease in the natural host: new strategy for vaccine development. *Journal of Virology* **75**(13): 6107–6114.

Fu, Z. F. 2005. Genetic comparison of the rhabdoviruses from animals and plants. *Current Topics in Microbiology and Immunology* **292**: 1–24.

Fuerst, T. R., Niles, E. G., Studier, F. W. and Moss, B. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **83**(21): 8122–8126.

Ge, P., Tsao, J., Schein, S., Green, T. J., Luo, M. and Zhou, Z. H. 2010. Cryo-EM model of the bullet-shaped vesicular stomatitis virus. *Science* **327**(5966): 689–693.

Geisbert, T. W., Geisbert, J. B., Leung, A., Addario-DiCaprio, K. M., Hensley, L. E., Grolla, A. and Feldmann, H. 2009. Single-injection vaccine protects nonhuman primates against infection with marburg virus and three species of ebola virus. *The Journal of Virology* **83**(14): 7296–7304.

Ghanem, A., Kern, A. and Conzelmann, K. K. 2011. Significantly improved rescue of rabies virus from cDNA plasmids. *European Journal of Cell Biology* **91**(1): 10–16.

Gomme, E. A., Wanjalla, C. N., Wirblich, C. and Schnell, M. J. 2011. Rabies virus as a research tool and viral vaccine vector. *Advances in Virus Research* **79**: 139–64.

Goodbourn, S. and Randall, R. E. 2009. The regulation of type I interferon production by paramyxoviruses. *Journal of Interferon & Cytokine Research*. **29**(9): 539–547.

Green, T. J., Zhang, X., Wertz, G. W. and Luo, M. 2006. Structure of the vesicular stomatitis virus nucleoprotein-RNA complex. *Science* **313**(5785): 357–360.

Harty, R. N., Brown, M. E., Hayes, F. P., Wright, N. T. and Schnell, M. J. 2001. Vaccinia virus-free recovery of vesicular stomatitis virus. *Journal of Molecular Microbiology and Biotechnology* **3**(4): 513–517.

Hornung, V., Ellegast, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., *et al*. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* **314**(5801): 994–997.

Huang, Y., Tang, Q., Nadin-Davis, S. A., Zhang, S., Hooper, C. D., Ming, P., Du, J., *et al*. 2010. Development of a reverse genetics system for a human rabies virus vaccine strain employed in China. *Virus Research* **149**(1): 28–35.

Inoue, K., Shoji, Y., Kurane, I., Iijima, T., Sakai, T. and Morimoto, K. 2003. An improved method for recovering rabies virus from cloned cDNA. *Journal of Virology Methods* **107**(2): 229–236.

Iseni, F., Garcin, D., Nishio, M., Kedersha, N., Anderson, P. and Kolakofsky, D. 2002. Sendai virus trailer RNA binds TIAR, a cellular protein involved in virus-induced apoptosis. *EMBO Journal* **21**(19): 5141–5150.

Ito, N., Takayama, M., Yamada, K., Sugiyama, M. and Minamoto, N. 2001. Rescue of rabies virus from cloned cDNA and identification of the pathogenicity-related gene: glycoprotein gene is associated with virulence for adult mice. *The Journal of Virology* **75**(19): 9121–9128.

Ito, N., Takayama-Ito, M., Yamada, K., Hosokawa, J., Sugiyama, M. and Minamoto, N. 2003. Improved recovery of rabies virus from cloned cDNA using a vaccinia virus-free reverse genetics system. *Microbiology and Immunology* **47**(8): 613–617.

Jackson, A. O., Dietzgen, R. G., Goodin, M. M., Bragg, J. N. and Deng, M. 2005a. Biology of plant rhabdoviruses. *Annual Review of Phytopathology* **43**: 623–660.

Johnson, J. E., Coleman, J. W., Kalyan, N. K., Calderon, P., Wright, K. J., Obregon, J., Ogin-Wilson, E., *et al*. 2009. In vivo biodistribution of a highly attenuated recombinant vesicular stomatitis virus expressing HIV-1 Gag following intramuscular, intranasal, or intravenous inoculation. *Vaccine*. **27**(22): 2930–2939.

Johnson, M. C., Simon, B. E., Kim, C. H. and Leong, J. A. 2000. Production of recombinant snakehead rhabdovirus: the NV protein is not required for viral replication. *The Journal of Virology* **74**(5): 2343–2350.

Justice, P. A., Sun, W., Li, Y., Ye, Z., Grigera, P. R. and Wagner, R. R. 1995. Membrane vesiculation function and exocytosis of wild-type and mutant matrix proteins of vesicular stomatitis virus. *The Journal of Virology* **69**(5): 3156–3160.

Kapadia, S. U., Rose, J. K., Lamirande, E., Vogel, L., Subbarao, K. and Roberts, A. 2005. Long-term protection from SARS coronavirus infection conferred by a single immunization with an attenuated VSV-based vaccine. *Virology.* **340**(2): 174–182.

Kapadia, S. U., Simon, I. D. and Rose, J. K. 2008. SARS vaccine based on a replication-defective recombinant vesicular stomatitis virus is more potent than one based on a replication-competent vector. *Virology.* **376**(1): 165–172.

Kelly, E. J., Nace, R., Barber, G. N. and Russell, S. J. 2010. Attenuation of vesicular stomatitis virus encephalitis through microRNA targeting. *The Journal of Virology* **84**(3): 1550–1562.

Klingen, Y., Conzelmann, K. K. and Finke, S. 2008. Double-labeled rabies virus: live tracking of enveloped virus transport. *Journal of Virology* **82**(1): 237–245.

Kondo, H., Maeda, T., Shirako, Y. and Tamada, T. 2006. Orchid fleck virus is a rhabdovirus with an unusual bipartite genome. *Journal of General Virology* **87**(Pt 8): 2413–2421.

Lafon, M. 2005. Rabies virus receptors. *Journal of Neurovirology* **11**(1): 82–87.

Lafon, M. 2008. Immune evasion, a critical strategy for rabies virus. *Dev Biol (Basel)* **131**: 413–419.

Lawson, N. D., Stillman, E. A., Whitt, M. A. and Rose, J. K. 1995. Recombinant vesicular stomatitis viruses from DNA. *Proceedings of the National Academy of Sciences of the United States of America* **92**(10): 4477–4481.

Le Mercier, P., Garcin, D., Hausmann, S. and Kolakofsky, D. 2002a. Ambisense sendai viruses are inherently unstable but are useful to study viral RNA synthesis. *The Journal of Virology* **76**(11): 5492–5502.

Le Mercier, P., Jacob, Y., Tanner, K. and Tordo, N. 2002b. A novel expression cassette of lyssavirus shows that the distantly related Mokola virus can rescue a defective rabies virus genome. *The Journal of Virology* **76**(4): 2024–2027.

Lech, P. J. and Russell, S. J. 2010. Use of attenuated paramyxoviruses for cancer therapy. *Expert Review on Vaccines* **9**(11): 1275–1302.

Li, J., Wang, J. T. and Whelan, S. P. 2006. A unique strategy for mRNA cap methylation used by vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America* **103**(22): 8493–8498.

Libersou, S., Albertini, A. A., Ouldali, M., Maury, V., Maheu, C., Raux, H., de, H. F., Roche, S., *et al*. 2010. Distinct structural rearrangements of the VSV glycoprotein drive membrane fusion. *Journal of Cell Biology* **191**(1): 199–210.

Lichty, B. D., Power, A. T., Stojdl, D. F. and Bell, J. C. 2004. Vesicular stomatitis virus: re-inventing the bullet. *Trends in Molecular Medicine* **10**(5): 210–216.

Lieber, A., Kiessling, U. and Strauss, M. 1989. High level gene expression in mammalian cells by a nuclear T7-phase RNA polymerase. *Nucleic Acids Research* **17**(21): 8485–8493.

Longdon, B., Obbard, D. J. and Jiggins, F. M. 2010. Sigma viruses from three species of Drosophila form a major new clade in the rhabdovirus phylogeny. *Proceedings of Society of Biological Sciences* **277**(1678): 35–44.

Luo, M., Green, T. J., Zhang, X., Tsao, J. and Qiu, S. 2007. Conserved characteristics of the rhabdovirus nucleoprotein. *Virus Research* **129**(2): 246–251.

Luytjes, W., Krystal, M., Enami, M., Pavin, J. D. and Palese, P. 1989. Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**(6): 1107–1113.

Ma, Y. and Li, J. 2011. Vesicular stomatitis virus as a vector to deliver virus-like particles of human norovirus: a new vaccine candidate against an important noncultivable virus. *The Journal of Virology* **85**(6): 2942–2952.

Majid, A. M., Barber, G. N., Ma, Y. and Li, J. 2011. Recombinant vesicular stomatitis virus (VSV) and other strategies in HCV vaccine designs and immunotherapy: vesicular stomatitis virus as a vector to deliver virus-like particles of human norovirus: a new vaccine candidate against an important noncultivable virus. *The Journal of Virology* **85**(6): 2942–2952.

Marschalek, A., Drechsel, L. and Conzelmann, K. K. 2012. The importance of being short: the role of rabies virus phosphoprotein isoforms assessed by differential IRES translation initiation. *European Journal of Cell Biology* **91**(1): 17–23.

Marschalek, A., Finke, S., Schwemmle, M., Mayer, D., Heimrich, B., Stitz, L. and Conzelmann, K. K. 2009. Attenuation of rabies virus replication and virulence

by picornavirus internal ribosome entry site elements. *The Journal of Virology* **83**(4): 1911–1919.

Mebatsion, T. 2001. Extensive attenuation of rabies virus by simultaneously modifying the dynein light chain binding site in the P protein and replacing Arg333 in the G protein. *The Journal of Virology* **75**(23): 11496–11502.

Mebatsion, T. and Conzelmann, K. K. 1996. Specific infection of CD4+ target cells by recombinant rabies virus pseudotypes carrying the HIV-1 envelope spike protein. *Proceedings of the National Academy of Sciences of the United States of America* **93**(21): 11366–11370.

Mebatsion, T., Finke, S., Weiland, F. and Conzelmann, K. K. 1997. A CXCR4/CD4 pseudotype rhabdovirus that selectively infects HIV-1 envelope protein-expressing cells. *Cell* **90**(5): 841–847.

Mebatsion, T., Konig, M. and Conzelmann, K. K. 1996. Budding of rabies virus particles in the absence of the spike glycoprotein. *Cell* **84**(6): 941–951.

Mebatsion, T., Schnell, M. J. and Conzelmann, K. K. 1995. Mokola virus glycoprotein and chimeric proteins can replace rabies virus glycoprotein in the rescue of infectious defective rabies virus particles. *Journal of Virology* **69**(3): 1444–1451.

Mebatsion, T., Weiland, F. and Conzelmann, K. K. 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. *Journal of Virology* **73**(1): 242–250.

Ming, P. G., Huang, Y., Tang, Q., Du, J. L., Tao, X. Y., Yan, J. X. and Hu, R. L. 2009. [Construction and analysis of full-length cDNA clone of rabies virus street strain]. *Bing. Du Xue. Bao* **25**(1): 17–22.

Miyamichi, K., Amat, F., Moussavi, F., Wang, C., Wickersham, I., Wall, N. R., Taniguchi, H., *et al*. 2011. Cortical representations of olfactory input by trans-synaptic tracing. *Nature* **472**(7342): 191–196.

Muik, A., Kneiske, I., Werbizki, M., Wilflingseder, D., Giroglou, T., Ebert, O., Kraft, A., *et al*. 2011. Pseudotyping vesicular stomatitis virus with lymphocytic choriomeningitis virus glycoproteins enhances infectivity for glioma cells and minimizes neurotropism. *The Journal of Virology* **85**(11): 5679–5684.

Naeve, C. W., Kolakofsky, C. M. and Summers, D. F. 1980. Comparison of vesicular stomatitis virus intracellular and virion ribonucleoproteins. *Journal of Virology* **33**(2): 856–865.

Niu, X., Wang, H. and Fu, Z. F. 2011. Role of chemokines in rabies pathogenesis and protection. *Advances in Virus Research* **79**: 73–89.

Obuchi, M., Fernandez, M. and Barber, G. N. 2003. Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity. *The Journal of Virology* **77**(16): 8843–8856.

Ogino, T. and Banerjee, A. K. 2007. Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Molecular Cell* **25**(1): 85–97.

Orbanz, J. and Finke, S. 2010. Generation of recombinant European bat lyssavirus type 1 and inter-genotypic compatibility of lyssavirus genotype 1 and 5 antigenome promoters. *Archives of Virology* **155**(10): 1631–1641.

Park, K. H., Huang, T., Correia, F. F. and Krystal, M. 1991. Rescue of a foreign gene by Sendai virus. *Proceedings of the National Academy of Sciences of the United States of America* **88**(13): 5537–5541.

Paterson, R. G., Russell, C. J. and Lamb, R. A. 2000. Fusion protein of the paramyxovirus SV5: destabilizing and stabilizing mutants of fusion activation. *Virology* **270**(1): 17–30.

Pattnaik, A. K., Ball, L. A., LeGrone, A. W. and Wertz, G. W. 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**(6): 1011–1020.

Pattnaik, A. K. and Wertz, G. W. 1990. Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs 42. *The Journal of Virology* **64**(6): 2948–2957.

Pattnaik, A. K. and Wertz, G. W. 1991. Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles. *Proceedings of the National Academy of Sciences of the United States of America* **88**(4): 1379–1383.

Perrotta, A. T. and Been, M. D. 1990. The self-cleaving domain from the genomic RNA of hepatitis delta virus: sequence requirements and the effects of denaturant. *Nucleic Acids Research* **18**(23): 6821–6827.

Prehaud, C., Lay, S., Dietzschold, B. and Lafon, M. 2003. Glycoprotein of nonpathogenic rabies viruses is a key determinant of human cell apoptosis. *Journal of Virology* **77**(19): 10537–10547.

Pringle, C. R. 1997. The order Mononegavirales: current status. *Archives of Virology* **142**(11): 2321–2326.

Qanungo, K. R., Shaji, D., Mathur, M. and Banerjee, A. K. 2004. Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. *Proceedings of the National Academy of Sciences of the United States of America* **101**(16): 5952–5957.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G. and Billeter, M. A. 1995. Rescue of measles viruses from cloned DNA. *EMBO Journal* **14**(23): 5773–5784.

Randall, R. E. and Goodbourn, S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *Journal of General Virology* **89**(Pt 1): 1–47.

Redinbaugh, M. G. and Hogenhout, S. A. 2005. Plant rhabdoviruses. *Current Topics in Microbiology and Immunology* **292**: 143–163.

Rieder, M., Brzózka, K., Pfaller, C. K., Cox, J. H., Stitz, L. and Conzelmann, K. K. 2011. Genetic dissection of interferon-antagonistic functions of rabies virus phosphoprotein: inhibition of interferon regulatory factor 3 activation is important for pathogenicity. *The Journal of Virology* **85**(2): 842–852.

Rieder, M. and Conzelmann, K. K. 2009. Rhabdovirus evasion of the interferon system. *Journal of Interferon and Cytokine Res*. **29**(9): 499–509.

Rolls, M. M., Webster, P., Balba, N. H. and Rose, J. K. 1994. Novel infectious particles generated by expression of the vesicular stomatitis virus glycoprotein from a self-replicating RNA. *Cell* **79**(3): 497–506.

Schneider, U., Naegele, M., Staeheli, P. and Schwemmle, M. 2003. Active borna disease virus polymerase complex requires a distinct nucleoprotein-to-phosphoprotein ratio but no viral X protein. *The Journal of Virology* **77**(21): 11781–11789.

Schnell, M. J., Buonocore, L., Boritz, E., Ghosh, H. P., Chernish, R. and Rose, J. K. 1998. Requirement for a non-specific glycoprotein cytoplasmic domain sequence to drive efficient budding of vesicular stomatitis virus. *EMBO Journal* **17**(5): 1289–1296.

Schnell, M. J., Buonocore, L., Kretzschmar, E., Johnson, E. and Rose, J. K. 1996. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. *Proceedings of the National Academy of Sciences of the United States of America* **93**(21): 11359–11365.

Schnell, M. J., Johnson, J. E., Buonocore, L. and Rose, J. K. 1997. Construction of a novel virus that targets HIV-1-infected cells and controls HIV-1 infection. *Cell* **90**(5): 849–857.

Schnell, M. J., Mebatsion, T. and Conzelmann, K. K. 1994. Infectious rabies viruses from cloned cDNA. *EMBO Journal* **13**(18): 4195–4203.

Schubert, M., Joshi, B., Blondel, D. and Harmison, G. G. 1992. Insertion of the human immunodeficiency virus CD4 receptor into the envelope of vesicular stomatitis virus particles. *The Journal of Virology* **66**(3): 1579–1589.

Schwartz, J. A., Buonocore, L., Suguitan, A. L., Jr., Silaghi, A., Kobasa, D., Kobinger, G., Feldmann, H., *et al*. 2010. Potent vesicular stomatitis virus-based avian influenza vaccines provide long-term sterilizing immunity against heterologous challenge. *The Journal of Virology* **84**(9): 4611–4618.

Seif, I., Coulon, P., Rollin, P. E. and Flamand, A. 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. *The Journal of Virology* **53**(3): 926–934.

Sharmeen, L., Kuo, M. Y., Dinter-Gottlieb, G. and Taylor, J. 1988. Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *The Journal of Virology* **62**(8): 2674–2679.

Shih, I. H. and Been, M. D. 2002. Catalytic strategies of the hepatitis delta virus ribozymes. *Annual Review of Biochemistry* **71**: 887–917.

Skall, H. F., Olesen, N. J. and Mellergaard, S. 2005. Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming: a review. *Journal of Fish Diseases* **28**(9): 509–529.

Smith, M. E., Koser, M., Xiao, S., Siler, C., McGettigan, J. P., Calkins, C., Pomerantz, R. J., *et al*. 2006. Rabies virus glycoprotein as a carrier for anthrax protective antigen. *Virology* **353**(2): 344–356.

Stepien, A. E., Tripodi, M. and Arber, S. 2010. Monosynaptic rabies virus reveals premotor network organization and synaptic specificity of cholinergic partition cells. *Neuron* **68**(3): 456–472.

Sutter, G., Ohlmann, M. and Erfle, V. 1995. Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Letters* **371**(1): 9–12.

Tao, L., Ge, J., Wang, X., Zhai, H., Hua, T., Zhao, B., Kong, D., *et al*. 2010. Molecular basis of neurovirulence of flury rabies virus vaccine strains: importance of the polymerase and the glycoprotein R333Q mutation. *The Journal of Virology* **84**(17): 8926–8936.

Theriault, S., Groseth, A., Artsob, H. and Feldmann, H. 2005. The role of reverse genetics systems in determining filovirus pathogenicity. *Archives of Virology Supplement* **19:** 157–177.

Thoulouze, M. I., Bouguyon, E., Carpentier, C. and Bremont, M. 2004. Essential role of the NV protein of Novirhabdovirus for pathogenicity in rainbow trout. *The Journal of Virology* **78**(8): 4098–4107.

Ugolini, G. 1995. Specificity of rabies virus as a transneuronal tracer of motor networks: transfer from hypoglossal motoneurons to connected second-order and higher order central nervous system cell groups. *Journal of Comparative Neurology* **356**(3): 457–480.

Ugolini, G. 2010. Advances in viral transneuronal tracing. *Journal of Neuroscience Methods* **194**(1): 2–20.

Walker, P. J. 2005. Bovine ephemeral fever in Australia and the world. *Current Topics in Microbiology and Immunology* **292**: 57–80.

Wall, N. R., Wickersham, I. R., Cetin, A., De La, P. M. and Callaway, E. M. 2010. Monosynaptic circuit tracing in vivo through CRE-dependent targeting and complementation of modified rabies virus. *Proceedings of the National Academy of Sciences of the United States of America* **107**(50): 21848–21853.

Weible, A. P., Schwarcz, L., Wickersham, I. R., Deblander, L., Wu, H., Callaway, E. M., Seung, H. S. and Kentros, C. G. 2010. Transgenic targeting of recombinant rabies virus reveals monosynaptic connectivity of specific neurons. *Journal of Neuroscience* **30**(49): 16509–16513.

Wen, Y., Wang, H., Wu, H., Yang, F., Tripp, R. A., Hogan, R. J. and Fu, Z. F. 2011. Rabies virus expressing dendritic cell-activating molecules enhances the innate and adaptive immune response to vaccination. *The Journal of Virology* **85**(4): 1634–1644.

Wertz, G. W., Perepelitsa, V. P. and Ball, L. A. 1998. Gene rearrangement attenuates expression and lethality of a nonsegmented negative strand RNA virus. *Proceedings of the National Academy of Sciences of the United States of America* **95**(7): 3501–3506.

Whelan, S. P., Ball, L. A., Barr, J. N. and Wertz, G. T. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proceedings of the National Academy of Sciences of the United States of America* **92**(18): 8388–8392.

Whelan, S. P., Barr, J. N. and Wertz, G. W. 2004. Transcription and replication of nonsegmented negative-strand RNA viruses. *Current Topics in Microbiology and Immunology* **283**: 61–119.

Whelan, S. P. and Wertz, G. W. 1999. The 5′ terminal trailer region of vesicular stomatitis virus contains a position-dependent cis-acting signal for assembly of RNA into infectious particles. *The Journal of Virology* **73**(1): 307–315.

Wickersham, I. R., Finke, S., Conzelmann, K. K. and Callaway, E. M. 2007a. Retrograde neuronal tracing with a deletion-mutant rabies virus. *Nature Methods* **4**(1): 47–49.

Wickersham, I. R., Lyon, D. C., Barnard, R. J., Mori, T., Finke, S., Conzelmann, K. K., Young, J. A. and Callaway, E. M. 2007b. Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron* **53**(5): 639–647.

Wiegand, M. A., Bossow, S., Schlecht, S. and Neubert, W. J. 2007. De novo synthesis of N and P proteins as a key step in Sendai viral gene expression. *Journal of Virology* **81**(24): 13835–13844.

Witko, S. E., Kotash, C. S., Nowak, R. M., Johnson, J. E., Boutilier, L. A., Melville, K. J., Heron, S. G., *et al*. 2006. An efficient helper-virus-free method for rescue of recombinant paramyxoviruses and rhadoviruses from a cell line suitable for vaccine development. *Journal of Virological Methods* **135**(1): 91–101.

Wu, L., Huang, T. G., Meseck, M., Altomonte, J., Ebert, O., Shinozaki, K., Garcia-Sastre, A., *et al*. 2008. rVSV(M Delta 51)-M3 is an effective and safe oncolytic virus for cancer therapy. *Human Gene Therapy* **19**(6): 635–647.

Wu, X. and Rupprecht, C. E. 2008. Glycoprotein gene relocation in rabies virus. *Virus Research*. **131**(1): 95–99.

Wyatt, L. S., Moss, B. and Rozenblatt, S. 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210**(1): 202–205.

Yonehara, K., Balint, K., Noda, M., Nagel, G., Bamberg, E. and Roska, B. 2011. Spatially asymmetric reorganization of inhibition establishes a motion-sensitive circuit. *Nature* **469**(7330): 407–410.

Zhao, L., Toriumi, H., Kuang, Y., Chen, H. and Fu, Z. F. 2009. The roles of chemokines in rabies virus infection: overexpression may not always be beneficial. *The Journal of Virology* **83**(22): 11808–11818.

Zinkernagel, R. M. 1997. Felix Hoppe-Seyler Lecture 1997. Protective antibody responses against viruses. *Biological Chemistry* **378**(8): 725–729.

6

Modification of measles virus and application to pathogenesis studies

Linda J. Rennick and W. Paul Duprex
Department of Microbiology, Boston University, School of Medicine and National Emerging Infectious Disease Laboratories, Boston University

6.1 Introduction

There are several excellent reviews on the molecular biology, immunology and pathogenesis of measles virus (MV) which have been published recently (De Swart, 2009; Gerlier and Valentin, 2009; Hahm, 2009; Navaratnarajah *et al*., 2009; Rima and Duprex, 2009; Schneider-Schaulies and Schneider-Schaulies, 2009; Sellin and Horvat, 2009; Yanagi *et al*., 2009; Schneider-Schaulies and Duprex, 2011). Readers are directed to these publications for further details of the topics which, for brevity, can only be summarised in the introduction below, particularly the adaptive (Schneider-Schaulies and Duprex, 2011) and innate (Rima and Duprex, 2011) immunological aspects of the disease. To complement these reviews we have judiciously selected representative recombinant (r) MVs and used them to illustrate the power of MV reverse genetics approaches in helping us to understand the pathology of the disease and the underlying biology of the virus.

6.2 Measles: the disease

Measles is typically a childhood infection of humans and is spread by the respiratory route (Griffin, 2007). The disease is characterised by a latent period of 10–14 days, a short prodromal phase of fever, coryza, cough and conjunctivitis, which is then followed by the appearance of the characteristic maculopapular rash. Rash onset coincides with the appearance of the immune response and initiation of virus clearance. Recovery is accompanied by lifelong immunity to reinfection (Panum,

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

1938). Monkeys exposed to infected humans or that have been experimentally infected with wild-type strains of MV develop a similar disease (van Binnendijk *et al*., 1995) and much of the more detailed knowledge of pathogenesis and sites of viral replication come from studies of non-human primates (De Swart, 2009). Significant progress has been made in understanding viral pathogenesis by harnessing optimal wild-type rMVs expressing fluorescent proteins and the measles monkey model and this serves to illustrate the power of reverse genetics (see below).

MV is spread via aerosol droplets and, historically, the virus has been proposed to target epithelial cells in respiratory surfaces (Griffin, 2007). However, recent experiments in our laboratory strongly suggest that the early target cells of MV are signalling lymphocyte activation molecule (SLAM), or more correctly CD150-expressing, alveolar macrophages or dendritic cells, which traffic the virus to bronchus associated lymphoid tissue (BALT) and/or regional lymph nodes (Lemon *et al*., 2011). Here they disseminate the infection to the plethora of CD150-expressing cells found in close proximity in the BALT, which provides the perfect environment for the amplification of a highly cell-associated, lymphotropic virus such as MV. Lymphocytes are the key cells infected in the blood, although monocytes have also been shown to be infected (Osunkoya *et al*., 1990; Esolen *et al*., 1993; de Swart *et al*., 2007), and these facilitate the systemic spread of the virus to organs throughout the body. Epithelial cells have a role in MV pathogenesis but at a later stage (Noyce *et al*., 2011) and it is proposed that epithelial cells in the respiratory surfaces facilitate the release of virus from the host, demonstrated by the fact that viruses which cannot enter these cells are not released (Leonard *et al*., 2008). Complications of MV infection can occur when the virus crosses the blood–brain barrier (by an unknown mechanism) where it can cause encephalitis and in a small number of cases the disease subacute sclerosing panencephalitis (SSPE) which is invariably fatal (Griffin, 2007; Schneider-Schaulies *et al*., 2003). Understanding MV entry and spread in the central nervous system (CNS) has also been facilitated by the use of rMV (see below).

6.3 Measles: the infectious agent

MV virions have a host cell derived lipid envelope and are highly pleomorphic, averaging in size from 120–250 nm. However, their diameters can reach up to 1000 nm, meaning that the cargo space varies significantly from 3×10^5 to $>10^7$ nm^3. As a result, they can accommodate more than one genome and many virions are functionally polyploid (Rager *et al*., 2002). Whether or not the virions have the same size distribution *in vivo* is unknown as they are highly cell-associated. The negative sensed genomic RNA is encapsidated with nucleoprotein (N) and associated with the large- (L) and phospho- (P) proteins, which together form the RNA-dependent RNA polymerase (RdRp) of the virus (Griffin, 2007). The hydrophobic matrix (M) protein is believed to act as the bridge between the encapsidated genome and the cytoplasmic tails of the two surface glycoproteins, the fusion (F) and

hemagglutinin (H) proteins, which protrude as 5–8 nm long spike-like projections from the envelope surface (Tahara *et al*., 2007b). The F and H glycoproteins are type I and type 2 proteins respectively. The M protein is not an integral membrane protein, but associates with the encapsidated genome within virions when observed by electron microscopy (Plate 3).

Classified as a morbillivirus, in the family *Paramyxoviridae* and order *Mononegavirales*, MV has a non-segmented, negative-sense, single-stranded RNA genome which is 15,894 nucleotides in length (Plate 3). The 3′ to 5′ genome encodes six major structural proteins within six transcription units (TUs) in the order N, P, M, F, H and L, Individual TUs are separated by an intergenic (Ig) trinucleotide spacer, 3′-GAA-5′ for all Ig spacers apart from that between H and L which is 3′-GCA-5′. These are not transcribed into mRNA during transcription by the RdRp. Each TU has a 5′ untranslated region (5′ UTR) upstream, and a 3′ UTR downstream, of the open reading frame (ORF). The 5′ and 3′ UTRs contain short, highly conserved gene start (GS) and gene end (GE) signals respectively which surround the Ig trinucleotide spacer. The GS signal is required for transcription initiation by the RdRp and the GE contains a polyadenylation signal on which the RdRp stutters, thereby adding a non-templated poly-A tail to the viral mRNA. These signals direct termination of one mRNA transcript and re-initiation at the downstream TU. The coding potential of the genome is further increased by the use of overlapping reading frames and a pseudotemplated transcription process, termed RNA editing, in the P gene (Cattaneo *et al*., 1989). This leads to expression of the non-structural C and V proteins respectively (Bellini *et al*., 1985; Cattaneo *et al*., 1989) and thus it is more formally correct to call this transcription unit P/C/V. It is possible that the P gene contains further coding potential. A protein, R, generated by ribosomal frameshifting during translation has been detected in infected cells, although a function has not been attributed to it (Liston and Briedis, 1995). Also a third open reading frame which overlaps the P and C open reading frames at the beginning of the P gene affects growth *in vitro* (Rennick *et al*., in preparation).

The gene order was originally inferred from Northern blot analysis of the mono- and poly-cistronic mRNA transcripts isolated from MV infected cells (Barrett and Underwood, 1985; Richardson *et al*., 1985; Dowling *et al*., 1986; Rima *et al*., 1986; Yoshikawa *et al*., 1986). Later they were determined to be correct by sequencing (Blumberg *et al*., 1988, Crowley *et al*., 1988, Alkhatib and Briedis, 1986, Bellini *et al*., 1986, Gerald *et al*., 1986, Richardson *et al*., 1986, Bellini *et al*., 1985, Rozenblatt *et al*., 1985). The six TUs are flanked by a 52 nucleotide leader (Le) sequence and a 37 nucleotide trailer (Tr) sequence at the 3′ and 5′ ends of the genome. The first 18 nucleotides of the MV Le and Tr show high sequence complementarity and this region is referred to as the ‘A box’. Due to this sequence complementarity, these regions are suggested to contain one of the elements of the promoter which is used when the RdRp functions as a replicase (Leppert *et al*., 1979; Horikami and Moyer, 1991). The genomic promoter (GP), located at the 3′ end of the genome, is a weaker promoter than the antigenomic promoter (AGP) which is located at the 3′ end of the antigenome and therefore the virus produces more copies of the negative-sensed

genomic RNA which is required for packaging into nascent virions. The replication promoter is bipartite and the RdRp also requires a motif within the N 5′ UTR; the 5′-$(GN_5)_3$-3′ motif, a stretch of conserved, equally spaced G nucleotides starting at position 79 of the genome, referred to as the 'B box' (Rennick *et al*., 2007; Walpita, 2004). An equivalent motif is also present as part of the AGP.

Studies initially performed with Sendai virus (SeV) minigenomes revealed that templates can only be efficiently replicated when their length is an exact multiple of six (Calain and Roux, 1993). This provides a functional basis for the previous observation that each N protein interacts with six nucleotides (one hexamer) of the genomic RNA (Egelman *et al*., 1989). This restriction has been termed the 'rule of six'. It has been shown that MV also adheres to this rule (Radecke *et al*., 1995; Sidhu *et al*., 1995). This has consequences for reverse genetics in that all rMV genomes must be engineered to have a total number of nucleotides which is a multiple of six.

As N proteins interact with six nucleotides of the genome, it means that the genome is likely to be exactly encapsidated with N subunits. This results in an exact nucleotide and N subunit match at the 3′ OH end of the genomic RNA, referred to as 3′ OH congruence, and this may be important in recognition of the GP. Alternatively, assuming that the six positions for interaction of N with the nucleotides are not equivalent, the nucleotide position relative to N (the N phase context) may be the critical factor. A study into whether the 3′ OH congruence or the N phase context was the critical factor was carried out for SeV (Vulliemoz and Roux, 2001). This study used minigenomes, for which the 3′ OH congruence of the GP could be discriminated from its N phase context. The 3′ OH congruence of the template was not critical for replication in cases where the N phase context of the promoter was conserved. However, when the N phase context was changed, so as to obliterate replication, the 3′ OH congruence could not restore replication. This strongly suggests that the application of the 'rule of six' depends on the recognition of nucleotides positioned in the proper hexameric phase. Phase is thought to be important in recognising the 'B box' with each conserved G nucleotide being in the same phase with respect to the N protein. Other elements which have been proposed to represent non-random positioning with regard to phase include the GE and GS signals and the editing site (Kolakofsky *et al*., 1998). A series of rMVs with modifications which shifted these regions into all possible phases exhibited an abnormal pattern of read-through at the M/F and F/H Ig boundaries (Billeter, 2000) and viruses showed some alteration in editing of the P/C/V gene. This suggests that the nucleotides participating in these signals might be recognised differently depending on their phase.

In the case of all non-segmented, negative-sensed, RNA viruses, the naked RNA genome is not infectious and is never found in that state in the cytoplasm. Instead, the genome is tightly encapsidated by N protein, forming a ribonucleoprotein (RNP) complex (Lamb and Parks, 2007). For MV the basic unit of infectivity consists of this RNP in complex with the RdRp and is most precisely termed the (−)RNP; this acts as a template for replication and transcription. The antigenome is encapsidated within the (+)RNP and this acts as a replicative intermediate functioning as

the template for the generation of progeny (−)RNPs. These are transported to the plasma membrane for incorporation into nascent virions. The fully encapsidated RNA forms a helical RNase resistant structure which is 1 μm long with a diameter of 18-21nm. Encapsidation of the basic unit of infectivity has consequences for the development of reverse genetics systems for the virus and it proved to be essential to appreciate the intricacies of the virus molecular biology for the successful recovery of rMVs (see below).

6.4 RNA synthesis: a tail of two processes

The MV RdRp must perform two distinct processes in order to produce progeny virus particles (Lamb and Parks, 2007). First, in primary transcription, the genes are transcribed to produce mRNAs, which are in turn translated by the host cellular machinery to produce the viral proteins. Sufficient amounts of nascent proteins are required for secondary transcription, genome replication and for the assembly of infectious virus particles. Second, the genome must be replicated to produce new genomes, which are encapsidated with N protein and packaged into progeny virus particles. Replication of the genome occurs in two steps. Initially a full-length antigenomic (+)RNP is generated from the incoming (−)RNP. The (+)RNP then acts as a template for the generation of full-length (−)RNPs. As a negative sense RNA virus, MV has to overcome the fact that there is no host RdRp capable of producing RNA from the (−)RNP. Additionally, it must employ mechanisms to prevent the formation of double stranded RNA intermediates, since these structures evoke an antiviral response in the host cell (reviewed in Gerlier and Valentin, 2009). MV overcomes these two difficulties by encoding an RdRp, which is incorporated into every virion to ensure infectivity (Baltimore *et al*., 1970), by packaging its genome as a (−)RNP, and by replicating its genome within a (+)RNP.

6.5 Transcription: starting, stopping, dropping off or starting again

There has been considerable debate as to whether the RdRp accesses the N GS signal directly or if it engages the (−)RNP at the extreme 3′ end and then scans to the GS signal thereby producing a short unencapsidated Le RNA. Such uncapped Le transcripts would be inherently unstable in infected cells and this could explain why they are difficult to detect. However, an *in vitro* study of MV transcription using purified virus has demonstrated that the RdRp can initiate transcription from the (−)RNP at the 3′ terminal nucleotide of the genome (an A nucleotide) and synthesise a Le RNA (Horikami and Moyer, 1991). This suggests, at least *in vitro*, that the RdRp accesses the 3′ end of the genome, and when it reaches the N GS signal it terminates synthesis of the Le RNA, and reinitiates to transcribe the N gene mRNA.

Free Le RNA has been detected in MV-infected A549 cells, but not in infected Vero cells (Crowley *et al*., 1988; Horikami and Moyer, 1991). This suggests that the stability of the leader RNA may be cell line dependent (Horikami and Moyer, 1995) or alternatively, that there is a host cell-dependent factor which determines the site of polymerase entry for transcription.

Studies using the prototypic non-segmented negative strand RNA virus, vesicular stomatitis virus (VSV), have shown that non-segmented negative strand RNA viruses employ a stop–start model of sequential transcription (reviewed in Barr *et al*., 2002). Transcription is obligatorily sequential (Abraham and Banerjee, 1976; Ball and White, 1976; Ball, 1977), discontinuous (Iverson and Rose, 1981) and polar (Villarreal *et al*., 1976). Transcription initiates at the 3′ end of the (−)RNP at the GS signal of the N gene, placing the N gene in the promoter proximal position. Each mRNA is synthesised in an order which reflects the position of the TU with respect to this single 3′ promoter. The abundance of mRNA transcripts decreases according to the relative distance of the TU from the 3′ end of the genome, resulting in many more mRNA transcripts from promoter proximal TUs such as the N and P/C/V genes than from promoter distal TUs, for example, the L gene. This phenomenon, known as transcription attenuation, reflects the sequential and polar transcription of the viral genome from the single promoter (Iverson and Rose, 1981) and also occurs for MV (Iverson and Rose, 1981; Schneider-Schaulies *et al*., 1989) and SeV (Homann *et al*., 1990). This attenuation is thought to occur by dissociation of transcriptionally active RdRp from the (−)RNP at the non-transcribed Ig spacers. Since the RdRp can only access downstream genes by first transcribing those upstream, this leads to the observed gradient of transcription. Sensitive Northern blotting studies have been used to measure the abundance of mRNA transcripts in infected cells and these confirmed the expected gradient in their abundance dependent on the position of the corresponding TU in the viral genome (Cattaneo *et al*., 1987; Schneider-Schaulies *et al*., 1989). A polar gradient of mRNA transcripts was also synthesised from purified MV *in vitro* (Horikami and Moyer, 1991). However, in this case the gradient was much steeper with little synthesis of the promoter distal genes suggesting that the *in vitro* reaction was either intrinsically inefficient, or that it required a cellular factor to enhance the processivity of the RdRp, or to promote polyadenylation or capping. It is likely that cellular factors have a part to play in MV RNA synthesis, for example, tubulin has been shown to stimulate RNA synthesis *in vitro* (Moyer *et al*., 1990) and promote maturation of progeny virus particles (Berghall *et al*., 2004).

6.6 From transcription to replication: the elusive switch

Translation of mRNA transcripts produced during primary transcription results in the intracellular accumulation of viral proteins. Historically it was believed that the major regulator of the switch from transcription to replication was the concentration of unassembled N protein, which is present as a pre-assembly complex with

P (N-P). Release of the N protein from this complex is required to encapsidate the nascent antigenomes and genomes produced by replication (Curran and Kolakofsky, 1999). The hypothesis is that this creates a self-regulatory mechanism in which, when the concentration of unassembled N-P complex is high, replication is favoured, and when the concentration falls, transcription is favoured. However, there is mounting evidence that the switch is not regulated in this manner. For example, a mutant SeV L protein has been reported which continues to make viral mRNA in the absence of replication; where high concentrations of unassembled N are present (Chandrika *et al*., 1995). Furthermore, human respiratory syncytial virus (HRSV) minigenome studies indicate that increased expression of N enhances both replication and transcription (Fearns *et al*., 1997). It has been suggested that the availability of N protein is just a prerequisite for replication and that other factors determine whether the (−)RNP functions as a template for transcription or replication. These may include either post-translational modifications of the P and L proteins (Hwang *et al*., 1999, Das *et al*., 1998, Pattnaik *et al*., 1997) or involvement of other viral and/or cellular factors. For HRSV, the absence of the M2-2 protein resulted in enhanced transcription and reduced replication (Bermingham and Collins, 1999), and the rabies virus (RABV) matrix protein has been shown to regulate the balance of virus transcription and replication (Finke *et al*., 2003). Tangentially, although possibly highly relevant, the first reverse genetics system used to recover rMV depends on cell lines which stably express the N and P proteins (see below). This illustrates that high concentrations of the N protein do not preclude the transcription, replication, assembly or egress of the virus or interfere dramatically with the virus life-cycle.

Regardless of the mechanism of the switch from transcription to replication a question which remains is, ‘How does the RdRp change from an entity that recognises the *cis*-acting signals for transcription of mRNA transcripts to one which is not responsive to them, and thereby replicates the same template?’ Current models suggest that the factor which determines whether the RdRp functions as a transcriptase or a replicase is whether or not the nascent RNA is encapsidated by N protein. For VSV it has been shown that unencapsidated Le RNA accumulates in cells early in infection, and unencapsidated Le RNA has also been found in certain SeV- and MV-infected cells (Crowley *et al*., 1988; Curran and Kolakofsky, 1991). Encapsidation of the nascent Le RNA is thought to be the anti-termination signal that causes the RdRp to ignore the first GS signal at the junction between the Le and the N gene, and go into a replicative mode. It has been assumed that the template is somehow altered, perhaps by N protein, to prevent the polymerase from recognising the *cis*-acting transcription signals. However, a natural mutant of VSV, which has a single mutation in the N gene, has been described which synthesises twice as much N gene mRNA as Le RNA (Chuang *et al*., 1997) leading to the suggestion that transcription initiates internally, directly at the N gene start signal. Following on from this, a model which postulates that there are two distinct forms of the RdRp complex: one for Le synthesis and one for internal initiation has been proposed. This is supported by a study which found that there is competition for the RdRp between the SeV N gene mRNA start site and the 3′ end of the genome

(Le Mercier *et al*., 2003). Another study on VSV has identified a novel tripartite polymerase complex which is involved in replication of the genome RNA (Gupta *et al*., 2003). Biochemical characterisation of this complex led the authors to propose that the transcriptase and replicase complexes of VSV are separate entities with the first being involved in producing capped mRNA transcripts (transcription), and the second being responsible for producing antigenomic RNA (replication). These studies suggest that the RdRp is predestined for either transcription or replication prior to accessing the (−)RNP, supporting the idea that the replicase and transcriptase are distinct separate entities.

All of these processes are important to understand for anyone wishing to develop and use reverse genetics systems for MV in particular and negative strand RNA viruses in general. This is perfectly illustrated in the 'eureka moment' which led to the recovery of a recombinant RABV (Schnell *et al*., 1994). Until then, negative strand RNA virologists were fixated on the generation of reverse genetics systems centred on genomic sense, full-length clones; they had no success. Recovery of a recombinant virus was only achieved upon the realisation that unencapsidated primary transcripts from such clones would be perfect reverse complements of the mRNA transcripts needed to generate the N, P and L proteins required for the assembly of a transcriptionally active (−)RNP. Only when an antigenomic, full-length clone was generated, thereby assembling a replication competent (+)RNP, was the first non-segmented negative sense RNA virus recovered (see below).

6.7 Getting in and getting out

There are three cell surface molecules which have been shown to act as MV entry receptors. Signalling lymphocyte activation molecule (SLAM), also known as CD150, is the molecule which best explains the lymphotropic nature of the wild-type virus, and much of the virus pathogenesis, since it is expressed on the activated T-cells, B-cells, macrophages, mature dendritic cells, platelets, and thymocytes shown to be infected during natural MV infections (Tatsuo *et al*., 2000). Very recently another receptor molecule, PVRL4, also known as nectin-4, has been identified, which explains the ability of wild-type MV to infect epithelial cells, which do not express CD150 (Noyce *et al*., 2011). The existence of an epithelial receptor (EpR) had been postulated since the identification of CD150 and residues on the MV H protein which interact with the EpR had been identified before the actual receptor molecule [(Leonard *et al*., 2008) see below]. During passage in non-disease relevant cell lines such as MRC-5 or chicken embryo fibroblast cells, vaccine strains of MV, by mutation of a handful of amino acids in the H glycoprotein, have evolved the ability to also use CD46 as an entry receptor (Naniche *et al*., 1993, Dörig *et al*., 1993). However, they still retain the ability to bind and use CD150 to enter cells and, although almost all nucleated cells in the human body express CD46 on their surface, vaccine strains target the same cells as wild-type virus during animal infection studies suggesting that CD46 is probably largely irrelevant *in vivo* (de Vries

et al., 2010). Thus the acquisition of CD46 use reflects tissue culture adaptation of the virus to cells lacking the true MV receptors. At this point it is not clear whether the ability to use CD46 plays a role in virus attenuation or whether it is purely a reflection of the empirical tissue culture adaptation process which was used to generate the vaccine viruses in the first place. The fact that vaccine viruses retain their ability to use CD150 and that they target the same cells as the wild-type viruses *in vivo,* suggests that their CD46 use is purely a by-product of the attenuation process and, although necessary for propagation of the vaccine *in vitro*, its use is not pathologically relevant.

Transport of the MV structural proteins within the cell, trafficking of the RNPs to the plasma membrane, and formation of nascent virions and their release are the least well understood aspects of the MV life-cycle. It is clear that the M protein is a key player in these processes and it is thought to induce condensation of the RNPs, which are subsequently recruited to the plasma membrane. In polarised cells it also mediates localisation of the F and H glycoproteins to the apical surface, which assists in particle production (Runkler *et al*., 2007). The M protein is also enriched in lipid rafts within the plasma membrane (Manie *et al*., 2000), and this has been shown to be essential for the release of virus-like particles from cells (Pohl *et al*., 2007). Once again rMVs have been invaluable tools in dissecting the cell biological aspects of the virus life-cycle, and viruses with point mutations in sorting signals which direct intracellular localisation, or ones with tagged viral proteins have helped to understand the spatial and temporal aspects of the infection.

6.8 Measles virus: reverse genetics

The development of reverse genetic systems for the *de novo* synthesis of negative sense RNA viruses from cloned cDNA has revolutionised the study of these viruses (reviewed in Conzelmann, 2004). The complexity and technical challenges associated with this achievement are probably best illustrated by the fact that positive sense RNA viruses were produced from cDNA clones nearly fifteen years before Conzelmann recovered the first non-segmented negative strand RNA virus (Taniguchi *et al*., 1978; Racaniello and Baltimore, 1981; Schnell *et al*., 1994). This is understandable as the genomic RNA of positive strand RNA viruses is infectious when transfected into cells meaning that for most, recombinant viruses can be generated by simply introducing the viral RNA or a full-length infectious cDNA clone into cells in the absence of additional viral protein factors. Several fundamental aspects of negative sense RNA virus biology make the process for these viruses much less straightforward (see above). The main problem encountered in producing negative sense RNA viruses from cDNA clones (virus ‘rescue’) is that the genomic RNA alone is not the biologically active entity for replication, transcription or translation. Regardless of how the RNA is generated, it must, at some point, be encapsidated by N protein. Even then it is not replication-competent until

it associates with the RdRp complex. Once assembled, this (+)RNP acts as the template for replication resulting in generation of (−)RNPs. Transcription of mRNAs, encoding the structural proteins required for assembly of the rMV virions, occurs from the (−)RNPs which may also be further replicated. This means that as well as generating or introducing a 15,894 nucleotide-long RNA into cells, the N, P and L proteins must be provided *in trans*, from a 'helper' virus, by co-transfection of expression plasmids or by stable expression within a cell. Generation of an rMV is further complicated by the fact that the virus will not tolerate additional nucleotides at the 3′ or 5′ termini of the RNA meaning that steps must be taken to ensure that the initial RNA molecule has the exact viral termini. Commensurate with this is the fact that the recombinant genomes will not be replicated unless the total number of nucleotides is a multiple of six, and there is also evidence that the phase of the *cis*-acting signals must be maintained in modified genomes.

Early attempts to rescue negative sense RNA viruses relied on the reconstitution of viral RNP complexes. The first virus for which this approach was successfully used was the segmented virus influenza A (Luytjes *et al*., 1989). RNA transcripts, generated *in vitro* and containing the authentic terminal sequences from an influenza A genome segment flanking the gene for chloramphenicol acetyl transferase (CAT), were encapsidated *in vitro* by purified influenza A virus N protein, and the viral polymerase proteins (PA, PB1 and PB2) to produce a synthetic RNP complex. After transfection of this RNP complex into influenza A virus-infected cells, the synthetic genome segment was successfully replicated and transcribed to generate CAT protein. Interestingly, this approach failed to rescue non-segmented, negative sense RNA viruses probably due to the tighter RNP complex structure in these viruses (Baudin *et al*., 1994). The first successful demonstration of rescue of a foreign gene for MV involved the use of a negative-sensed minigenome; the MV 3′ and 5′ non-coding termini (NCT) consisting of the Le and Tr (containing the GP and AGP promoters) and the N 5′ UTR and L 3′ UTR (containing the N GS and the L GE signals), flanking the ORF for the CAT reporter gene (Sidhu *et al*., 1995). These sequences were cloned into a plasmid designed to produce an *in vitro* minigenome RNA transcript of equivalent polarity to the genome but lacking all of the viral genes. The RNA was expressed using a T7 promoter (Plate 3). Generation of a precise 3′ terminus is important for the subsequent replication of the minigenome inside cells. This was achieved by incorporating a hepatitis δ ribozyme sequence into the minigenome clone. The hepatitis δ ribozyme sequence is ideal for this purpose, in that only sequences downstream of the cleavage site are required for the autocatalytic activity (Perrotta and Been, 1991). Therefore upon *in vitro* transcription and ribozyme folding there is an autocatalytic, intramolecular cleavage event within the nascent RNA transcript producing RNA molecules with the exact 3′ end of the virus minigenome (Rosenstein and Been, 1991). The difference between this and the influenza rescue described above was that the RNP complex was not formed outside of the cell. Instead the synthetic minigenomic RNA was transcribed *in vitro* and transfected into MV infected cells and the RNP complex was assembled inside the cell.

Expression of CAT activity from the minigenome suggested this approach might be extended and/or adapted to allow the recovery of an infectious rMV and, with reference to the RABV system, a full-length, antigenomic cDNA clone of MV, p(+)MV, was generated (Radecke *et al*., 1995). This clone was largely based on a laboratory-adapted, Edmonston-derived MV and, similar to the CAT-expressing minigenome, it contained a T7 RNA polymerase promoter prior to the first nucleotide of the genome and a hepatitis δ ribozyme sequence after the Tr region (Plate 3). Rather than generate the antigenomic RNA *in vitro* and risk its degradation upon transfection, transcripts were expressed directly from p(+)MV within 293 cells which stably expressed T7 RNA polymerase and the MV N and P proteins (293-3-46 cells). An additional plasmid (pEMC-La) was used to express the L protein from T7 generated transcripts and an encephalomyocarditis virus (EMCV) internal ribosome entry sequence (IRES) ensured that acceptable amounts of the 2183 amino acid protein was expressed in the 293-3-46 cells. Expression of the N, P and L proteins within the 293 cells negated the need to drive the rescue with a superinfecting, helper MV which tends to complicate the identification of the actual rMV. Recovery of the first rMV, designated Edtag due to the inclusion of three silent nucleotide changes in the genome, was a major achievement and this virus and the underlying system has been used extensively by many groups around the world. In many ways this original MV reverse genetics system underpins the subsequent second and third generation derivations making its impact highly significant within the MV field (see Section 6.8).

Recovery of non-segmented negative strand RNA viruses is tremendously inefficient and it requires significant efforts to obtain rescue events in every transfected well. This is illustrated by the fact that although the system was developed over fifteen years ago, only a few laboratories routinely recover rMVs. There has been a continual drive to optimise and modify the original protocol to try and improve the efficiency of rescue, although for the most part the fundamental aspects remain unchanged. Key modifications include using an alternative source of T7 RNA polymerase, typically from recombinant poxviruses (Fuerst *et al*., 1986; Wyatt *et al*., 1995; Britton *et al*., 1996). The key advantage of using a poxvirus to express T7 RNA polymerase is that recovery of the rMV is not restricted to the 293-3-46 cell line, something which becomes highly relevant when one wishes to recover wild-type viruses which only use CD150 as a cellular receptor. This also permits expression of the N, P and L proteins from co-transfected plasmids which allows protein amounts to be titrated and optimised (Schneider *et al*., 1997b). In the first instance, plasmids containing a T7 RNA polymerase promoter and EMCV IRES were used (pEMC-Na, pEMC-Pa and pEMC-La) although more recently N, P and L have been expressed under the control of the highly efficient, constitutive, chicken actin promoter (Martin *et al*., 2006). Further tweaks, such as the use of DNA replication and caspase inhibitors (Kovacs *et al*., 2003) or the introduction of a heat-shock step (Parks *et al*., 1999), have marginally improved recovery. How effective and commonly used such approaches are and whether they have been adopted by the majority of laboratories performing MV rescue is not clear.

The recombinant vaccinia virus encoding T7 RNA polymerase (vTF7-3) has been used as a source of T7 RNA polymerase for a wide range of applications as it expresses high levels of the polymerase (Fuerst *et al*., 1986). However, there are certain drawbacks when it is used to recover rMVs, for example, it is fully replication competent in the cells used for virus rescue, it exhibits significant cytopathogenicity and therefore must be removed from the rescued virus stocks, it has unknown pathogenicity in humans, and it can lead to homologous recombination between the full-length antigenomic plasmid and the helper plasmids. As a result, some laboratories use host-range restricted vaccinia viruses engineered to express T7 RNA polymerase such as modified vaccinia Ankara (MVA-T7), or fowlpox virus (FP-T7) (Wyatt *et al*., 1995; Britton *et al*., 1996; Schneider *et al*., 1997b). These viruses are less cytopathic and do not typically produce progeny virus within the cells used for rescue. They are grown in primary chicken embryo fibroblasts or, in the case of MVA-T7, baby hamster kidney (BHK) cells. Another alternative is the recombinant vaccinia virus LO-T7-1, which is based on the Lister vaccine strain, which was used as a vaccine in the World Health Organization smallpox eradication programme (Nakatsu *et al*., 2006a). Nonetheless recombination is still an issue and it is important to ensure that any site-directed mutations are maintained in the rMV by consensus sequencing of reverse transcription (RT)/ polymerase chain reaction (PCR) amplicons generated from pass 4 (P4) stocks.

Some groups have attempted to remove T7-expressing poxviruses from the rescue system entirely, either by supplying the polymerase from a transfected plasmid, or by employing a variation on the original procedure of using cells stably expressing the polymerase, for example, BSR-T7 cells (Buchholz *et al*., 1999), a BHK cell derivative. Again there are advantages and disadvantages with any approach and it is important to take into consideration that there may be unknown intrinsic antiviral mechanisms within, or host factors missing from rodent cells which could have a significant impact on the replication, transcription and assembly of human viruses. In general, we tend to dismiss, or not even consider the possibility that T7 RNA polymerase may introduce mutations into the primary transcripts which may have a significant bearing on the phenotype of the rMV. To that end efforts have been made to remove T7 RNA polymerase from the system entirely and drive transcription using a eukaryotic RNA polymerase II promoter (Martin *et al*., 2006). This approach has been augmented by the insertion of a hammerhead ribozyme immediately following the Pol II promoter and just before the start of the virus genome which autocatalytically cleaves the primary transcript to generate the exact 5′ non-coding terminus of the antigenomic RNA.

6.8.1 First, second, and third generation reverse genetics systems

The p(+)MV-based reverse genetics system used to recover Edtag (Radecke *et al*., 1995) has been widely used by many groups to generate a wide range of rMVs (see below). The virus is mostly based on a laboratory-adapted strain of measles,

Edmonston B, although some additional clones were used in the construction of p(+)MV. Over time Edtag has been found to contain nucleotide changes that are not found in other sequences from the Edmonston lineage. Sequence comparison carried out between Edtag and the vaccine strains Moraten, Schwarz, Edmonston-Zagreb (EZ), Rubeovax, and AIK-C (GenBank accession numbers AF266287, AF266291, AF266290, AF266289, and AF266286, respectively) indicates that as well as some non-coding changes, there were eleven amino acid changes in Edtag that were not present in at least one of the vaccine strains: P/V (Y110H), P (L246S and Y306H), V (C272R and Y291H), M (R175G), F (M94V), H (E492G), and L (E429D, R1629Q, and N1805S). The changes in the M, F, and H proteins have been investigated previously and it has been suggested that these changes are compensatory to allow Edtag to grow and spread efficiently (Plemper and Compans, 2003; Tahara *et al*., 2005; Tahara *et al*., 2007a; Tahara *et al*., 2007b). At least one change in the L protein (N1805S) is close to a predicted conserved domain although whether this has any phenotypic effect has not been determined (Zhang *et al*., 2010).

Recognising the possible risks associated with p(+)MV, some groups have modified the original plasmid to attempt to improve the system. Two groups directly modified the original p(+)MV to convert some of the Edtag specific amino acid changes to more representative Edmonston-like equivalents, one replacing the P/C/V gene which encodes proteins which have been shown to be deficient for antagonism of the interferon system (Devaux *et al*., 2007) with an EZ vaccine P gene (de Vries *et al*., 2010), and the other replacing the N, P, M and F genes with those from the vaccine Moraten and converting some of the other changes by site-directed mutagenesis; this clone retained some of the Edtag specific changes in L (Devaux *et al*., 2007). In order to work with authentic vaccine viruses, other groups have generated completely new full-length clones based either on the Schwartz vaccine strain (Combredet *et al*., 2003) or the EZ vaccine (Duprex *et al*., in preparation).

Other groups have focused on developing systems to allow the manipulation of wild-type viruses with a view to getting a more comprehensive understanding of MV pathogenesis. The first system for the generation of a wild-type rMV completely from cDNA (Takeda *et al*., 2000) was based on the Japanese isolate of MV, IC-B (Kobune *et al*., 1990). This system was generated from a virus which was originally isolated from a patient in 1984 and passaged on B95a cells. Although B95a is a transformed marmoset cell line, it comes close to a disease-relevant cell line as it expresses CD150 and supports the replication of clinical isolates. Importantly, rescue of the wild-type virus was carried out in the same cells. A second wild-type reverse genetics system (Lemon *et al*., 2011) has been generated based on a currently circulating African strain of MV isolated from a patient in Khartoum, Sudan (designated MV^{KS}; El Mubarak *et al*., 2000; El Mubarak *et al*., 2002; El Mubarak *et al*., 2004). The key difference between this system and the earlier Japanese version is that the MV^{KS}-based system was generated following consensus sequencing of RT/PCR amplicons of the viral genome obtained directly from a clinical specimen passaged only several times in a human B cell line (B-LCL). This approach has been taken as passage of a virus in transformed cells can lead to the generation

of mutations that are not present in the natural circulating virus population which may in turn attenuate the virus and have an impact on our understanding of virus pathogenesis.

It is our contention that pathogenesis and attenuation are two sides of the same coin and we argue that in the post-empirical world of vaccine development it is vital to understand both aspects for the highly efficacious existing vaccines. Such knowledge should prove beneficial in the end game of MV eradication and for those who aim to use the virus as a vaccine vector, harness it as a replicating virus for gene therapy or those attempting to rationally attenuate similar viruses or new and emerging viruses. Measles still remains an important disease and the virus is a pathogen which warrants investigation both *in vitro* at the molecular level and *in vivo* in appropriate animal models. It provides an excellent model of a systemic virus infection for a lymphotropic, epitheliotropic, endotheliotropic and neurotropic virus and as a finely attuned pathogen we have still much to learn from how it manipulates both the intracellular environment and the host to represent one of the most infectious, highly transmissible, human infectious agents known.

6.8.2 Application to pathogenesis studies

The generation of the original MV reverse genetics system by Martin Billeter (Radecke *et al*., 1995) has transformed the field of MV study. Many rMVs have been generated from the original p(+)MV clone, or from second generation (improved Edtag) or third generation (vaccine and wild-type) clones based on that system. These viruses fall into seven general categories:

1. Viruses in which TUs are replaced with those from other MV strains or other viruses.
2. Viruses which contain additional non-MV derived sequences within one or more additional TUs.
3. Viruses in which an ORF has been modified by site-directed mutagenesis.
4. Viruses in which viral proteins have been extended by insertion of short epitope tags or complete foreign ORFs.
5. Viruses with modifications in the *cis*-acting signals.
6. Viruses in which complete genes are deleted.
7. Viruses in which the single-stranded genome has been segmented.

Examples of studies using viruses falling into each of these categories are shown in Table 6.1. This list is by no means exhaustive and is meant to highlight the diversity of rMVs which have been generated, and how the use of reverse genetics has advanced, and in some cases fundamentally changed, our understanding of MV biology and pathogenesis. Examples from each category are discussed in more

Table 6.1 Measles virus reverse genetics: systems and recombinant viruses.

	Strain		Virus type	Reference	Year
Rescue systems	Edtag		Laboratory-adapted	Radecke *et al*., 1995	1995
	IC-B		Wild-type	Takeda *et al*., 2000	2000
	KS		Wild-type	Lemon *et al*., 2011	2011
	Modified Edtag (Edmonston P)		Laboratory-adapted	de Vries *et al*., 2010	2010
	Modified Edtag (Moraten-like)		Laboratory-adapted	Devaux *et al*., 2007	2007
	Modified Edtag (Pol II-driven)		Laboratory-adapted	Martin *et al*., 2006	2006
	Schwarz		Vaccine	Combredet *et al*., 2003	2003
	AIK-C		Vaccine	Nakayama *et al*., 2001	2001
	Moraten		Vaccine	Liniger *et al*., 2009	2009
	SI (SSPE)		SSPE-isolate	Seki *et al*., 2011	2011
	TU/s or ORF/s (virus strain)	System	Aspect of MV virology	Reference	Year
Replacement with other MV strains or other viruses	F (SSPE-isolate)	IC-B	Neuropathogenesis	Ayata *et al*., 2010	2010
	H (CAM/RB)	Edtag	Neuropathogenesis	Duprex *et al*., 1999a; Moeller *et al*., 2001, Moeller-Ehrlich *et al*., 2007	1999 2001 2007
	M (SSPE-isolate)	Edtag	Persistence	Patterson *et al*., 2001	2001
	H (CAM/RB)	Edtag	Persistence	Schubert *et al*., 2006	2006
	H (WTF)	Edtag	Assembly and spread	Runkler *et al*., 2009	2009
	L (Edtag)	IC-B	Attenuation	Takeda *et al*., 2008	2008
	F and H (T11Ve-23)	IC-B	Cell adaption	Okada *et al*., 2009	2009
	M, F, H and L (CAM-70)	IC-B	Cell adaption	Sharma *et al*., 2009	2009
	M and L (Edtag)	IC-B	Cell adaption	Tahara *et al*., 2005	2005
	H (MVi/Tokyo.JPN/87-K)	AIK-C	Cell adaption	Kumada *et al*., 2004	2004
	P (AIK-C/Edmonston)	AIK-C	Temperature sensitivity	Komase *et al*., 2006	2006

	H (WTF)	Edtag	Entry and spread	Schneider *et al*., 2002	2002
	H (Edtag; IC-B)	Edtag; IC-B	Entry and spread	Takeuchi *et al*., 2002	2002
	H and F (WTF)	Edtag	Entry and spread	Ohgimoto *et al*., 2001	2001
	H and F (WTF)	Edtag	Entry and spread	Johnston *et al*., 1999	1999
	Delta-F/H +VSV G	Edtag	Immunology	Shishkova *et al*., 2007	2007
	Delta-F/H +VSV G	Edtag	Immunology	Fehr *et al*., 1998	1998
	Delta-F/H +VSV G	Edtag	Immunosuppression	Schlender *et al*., 1996	1996
	TU/s or ORF/s (virus strain)	System	Aspect of MV virology	Reference	Year
Insertion of an additional transcription unit	EGFP	Edtag	Spread and attenuation	Duprex *et al*., 2000	2000
	EGFP	Edtag	Spread and attenuation	Duprex *et al*., 1999b	1999
	EGFP	rMV	Spread and attenuation	de Vries *et al*., 2010	2010
	EGFP	IC-B	Entry and spread	Hashimoto *et al*., 2002	2002
	EGFP	Edtag	Neuropathogenesis	Ludlow *et al*., 2008	2008
	EGFP	KS	Pathogenesis	Lemon *et al*., 2011	2011
	EGFP	KS	Pathogenesis	de Swart *et al*., 2007	2007
	DsRed	Edtag	Super-infection immunity	Ludlow *et al*., 2005	2005
	Cre recombinase	IC-B	Cell adaption	Iwasaki and Yanagi, 2011	2011
	MuV (HN; F)/SIV-1 (env; gag; pol)	Edtag	Vaccinology	Wang *et al*., 2001	2001
	HBV (sAG)	Edtag	Vaccinology	Singh *et al*., 1999	1999
	Helicobacter pylori (neutrophil activating molecule)	Edtag	Vaccinology	Iankov *et al*., 2011	2011

(*continued*)

Table 6.1 (*Continued*)

	TU/s or ORF/s	System	Aspect of MV virology	Reference	Year
	IL-2	Edtag	Vaccinology	Hoffman *et al*., 2003	2003
	Dengue (tetravalent antigen)	Schwarz	Vaccinology	Brandler *et al*., 2010	2010
	HIV-1-derived CTL polyepitope	Schwarz	Vaccinology	Lorin *et al*., 2005	2005
	HIV-1-derived CTL polyepitope	Schwarz	Vaccinology	Lorin *et al*., 2004	2004
	HIV-1 (gp160)	Schwarz	Vaccinology	Guerbois *et al*., 2009	2009
	HRSV (F)	AIK-C	Vaccinology	Sawada *et al*., 2011	2011
	HIV-1 (gag-pol fusion; gp140)	Moraten	Vaccinology	Liniger *et al*., 2009	2009
	EGF and IGF1	Edtag	Gene therapy	Schneider *et al*., 2000	2000
	CEA	Edtag	Gene therapy	Galanis *et al*., 2010	2010
	CEA	Edtag	Gene therapy	Hammond *et al*., 2001	2001
	CEA/sodium iodide symporter (NIS)	Edtag	Gene therapy	Hasegawa *et al*., 2006	2006
	NIS	Edtag	Gene therapy	Dingli *et al*., 2004	2004
	ssFv CD38	Edtag	Gene therapy	Peng *et al*., 2003	2003
	scEGFR	Edtag	Gene therapy	Paraskevakou *et al*., 2007	2007
	TU/s or ORF/s	System	Aspect of MV virology	Reference	Year
SDM modification of viral proteins	H	Edtag	Entry	Vongpunsawad *et al*., 2004	2004
	H	IC-B	Entry	Leonard *et al*., 2008	2008
	H	IC-B	Entry	Seki *et al*., 2006	2006
	V	Moraten-like IC-B	Attenuation	Devaux *et al*., 2011	2011
	V and C	IC-B	Attenuation	Nakatsu *et al*., 2008	2008

	N	IC-B	RNA synthesis and assembly	Iwasaki *et al*., 2009	2009
	F and H	Edtag	Assembly and spread	Cathomen *et al*., 1998b	1998
	F and H	Edtag	Assembly and spread	Runkler *et al*., 2008	2008
	F and H	Edtag	Assembly and spread	Moll *et al*., 2002	2002
	M	IC-B	Assembly and spread	Tahara *et al*., 2007b	2007
	F	Edtag	Spread	Plemper and Compans, 2003	2003
	F	Edtag	Spread	Maisner *et al*., 2000	2000
	F	AIK-C	Temperature sensitivity and spread	Nakayama *et al*., 2001	2001
	H	IC-B	Cell adaption	Tahara *et al*., 2007a	2007
	F	Edtag	Gene therapy	Springfeld *et al*., 2006	2006
	L	Edtag	Antiviral therapy	Yoon *et al*., 2009	2009
	Protein and insertion	**System**	**Aspect of MV virology**	**Reference**	**Year**
Extension of viral proteins	L (EGFP and myc epitope)	Edtag	RNA synthesis	Duprex *et al*., 2002	2002
	V (Strep tag)	Schwarz	Host protein interactions	Komarova *et al*., 2011	2011
	P (EGFP)	Edtag	Assembly	Devaux and Cattaneo, 2004	2004
	UTR	**System**	**Aspect of MV virology**	**Reference**	**Year**
Modifications in the cis-acting UTRs	M/F	IC-B	Replication and cytopathogenicity	Takeda *et al*., 2005	2005
	F	Edtag	Translation	Cathomen *et al*., 1995	1995
	TU/s or ORF/s	**System**	**Aspect of MV virology**	**Reference**	**Year**
Gene deletions	Delta-M	Edtag	Assembly and spread	Cathomen *et al*., 1998a	1998

(*continued*)

Table 6.1 (*Continued*)

	TU/s or ORF/s	System	Aspect of MV virology	Reference	Year
	Delta-V	IC-B	Innate immunity	Ikegame *et al*., 2010	2010
	Delta-V	Moraten-like	Innate immunity	Cruz *et al*., 2006	2006
	Delta-C	Edtag	Innate immunity	Shaffer *et al*., 2003	2003
	Delta-C	IC-B	Translation and innate immunity	Nakatsu *et al*., 2006b	2006
	Delta-C	Edtag	Attenuation	Escoffier *et al*., 1999	1999
	Delta-C	IC-B	Attenuation	Takeuchi *et al*., 2005	2005
	Delta -V	Edtag	Pathogenesis	Valsamakis *et al*., 1998	1998
	Delta-V	Edtag	Pathogenesis and RNA synthesis	Tober *et al*., 1998	1998
	Delta-V	Edtag	RNA synethesis	Schneider *et al*., 1997a	1997
	Modification	System	Aspect of MV virology	Reference	Year
Genome modifications	Split genome	IC-B	Expression of foreign proteins and vaccinology	Takeda *et al*., 2006	2006
	Multiple ATUs	Edtag	Expression of foreign proteins and vaccinology	Zuniga *et al*., 2007	2007
	Polyploid genome	Edtag	Molecular biology	Rager *et al*., 2002	2002

detail below and we have sectionalised them, first into those which express fluorescent proteins, as these rMVs have had a tremendous impact on understanding the following various aspects of the virus life-cycle, then into seven aspects of the MV life-cycle and finally we focus on some of the more applied aspects such as vaccinology and gene therapy.

6.8.3 Viruses expressing enhanced green fluorescent protein from an additional transcription unit

One of the most fruitful advances to come out of the development of reverse genetics for MV was the generation of a virus which expresses enhanced green fluorescent protein (EGFP) when the virus replicates within a cell (Duprex *et al*., 1999b). This was achieved by inserting an additional transcription unit (ATU) containing the EGFP ORF in the promoter proximal position, that is, upstream of the N gene in the Edtag backbone. This position was chosen as, due to the transcription gradient, it was predicted to give rise to the greatest number of mRNAs transcripts for the reporter gene. Furthermore, as the ATU is upstream of all the other viral TUs, the ratio of mRNA transcripts, and hence viral proteins, should remain unchanged relative to the parental, unmodified virus. The virus (MVeGFP) was successfully rescued and when live, virus-infected cells were exposed to UV light and viewed with appropriate filters, the EGFP emitted green fluorescence, illuminating the infected cells. The first application for the virus was to infect neuronal cells and observe the cell-to-cell spread of the virus over time (Duprex *et al*., 1999b). Expression of EGFP resulted in a virus which could be detected at very early time-points post-infection, even in single living cells, and could be tracked in those cells in real time. The presence of the EGFP meant that even tiny processes extending from virus-infected cells could be seen. The virus was used to infect transgenic mice expressing CD46 (Mrkic *et al*., 1998) via the intracerebral route and it was detected in the CNS (Duprex *et al*., 2000). These studies were extended to infections of non-transgenic mice with an rMV expressing the H glycoprotein from a rodent brain-adapted virus which also expressed EGFP and this emphasised the exquisite sensitivity of the approach (Ludlow *et al*., 2008). The virus expressing EGFP was used to infect mice intracerebrally and the infection was allowed to progress until the first signs of clinical symptoms became apparent. At this point animals were sacrificed and the brains sectioned using a vibratome, to produce up to 200 μm sections, and the EGFP fluorescence was imaged by confocal scanning laser microscopy (CSLM) which allows high resolution imaging of single optical planes within a thicker section. The single optical planes can then be ‘stacked’ to form a representation of fluorescence present within the entire section imaged. EGFP fluorescence was readily detected in the brain sections, and again even single infected cells could be detected. The increased thickness of the vibratome-cut sections in comparison to more conventional, thinner, microtome-cut sections enabled easier detection of extended cellular processes connecting MV-infected neurones in the mouse

brain; virus could be tracked throughout multiple cell layers and axonal tracts could be detected between cell bodies that would have been difficult to detect by conventional methods.

Equivalent third generation rMVs have been generated for the IC-B and MV^{KS} wild-type virus strains (Hashimoto *et al*., 2002). These have shown great utility in studies of viral pathogenesis in macaques (Takeuchi *et al*., 2005; de Swart *et al*., 2007; de Vries *et al*., 2010; Lemon *et al*., 2011). In particular, they have permitted investigation of the early target cells of MV (Lemon *et al*., 2011), which has led to a re-evaluation of the text-book accounts of MV pathogenesis (see above) and have shown the importance of the non-structural C protein (Takeuchi *et al*., 2005).

6.8.4 Cell entry

One area of MV biology which has benefited immensely from recombinant viruses is the study of the H glycoprotein and its interaction with the viral entry receptors. One such study used MVeGFP to identify specific residues which were necessary for the interaction of the H glycoprotein with the two known receptors at the time, CD150 and CD46 (Vongpunsawad *et al*., 2004). Rounds of iterative mutagenesis, alongside functional analysis of the mutant proteins following transient transfection of eukaryotic expression vectors, were used to identity candidate amino acids within the H glycoprotein which lacked the ability to interact with one of the receptor molecules. The mutations that conferred this phenotype were transferred into p(+)MV to allow generation of recombinant viruses which are selectively 'blind' to one of the receptors, or at the least had significantly diminished binding (Vongpunsawad *et al*., 2004). Subsequently, the ectodomain (residues 149–617) of the MV H glycoprotein was crystallised (Colf *et al*., 2007, Hashiguchi *et al*., 2007) revealing a disulfide linked dimer configuration with each molecule having a cuboidal β-propeller structure composed of six antiparallel β-sheets (four-stranded) with two glycosylated asparagine residues shielding the globular head, which closely resembled early structural models proposed for the MV H proteins based on the crystal structures of other paramyxovirus attachment proteins (Langedijk *et al*., 1997; Vongpunsawad *et al*., 2004). Also, more recently the crystal structure of the H glycoprotein in complex with CD150 (Hashiguchi *et al*., 2011) or CD46 (Santiago *et al*., 2010) has been solved at atomic resolution. The amino acids important for H glycoprotein binding to CD150 or CD46 have been mapped onto these structures and are clustered and strategically positioned in the unshielded area of the globular head. A recombinant virus which was based on the wild-type IC-B virus, and bound CD150 less efficiently, was used to infect rhesus monkeys (Leonard *et al*., 2010), The rMV was attenuated and induced a strong adaptive immune response.

Before the identification of PVRL4 as an entry receptor for MV (Noyce *et al*., 2011), a similar process of iterative mutagenesis of the H glycoprotein was used to generate an rMV which was "blind" to the, at that time, putative EpR (Leonard

et al., 2008). When this virus was used to infect rhesus monkeys, it was found that although it could spread systemically, it was not shed from the animals, implicating the unknown EpR as an important factor in MV pathogenesis. Thus reverse genetics systems played a critical role in the identification of the two most pathologically relevant MV receptors.

6.8.5 Viruses expressing enhanced green fluorescent protein fused to viral proteins

The L protein of MV is a large, 2183 amino acid (approximately 250 kDa) protein. Although there is no direct evidence, it is assumed that it contains the viral polymerase activity, and that it is responsible for activities such as capping, methylation and polyadenylation during mRNA synthesis. Sequence alignments of the L proteins from different morbilliviruses reveal two highly variable regions, termed 'hinges' (McIlhatton *et al*., 1997), and it is thought that they may form the boundaries between different domains of the protein. These hinge regions were probed to determine if they would tolerate the insertion of small epitope tags or larger reporter ORFs to determine their flexibility and to see if such a manipulation might have an effect on the rate at which the RdRp transcribed and replicated the genomic RNA (Duprex *et al*., 2002). Two recombinant viruses were generated. The first contained an insertion of a c-myc epitope tag into the second hinge region of the Edtag L protein (a similar insertion into the first hinge region resulted in a non-functional protein in a preliminary minigenome assessment). When this small insertion was tolerated, the ORF for EGFP was tested in the same position. This resulted in a virus which encoded an L protein that could be detected and tracked in living cells by virtue of the autofluorescent tag. When the growth kinetics of the two recombinant viruses were compared with the parental Edtag virus, the virus with the c-myc tag grew to an equivalent titre and at an equivalent rate. However, the virus with the EGFP insertion grew to a lower titre and the growth kinetics lagged behind the other viruses by approximately five hours. It was surmised that this could provide a novel means to irreversibly and rationally attenuate viruses by permanently modulating the transcription and replication of the recombinant virus; the rationale being that as the EGFP was embedded within the L protein, the virus would not be able to ablate EGFP expression by, for example, introduction of a stop codon within the EGFP sequence since this would also result in the ablation of full-length L protein production.

A follow-on study using a similarly modified rinderpest virus, which is closely related to MV, confirmed that this approach led to rational attenuation of the virus in cattle, the natural host of rinderpest virus (Brown *et al*., 2005). A second study further investigated the dynamics of RNA synthesis during infection of the rMV containing the EGFP insertion in the L protein (Plumet *et al*., 2005). This showed that the modified polymerase has an elongation speed which is approximately five

times slower than the parental unmodified protein although the transcription gradient displayed by the modified protein is unchanged relative to the unmodified version.

Addition of the EGFP to the amino terminus of the P and V proteins permitted the detection of these proteins in living cells and abolished the expression of the C protein (Devaux and Cattaneo, 2004). This led to a decrease in virus release which was partially restored when the C protein was expressed from an ATU located between the H and L genes demonstrating C functions as an infectivity factor.

6.8.6 The impact of the M/F non-coding regions on viral replication and gene expression

The 3′ UTR of the M gene and the 5′ UTR of the F gene are very different from all the other MV UTRs. The total combination of M 3′ UTR, Ig and F 5′ UTR is 1012 nucleotides, comprising 426 nucleotides for the M 3′ UTR and 583 nucleotides for the F 5′ UTR together constituting 6.4% of the total genome length. In comparison, the other MV non-coding TU boundaries range from 107 to 160 nucleotides. The M/F TU boundary is very GC rich, with a relatively high level of predicted secondary structure (Buckland *et al*., 1987). The 5′ UTR of the F gene has some interesting properties. The region has an unusually high number of cytosine residues (44%; including a $G_7C_7A_7$ tract) with an overall GC content of 64% (Buckland *et al*., 1987), and a high degree of predicted secondary structure (Richardson *et al*., 1986). It has been suggested that the F 5′ UTR acts as a focusing factor, directing translation initiation to the second of three clustered in frame AUG codons (Cathomen *et al*., 1995). However, *in vivo* and *in vitro* studies have shown that mRNA transcripts with a high potential to form stable secondary structure in the 5′ UTR tend to be translated inefficiently (Kozak, 1991a, 1991b). It has also been shown that all known mRNA transcripts encoding ribosomal proteins have a short (5–14 nucleotide) oligopyrimidine tract at their 5′ end which has been associated with underutilisation of the mRNA in translation (Pain, 1996). The F 5′ UTR contains multiple C_5 tracts towards the 5′ end, which could lead to a negative effect on translation of the F mRNA transcripts. A study of the phenotype of the Edtag-based rMV, del5F (Valsamakis *et al*., 1998), which contains a 504 nucleotide deletion in the F 5′ UTR, in the severe combined immunodeficiency disease (SCID) human thymus and liver mouse model, which reproduces *in vivo* virulence phenotypes of MV (Auwaerter *et al*., 1996), found that deletion of that region resulted in decreased peak virus production and a small change in the kinetics of viral growth. The conclusion was that the F 5′ UTR was not absolutely required for MV replication in that system, and deletion of the sequence led to a change in the level, but not the abrogation of F glycoprotein expression, possibly by affecting translation. A similar study was carried out with the wild-type IC-B strain by generating a series of rMVs having alterations or deletions in the UTRs (Takeda *et al*., 2005). Like the previous Edtag based study, this study also concluded that the regions were not essential

for virus replication, but that they did regulate replication and cytopathogenicity by modulating M and F protein levels. It was shown that the M 3′ UTR had the ability to increase M protein levels, whereas the F 5′ UTR decreased F glycoprotein levels which inhibited viral replication and reduced cytopathogenicity. This study speculated that the reduction in cytopathogenicity meditated by the long F 5′ UTR may actually be advantageous for the fitness of the virus.

6.8.7 Understanding the persistence and neurotropic potential of MV

SSPE is a rare, progressive, and invariably fatal persistent MV infection of the CNS and represents a paradigm for the long-term persistence of a human RNA virus. In SSPE cases the M protein is either absent (Cattaneo and Billeter, 1992), or when present, is not associated with budding structures *in vivo* and is unable to bind RNPs *in vitro* (Hirano *et al*., 1992; Hirano *et al*., 1993). The M gene in MVs isolated from SSPE cases is hypermutated due to the biased replacement of many uridine by cytidine resides (Bass *et al*., 1989). This leads to very little or no functional M protein expression due to disruption of the start initiation codon, alteration of functionally important amino acids or introduction of a stop codon within the ORF. Adenosine deaminase has been implicated in the development of this biased hypermutation (Bass, 2002) and subsequently the development of SSPE. Persistence in neural cell cultures, and superinfection immunity, have been studied using two antigenically identical rMVs which are phenotypically distinct, as they express either a green or red fluorescent reporter protein (Ludlow *et al*., 2005). The exquisite sensitivity and possibility of being able to detect single infected cells in the absence of any overt cytopathic effect showed that superinfection immunity was virtually absolute. Importantly infectious virus was not released from the persistently infected cells and the virus remained highly cell associated. An *in vivo* persistence model in immunocompetent mice (C57 and Balb/c) using EGFP-expressing viruses has been developed to understand virus distribution in the CNS and identify foci of infection up to 50 days post-infection (Schubert *et al*., 2006).

Since the M protein is completely absent in virus sequences isolated from some cases of SSPE, an rMV which lacks the protein was generated to explore what effects this has on virus replication, propagation and pathogenesis (Cathomen *et al*., 1998a). Removal of 960 nucleotides of M coding sequence from Edtag produced an rMV which retained only 15 amino acids of M protein. Although the protein is not absolutely required for virus spread, its absence dramatically affected propagation of the virus *in vitro* and it exhibited a hyper-fusogenic phenotype. Compared to Edtag, four logs less infectious particles were released, suggesting it was defective for assembly and egress and the rMV remained mostly cell associated inducing enhanced fusion of virus-infected cells with neighbouring cells. Upon inoculation of the rMV into the brain of a transgenic mouse engineered to express CD46, it propagated more deeply into the brain parenchyma than Edtag. As the M gene is

dramatically disrupted in viruses isolated from SSPE patients, there was a question as to whether these viruses are infectious and the mutations enable them to persist and thereby cause the prolonged neurodegenerative disease, or they represent a non-infectious end-stage result of the progressive infection. To address this, a recombinant virus was generated where the M gene of Edtag was replaced with that from an SSPE case (Patterson *et al*., 2001). This recombinant virus was inoculated directly into the brain of a CD46 expressing transgenic mouse. Even though the M gene was heavily disrupted by biased hypermutation, the virus was infectious and produced a protracted, progressive infection, resulting in death as long as 30 to 50 days after the inoculation, suggesting that the altered M protein has a direct role in disease pathogenesis. Insertion of the M, F and H genes from the Osaka-2 SSPE strain of MV into the wild-type IC-B background showed that the F glycoprotein was the major determinant of neurovirulence in a hamster model (Ayata *et al*., 2010). This finding, which was linked to a single amino acid, is probably more clinically relevant as genotype A vaccine viruses have never been associated with SSPE cases. It would be interesting to know if this mutation facilitates entry into the rodent CNS using a novel receptor. Specific mutations in the H glycoprotein have also been shown to affect entry and spread in the rodent CNS in the Edtag background (Duprex *et al*., 1999a; Moeller *et al*., 2001; Moeller-Ehrlich *et al*., 2007).

6.8.8 Evading the immune response

In addition to the P protein, the P/C/V gene also encodes two non-structural proteins, C and V. The V protein is produced from an alternative mRNA transcript generated by the non-templated co-transcriptional insertion of a single G nucleotide at a specific position on the P/C/V gene. The V protein shares the same initiation codon as P, as well as the amino-terminal 231 amino acids of the P protein but the presence of the additional nucleotide at position 751 of the edited mRNA transcript leads to the ribosome shifting to an alternative reading frame resulting in the last 276 amino acids, normally encoded by the P mRNA, being replaced with a cysteine-rich domain of 68 amino acids (Cattaneo *et al*., 1989). The unique carboxyl terminus of V has zinc-binding properties (Liston and Briedis, 1994). The C protein is produced from an alternative initiation codon present in the P and V mRNA transcripts just downstream of that for P and V in an overlapping ORF. The initiation context of the start codon for P/V is sub-optimal which is thought to allow some ribosomes to scan through the P initiation codon and initiate protein synthesis at the C protein start codon. The C protein is a basic protein of 186 amino acids. Both V and C interact with cellular proteins (Liston *et al*., 1995). The C and V proteins are non-structural, and one of the first questions posed after the successful generation of the Edtag reverse genetics system was, ‘Are they necessary for MV replication?’

A number of rMVs have been generated to address this question. In the first C protein synthesis was abrogated by the introduction of two point mutations into the P/C/V gene which converted the C initiation codon to ACG and introduced a stop codon into the C ORF just downstream (Radecke and Billeter, 1996). Importantly, both changes were silent in the overlapping P protein ORF. A viable virus was rescued which replicated *in vitro* without obvious growth impairment. However, it was noted that the virus causes a smaller plaque phenotype. Importantly, the growth of the virus was impaired in primary human peripheral blood mononuclear cells (Shaffer *et al*., 2003), but in the presence of antibodies to interferon, it grew to the same titres as the parental Edtag virus, suggesting that the C protein plays a role in MV pathogenesis by inhibiting interferon signalling.

Other rMVs have been generated to investigate the function of the V protein (Schneider *et al*., 1997a). This study concentrated on the editing site within the P/C/V gene and examined the consequences of altering it on protein expression and virus replication. Six different modifications were generated including nucleotide changes and insertions. In the case of insertions, nucleotides were also removed at the H/L TU boundary to ensure that the resulting rMV genomes were a multiple of six. Several rMVs were successfully rescued and readily propagated, others appeared to rescue, but could only be propagated by supplying the P protein *in trans*, suggesting that they were not viable. This illustrates the point that in systems where plasmids are used to supply the N, P and L proteins, rMVs must be passaged a number of times to ensure that these plasmids are no longer present and therefore do not support the replication of a non-viable virus. Two of the viable viruses did not produce any V protein, whereas one overexpressed it relative to the parental Edtag. Regardless of whether V was not expressed or overexpressed, the viruses all grew similarly to the parental Edtag virus when their growth kinetics were analyzed, although the virus overexpressing V produced generally smaller syncytia. These two studies suggested that the C and V proteins were non-essential for virus replication in tissue culture and it was postulated that these proteins were virulence factors *in vivo*. This is somewhat at variance with the report that C functions as an infectivity factor (Devaux and Cattaneo, 2004).

The same viruses were investigated in small animal models (Tober *et al*., 1998; Valsamakis *et al*., 1998). The first study utilised SCID mice surgically implanted with human thymus/liver tissue in a small animal model which mirrors the virulence of wild-type MV isolated from humans. Inoculation of the virus lacking C expression into the implant resulted in less efficient virus growth with decreased replication in peripheral blood mononuclear cells and epithelial cells of the thymus, suggesting an important *in vivo* role for the protein (Valsamakis *et al*., 1998; Escoffier *et al*., 1999). Similar inoculation of the viruses with modified V expression into the same model showed that absence of V delayed and prolonged virus replication in the human thymic epithelial cells, whereas V overexpression was associated with more rapid replication (Valsamakis *et al*., 1998). Absence or overexpression of V also decreased MV replication in the lungs of cotton rats (Tober *et al*., 1998).

These studies showed that the C and V proteins are necessary for growth of the virus *in vivo*.

More recently, the C and V proteins have been shown to be important antagonists against interferon induction within virus infected cells, and the response of these cells to interferon signalling [reviewed in (Gerlier and Valentin, 2009)]. The amino terminus of the V protein, and therefore that of the P protein, binds the signal transducer and activator of transcription (STAT) factors to inhibit the phosphorylation of STAT1 in response to interferon induced signalling within the cell, and block the subsequent nuclear translocation of dimerised STAT1. The carboxyl terminus of V binds and blocks oligomerisation of melanoma differentiation-associated gene 5 (MDA5), which in turn prevents downstream signalling and inhibits induction of interferon (Childs *et al*., 2009). There are amino acid changes specific to the Edtag V protein which makes the Edtag V unable to carry out these antagonistic roles. An rMV containing the P gene from Edtag showed that the substitution of tyrosine to histidine at position 110 within the shared amino terminal domain of the V and P proteins abrogated the ability of these proteins to block the nuclear translocation of STAT1, in response to interferon induced signalling within the cell (Devaux *et al*., 2007). The substitution of histidine for cysteine at position 272 of the V protein carboxyl terminus renders the protein unable to bind MDA5, and consequently it was unable to antagonise MDA5-induced interferon induction (Takaki *et al*., 2011). This impacts on the results of the modified Edtag V studies described above, since the V protein is already defective for these key functions before it is modified. However, they still illustrate some of the other properties of the virus, for example, V protein production in response to changes in the editing site, and these studies still stand as early, elegant examples of the power of the new technology at the time.

The C protein was further investigated using the IC-B reverse genetics system (Takeuchi *et al*., 2005). Point mutations were made in the ORF encoding C, introducing stop codons into the second and fifth amino acid positions, leading to the ablation of C protein expression. When this rMV was used to infect permissive cells, its growth was restricted relative to the parental IC-B virus, particularly at late stages of infection. Some of the results suggested that C may prevent cell death. Cynomolgus macaques infected with the virus had 1000-fold fewer infected cells in the thymus compared to animals infected with IC-B. Immunohistochemical staining of tissues which commonly show high levels of MV antigen revealed a dramatic decrease of antigen in animals infected with the modified virus. A follow-up study using the same rMV revealed that ablation of C protein expression from the IC-B virus resulted in interferon regulatory factor 3 activation in cells, leading to the induction of interferon in response to infection (Nakatsu *et al*., 2006b). The absence of C also led to the inhibition of protein translation by allowing phosphorylation of eukaryotic translation initiation factor eIF-2α. More recently this rMV has been used to investigate the functionality of the Edtag C protein (Nakatsu *et al*., 2009). The Edtag C protein sequence is identical to that of the C protein encoded in the licensed MV vaccine EZ, and it has been hypothesised that changes between

wild-type and vaccine C proteins contributes to the attenuation of vaccine viruses. The new rMV combined the mutation of the nucleotides in the P/C/V gene to ablate wild-type IC-B C expression, with insertion of the ORF for Edtag C protein as an ATU between the H and L genes. Addition of the Edtag C protein resulted in an rMV which was again able to counteract the induction of interferon and block the inhibition of protein translation, like the parental IC-B virus. This investigation of the Edtag C protein by reverse genetics has revealed that even though Edtag is a highly attenuated MV strain, it encodes a fully functional C protein.

6.8.9 Virus interactions with the host cell

MV relies on the host cell machinery for virus replication, assembly and egress, hijacking the apparatus for its own benefit. Conversely, cells have evolved a battery of defences to fight back against viruses. Two recent studies elegantly illustrate these two different, but ultimately overlapping aspects of MV-host cell interaction.

Murine cells are refractory to MV infection. The murine epithelial (MODE-K) cell line is non-permissive for MV replication (Vincent *et al*., 2002) and the block has been linked to the level of RNA synthesis and protein expression. Further study utilising a MV minigenome revealed that there was a post-entry block of MV replication which affected initial transcription from the incoming RNPs. Infection of human/mouse somatic hybrid clones, which expressed human CD46, revealed that some cells were permissive for MV replication whereas others were not. The authors concluded that a cellular factor (or factors, as yet unknown) can control MV transcription, and this control may involve stabilisation of the incoming viral polymerase templates. However, it might also be possible that the hybrid clones that were permissive to MV lacked a restriction factor present in the mouse cells, or some combination of both aspects.

Although mouse models are used for MV pathogenesis studies, they are either transgenic mice which have the interferon α/β receptor knocked out to allow the virus to be able to replicate efficiently (Horvat *et al*., 1996; Blixenkrone-Moller *et al*., 1998; Mrkic *et al*., 2000; Hahm *et al*., 2003; Sellin *et al*., 2006; Ohno *et al*., 2007), or the viruses are inoculated via unnatural routes into immunocompetent animals (see above) to look at localised effects. Neither of these approaches gives rise to a fully systemic infection and the infection is rather limited. A recent study sought to address whether MV lacks a factor which would allow it to replicate in mouse cells (Iwasaki and Yanagi, 2011). This study utilised the C protein from SeV (also known as murine parainfluenza virus 1). The natural host for SeV is the mouse and the C protein has been shown to be an important interferon antagonist. This study sought to investigate whether expression of the C protein of SeV would allow wild-type MV to efficiently infect murine cells. At this stage it would have been most straightforward to engineer an rMV which expressed the SeV C protein from an ATU. However, the authors decided against this as the SeV C protein is a potent antagonist of human and mouse interferon and, as such, there was a possible

risk that such an rMV would be more pathogenic for humans than the currently circulating MVs. This illustrates the point that just because it is technically possible to generate a recombinant virus, due diligence must be paid to the safety implications. Instead, this study utilised the Cre-loxP system to induce SeV C protein expression in virus-infected cells. The rMV was engineered to express Cre recombinase, and the cells contained a SeV C expression cassette flanked with loxP sequences, such that they only expressed the MV C protein when Cre recombinase was expressed in the same cell. When the rMV infected the cells, it produced Cre recombinase inducing the expression of SeV C by the cells which removed the block to MV replication, and the virus grew efficiently in the mouse cells.

The second study identified host proteins used by MV to benefit replication or which are targeted by the virus to prevent them from restricting the infection (Komarova *et al*., 2011). The study concentrated on the interactions of the V protein as a proof of principle, since it is already known to interact with host proteins. The V protein was expressed from an ATU between the P and M genes of an rMV based on the Schwarz vaccine strain. The V protein contained an extension at the amino terminus comprising a Strep tag and a tobacco etch virus (TEV) protease cleavage site. Unmodified V protein was also expressed from the unmodified P/C/V gene. When the rMV infected cells the Strep-tagged V protein was expressed along with all the usual protein products. Assuming that the presence of the amino terminal extension does not affect the critical interactions the V protein makes with host factors, the presence of the Strep tag allowed purification of the V protein complexes by modified tandem affinity chromatography. Mass spectrometry analysis was used to identify the other proteins in the complexes. The analysis revealed 245 proteins including four proteins which are already known to interact with the V protein.

6.8.10 Assembly and release

Study of the assembly and release of virus particles is an area of MV biology which has been somewhat neglected. Studies have primarily focused on the M protein, since it mediates contact between the RNP and the F and H transmembrane glycoproteins. An rMV based on Edtag containing the ATU for EGFP upstream of N was generated to express an M protein in which a highly conserved valine residue, which is often mutated in viruses isolated from cases of SSPE, was replaced with either an alanine or cysteine residue (Runkler *et al*., 2007). Substitution with cysteine resulted in a stable M protein, while substitution with alanine resulted in an M protein that was rapidly degraded. In virus-infected cells, the rMV with cysteine behaved the same way as Edtag, whereas the virus with the alanine substitution produced more cell-to-cell fusion and released 10- to 100-fold fewer virions into the supernatant, demonstrating that this substitution interfered with the function of the M protein in virus assembly and release. Further investigation revealed that although the modified M protein could bind the N protein and that its membrane

association was only slightly less efficient, the altered protein was unable to accumulate at the cell surface. Consequently transport of the (−)RNP to the surface was impeded, which in turn affected egress. A second study examined the consequences of M and N interaction in the wild-type IC-B virus containing the EGFP ATU upstream of N (Iwasaki *et al*., 2009). An rMV was generated containing an amino acid deletion at the carboxyl terminus of N which resulted in ablation of the N–M interaction. This rMV grew less efficiently than IC-B, leading to the suggestion that because M protein has an intrinsic ability to associate with the plasma membrane, it may retain the (−)RNP in that locale by interacting with the N protein in order to promote virus assembly, particle production and virus release.

6.8.11 Vaccinology

A safe, efficacious vaccine for MV has been in use from the late 1960s. Surprisingly, little is known about why the virus is attenuated *in vivo* and there are relatively few changes that are shared among all the vaccine strains of MV when compared to their parental wild-type sequences (Bankamp *et al*., 2011). However, it should be noted that some alterations may result from the adaptation of the virus to the unnatural cell types it was forced to grow in rather than being key players in attenuation *per se*. Reverse genetics has allowed researchers to revisit the vaccine virus sequences to try to elucidate the molecular basis of attenuation.

One such study used the full-length genome sequences for the wild-type IC-B and attenuated Edtag viruses to generate a series of rMVs containing swaps of the Edtag sequences into the wild-type IC-B background (Tahara *et al*., 2005). Nine rMVs were generated, swapping genomic regions containing one to almost three TUs at a time, to cover the entire length of the Edtag genome. The study found that regions in the H and L genes of Edtag conferred the ability for the wild-type rMV to grow efficiently in African green monkey kidney Vero cells, which are an unnatural target of MV, but that this was at a cost of decreased ability to replicate in B cells, a natural target of MV. This study was extended to look at other properties of the rMVs containing single gene swaps from Edtag into IC-B, and it was shown that viruses containing the P and L TUs from Edtag spread less efficiently and produced smaller areas of fusion in plaque assays (Takeda *et al*., 2008). Equivalent viruses containing the P gene (modified to correct the Edtag specific P/V 110 and V 272 changes back to Edmonston vaccine-specific amino acids) or the L gene from Edtag, and expressing *Renilla* luciferase from a promoter proximal ATU, were used to infect mice expressing CD150. Quantification of luciferase produced in the spleen of infected animals revealed 10,000- and 200-fold reduced levels of the enzyme from the viruses containing the Edtag P or L genes respectively when compared to the parental IC-B virus, indicating that these proteins contribute to the *in vivo* attenuation phenotype of the Edmonston lineage vaccines.

Since MV vaccines have been so successful in reducing the number of MV cases (WHO, 2009), and rMVs have been demonstrated to be surprisingly genetically

stable (Rima and Duprex, 2011), they have received interest in their potential as replicating vectors to deliver epitopes for disease causing entities that have no safe, efficacious vaccine of their own. Among many other examples, attenuated MV has been modified to deliver antigens for hepatitis B virus, simian and human immunodeficiency viruses, flaviviruses and some bacterial infections from ATUs within the genome (reviewed in Billeter *et al*., 2009; and see Table 6.1). The antigens chosen and the mode of vaccine delivery probably need to be refined as the growth characteristics of vaccine viruses can be modified during genetic manipulation. Nonetheless, using MV as a vector to deliver foreign material is an attractive goal and scientists have been working to maximise the potential of this. Viruses that contain an ATU from which a foreign protein is expressed as a transgene have been invaluable in MV research (see above). There is a question as to what the limit is in terms of the amount of foreign material the rMV can tolerate, both in terms of basic size of the genome, and the effects on replication/transcription. A virus has been generated which sought to test these limits (Zuniga *et al*., 2007). This virus, dubbed the 'triporter', expresses additional material from three separate ATUs in a modified Edtag context. The first is upstream of N at the 3′ end of the genome and expresses EGFP, the second is between the P and M TUs and expresses β-galactosidase, and the third is between the H and L TUs and expresses CAT. Collectively these three ATUs increase the genome size by over 25% and introduce three additional Ig sequences where the RdRp can detach from the (−)RNP which in turn may increase transcription attenuation for TUs further down the genome. Nevertheless the rMV could be rescued, replicated efficiently in cells and all three reporter proteins could be detected. To test the utility of this system in delivering foreign antigens *in vivo*, a second virus was generated which expressed two proteins of simian immunodeficiency virus (SIV) from two of the ATUs; between P and M, and between H and L. Infection of CD46 expressing transgenic mice lacking the interferon α/β receptor led to the induction of strong and enduring humoral immune responses to SIV.

Given that polyploidy has been observed in MV virions (Rager *et al*., 2002) and 'triporter' viruses can be generated, it was logical to hypothesise that MV could exist as a segmented virus. This has been shown to be possible using the wild-type IC-B strain (Takeda *et al*., 2006). Three separate genome segments were generated. The first segment expressed β-galactosidase from the promoter proximal position, with the N and P genes being present in the second and third positions. The second segment expressed the fluorescent reporter protein DsRed from the promoter proximal position with M and F proteins being expressed from the second and third genes of that segment. The third segment expressed EGFP from the first position with the H and L proteins expressed from the second and third genes. Replicating genome segments were recovered which replicated well in cultured cells and the three segments were efficiently encapsidated and packaged into virions even in the absence of a known mechanism to ensure that each virion contained a copy of each segment. The reporter genes were stably maintained upon passage. Such viruses may

permit the development of novel vaccination strategies and may also offer insights into aspects of RNP packaging.

The Molecular Medicine Program at the Mayo Clinic, USA, has made seminal contributions to the development of MV as a replicating vector for gene therapy and there are currently a number of rMVs being tested in clinical trials (Galanis, 2010; Galanis *et al*., 2010). From a molecular virological perspective many of the viruses which have been produced elegantly illustrate the genomic fluidity of MV. For example, rMVs which express an H glycoprotein fused to soluble antibodies which recognise tumour-specific markers such as common embryonic antigen, CD38 or epidermal growth factor receptors (Hammond *et al*., 2001; Peng *et al*., 2003; Nakamura *et al*., 2004; Allen *et al*., 2006) and ones which restrict maturation of the F glycoprotein to locations where matrix metalloproteinases by modification of the furin cleave site (Springfeld *et al*., 2006) have been generated. A synergistic approach in which the rMV expresses the sodium iodine symporter (NIS) allows the virus to be localised following the administration of radioactive isotopes (Studebaker *et al*., 2010) and the oncolytic activity can be enhanced by higher energy β or γ emitters (Msaouel *et al*., 2009). Although beyond the scope of this chapter it will be fascinating to see how these approaches develop in the next few years.

6.9 Future perspectives

It is exciting to see how the original MV reverse genetics system developed by Martin Billeter in 1995 has spawned many progeny, both in terms of recombinant viruses and second and third generation systems. Nonetheless, for the most part these represent refinements and although the cDNA clone and the resulting rMV designated Edtag should probably be retired from general use, given the complex assembly history and particular phenotype, this clone and virus have served the MV community well. There are now two well-characterised systems from which wild-type rMVs can be rescued, representing clinical isolates from the developing and the developed world. Most importantly these have been shown to be pathogenic in the most appropriate animal model, the macaque. This is paralleled by systems which can be used to recover the key vaccine viruses, Moraten, Schwartz and Edmonston-Zagreb. It must never be forgotten that paramyxoviruses are the products of the cells in which they are grown and there is work to be done to understand the impact of the cellular substrate on important parameters such as thermostability, genome stability, mutability and particle to plaque forming unit ratios, to mention but a few. Given the number of rMVs which have been generated no such chapter can be comprehensive, and rather than list viruses, we have tried to use judiciously selected examples to illustrate the impact genome manipulation has had on our understanding of the molecular biology, cell biology, immunology, biochemistry and ultimately MV disease pathogenesis. What is intriguing is the molecular basis of virus attenuation is still not fully understood and although certain mutations

have an effect, the specific mutations in the vaccines which have made them some of the safest and most efficacious licensed products remains unidentified. This will be of practical value as recognising which mutations are responsible for the conversion of a wild-type to an attenuated MV should allow the updating of the existing vaccine strains, which were derived from MV isolates 40 years ago, to more closely resemble currently circulating MV strains without interfering with attenuation traits (Norrby, 1995).

Acknowledgements

We thank Martin Ludlow, Kate Sawatzki, Rik de Swart (RdS), Rory de Vries and Rachel Fearns for helpful comments and critique of the manuscript and figures. Macaque viral pathogenesis studies, as illustrated by the macroscopic image, form part of an ongoing collaboration between RdS (Erasmus, MC Rotterdam) and WPD and we acknowledge ZonMw (grant #91208012), MRC (grant #G0801001) and FNIH (grant #DUPREX09GCGH0) for funding these studies. We acknowledge our ongoing collaboration with Stephen McQuaid (Queen's University of Belfast), recognising his exceptional skills in immunohistochemistry and immunocytochemistry, and thank him for the photomicrograph of the infected epithelial cells in the macaque. We are also very grateful to Sarah Butcher and Lassi Liljeroos (Structural Virology Programme, University of Helsinki) for providing us with the electron tomogram of measles and for their novel insights into the morphology of the virion.

References

Abraham, G. and Banerjee, A. K. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America*, **73**: 1504–1508.

Alkhatib, G. and Briedis, D. J. 1986. The predicted primary structure of the measles virus Hemagglutinin. *Virology* **150**: 479–490.

Allen, C., Vongpunsawad, S., Nakamura, T., James, C. D., Schroeder, M., Cattaneo, R., Giannini, C., *et al*. 2006. Retargeted oncolytic measles strains entering via the EGFRvIII receptor maintain significant antitumor activity against gliomas with increased tumor specificity. *Cancer Research* **66**: 11840–11850.

Auwaerter, P. G., Kaneshima, H., Mccune, J. M., Wiegand, G. and Griffin, D. E. 1996. Measles virus infection of thymic epithelium in the SCID-hu mouse leads to thymocyte apoptosis. *The Journal of Virology* **70**: 3734–3740.

Ayata, M., Takeuchi, K., Takeda, M., Ohgimoto, S., Kato, S., Sharma, L. B., Tanaka, M., *et al*. 2010. The F gene of the Osaka-2 strain of measles virus derived from a case of subacute sclerosing panencephalitis is a major determinant of neurovirulence. *Journal of Virology* **84**: 11189–11199.

Ball, L. A. 1977. Transcriptional mapping of vesicular stomatitis virus in vivo. *Journal of Virology* **21**: 411–414.

Ball, L. A. and White, C. N. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America* **73**: 442–446.

Baltimore, D., Huang, A. S. and Stampfer, M. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus, II: an RNA polymerase in the virion. *Proceedings of the National Academy of Sciences of the United States of America*, **66**: 572–576.

Bankamp, B., Takeda, M., Zhang, Y., Xu, W. and Rota, P. A. 2011. Genetic characterization of measles vaccine strains. *Journal of Infectious Diseases* **204** Suppl 1, S533–S548.

Barr, J. N., Whelan, S. P. and Wertz, G. W. 2002. Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Biochimica et Biophysica Acta* **1577**: 337–353.

Barrett, T. and Underwood, B. 1985. Comparison of messenger RNAs induced in cells infected with each member of the morbillivirus group. *Virology* **145**: 195–199.

Bass, B. L. 2002. RNA editing by adenosine deaminases that act on RNA. *Annual Review of Biochemistry* **71**: 817–846.

Bass, B. L., Weintraub, H., Cattaneo, R. and Billeter, M. A. 1989. Biased hypermutation of viral RNA genomes could be due to unwinding/modification of double-stranded RNA. *Cell* **56**: 331.

Baudin, F., Bach, C., Cusack, S. and Ruigrok, R. W. 1994. Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. *EMBO Journal* **13**: 3158–65.

Bellini, W. J., Englund, G., Richardson, C. D., Rozenblatt, S. and Lazzarini, R. A. 1986. Matrix genes of measles virus and canine distemper virus: cloning, nucleotide sequences, and deduced amino acid sequences. *The Journal of Virology* **58**: 408–416.

Bellini, W. J., Englund, G., Rozenblatt, S., Arnheiter, H. and Richardson, C. D. 1985. Measles virus P gene codes for two proteins. *The Journal of Virology* **53**: 908–919.

Berghall, H., Wallen, C., Hyypia, T. and Vainionpaa, R. 2004. Role of cytoskeleton components in measles virus replication. *Archives of Virology* **149**: 891–901.

Bermingham, A. and Collins, P. L. 1999. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 11259–11264.

Billeter, M. A. 2000. Reverse genetics with measles virus to define viral protein and genome function. In Mahy, B. W. and Kolakofsky, D. (eds) *NSV: 11th International Conference on Negative Strand Viruses*.

Billeter, M. A., Naim, H. Y. and Udem, S. A. 2009. Reverse genetics of measles virus and resulting multivalent recombinant vaccines: applications of

recombinant measles viruses. *Current Topics in Microbiology and Immunology* **329**: 129–162.

Blixenkrone-Moller, M., Bernard, A., Bencsik, A., Sixt, N., Diamond, L. E., Logan, J. S. and Wild, T. F. 1998. Role of CD46 in measles virus infection in CD46 transgenic mice. *Virology* **249**: 238–248.

Blumberg, B. M., Crowley, J. C., Silverman, J. I., Menonna, J., Cook, S. D. and Dowling, P. C. 1988. Measles virus L protein evidences elements of ancestral RNA polymerase. *Virology* **164**: 487–497.

Brandler, S., Ruffie, C., Najburg, V., Frenkiel, M. P., Bedouelle, H., Despres, P. and Tangy, F. 2010. Pediatric measles vaccine expressing a dengue tetravalent antigen elicits neutralizing antibodies against all four dengue viruses. *Vaccine* **28**: 6730–6739.

Britton, P., Green, P., Kottier, S., Mawditt, K. L., Penzes, Z., Cavanagh, D. and Skinner, M. A. 1996. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. *The Journal of General Virology* **77**(Pt 5): 963–967.

Brown, D. D., Rima, B. K., Allen, I. V., Baron, M. D., Banyard, A. C., Barrett, T. and Duprex, W. P. 2005. Rational attenuation of a morbillivirus by modulating the activity of the RNA-dependent RNA polymerase. *The Journal of Virology* **79**: 14330–14338.

Buchholz, U. J., Finke, S. and Conzelmann, K. K. 1999. Generation of bovine respiratory syncytial virus (bRSV) from cDNA: bRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional bRSV genome promoter. *Journal of Virology* **73**: 251–259.

Buckland, R., Gerald, C., Barker, R. and Wild, T. F. 1987. Fusion glycoprotein of measles virus: nucleotide sequence of the gene and comparison with other paramyxoviruses. *Journal of General Virology* **68**(Pt 6): 1695–1703.

Calain, P. and Roux, L. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *Journal of Virology* **67**: 4822–4830.

Cathomen, T., Buchholz, C. J., Spielhofer, P. and Cattaneo, R. 1995. Preferential initiation at the second AUG of the measles virus F mRNA: a role for the long untranslated region. *Virology* **214**: 628–632.

Cathomen, T., Mrkic, B., Spehner, D., Drillien, R., Naef, R., Pavlovic, J., Aguzzi, A., *et al*. 1998a. A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation. *EMBO Journal* **17**: 3899–3908.

Cathomen, T., Naim, H. Y. and Cattaneo, R. 1998b. Measles viruses with altered envelope protein cytoplasmic tails gain cell fusion competence. *The Journal of Virology* **72**: 1224–1234.

Cattaneo, R. and Billeter, M. A. 1992. Mutations and A/I hypermutations in measles virus persistent infections. *Current Topics in Microbiology and Immunology* **176**: 63–74.

Cattaneo, R., Kaelin, K., Baczko, K. and Billeter, M. A. 1989. Measles virus editing provides an additional cysteine-rich protein. *Cell* **56**: 759–764.

Cattaneo, R., Rebmann, G., Schmid, A., Baczko, K., ter Meulen, V. and Billeter, M. A. 1987. Altered transcription of a defective measles virus genome derived from a diseased human brain. *EMBO Journal* **6**: 681–688.

Chandrika, R., Horikami, S. M., Smallwood, S. and Moyer, S. A. 1995. Mutations in conserved domain I of the Sendai virus L polymerase protein uncouple transcription and replication. *Virology* **213**: 352–363.

Childs, K. S., Andrejeva, J., Randall, R. E. and Goodbourn, S. 2009. Mechanism of MDA-5 inhibition by paramyxovirus V proteins. *Journal of Virology* **83**: 1465–1473.

Chuang, J. L., Jackson, R. L. and Perrault, J. 1997. Isolation and characterization of vesicular stomatitis virus PoIR revertants: polymerase readthrough of the leader-N gene junction is linked to an ATP-dependent function. *Virology* **229**: 57–67.

Colf, L. A., Juo, Z. S. and Garcia, K. C. 2007. Structure of the measles virus hemagglutinin. *Nature Structural & Molecular Biology* **14**: 1227–1228.

Combredet, C., Labrousse, V., Mollet, L., Lorin, C., Delebecque, F., Hurtrel, B., McClure, H., *et al*. 2003. A molecularly cloned Schwarz strain of measles virus vaccine induces strong immune responses in macaques and transgenic mice. *The Journal of Virology* **77**: 11546–11554.

Conzelmann, K. K. 2004. Reverse Genetics Of Mononegavirales. *Current Topics in Microbiology and Immunology* **283**: 1–41.

Crowley, J. C., Dowling, P. C., Menonna, J., Silverman, J. I., Schuback, D., Cook, S. D. and Blumberg, B. M. 1988. Sequence variability and function of measles virus 3′ and 5′ ends and intercistronic regions. *Virology* **164**: 498–506.

Cruz, C. D., Palosaari, H., Parisien, J. P., Devaux, P., Cattaneo, R., Ouchi, T. and Horvath, C. M. 2006. Measles virus V protein inhibits p53 family member p73. *Journal of Virology* **80**: 5644–5650.

Curran, J. A. and Kolakofsky, D. 1991. Rescue of a Sendai virus DI genome by other parainfluenza viruses: implications for genome replication. *Virology* **182**: 168–176.

Curran, J. A. and Kolakofsky, D. 1999. Replication of paramyxoviruses. *Advances in Virus Research* **54**: 403–422.

Das, T., Mathur, M., Gupta, A. K., Janssen, G. M. and Banerjee, A. K. 1998. RNA polymerase of vesicular stomatitis virus specifically associates with translation elongation factor-1 alphabetagamma for its activity. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 1449–1454.

De Swart, R. L. 2009. Measles studies in the macaque model. *Current Topics in Microbiology and Immunology* **330**: 55–72.

De Swart, R. L., Ludlow, M., De Witte, L., Yanagi, Y., Van Amerongen, G., McQuaid, S., Yuksel, S., *et al*. 2007. Predominant infection of CD150+ lymphocytes and dendritic cells during measles virus infection of macaques. *PLoS Pathogens* **3**: e178.

De Vries, R. D., Lemon, K., Ludlow, M., McQuaid, S., Yuksel, S., Van Amerongen, G., Rennick, L. J., *et al*. 2010. In vivo tropism of attenuated and pathogenic

measles virus expressing green fluorescent protein in macaques. *Journal of Virology* **84**: 4714–4724.

Devaux, P. and Cattaneo, R. 2004. Measles virus phosphoprotein gene products: conformational flexibility of the P/V protein amino-terminal domain and C protein infectivity factor function. *Journal of Virology* **78**: 11632–11640.

Devaux, P., Hudacek, A. W., Hodge, G., Reyes-Del Valle, J., Mcchesney, M. B. and Cattaneo, R. 2011. A recombinant measles virus unable to antagonize STAT1 function cannot control inflammation and is attenuated in rhesus monkeys. *Journal of Virology* **85**: 348–356.

Devaux, P., von Messling, M., V, Songsungthong, W., Springfeld, C. and Cattaneo, R. 2007. Tyrosine 110 in the measles virus phosphoprotein is required to block STAT1 phosphorylation. *Virology*, **360**: 72–83.

Dingli, D., Peng, K. W., Harvey, M. E., Greipp, P. R., O'Connor, M. K., Cattaneo, R., Morris, J. C. and Russell, S. J. 2004. Image-guided radiovirotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. *Blood* **103**: 1641–1646.

Dörig, R. E., Marcil, A., Chopra, A. and Richardson, C. D. 1993. The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell* **75**: 295–305.

Dowling, P. C., Blumberg, B. M., Menonna, J., Adamus, J. E., Cook, P., Crowley, J. C., Kolakofsky, D. and Cook, S. D. 1986. Transcriptional map of the measles virus genome. *Journal of General Virology* **67**(Pt 9): 1987–1992.

Duprex, W. P., Collins, F. M. and Rima, B. K. 2002. Modulating the function of the measles virus RNA-dependent RNA polymerase by insertion of green fluorescent protein into the open reading frame. *Journal of Virology* **76**: 7322–8.

Duprex, W. P., Duffy, I., McQuaid, S., Hamill, L., Cosby, S. L., Billeter, M. A., Schneider-Schaulies, J., *et al*. 1999a. The H gene of rodent brain-adapted measles virus confers neurovirulence to the Edmonston vaccine strain. *Journal of Virology* **73**: 6916–6922.

Duprex, W. P., McQuaid, S., Hangartner, L., Billeter, M. A. and Rima, B. K. 1999b. Observation of measles virus cell-to-cell spread in astrocytoma cells by using a green fluorescent protein-expressing recombinant virus. *The Journal of Virology* **73**: 9568–9575.

Duprex, W. P., McQuaid, S., Roscic-Mrkic, B., Cattaneo, R., McCallister, C. and Rima, B. K. 2000. In vitro and in vivo infection of neural cells by a recombinant measles virus expressing enhanced green fluorescent protein. *Journal of Virology* **74**: 7972–9.

Egelman, E. H., Wu, S. S., Amrein, M., Portner, A. and Murti, G. 1989. The Sendai virus nucleocapsid exists in at least four different helical states. *The Journal of Virology* **63**: 2233–2243.

El Mubarak, H. S., Van De Bildt, M. W., Mustafa, O. A., Vos, H. W., Mukhtar, M. M., Groen, J., El Hassan, A. M., *et al*. 2000. Serological and virological characterization of clinically diagnosed cases of measles in suburban Khartoum. *Journal of Clinical Microbiology* **38**: 987–91.

El Mubarak, H. S., Van De Bildt, M. W.G., Mustafa, O. A., Vos, H. W., Mukhtar, M. M., Ibrahim, S. A., Andeweg, A. C., El Hassan, A. M., *et al*. 2002. Genetic characterisation of wild type measles viruses circulating in suburban Khartoum, 1997–2000. *Journal of General Virology* **83**: 1437–1443.

El Mubarak, H. S., Yüksel, S., Mustafa, O. M., Ibrahim, S. A., Osterhaus, A. D.M. E. and De Swart, R. L. 2004. Surveillance of measles in Sudan using filter paper blood samples. *Journal of Medical Virology* **73**: 624–630.

Escoffier, C., Manie, S., Vincent, S., Muller, C. P., Billeter, M. and Gerlier, D. 1999. Nonstructural C protein is required for efficient measles virus replication in human peripheral blood cells. *The Journal of Virology* **73**: 1695–1698.

Esolen, L. M., Ward, B. J., Moench, T. R. and Griffin, D. E. 1993. Infection of monocytes during measles. *The Journal of Infectious Diseases* **168**: 47–52.

Fearns, R., Peeples, M. E. and Collins, P. L. 1997. Increased expression of the N protein of respiratory syncytial virus stimulates minigenome replication but does not alter the balance between the synthesis of mRNA and antigenome306. *Virology* **236**: 188–201.

Fehr, T., Naim, H. Y., Bachmann, M. F., Ochsenbein, A. F., Spielhofer, P., Bucher, E., Hengartner, H., *et al*. 1998. T-cell independent IgM and enduring protective IgG antibodies induced by chimeric measles viruses. *Nature Medicine* **4**: 945–948.

Finke, S., Mueller-Waldeck, R. and Conzelmann, K. K. 2003. Rabies virus matrix protein regulates the balance of virus transcription and replication. *Journal of General Virology* **84**: 1613–1621.

Fuerst, T. R., Niles, E. G., Studier, F. W. and Moss, B. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 8122–8126.

Galanis, E. 2010. Therapeutic potential of oncolytic measles virus: promises and challenges. *Clinical Pharmacology and Therapeutics* **88**: 620–5.

Galanis, E., Hartmann, L. C., Cliby, W. A., Long, H. J., Peethambaram, P. P., Barrette, B. A., Kaur, J. S., Haluska, P. J., Jr., *et al*. 2010. Phase I trial of intraperitoneal administration of an oncolytic measles virus strain engineered to express carcinoembryonic antigen for recurrent ovarian cancer. *Cancer Research* **70**: 875–882.

Gerald, C., Buckland, R., Barker, R., Freeman, G. and Wild, T. F. 1986. Measles virus haemagglutinin gene: cloning, complete nucleotide sequence analysis and expression in COS cells. *Journal of General Virology* **67**(Pt 12): 2695–2703.

Gerlier, D. and Valentin, H. 2009. Measles virus interaction with host cells and impact on innate immunity. *Current Topics in Microbiology and Immunology* **329**: 163–91.

Griffin, D. E. 2007. Measles virus. In Knipe D. M. and Howley, P. M. (eds) *Fields Virology*. 5th edition. Lippincott, Williams and Wilkins: Philadelphia, PA.

Guerbois, M., Moris, A., Combredet, C., Najburg, V., Ruffie, C., Fevrier, M., Cayet, N., *et al*. 2009. Live attenuated measles vaccine expressing HIV-1 Gag virus like

particles covered with gp160DeltaV1V2 is strongly immunogenic. *Virology* **388**: 191–203.

Gupta, A. K., Shaji, D. and Banerjee, A. K. 2003. Identification of a novel tripartite complex involved in replication of vesicular stomatitis virus genome RNA. *The Journal of Virology* **77**: 732–738.

Hahm, B. 2009. Hostile communication of measles virus with host innate immunity and dendritic cells. *Current Topics in Microbiology and Immunology* **330**: 271–287.

Hahm, B., Arbour, N., Naniche, D., Homann, D., Manchester, M. and Oldstone, M. B. 2003. Measles virus infects and suppresses proliferation of T lymphocytes from transgenic mice bearing human signaling lymphocytic activation molecule. *The Journal of Virology* **77**: 3505–3515.

Hammond, A. L., Plemper, R. K., Zhang, J., Schneider, U., Russell, S. J. and Cattaneo, R. 2001. Single-chain antibody displayed on a recombinant measles virus confers entry through the tumor-associated carcinoembryonic antigen. *The Journal of Virology* **75**: 2087–2096.

Hasegawa, K., Pham, L., O'connor, M. K., Federspiel, M. J., Russell, S. J. and Peng, K. W. 2006. Dual therapy of ovarian cancer using measles viruses expressing carcinoembryonic antigen and sodium iodide symporter. *Clinical Cancer Research* **12**: 1868–1875.

Hashiguchi, T., Kajikawa, M., Maita, N., Takeda, M., Kuroki, K., Sasaki, K., Kohda, D., *et al*. 2007. Crystal structure of measles virus hemagglutinin provides insight into effective vaccines. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 19535–19540.

Hashiguchi, T., Ose, T., Kubota, M., Maita, N., Kamishikiryo, J., Maenaka, K. and Yanagi, Y. 2011. Structure of the measles virus hemagglutinin bound to its cellular receptor SLAM. *Nature Structural and Molecular Biology* **18**: 135–41.

Hashimoto, K., Ono, N., Tatsuo, H., Minagawa, H., Takeda, M., Takeuchi, K. and Yanagi, Y. 2002. SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. *The Journal of Virology* **76**: 6743–6749.

Hirano, A., Ayata, M., Wang, A. H. and Wong, T. C. 1993. Functional analysis of matrix proteins expressed from cloned genes of measles virus variants that cause subacute sclerosing panencephalitis reveals a common defect in nucleocapsid binding. *The Journal of Virology* **67**: 1848–1853.

Hirano, A., Wang, A. H., Gombart, A. F. and Wong, T. C. 1992. The matrix proteins of neurovirulent subacute sclerosing panencephalitis virus and its acute measles virus progenitor are functionally different. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 8745–8749.

Hoffman, S. J., Polack, F. P., Hauer, D. A., Singh, M., Billeter, M. A., Adams, R. J. and Griffin, D. E. 2003. Vaccination of rhesus macaques with a recombinant measles virus expressing interleukin-12 alters humoral and cellular immune responses. *Journal of Infectious Diseases* **188**: 1553–1561.

Homann, H. E., Hofschneider, P. H. and Neubert, W. J. 1990. Sendai virus gene expression in lytically and persistently infected cells. *Virology* **177**: 131–140.

Horikami, S. M. and Moyer, S. A. 1991. Synthesis of leader RNA and editing of the P mRNA during transcription by purified measles virus. *The Journal of Virology* **65**: 5342–5347.

Horikami, S. M. and Moyer, S. A. 1995. Structure, transcription, and replication of measles virus. *Current Topics in Microbiology and Immunology* **191**: 35–50.

Horvat, B., Rivailler, P., Varior-Krishnan, G., Cardoso, A., Gerlier, D. and Rabourdin-Combe, C. 1996. Transgenic mice expressing human measles virus (MV) receptor CD46 provide cells exhibiting different permissivities to MV infections. *The Journal of Virology* **70**: 6673–6681.

Hwang, L. N., Englund, N., Das, T., Banerjee, A. K. and Pattnaik, A. K. 1999. Optimal replication activity of vesicular stomatitis virus RNA polymerase requires phosphorylation of a residue(s) at carboxy-terminal domain II of its accessory subunit, phosphoprotein P. *Journal of Virology* **73**: 5613–20.

Iankov, I. D., Haralambieva, I. H. and Galanis, E. 2011. Immunogenicity of attenuated measles virus engineered to express Helicobacter pylori neutrophil-activating protein. *Vaccine* **29**: 1710–1720.

Ikegame, S., Takeda, M., Ohno, S., Nakatsu, Y., Nakanishi, Y. and Yanagi, Y. 2010. Both RIG-I and MDA5 RNA helicases contribute to the induction of alpha/beta interferon in measles virus-infected human cells. *Journal of Virology* **84**: 372–379.

Iverson, L. E. and Rose, J. K. 1981. Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. *Cell* **23**: 477–484.

Iwasaki, M., Takeda, M., Shirogane, Y., Nakatsu, Y., Nakamura, T. and Yanagi, Y. 2009. The matrix protein of measles virus regulates viral RNA synthesis and assembly by interacting with the nucleocapsid protein. *Journal of Virology* **83**: 10374–10383.

Iwasaki, M. and Yanagi, Y. 2011. Expression of the Sendai (murine parainfluenza) virus C protein alleviates restriction of measles virus growth in mouse cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 15384–15389.

Johnston, I. C., Ter, M., V, Schneider-Schaulies, J. and Schneider-Schaulies, S. 1999. A recombinant measles vaccine virus expressing wild-type glycoproteins: consequences for viral spread and cell tropism. *The Journal of Virology* **73**: 6903–6915.

Kobune, F., Sakata, H. and Sugiura, A. 1990. Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. *The Journal of Virology* **64**: 700–705.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. and Roux, L. 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *Journal of Virology* **72**: 891–899.

Komarova, A. V., Combredet, C., Meyniel-Schicklin, L., Chapelle, M., Caignard, G., Camadro, J. M., *et al*. 2011. Proteomic analysis of virus–host interactions

in an infectious context using recombinant viruses. *Molecular and Cellular Proteomics* **10**(12):M110.007443.

Komase, K., Nakayama, T., Iijima, M., Miki, K., Kawanishi, R. and Uejima, H. 2006. The phosphoprotein of attenuated measles AIK-C vaccine strain contributes to its temperature-sensitive phenotype. *Vaccine* **24**: 826–834.

Kovacs, G. R., Parks, C. L., Vasilakis, N. and Udem, S. A. 2003. Enhanced genetic rescue of negative-strand RNA viruses: use of an MVA-T7 RNA polymerase vector and DNA replication inhibitors. *Journal of Virology Methods* **111**: 29–36.

Kozak, M. 1991a. Effects of long 5′ leader sequences on initiation by eukaryotic ribosomes in vitro. *Gene Expression* **1**: 117–125.

Kozak, M. 1991b. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *Journal of Biological Chemistry* **266**: 19867–19870.

Kumada, A., Komase, K. and Nakayama, T. 2004. Recombinant measles AIK-C strain expressing current wild-type hemagglutinin protein. *Vaccine* **22**: 309–316.

Lamb, R. A. and Parks, G. D. 2007. Paramyxoviridae: the viruses and their replication. In Knipe, D. M. and Howley, P. M. (eds) *Fields Virology*. Lippincott, Williams and Wilkins: Philadelphia, PA.

Langedijk, J. P., Daus, F. J. and Van Oirschot, J. T. 1997. Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin. *The Journal of Virology* **71**: 6155–6167.

Le Mercier, P., Garcin, D., Garcia, E. and Kolakofsky, D. 2003. Competition between the Sendai virus N mRNA start site and the genome 3′-end promoter for viral RNA polymerase. *The Journal of Virology* **77**: 9147–9155.

Lemon, K., De Vries, R. D., Mesman, A. W., McQuaid, S., Van Amerongen, G., Yuksel, S., Ludlow, M., Rennick, L. J., *et al*. 2011. Early target cells of measles virus after aerosol infection of non-human primates. *PLoS Pathogens* **7**: e1001263.

Leonard, V. H., Hodge, G., Reyes-Del, V. J., Mcchesney, M. B. and Cattaneo, R. 2010. Measles virus selectively blind to signaling lymphocytic activation molecule (SLAM; CD150) is attenuated and induces strong adaptive immune responses in rhesus monkeys. *Journal of Virology* **84**: 3413–3420.

Leonard, V. H., Sinn, P. L., Hodge, G., Miest, T., Devaux, P., Oezguen, N., Braun, W., Mccray, P. B., *et al*. 2008. Measles virus blind to its epithelial cell receptor remains virulent in rhesus monkeys but cannot cross the airway epithelium and is not shed. *Journal of Clinical Investigations* **118**: 2448–2458.

Leppert, M., Rittenhouse, L., Perrault, J., Summers, D. F. and Kolakofsky, D. 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. *Cell* **18**: 735–747.

Liniger, M., Zuniga, A., Morin, T. N., Combardiere, B., Marty, R., Wiegand, M., Ilter, O., *et al*. 2009. Recombinant measles viruses expressing single or multiple antigens of human immunodeficiency virus (HIV-1) induce cellular and humoral immune responses. *Vaccine* **27**: 3299–3305.

Liston, P. and Briedis, D. J. 1994. Measles virus V protein binds zinc. *Virology* **198**: 399–404.

Liston, P. and Briedis, D. J. 1995. Ribosomal frameshifting during translation of measles virus P protein mRNA is capable of directing synthesis of a unique protein. *The Journal of Virology* **69**: 6742–6750.

Liston, P., Diflumeri, C. and Briedis, D. J. 1995. Protein interactions entered into by the measles virus P, V, and C proteins. *Virus Research* **38**: 241–259.

Lorin, C., Delebecque, F., Labrousse, V., Da Silva, L., Lemonnier, F., Brahic, M. and Tangy, F. 2005. A recombinant live attenuated measles vaccine vector primes effective HLA-A0201-restricted cytotoxic T lymphocytes and broadly neutralizing antibodies against HIV-1 conserved epitopes. *Vaccine* **23**: 4463–4472.

Lorin, C., Mollet, L., Delebecque, F., Combredet, C., Hurtrel, B., Charneau, P., Brahic, M. and Tangy, F. 2004. A single injection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. *The Journal of Virology* **78**: 146–157.

Ludlow, M., Duprex, W. P., Cosby, S. L., Allen, I. V. and McQuaid, S. 2008. Advantages of using recombinant measles viruses expressing a fluorescent reporter gene with vibratome slice technology in experimental measles neuropathogenesis. *Neuropathology and Applied Neurobiology* **34**: 424–434.

Ludlow, M., McQuaid, S., Cosby, S. L., Cattaneo, R., Rima, B. K. and Duprex, W. P. 2005. Measles virus superinfection immunity and receptor redistribution in persistently infected NT2 cells. *The Journal of General Virology* **86**: 2291–2303.

Luytjes, W., Krystal, M., Enami, M., Pavin, J. D. and Palese, P. 1989. Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**: 1107–1113.

Maisner, A., Mrkic, B., Herrler, G., Moll, M., Billeter, M. A., Cattaneo, R. and Klenk, H. D. 2000. Recombinant measles virus requiring an exogenous protease for activation of infectivity. *Journal of General Virology* **81**: 441–449.

Manie, S. N., Debreyne, S., Vincent, S. and Gerlier, D. 2000. Measles virus structural components are enriched into lipid raft microdomains: a potential cellular location for virus assembly. *The Journal of Virology* **74**: 305–311.

Martin, A., Staeheli, P. and Schneider, U. 2006. RNA polymerase II-controlled expression of antigenomic RNA enhances the rescue efficacies of two different members of the Mononegavirales independently of the site of viral genome replication. *Journal of Virology* **80**: 5708–5715.

Moeller-Ehrlich, K., Ludlow, M., Beschorner, R., Meyermann, R., Rima, B. K., Duprex, W. P., Niewiesk, S. and Schneider-Schaulies, J. 2007. Two functionally linked amino acids in the stem 2 region of measles virus haemagglutinin determine infectivity and virulence in the rodent central nervous system. *The Journal of General Virology* **88**: 3112–3120.

Moeller, K., Duffy, I., Duprex, P., Rima, B., Beschorner, R., Fauser, S., Meyermann, R., *et al*. 2001. Recombinant measles viruses expressing altered hemagglutinin

(H) genes: functional separation of mutations determining H antibody escape from neurovirulence. *Journal of Virology* **75**: 7612–7620.

Moll, M., Klenk, H. D. and Maisner, A. 2002. Importance of the cytoplasmic tails of the measles virus glycoproteins for fusogenic activity and the generation of recombinant measles viruses. *The Journal of Virology* **76**: 7174–7186.

Moyer, S. A., Baker, S. C. and Horikami, S. M. 1990. Host cell proteins required for measles virus reproduction. *Journal of General Virology* **71**(Pt 4): 775–783.

Mrkic, B., Odermatt, B., Klein, M. A., Billeter, M. A., Pavlovic, J. and Cattaneo, R. 2000. Lymphatic dissemination and comparative pathology of recombinant measles viruses in genetically modified mice. *The Journal of Virology* **74**: 1364–1372.

Mrkic, B., Pavlovic, J., Rulicke, T., Volpe, P., Buchholz, C. J., Hourcade, D., Atkinson, J. P., Aguzzi, A. and Cattaneo, R. 1998. Measles virus spread and pathogenesis in genetically modified mice. *The Journal of Virology* **72**: 7420–7427.

Msaouel, P., Iankov, I. D., Allen, C., Aderca, I., Federspiel, M. J., Tindall, D. J., Morris, J. C., *et al*. 2009. Noninvasive imaging and radiovirotherapy of prostate cancer using an oncolytic measles virus expressing the sodium iodide symporter. *Molecular Therapy: The Journal of the American Society of Gene Therapy* **17**: 2041–2048.

Nakamura, T., Peng, K. W., Vongpunsawad, S., Harvey, M., Mizuguchi, H., Hayakawa, T., Cattaneo, R. and Russell, S. J. 2004. Antibody-targeted cell fusion. *Nature Biotechnology* **22**: 331–336.

Nakatsu, Y., Takeda, M., Iwasaki, M. and Yanagi, Y. 2009. A highly attenuated measles virus vaccine strain encodes a fully functional C protein. *Journal of Virology* **83**: 11996–12001.

Nakatsu, Y., Takeda, M., Kidokoro, M., Kohara, M. and Yanagi, Y. 2006a. Rescue system for measles virus from cloned cDNA driven by vaccinia virus Lister vaccine strain. *Journal of Virological Methods* **137**: 152–155.

Nakatsu, Y., Takeda, M., Ohno, S., Koga, R. and Yanagi, Y. 2006b. Translational inhibition and increased interferon induction in cells infected with C protein-deficient measles virus. *Journal of Virology* **80**: 11861–11867.

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M. and Yanagi, Y. 2008. Measles virus circumvents the host interferon response by different actions of the C and V proteins. *Journal of Virology* **82**: 8296–8306.

Nakayama, T., Komase, K., Uzuka, R., Hoshi, A. and Okafuji, T. 2001. Leucine at position 278 of the AIK-C measles virus vaccine strain fusion protein is responsible for reduced syncytium formation. *Journal of General Virology* **82**: 2143–2150.

Naniche, D., Varior-Krishnan, G., Cervoni, F., Wild, T. F., Rossi, B., Rabourdin-Combe, C. and Gerlier, D. 1993. Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *The Journal of Virology* **67**: 6025–6032.

Navaratnarajah, C. K., Leonard, V. H. and Cattaneo, R. 2009. Measles virus glycoprotein complex assembly, receptor attachment, and cell entry. *Current Topics in Microbiology and Immunology* **329**: 59–76.

Norrby, E. 1995. The paradigms of measles vaccinology. *Current Topics in Microbiology and Immunology* **191**: 167–80.

Noyce, R. S., Bondre, D. G., Ha, M. N., Lin, L. T., Sisson, G., Tsao, M. S. and Richardson, C. D. 2011. Tumor cell marker PVRL4 (Nectin 4) is an epithelial cell receptor for measles virus. *PLoS Pathogens* **7**: e1002240.

Ohgimoto, S., Ohgimoto, K., Niewiesk, S., Klagge, I. M., Pfeuffer, J., Johnston, I. C., Schneider-Schaulies, J., *et al*. 2001. The haemagglutinin protein is an important determinant of measles virus tropism for dendritic cells in vitro. *Journal of General Virology* **82**: 1835–1844.

Ohno, S., Ono, N., Seki, F., Takeda, M., Kura, S., Tsuzuki, T. and Yanagi, Y. 2007. Measles virus infection of SLAM (CD150) knockin mice reproduces tropism and immunosuppression in human infection. *Journal of Virology* **81**: 1650–1659.

Okada, H., Itoh, M., Nagata, K. and Takeuchi, K. 2009. Previously unrecognized amino acid substitutions in the hemagglutinin and fusion proteins of measles virus modulate cell-cell fusion, hemadsorption, virus growth, and penetration rate. *Journal of Virology* **83**: 8713–8721.

Osunkoya, B. O., Ukaejiofo, E. O., Ajayi, O. and Akinyemi, A. A. 1990. Evidence that circulating lymphocytes act as vehicles or viraemia in measles. *West African Journal of Medicine* **9**: 35–39.

Pain, V. M. 1996. Initiation of protein synthesis in eukaryotic cells. *European Journal of Biochemistry* **236**: 747–771.

Panum, P. 1938. Observations made during the epidemic of measles on the Faroe Islands in the year 1846. *Med Classics* **3**: 829–886.

Paraskevakou, G., Allen, C., Nakamura, T., Zollman, P., James, C. D., Peng, K. W., Schroeder, M., *et al*. 2007. Epidermal growth factor receptor (EGFR)-retargeted measles virus strains effectively target. *Molecular Therapy* **15**: 677–686.

Parks, C. L., Lerch, R. A., Walpita, P., Sidhu, M. S. and Udem, S. A. 1999. Enhanced measles virus cDNA rescue and gene expression after heat shock. *The Journal of Virology* **73**: 3560–3566.

Patterson, J. B., Cornu, T. I., Redwine, J., Dales, S., Lewicki, H., Holz, A., Thomas, D., Billeter, M. A. and Oldstone, M. B. 2001. Evidence that the hypermutated M protein of a subacute sclerosing panencephalitis measles virus actively contributes to the chronic progressive CNS disease. *Virology* **291**: 215–225.

Pattnaik, A. K., Hwang, L., Li, T., Englund, N., Mathur, M., Das, T. and Banerjee, A. K. 1997. Phosphorylation within the amino-terminal acidic domain I of the phosphoprotein of vesicular stomatitis virus is required for transcription but not for replication. *Journal of Virology* **71**: 8167–8175.

Peng, K. W., Donovan, K. A., Schneider, U., Cattaneo, R., Lust, J. A. and Russell, S. J. 2003. Oncolytic measles viruses displaying a single-chain antibody against CD38, a myeloma cell marker. *Blood* **101**: 2557–2562.

Perrotta, A. T. and Been, M. D. 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. *Nature* **350**: 434–436.

Plemper, R. K. and Compans, R. W. 2003. Mutations in the putative HR-C region of the measles virus F2 glycoprotein modulate syncytium formation. *The Journal of Virology* **77**: 4181–4190.

Plumet, S., Duprex, W. P. and Gerlier, D. 2005. Dynamics of viral RNA synthesis during measles virus infection. *Journal of Virology* **79**: 6900–6908.

Pohl, C., Duprex, W. P., Krohne, G., Rima, B. K. and Schneider-Schaulies, S. 2007. Measles virus M and F proteins associate with detergent-resistant membrane fractions and promote formation of virus-like particles. *The Journal of General Virology* **88**: 1243–1250.

Racaniello, V. R. and Baltimore, D. 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**: 916–919.

Radecke, F. and Billeter, M. A. 1996. The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cells. *Virology* **217**: 418–421.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G. and Billeter, M. A. 1995. Rescue of measles viruses from cloned DNA. *EMBO Journal* **14**: 5773–5784.

Rager, M., Vongpunsawad, S., Duprex, W. P. and Cattaneo, R. 2002. Polyploid measles virus with hexameric genome length. *EMBO Journal* **21**: 2364–72.

Rennick, L. J., Duprex, W. P. and Rima, B. K. 2007. Measles virus minigenomes encoding two autofluorescent proteins reveal cell-to-cell variation in reporter expression dependent on viral sequences between the transcription units. *Journal of General Virology* **88**: 2710–2718.

Richardson, C. D., Berkovich, A., Rozenblatt, S. and Bellini, W. J. 1985. Use of antibodies directed against synthetic peptides for identifying cDNA clones, establishing reading frames, and deducing the gene order of measles virus. *The Journal of Virology* **54**: 186–193.

Richardson, C., Hull, D., Greer, P., Hasel, K., Berkovich, A., Englund, G., Bellini, W., *et al,* 1986. The nucleotide sequence of the mRNA encoding the fusion protein of measles virus (Edmonston strain): a comparison of fusion proteins from several different paramyxoviruses. *Virology* **155**: 508–523.

Rima, B. K., Baczko, K., Clarke, D. K., Curran, M. D., Martin, S. J., Billeter, M. A. and ter Meulen, V. 1986. Characterization of clones for the sixth (L) gene and a transcriptional map for morbilliviruses. *Journal of General Virology* **67**(Pt 9): 1971–1978.

Rima, B. K. and Duprex, W. P. 2009. The measles virus replication cycle. *Current Topics in Microbiology and Immunology* **329**: 77–102.

Rima BK. and Duprex WP. 2011. New concepts in measles virus replication: getting in and out in vivo and modulating the host cell environment. *Virus Research* **162**(1–2): 47–62.

Rosenstein, S. P. and Been, M. D. 1991. Evidence that genomic and antigenomic RNA self-cleaving elements from hepatitis delta virus have similar secondary structures. *Nucleic Acids Research* **19**: 5409–5416.

Rozenblatt, S., Eizenberg, O., Ben Levy, R., Lavie, V. and Bellini, W. J. 1985. Sequence homology within the morbilliviruses. *The Journal of Virology* **53**: 684–690.

Runkler, N., Dietzel, E., Carsillo, M., Niewiesk, S. and Maisner, A. 2009. Sorting signals in the measles virus wild-type glycoproteins differently influence virus spread in polarized epithelia and lymphocytes. *The Journal of General Virology* **90**: 2474–2482.

Runkler, N., Dietzel, E., Moll, M., Klenk, H. D. and Maisner, A. 2008. Glycoprotein targeting signals influence the distribution of measles virus envelope proteins and virus spread in lymphocytes. *Journal of General Virology* **89**: 687–696.

Runkler, N., Pohl, C., Schneider-Schaulies, S., Klenk, H. D. and Maisner, A. 2007. Measles virus nucleocapsid transport to the plasma membrane requires stable expression and surface accumulation of the viral matrix protein. *Cell Microbiology* **9**: 1203–1214.

Santiago, C., Celma, M. L., Stehle, T. and Casasnovas, J. M. 2010. Structure of the measles virus hemagglutinin bound to the CD46 receptor. *Nature Structural and Molecular Biology* **17**: 124–129.

Sawada, A., Komase, K. and Nakayama, T. 2011. AIK-C measles vaccine expressing fusion protein of respiratory syncytial virus induces protective antibodies in cotton rats. *Vaccine* **29**: 1481–1490.

Schlender, J., Schnorr, J. J., Spielhoffer, P., Cathomen, T., Cattaneo, R., Billeter, M. A., ter Meulen, V. and Schneider-Schaulies, S. 1996. Interaction of measles virus glycoproteins with the surface of uninfected peripheral blood lymphocytes induces immunosuppression in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 13194–13199.

Schneider, H., Kaelin, K. and Billeter, M. A. 1997a. Recombinant measles viruses defective for RNA editing and V protein synthesis are viable in cultured cells. *Virology* **227**: 314–322.

Schneider, H., Spielhofer, P., Kaelin, K., Dotsch, C., Radecke, F., Sutter, G. and Billeter, M. A. 1997b. Rescue of measles virus using a replication-deficient vaccinia-T7 vector. *Journal of Virological Methods* **64**: 57–64.

Schneider, U., Bullough, F., Vongpunsawad, S., Russell, S. J. and Cattaneo, R. 2000. Recombinant measles viruses efficiently entering cells through targeted receptors. *The Journal of Virology* **74**: 9928–9936.

Schneider, U., von Messling, V., Devaux, P. and Cattaneo, R. 2002. Efficiency of measles virus entry and dissemination through different receptors. *The Journal of Virology* **76**: 7460–7467.

Schneider-Schaulies, J., ter Meulen, V. and Schneider-Schaulies, S. 2003. Measles infection of the central nervous system. *Journal of Neurovirology* **9**: 247–252.

Schneider-Schaulies, S. and Duprex, W. P. 2011. Measles virus. *The Biology of Paramyxoviruses*.In: Samal, S. K. (ed). Caister Academic Press:Norfolk, United Kingdom.

Schneider-Schaulies, S., Liebert, U. G., Baczko, K., Cattaneo, R., Billeter, M. and ter Meulen, V. 1989. Restriction of measles virus gene expression in acute and subacute encephalitis of Lewis rats. *Virology* **171**: 525–534.

Schneider-Schaulies, S. and Schneider-Schaulies, J. 2009. Measles virus-induced immunosuppression. *Current Topics in Microbiology and Immunology* **330**: 243–269.

Schnell, M. J., Mebatsion, T. and Conzelmann, K. K. 1994. Infectious rabies viruses from cloned cDNA. *EMBO Journal* **13**: 4195–4203.

Schubert, S., Moller-Ehrlich, K., Singethan, K., Wiese, S., Duprex, W. P., Rima, B. K., Niewiesk, S. and Schneider-Schaulies, J. 2006. A mouse model of persistent brain infection with recombinant Measles virus. *Journal of General Virology* **87**: 2011–2019.

Seki, F., Takeda, M., Minagawa, H. and Yanagi, Y. 2006. Recombinant wild-type measles virus containing a single N481Y substitution in its haemagglutinin cannot use receptor CD46 as efficiently as that having the haemagglutinin of the Edmonston laboratory strain. *Journal of General Virology* **87**: 1643–1648.

Seki, F., Yamada, K., Nakatsu, Y., Okamura, K., Yanagi, Y., Nakayama, T., Komase, K. and Takeda, M. 2011. The SI strain of measles virus derived from an SSPE patient possesses typical genome alterations and unique amino acid changes that modulate receptor specificity and reduce membrane fusion activity. *Journal of Virology*. **85**: 11871–11882.

Sellin, C. I., Davoust, N., Guillaume, V., Baas, D., Belin, M. F., Buckland, R., Wild, T. F. and Horvat, B. 2006. High pathogenicity of wild-type measles virus infection in CD150 (SLAM) transgenic mice. *Journal of Virology* **80**: 6420–6429.

Sellin, C. I. and Horvat, B. 2009. Current animal models: transgenic animal models for the study of measles pathogenesis. *Current Topics in Microbiology and Immunology* **330**: 111–127.

Shaffer, J. A., Bellini, W. J. and Rota, P. A. 2003. The C protein of measles virus inhibits the type I interferon response. *Virology* **315**: 389–397.

Sharma, L. B., Ohgimoto, S., Kato, S., Kurazono, S., Ayata, M., Takeuchi, K., Ihara, T. and Ogura, H. 2009. Contribution of matrix, fusion, hemagglutinin, and large protein genes of the CAM-70 measles virus vaccine strain to efficient growth in chicken embryonic fibroblasts. *Journal of Virology* **83**: 11645–11654.

Shishkova, Y., Harms, H., Krohne, G., Avota, E. and Schneider-Schaulies, S. 2007. Immune synapses formed with measles virus-infected dendritic cells are unstable and fail to sustain T cell activation. *Cell Microbiology* **9**: 1974–1986.

Sidhu, M. S., Chan, J., Kaelin, K., Spielhofer, P., Radecke, F., Schneider, H., Masurekar, M., *et al*. 1995. Rescue of synthetic measles virus minireplicons: measles genomic termini direct efficient expression and propagation of a reporter gene. *Virology* **208**: 800–807.

Singh, M., Cattaneo, R. and Billeter, M. A. 1999. A recombinant measles virus expressing hepatitis B virus surface antigen induces humoral immune responses in genetically modified mice. *The Journal of Virology* **73**: 4823–4828.

Springfeld, C., von Messling, V., Frenzke, M., Ungerechts, G., Buchholz, C. J. and Cattaneo, R. 2006. Oncolytic efficacy and enhanced safety of measles virus activated by tumor-secreted matrix metalloproteinases. *Cancer Research* **66**: 7694–7700.

Studebaker, A. W., Kreofsky, C. R., Pierson, C. R., Russell, S. J., Galanis, E. and Raffel, C. 2010. Treatment of medulloblastoma with a modified measles virus. *Neuro-oncology* **12**: 1034–42.

Tahara, M., Takeda, M., Seki, F., Hashiguchi, T. and Yanagi, Y. 2007a. Multiple amino acid substitutions in hemagglutinin are necessary for wild-type measles virus to acquire the ability to use receptor CD46 efficiently. *Journal of Virology* **81**: 2564–2572.

Tahara, M., Takeda, M. and Yanagi, Y. 2005. Contributions of matrix and large protein genes of the measles virus Edmonston strain to growth in cultured cells as revealed by recombinant viruses. *Journal of Virology* **79**: 15218–15225.

Tahara, M., Takeda, M. and Yanagi, Y. 2007b. Altered interaction of the matrix protein with the cytoplasmic tail of hemagglutinin modulates measles virus growth by affecting virus assembly and cell-cell fusion. *Journal of Virology* **81**: 6827–6836.

Takaki, H., Watanabe, Y., Shingai, M., Oshiumi, H., Matsumoto, M. and Seya, T. 2011. Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN-beta-inducing potential. *Molecular Immunology* **48**: 497–504.

Takeda, M., Nakatsu, Y., Ohno, S., Seki, F., Tahara, M., Hashiguchi, T. and Yanagi, Y. 2006. Generation of measles virus with a segmented RNA genome. *Journal of Virology* **80**: 4242–4248.

Takeda, M., Ohno, S., Seki, F., Nakatsu, Y., Tahara, M. and Yanagi, Y. 2005. Long untranslated regions of the measles virus M and F genes control virus replication and cytopathogenicity. *Journal of Virology* **79**: 14346–14354.

Takeda, M., Ohno, S., Tahara, M., Takeuchi, H., Shirogane, Y., Ohmura, H., Nakamura, T. and Yanagi, Y. 2008. Measles viruses possessing the polymerase protein genes of the Edmonston vaccine strain exhibit attenuated gene expression and growth in cultured cells and SLAM knock-in mice. *Journal of Virology* **82**: 11979–11984.

Takeda, M., Takeuchi, K., Miyajima, N., Kobune, F., Ami, Y., Nagata, N., Suzaki, Y., *et al*. 2000. Recovery of pathogenic measles virus from cloned cDNA. *Journal of Virology* **74**: 6643–6647.

Takeuchi, K., Takeda, M., Miyajima, N., Ami, Y., Nagata, N., Suzaki, Y., Shahnewaz, J., Kadota, S. and Nagata, K. 2005. Stringent requirement for the C protein of wild-type measles virus for growth both in vitro and in macaques. *Journal of Virology* **79**: 7838–7844.

Takeuchi, K., Takeda, M., Miyajima, N., Kobune, F., Tanabayashi, K. and Tashiro, M. 2002. Recombinant wild-type and edmonston strain measles viruses bearing heterologous H proteins: role of H protein in cell fusion and host cell specificity. *Journal of Virology* **76**: 4891–4900.

Taniguchi, T., Palmieri, M. and Weissmann, C. 1978. QB DNA-containing hybrid plasmids giving rise to QB phage formation in the bacterial host. *Nature* **274**: 223–228.

Tatsuo, H., Ono, N., Tanaka, K. and Yanagi, Y. 2000. SLAM (CDw150) is a cellular receptor for measles virus. *Nature* **406**: 893–897.

Tober, C., Seufert, M., Schneider, H., Billeter, M. A., Johnston, I. C., Niewiesk, S., ter Meulen, V. and Schneider-Schaulies, S. 1998. Expression of measles virus V protein is associated with pathogenicity and control of viral RNA synthesis. *The Journal of Virology* **72**: 8124–8132.

Valsamakis, A., Schneider, H., Auwaerter, P. G., Kaneshima, H., Billeter, M. A. and Griffin, D. E. 1998. Recombinant measles viruses with mutations in the C, V, or F gene have altered growth phenotypes in vivo. *The Journal of Virology* **72**: 7754–7761.

Van Binnendijk, R. S., Van der Heijden, R. W. and Osterhaus, A. D. 1995. Monkeys in measles research. *Current Topics in Microbiology and Immunology* **191**: 135–148.

Villarreal, L. P., Breindl, M. and Holland, J. J. 1976. Determination of molar ratios of vesicular stomatitis virus induced RNA species in BHK21 cells. *Biochemistry* **15**: 1663–7.

Vincent, S., Tigaud, I., Schneider, H., Buchholz, C. J., Yanagi, Y. and Gerlier, D. 2002. Restriction of measles virus RNA synthesis by a mouse host cell line: trans-complementation by polymerase components or a human cellular factor(s). *The Journal of Virology* **76**: 6121–6130.

Vongpunsawad, S., Oezgun, N., Braun, W. and Cattaneo, R. 2004. Selectively receptor-blind measles viruses: Identification of residues necessary for SLAM- or CD46-induced fusion and their localization on a new hemagglutinin structural model. *The Journal of Virology* **78**: 302–313.

Vulliemoz, D. and Roux, L. 2001. 'Rule of six': how does the Sendai virus RNA polymerase keep count? *The Journal of Virology* **75**: 4506–4518.

Walpita, P. 2004. An internal element of the measles virus antigenome promoter modulates replication efficiency. *Virus Research* **100**: 199–211.

Wang, Z., Hangartner, L., Cornu, T. I., Martin, L. R., Zuniga, A., Billeter, M. A. and Naim, H. Y. 2001. Recombinant measles viruses expressing heterologous antigens of mumps and simian immunodeficiency viruses. *Vaccine* **19**: 2329–2336.

WHO 2009. Global reductions in measles mortality 2000–2008 and the risk of measles resurgence. *Weekly Epidemiological Record* **84**: 509–516.

Wyatt, L. S., Moss, B. and Rozenblatt, S. 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210**: 202–205.

Yanagi, Y., Takeda, M., Ohno, S. and Hashiguchi, T. 2009. Measles virus receptors. *Current Topics in Microbiology and Immunology* **329**: 13–30.

Yoon, J. J., Krumm, S. A., Ndungu, J. M., Hoffman, V., Bankamp, B., Rota, P. A., Sun, A., Snyder, J. P. and Plemper, R. K. 2009. Target analysis of the

experimental measles therapeutic AS-136A. *Antimicrobial Agents and Chemotherapy* **53**: 3860–3870.

Yoshikawa, Y., Mizumoto, K. and Yamanouchi, K. 1986. Characterization of messenger RNAs of measles virus. *Journal of General Virology* **67**(Pt 12): 2807–2812.

Zhang, X., Wei, Y., Ma, Y., Hu, S. and Li, J. 2010. Identification of aromatic amino acid residues in conserved region VI of the large polymerase of vesicular stomatitis virus is essential for both guanine-N-7 and ribose 2′-O methyltransferases. *Virology* **408**: 241–52.

Zuniga, A., Wang, Z., Liniger, M., Hangartner, L., Caballero, M., Pavlovic, J., Wild, P., *et al*. 2007. Attenuated measles virus as a vaccine vector. *Vaccine* **25**: 2974–2983.

7

Bunyavirus reverse genetics and applications to studying interactions with host cells

Richard M. Elliott
Biomedical Sciences Research Complex, School of Biology, University of St Andrews

7.1 Introduction: the family *Bunyaviridae*

The family *Bunyaviridae* was formally established by the International Committee on Taxonomy of Viruses in 1975 (Porterfield *et al*., 1975) and is currently the largest grouping of RNA viruses. The family takes its name from Bunyamwera virus (BUNV) which was originally isolated from *Aedes* mosquitoes in the Semliki Forest, Uganda (Smithburn *et al*., 1946). Over 350 distinct virus isolates have been classified within the *Bunyaviridae*, whose members are characterised by a tripartite single-stranded RNA genome that encodes viral proteins using a negative- or ambisense strategy. Viruses replicate in the cytoplasm and characteristically assemble intracellularly at the Golgi complex (Nichol *et al*., 2005). Bunyavirus particles are composed of just four structural proteins (Figure 7.1), two external virion surface glycoproteins (Gn and Gc) that are encoded by the M (medium-sized) genome RNA segment, and two internal proteins, N, the nucleocapsid protein encoded by the S (small) segment, and L, the viral RNA-dependent RNA polymerase, that is coded for by the L (large) genome segment. In addition, most viruses also encode nonstructural proteins though their presence differs not only between genera but also between viruses in the same genus. The three RNA segments are encapsidated by N protein to form ribonucleoprotein complexes (RNP), and the RNPs are the functional templates for all viral RNA synthetic events (Plyusnin and Elliott, 2011).

Currently the family *Bunyaviridae* is subdivided into five genera, *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus*. Viruses in each genus are thus referred to as orthobunyaviruses, hantaviruses, etc. while the term bunyavirus is used when referring to viruses in the family as a whole. Classification into an individual genus is based on serological relationships, the pattern of sizes of viral RNAs and structural proteins, and the consensus terminal nucleotide sequence

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

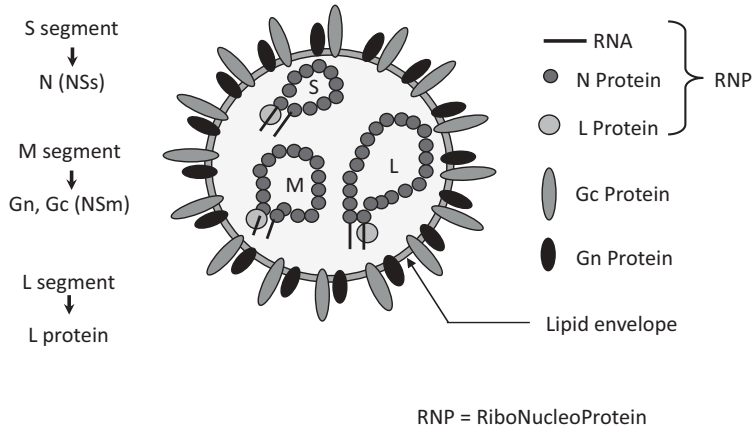

Figure 7.1 Schematic of a bunyavirus particle and coding assignment of the three genome segments.

of the viral genome segments (Nichol *et al*., 2005). Orthobunyaviruses, nairoviruses and phleboviruses are arboviruses (arthropod borne viruses) whose natural life-cycle involves replication in blood-feeding arthropods (mainly mosquitoes, midges, sandflies or ticks) and warm-blooded vertebrate species. Tospoviruses are plant pathogens and are also transmitted by arthropods, in their case, by different thrips species. Hantaviruses are not vectored by arthropods but are maintained in nature as persistent infections of rodents and insectivores, and are transmitted to humans in infected excretions. Viruses in all five genera impinge on human health and well-being, either directly in causing disease or indirectly in causing disease of livestock or crop plants (Plyusnin and Elliott, 2011). Table 7.1 lists some of the important members of the family.

7.2 Bunyavirus replication

The bunyavirus replication cycle is similar to that of other negative-sense RNA viruses (Elliott, 2005), the principal stages being as follows: (1) attachment, mediated by an interaction of either or both viral glycoproteins and host receptors, followed by entry through receptor mediated endocytosis; (2) uncoating, by acidification of endocytic vesicles, and fusion of viral membranes with endosomal membranes to release the RNPs; (3) primary transcription of genomic RNAs to yield mRNA species using host cell-derived primers and the virion-associated polymerase; (4) translation of viral mRNAs; (5) synthesis and encapsidation of antigenomes (full-length postive sense complementary RNA); (6) synthesis of progeny vRNAs (genome replication) from antigenome-containing RNPs; (7) virion assembly and morphogenesis by budding into the Golgi cisterna; and

Table 7.1 Selected important pathogens in the family *Bunyaviridae*.

Genus/virus	Disease	Principal vector	Distribution
Orthobunyavirus			
Akabane	Cattle: abortion and congenital defects	Midge	Africa, Asia, Australia
Cache Valley	Sheep, cattle: congenital defects	Mosquito	N America
La Crosse	Human: encephalitis	Mosquito	N America
Ngari	Human: hemorrhagic fever	Mosquito	Africa
Oropouche	Human: fever	Midge	S. America
Tahyna	Human: fever	Mosquito	Europe
Hantavirus			
Hantaan	Human: severe haemorrhagic fever with renal syndrome (HFRS), fatality 5-15%	Field mouse	Eastern Europe, Asia
Seoul	Human: moderate HFRS, fatality 1%	Rat	Worldwide
Puumala	Human: mild HFRS, fatality 0.1%	Bank vole	Western Europe
Sin Nombre	Human: hantavirus cardiopulmonary syndrome, fatality 50%	Deer mouse	N America
Nairovirus			
Crimean-Congo haemorrhagic fever	Human: haemorrhagic fever, fatality 20–80%	Tick	Eastern Europe, Africa, Asia
Nairobi sheep disease	Sheep, goat: fever, haemorrhagic gastroenteritis, abortion	Tick, mosquito	Africa, Asia
Phlebovirus			
Rift Valley fever	Human: encephalitis, haemorrhagic fever, retinitis, fatality 1–10% Domestic ruminants: necrotic hepatitis, haemorrhage, abortion	Mosquito	Africa
Naples sandfly fever	Human: fever	Sandfly	Europe, Africa
Sicilian sandfly fever	Human: fever	Sandfly	Europe, Africa
Tospovirus			
Tomato spotted wilt	Plants: over 650 species, various symptoms	Thrips	Worldwide

(8) fusion of cytoplasmic vesicles containing viruses with the plasma membrane and release of mature virions (Plyusnin and Elliott, 2011).

Host cell receptors have not been identified for the vast majority of bunyaviruses; β3 and β1 integrins have been associated with entry of pathogenic and non-pathogenic hantaviruses, respectively, into endothelial cells (Gavrilovskaya *et al*., 1998; Gavrilovskaya *et al*., 1999), and DC-SIGN has been shown to mediate

phlebovirus binding, entry and infection of dendritic cells (Lozach *et al*., 2011). Bunyavirus mRNA synthesis resembles that of influenza viruses in that it is primed by cap-containing oligonucleotides cleaved from the 5′ ends of host messages (Patterson *et al*., 1984). In contrast to influenza viruses (where cap-snatching occurs in the nucleus; Plotch *et al*., 1981), bunyavirus transcription takes place in the cytoplasm and utilises mature host mRNAs as primer donors. The endonuclease activity responsible has recently been mapped to a domain at the N terminus of the L protein (Reguera *et al*., 2010). For viruses that have ambi-sense genomes segments, the protein encoded in the positive-sense orientation in the genomic RNA is translated from a specific sub-genomic mRNA that is transcribed from the antigenome, step (5) above. Potentially this allows some degree of temporal control on gene expression in that the 'positive-sense' protein would be synthesised relatively late in the infectious cycle, after the onset of genome replication (Elliott, 2005).

Detailed electron microscopic analysis of BUNV-infected cells has allowed description of the 'viral factory' that is built around the Golgi complex where replication occurs (Novoa *et al*., 2005). The factory is composed of repetitive units of Golgi stacks, mitochondria, components of the rough ER and virus-derived tubular structures with globular heads. Advanced imaging techniques and 3-D reconstructions indicated that the tubes link cellular organelles to the Golgi, thus providing a route for cellular factors required for genome replication. A model was proposed in which viral RNPs are transported from sites of replication in the globular domain of the tubes to the cytoplasm where they condense on Golgi membranes, modified by the insertion of Gn and Gc, and promote budding of particles into Golgi-derived vesicles (Fontana *et al*., 2008).

7.3 History of bunyavirus reverse genetics

In the negative-sense RNA virus field, the term 'reverse genetics' encompasses two major experimental approaches, first, 'minigenome' or 'minireplicon' systems in which genome analogues, usually containing a reduced number of viral genes and the inclusion of an easily measurable reporter gene, are transcribed and replicated by co-expressed viral proteins, and, second, 'rescue' systems whereby infectious viruses, containing specific genetic alterations, are recovered (rescued) from cDNA copies of the viral genomes. A major technical obstacle to overcome for the majority of negative-sense RNA viruses was devising a way to generate the RNP template for viral transcription and replication: simply mixing viral RNA and N protein in a test-tube did not result in a functional RNP. Technical advances that aided the field were the characterisation and subsequent commercial availability of bacteriophage RNA polymerases, such as those from T7 or SP6, that allowed transcription of RNA from cDNA clones, and the characterisation of ribozymes that allowed precise trimming of RNA transcripts to maintain the exact termini as found in authentic viral RNAs.

The recovery of infectious BUNV from plasmid DNA was published in 1996, and this was the first occasion that any segmented negative-sense virus had been

rescued entirely from cloned cDNA. At the time we thought this a most significant breakthrough and as such tried to get it published in *Science* and *Nature* but without success. The latter advised that the paper was more suited to a 'specialized virology journal', advice we ignored: we submitted the manuscript to *PNAS* where it was duly accepted (Bridgen and Elliott, 1996). Indeed, the editors of *PNAS* considered the paper sufficiently noteworthy that they commissioned a Commentary in the same issue (Rose, 1996).

It is worthwhile digressing to understand the background that led to the successful development of BUNV rescue as this may help others when trying to devise similar protocols for their pet bunyavirus. Key is confidence that a cDNA clone(s) is truly representative of the authentic virus sequence. As is well documented, viral RNA polymerases lack proof-reading functions, thus genome replication is highly error prone, leading to the quasi-species concept of a population of related but non-identical genomes within a virus stock (Drake and Holland, 1999). In addition, errors can be introduced during reverse transcription and/or PCR when generating cDNAs, and therefore there is a possibility that any individual cDNA clone may not represent a functional sequence. Therefore, it is preferable to generate a consensus sequence based on multiple cDNA clones or to match the sequence of a cDNA with the population (consensus) sequence of the viral genomic RNA, or alternatively to establish functional assays for the encoded proteins.

With regard to BUNV a functional assay for the polymerase was developed which involved transfecting cells expressing T7 RNA polymerase from a recombinant vaccinia virus, first, with L segment cDNA (L gene under T7 promoter control), followed by purified viral RNPs, and then extracting total cellular RNA to assess new genome synthesis by Northern blotting (Jin and Elliott, 1991). This was tedious but proved that the L segment cDNA (that, incidentally, had been cloned by 'conventional' methods in the pre-PCR era; Elliott, 1989) encoded an L protein that had RNA synthesis activity. In turn, this led to creation of the minigenome system for BUNV, using minigenome RNAs synthesised *in vitro* and transfected into cells expressing BUNV N and L proteins again by the vaccinia virus-T7 system (Dunn *et al*., 1995).

The next stage was to attempt virus rescue. Previous studies on BUNV glycoproteins expressed from cDNA clones provided reasonable evidence that the cDNA contained functional sequences in that correct processing of the glycoprotein precursor was observed, and the expressed proteins were targeted to the Golgi (Nakitare and Elliott, 1993, Lappin *et al*., 1994). Therefore, again using the vaccinia virus-T7 polymerase system, cells were transfected with a mixture of three 'expression plasmids' or 'helper plasmids' (the BUNV protein coding sequences under control of encephalomyocarditis virus internal ribosome entry sequence (IRES) to ensure efficient protein expression) followed by a mixture of three 'transcription plasmids' (full-length BUNV cDNAs flanked by T7 promoter and hepatitis delta virus ribozyme sequences). It was anticipated that any rescued BUNV would be swamped by a background of vaccinia virus and hence the supernatant was passaged through mosquito cells (which permit bunyavirus but not vaccinia virus replication)

followed by plaque isolation of the recombinant BUNV bunyavirus on mammalian cells. No recombinant viruses were generated when the transcription plasmids expressed genome-sense RNAs, but a few plaques (10 to 100 per 10^7 transfected cells) were obtained when the transcription plasmids expressed antigenome RNAs. Looking back the procedure seems rather heroic – it took nearly 3 weeks and generated only small amounts of recombinant virus. Despite its inefficiency, the system was used to generate a recombinant BUNV that did not express the NSs protein (rBUNdelNSs; Bridgen *et al*., 2001), which proved invaluable in determining the function of this non-structural protein (see Section 7.7.5). We suspected the low efficiency of the system was the use of vaccinia virus to provide T7 RNA polymerase; bunyaviruses mature at the Golgi (reviewed by Pettersson and Melin, 1996; Plyusnin and Elliott, 2011) and the vaccinia virus is wrapped by a double membrane that is probably derived from the Golgi (Roberts and Smith, 2008). We thought that if there were competition in the Golgi between the two viruses, the vaccinia virus could win out. It became clear that if we wanted to recover mutant viruses that were markedly less fit than wild type, the inefficiency of the system would be a significant impediment. Therefore, a survey of different ways of expressing T7 RNA polymerase in cells was conducted, and we found the BSR/T7-5 line (Buchholz *et al*., 1999) to be the most efficient for generating infectious virus (Lowen *et al*., 2004).

Meanwhile we had improved the BUNV minigenome system by transfecting cells with a plasmid expressing the minigenome rather than *in vitro* transcribed RNA (Weber *et al*., 2001). We then adapted this protocol in an effort to check the functionality of the transcription plasmids, as we found occasionally that batches of plasmid did not produce infectious virus in the rescue system. We showed that expression of the L and S segment transcription plasmids supported minigenome transcription and replication at a level about 10% of that achieved by the IRES-driven expression plasmids (Lowen *et al*., 2004), even though the T7-transcribed antigenome RNAs would not be 5′capped or 3′polyadenylated and thus were not expected to be active as mRNAs. This led to the idea of just transfecting cells with the three transcription plasmids and omitting the expression plasmids. This generated infectious virus at the first attempt (Lowen *et al*., 2004). The system is extremely efficient, routinely generating 10^7–10^8 pfu of wild-type BUNV from a 60mm diameter dish of cells transfected with 1μg of each plasmid.

7.4 Minigenome systems for bunyaviruses

Minigenome systems for negative-sense viruses have employed both cytoplasmic RNA polymerase (mainly T7 polymerase) and nuclear (cellular RNA polymerase I, pol-I) to drive minigenome synthesis (Neumann and Kawaoka, 2004), the latter perhaps counter-intuitively for those viruses that do not have a nuclear phase in their life-cycle. However, both polymerases have been used to establish minigenome

Table 7.2 Summary of available reverse genetics systems for bunyaviruses[1].

Genus/Virus	Minigenome assay	VLP assay	Virus rescue	
			T7 RNA polymerase	RNA polymerase I
Orthobunyavirus				
Akabane				Ogawa *et al*., 2007
Bunyamwera	Dunn *et al*., 1995 Weber *et al*., 2000 Kohl *et al*., 2004b	Shi *et al*., 2007 Eifan and Elliott, 2009	Bridgen and Elliott, 1996 Lowen *et al*., 2004	
La Crosse	Blakqori *et al*., 2003	Soldan *et al*., 2010	Blakqori and Weber, 2005	
Hantavirus				
Hantaan	Flick *et al*., 2003a Zhang *et al*., 2008			
Nairovirus				
Crimean-Congo haemorrhagic fever	Flick *et al*., 2003b Bergeron *et al*., 2010			
Phlebovirus				
Rift Valley fever	Lopez *et al*., 1995 Ikegami *et al*., 2005	Habjan *et al*., 2009a Terasaki *et al*., 2011 Piper *et al*., 2011	Ikegami *et al*., 2006 Gerrard *et al*., 2007 Habjan *et al*., 2008 Billecocq *et al*., 2008	Habjan *et al*., 2008 Billecocq *et al*., 2008
Toscana	Accardi *et al*., 2001			
Uukuniemi	Flick and Pettersson, 2001	Overby *et al*., 2006		

Note: [1]Only the primary reference or references reporting a significant change are noted.

systems for representatives of the four genera containing animal-infecting bunyaviruses (Table 7.2), but so far no minigenome assay has been reported for tospoviruses. A further development was the creation of a BUNV minigenome system in *Aedes albopictus* mosquito cells, though this shows much lower activity compared to mammalian cell system, largely because of the relatively low plasmid transfection efficiencies of mosquito cells (Kohl *et al*., 2004b).

Minigenome systems have been used to confirm that only N and L proteins are required for viral RNA synthesis (Dunn *et al*., 1995; Lopez *et al*., 1995; Accardi *et al*., 2001; Flick and Pettersson, 2001; Blakqori *et al*., 2003; Ikegami *et al*., 2005;

Gauliard *et al*., 2006). For both BUNV and La Crosse orthobunyavirus (LACV) minigenome systems, expression of NSs was found to down-regulate reporter gene expression. This inhibition was also observed when NSs proteins from heterologous viruses were used in conjunction with the BUNV-based system, suggesting that NSs may have some role in controlling viral RNA synthesis (Weber *et al*., 2001; Blakqori *et al*., 2003). Contrasting resulting have been reported for Rift Valley fever phlebovirus (RFVFV) NSs – Ikegami *et al*. (2005) reported that NSs enhanced minigenome activity whereas others observed an inhibitory effect, like the orthobunyavirus NSs proteins (Bouloy and Weber, 2010; Brennan *et al*., 2011a). These discrepant results may reflect differences in experimental protocol, such as the type of cells in which the assay was performed.

Development of minigenome systems for hantaviruses and nairoviruses has been more problematic. Two reports describe a Hantaan virus minigenome system using the RNA polymerase I approach (Flick *et al*., 2003a; Zhang *et al*., 2008) though in both cases background levels appear high and the positive result (that is, when viral L and N proteins are expressed) gives reporter gene activity significantly lower than that described with orthbunya- or phlebo-virus systems. There are two reports on minigenome systems for Crimean-Congo haemorrhagic fever nairovirus (CCHFV). The first used virus infection to supply viral proteins and hence was limited in its application as the work had to be performed under BSL4 containment (Flick *et al*., 2003b). A more convenient system using plasmid expression of helper proteins was described by Bergeron *et al*. (2010). Interestingly, the system also gave unacceptably high background reporter gene activity if the plasmid-encoding minigenome was transfected into cells (from either T7 or pol-I promoters), but gave robust signals if *in vitro* transcribed minigenome RNA was transfected. The authors speculate that CCHFV-specific sequences somehow enhanced cryptic promoter activity in the transfected plasmid DNA. The system was used to demonstrate that the ovarian tumour protease domain that is unique for nairovirus L proteins was not required for RNA synthesis function.

7.5 Virus-like particle production

An extension of the minigenome assay is the production of infectious virus-like particles (VLP) that can package a minigenome. VLPs are produced by co-expression of the viral glycoproteins, along with N and L proteins, and although VLPs are capable of infecting new cells, they cannot spread or produce progeny as the glycoprotein-encoding genome segment is not packaged. VLPs have been developed for a number of bunyaviruses (Table 7.2) and used to study the requirements for genome packaging, both at the protein level (Overby *et al*., 2006; Shi *et al*., 2006; Overby *et al*., 2007a; Overby *et al*., 2007b; Shi *et al*., 2007; Shi *et al*., 2009; Katz *et al*., 2010) and genome sequence level (Terasaki *et al*., 2011). Most reports indicate that passage of VLPs required the target cells to express the viral N and L proteins, though BUNV VLPs containing a *Renilla* luciferase minigenome

were capable of autonomous expression without the need for exogenous viral proteins (Eifan and Elliott, 2009). From a practical standpoint, RVFV VLPs show promise as candidate vaccines (Naslund *et al*., 2009; Mandell *et al*., 2010; Pichlmair *et al*., 2010).

7.6 Rescue systems for bunyaviruses

The T7 polymerase-based experimental protocol has subsequently been adopted for the rescue of LACV (Blakqori and Weber, 2005) and RVFV (Ikegami *et al*., 2006; Bird *et al*., 2007; Gerrard *et al*., 2007a; Billecocq *et al*., 2008; Habjan *et al*., 2008) (Table 7.2). Recovery of LACV is informative on two levels. First, the strict requirement of a fully functional polymerase clone was apparent by the observation that an L clone that produced activity in the minigenome system was not competent to rescue infectious virus, and had to be modified to match the consensus sequence of three independent GenBank entries (Blakqori *et al*., 2003; Blakqori and Weber, 2005; G. Blakqori, pers. comm.). Indeed, in a side-by-side comparison of BUNV and LACV minigenome systems using the original LACV L cDNA clone, we found the BUNV clones to show considerably more reporter activity (A. McLees and R. M. Elliott, unpublished data). This highlights the need for cDNA clones having the correct (functional) sequence, and also for appropriate assays to monitor their functionality. Second, rescue of La Crosse virus was unsuccessful if plasmids expressing helper viral proteins were co-transfected and only worked when just the three transcription plasmids were transfected (Blakqori and Weber, 2005). This suggests that the ratios of viral proteins and viral RNAs can be critical for successful generation of functional RNPs and subsequent initiation of the infectious cycle. Akabane orthobunyavirus (AKAV) was rescued using the pol-I system (Ogawa *et al*., 2007), and in contrast to other bunyavirus rescues, recombinant virus was found to display slight phenotypic differences from authentic virus. This suggests that the cDNA clones used to derive the virus might not be exact matches to the authentic virus genome or that the recombinant virus was more clonal than authentic virus. RVFV has been recovered by both T7 and pol-I approaches, and for the former the three plasmid approach was also successful. Little difference in rescue efficiency was observed between T7 and pol-I systems for RVFV (Billecocq *et al*., 2008; Habjan *et al*., 2008).

7.7 Application of reverse genetics to study bunyavirus replication

The remainder of this chapter describes the application of rescue technology to explore bunyavirus gene function and viral replication. While the emphasis will be on descriptions of viruses carrying defined mutations in their genomes, it should

be appreciated that experiments using minigenome and VLP assays often underpin these studies and inform which mutant viruses should be produced.

7.7.1 Attenuation of bunyavirus replication by modification of noncoding sequences

Each bunyavirus genome segment comprises 3′ and 5′ noncoding (or untranslated) regions, NCR, that flank the coding sequence for the viral protein. In addition, in ambisense genome segments there is a noncoding intergenic sequence between the coding regions that signals transcription termination. Apart from the conserved very terminal 8–11 bases at both ends of the RNA segments, NCRs are highly variable in length and sequence between segments, both within one virus and between analogous segments of different viruses. The NCRs contain the N protein encapsidation signal, viral promoters, mRNA transcription termination signals and probably RNP-packaging signals, and thus it is remarkable the degree of variation observed in the NCRs for these common functions. Results of minigenome studies indicated that the NCR of the L, M and S segments had different activities, suggesting that promoter strength of the three segments differed (Barr *et al*., 2003; Flick *et al*., 2004; Kohl *et al*., 2004a; Gauliard *et al*., 2006). By reverse genetics a virus was created in which the NCRs on the L segment of BUNV were replaced with those of the M segment (which had been shown to be the most active promoter; Kohl *et al*., 2004a). The virus, termed MLM, thus had wild-type S and M segments and a chimeric L segment (Lowen *et al*., 2005). This virus was attenuated not only in cultured cells but also in mice. Perhaps surprisingly, although the chimeric L segment contained a high activity promoter sequence, the levels of L segment RNAs and of L protein were markedly reduced in infected cells, and the virus showed a much higher particle-to-infectivity ratio than wild-type BUNV. A second study with BUNV showed that internal deletions within the S segment NCRs also attenuated virus replication (Lowen and Elliott, 2005). More recently, we have developed viruses having deletions in the NCRs of all three segments that are so attenuated they no longer cause cytopathic effects in cultured cells (B. Mazel-Sanchez and R.M. Elliott, unpublished data). These results suggest that attenuating replication thorough rearrangement or deletion of NRC could be used to develop live attenuated candidate vaccines.

A further example of the remarkable plasticity of bunyavirus genomes is exemplified by the conversion of RVFV from a three-segmented to a two-segmented virus (Brennan *et al*., 2011b). As discussed in detail below (Section 7.7.5) the NSs protein of RVFV is dispensable for growth in cell culture and NSs-deleted viruses are attenuated in immunocompetent cells and animals. The NSs locus can be used to express foreign genes such as GFP or luciferase (Ikegami *et al*., 2006; Billecocq *et al*., 2008; Bird *et al*., 2008; Kuri *et al*., 2010). We asked whether the virus glycoprotein coding sequences could be inserted in place of NSs, and were successful in rescuing a two-segmented virus whose genome comprised a wild-type L segment

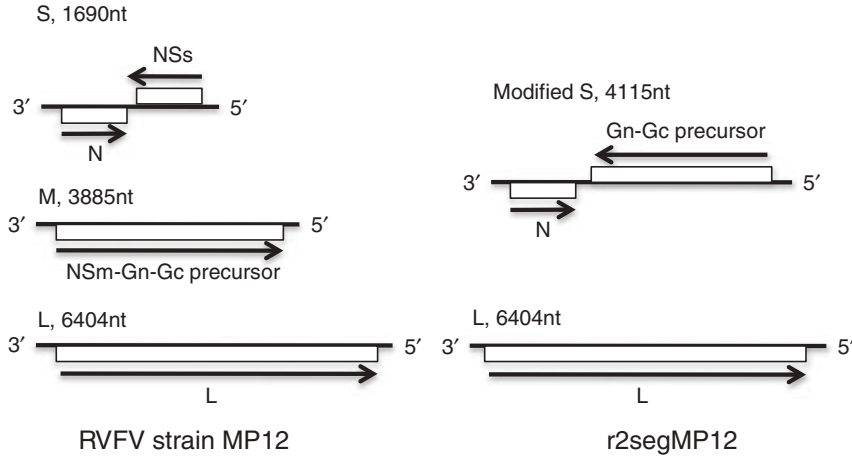

Figure 7.2 Genome organisation of Rift Valley fever virus and the two segmented recombinant called r2segMP12. The RNA segments are represented as the virion sense and the coding regions indicated by open boxes. mRNAs are indicated as arrows. The sizes of the genome segments are based on the MP12 strain of RVFV.

and a hybrid S segment expressing N and glycoproteins (Figure 7.2). The virus thus has an S segment genome arrangement similar to that of arenaviruses (Buchmeier *et al*., 2007). The two-segmented virus was further attenuated compared to a three-segmented NSs deletant virus, but was genetically stable on repeated passage. Interestingly, it was shown that the two-segment virus could act as a vector to express a foreign gene cloned between the M segment NCR. As proof of principal, the coding sequence for GFP was inserted into the M segment in place of the viral glycoprotein precursor sequence and a virus rescued that contained three RNA segments. The virus expressed GFP in infected cells and was stable on repeated passage (Brennan *et al*., 2011b). This opens the door to expressing immunogenic proteins that might give rise to protective antibodies against other pathogens leading to the possibility of bivalent vaccines against RVFV and another disease.

7.7.2 Mutation of nucleocapsid protein

In addition to encapsidating the viral RNA segment, the bunyavirus N protein interacts with itself to form homo-oligomers, with the viral L protein, with the carboxy terminal tail(s) of either or both of the virus glycoproteins (see later), and probably with cellular proteins. A large-scale mutagenic analysis of the BUNV N protein was carried out to define functional domains in the N in advance of a 3-D structural determination. Using a combination of random and targeted mutagenesis techniques, approximately half of the residues in the 233 amino acid BUNV N protein were individually changed (Eifan and Elliott, 2009). The mutant N proteins were assessed

in the minigenome system and displayed a range of activities compared to wild-type N. (An NSs-deleted cDNA was used to ensure that any effects noted could be attributed to the N protein.) On the basis of these results, some mutants were selected for further study, and 57 recombinant viruses were generated carrying single point mutations in the N protein. The viruses showed a range of titre (from 10^3 to 10^8 pfu/ml) and plaque size in cell culture. Four temperature-sensitive mutants were obtained that fell into two groups, one whose lesions affected genome synthesis and the other that had defects in both genome and antigenome, but not mRNA, synthesis. These results confirm the crucial role played by N in RNA production. Interestingly, out of >300 ts mutants of different bunyaviruses previously obtained by conventional virological procedures (for example, growth in the presence of chemical mutagens), only a single mutation had been mapped to the S segment (Pringle, 1996). Obtaining four ts mutants mapping to the BUNV S segment in this study is further testament to the power of reverse genetics. Based on the behaviour of mutant N proteins, a preliminary domain map was constructed (Eifan and Elliott, 2009).

7.7.3 Epitope tagging of the L protein

Study of the intracellular distribution and other properties of the L protein has been hampered by the small amounts of L made during infection and the lack of specific antibodies. The situation has been improved by the recovery of viable BUNV and RVFV that express L protein tagged with the V5 epitope of parainfluenza virus type 5 (Shi and Elliott, 2009; Brennan *et al*., 2011a). In both cases, the 14-amino acid epitope was inserted in a region towards the C terminus of L that was variable in sequence when comparing different L proteins and predicted to be on the surface of the protein. The effect of inserting the epitope in the minigenome system was first assessed, and then infectious viruses were recovered by reverse genetics. For both BUNV and RVFV, insertion of the epitope resulted in a degree of attenuation in tissue culture, but the recombinant L protein could easily be detected using high avidity anti-V5 antibodies by both western blotting and immunofluorescent staining of infected cells. The recombinant viruses will be valuable tools to further investigate the role of the L protein in the viral life-cycle.

7.7.4 NSm protein

The orthobunyavirus NSm protein is sandwiched between Gn and Gc in the precursor polyprotein encoded by the M segment. NSm localises independently of other viral proteins to the Golgi (Nakitare and Elliott, 1993), behaves as an integral membrane protein (Shi *et al*., 2006), and has been suggested to have a possible role in virus assembly. Rescue experiments were attempted with cDNA clones containing deletions of different regions of BUNV NSm, and the results showed that the N-terminal domain of NSm was essential for virus replication (Shi *et al*., 2006).

Furthermore, the hydrophobic C-terminus of NSm was suggested to act as an internal signal sequence for Gc. It was possible to insert the coding sequence for green fluorescent protein (GFP) in-frame in an internally deleted NSm protein and recover viable virus. The virus expressed chimeric GFP-NSm protein that localised to the Golgi similar to authentic NSm.

The situation with NSm proteins of phleboviruses is more complicated. Some phleboviruses like Uukuniemi virus (UUKV) do not encode NSm, Punta Totro and Toscana viruses encode a 30 kDa NSm protein at the N-terminus of the precursor, while RVFV produces two NSm proteins called NSm1 (78kDa) and NSm2 (14kDa) (Bouloy, 2011). The RVFV M segment-encoded precursor contains five in-frame AUG codons at its N-terminus. The first AUG codon initiates translation of NSm1 and the second AUG is used to produce NSm2. The sequences for Gn and Gc follow those of NSm2, while NSm1 actually incorporates Gn sequences (Gerrard and Nichol, 2007). Using reverse genetics, recombinant viruses have been produced, by substitution mutations in the AUG codon, that do not synthesise the NSm proteins. The viruses showed similar growth properties to the parent in mammalian and insect cells, and produced similar sized plaques (Won *et al*., 2006). A further NSm-deletion mutant, where most of the NSm coding region was actually excised from the M segment, however, produced larger plaques than the parent and induced more extensive cell death than the parent (Won *et al*., 2007). It was shown that the mutant virus induced apoptosis via the caspsase 3, 8 and 9 pathways. These data suggest that the role of NSm is antiapopotic. It should be noted that all these mutant viruses used the MP12 attenuated candidate vaccine strain of RVFV. In contrast, recombinant RVFV with analogous deletions of NSm but based on a virulent strain (ZH501) did not show plaque phenotype differences compared to the parental strain (Gerrard *et al*., 2007b) and the mutant virus, although attenuated, was still able to cause lethal disease in a rat model (Bird *et al*., 2007) Some rats infected with the mutant virus showed delayed onset RVF neurological disease in the absence of high titre viraemia, a clinical feature observed in some infections of livestock and humans (Bird *et al*., 2007). It will be of interest to perform side-by-side comparisons of MP12 and ZH501 mutant viruses in rats to determine whether amino acids differences in other viral genes may modulate the effect of deleting NSm.

7.7.5 Role of NSs protein

The first mutant bunyavirus produced by reverse genetics was a recombinant BUNV lacking the NSs protein (Bridgen *et al*., 2001). The orthobunyavirus S segment encodes N and NSs proteins in overlapping reading frames. Both proteins are translated from the same mRNA as the result of alternate AUG codon usage. The overlapping coding strategy makes wide-scale mutagenic analysis of NSs difficult as a mutation in NSs sequence could also change an amino acid in N. However, it was possible to ablate NSs expression without changing the amino acid sequence of N (Bridgen *et al*., 2001) and the generation of a recombinant virus lacking

NSs, rBUNdelNSs, led to the elucidation of NSs as having a major role in antagonising the host innate immune response (Weber *et al*., 2002; Kohl *et al*., 2003). Subsequently, recombinant LACV and AKAV lacking NSs expression were also generated (Blakqori and Weber, 2005; Ogawa *et al*., 2007). BUNV and LACV NSs-deletion mutants are highly attenuated in interferon competent mammalian cell lines and in mice, identifying NSs as a major virulence factor. Recombinant AKAV lacking NSs was also attenuated in mice (Ogawa *et al*., 2007). NSs-deficient viruses are strong inducers of IFN and are unable to shut off host cell protein synthesis (Bridgen *et al*., 2001; Thomas *et al*., 2004; Blakqori *et al*., 2007; Hart *et al*., 2009). NSs inhibits host RNA polymerase II transcription, thereby globally down-regulating host mRNA production, including those for interferon and thus activation of innate immune responses. BUNV NSs blocks phosphorylation of RNAPII and this is probably achieved by interacting with MED8, a critical component of Mediator that is involved in controlling cellular transcription (Leonard *et al*., 2006). The interacting domain in NSs was mapped to the C-terminus, and a recombinant virus expressing a truncated NSs protein missing this region showed a similar phenotype to the virus lacking NSs entirely (Leonard *et al*., 2006). However, further studies showed that a virus expressing an N-terminally deleted NSs that retained the MED8 interacting domain was also unable to block RNA polymerase II transcription, indicating that both interaction with MED8 and an as yet unidentified function in the N-terminus of NSs are required for the virus to overcome host antiviral defences (van Knippenberg *et al*., 2010). LACV NSs behaves slightly differently and causes proteasomal degradation of RNA polymerase II in a manner resembling the DNA damage response (Verbruggen *et al*., 2011). Despite the overall similarity in inhibiting cellular transcription, BUNV and LACV NSs differ with respect to apoptosis. BUNVdelNSs induced apoptosis more quickly than wt BUNV whereas the analogous LACV mutant was less apoptogenic than its corresponding wild-type parent (Kohl *et al*., 2003; Blakqori and Weber, 2005).

RVFV NSs was also identified as an IFN antagonist, initially through studies on a naturally occurring variant called Clone 13 that contains a large deletion in NSs (Muller *et al*., 1995). Like their orthobunyavirus counterparts, RVFV engineered to delete the NSs gene is attenuated in IFN-competent cells and animals (Ikegami *et al*., 2006; Bird *et al*., 2008; Habjan *et al*., 2009b; Brennan *et al*., 2011b). RVFV NSs interacts with a number of cellular proteins including SAP30, part of the Sin3A/NCoR/HDAC repressor complex that is involved in regulation of gene transcription. NSs was shown to be recruited with the repressor complex to the IFNβ promoter, and thus specifically prevented transcriptional activation of the IFNβ gene. A virus in which the SAP30 interacting domain in NSs was deleted was unable to antagonise IFNβ expression and was attenuated in a mouse model (Le May *et al*., 2008). RVFV NSs can also inhibit global cellular transcription though this occurs somewhat later in the infectious cycle than the early SAP30-dependent inhibition of IFNβ transcription. RVFV NSs interacts with the p44 subunit of TFIIH and sequesters it, thereby interrupting the supply of this crucial transcription factor (Le May *et al*., 2004). In addition, NSs interacts with the p62 component of

TFIIH resulting in its degradation (Kalveram *et al*., 2011). A novel activity of RVFV NSs was its ability to affect cellular translation by specific degradation of double-stranded RNA-dependent protein kinase (PKR), thereby preventing phosphorylation of eukaryotic initiation factor 2a (Habjan *et al*., 2009b; Ikegami *et al*., 2009). Recombinant RVFV in which its NSs was replaced with that from another phlebovirus (sandfly fever Sicilian) or the orthobunyavirus LACV were unable to degrade PKR, though the recombinant viruses were still capable of suppressing IFN induction (Habjan *et al*., 2009b). The relative ease with which recombinant RVFV can be created in the laboratory should enable detailed domain mapping studies on NSs to be performed in the future.

7.7.6 Modification of viral glycoproteins

The orthobunyavirus glycoproteins are type I integral membrane proteins that are characteristically markedly difference in size, Gn being about 35kD and Gc about 110kDa. The Golgi targeting signal has been mapped to the transmembrane domain of Gn, and Gc has to heterodimerise with Gn for transport to this organelle (Lappin *et al*., 1994; Shi *et al*., 2004). The glycoproteins contain few N-linked glycosylation sites, and for BUNV there is a single site on Gn (at N residue 60) and two sites on Gc (N624 and N1169) (Shi *et al*., 2005). Comparison of different orthobunyavirus glycoproteins sequences indicates that the sites at N60 and N1169 are well conserved among different viruses, while the site at N624 in Gc is conserved among viruses serologically related to BUNV. The importance of the glycosylation sites was investigated by creating recombinant viruses with the sites deleted; glycan addition N60 was shown to be essential for virus viability, whereas either or both glycosylation sites in Gc could be eliminated and viable viruses generated. However, glycan addition at both sites contributed to efficient virus infection (Shi *et al*., 2005).

Bunyaviruses do not encode an equivalent of a matrix protein to maintain virion stability, as found for other negative sense RNA viruses, and it has long been suggested that interaction between either or both of the cytoplasmic tails of the viral glycoproteins might interact with the RNP. This notion has been supported by studies using VLP assays to demonstrate that VLP formation is prevented when the cytoplasmic tails of viral glycoproteins are mutated. Reverse genetics showed that the cytoplasmic tails of BUNV were highly sensitive to mutation and only a couple of recombinant viruses containing alanine substitution mutations in the tail of Gc were recovered, though these were markedly attenuated and produced small plaques in cultured cells (Shi *et al*., 2007). The importance of the cytoplasmic tails of UUKV glycoproteins in assembly was also demonstrated, by exploiting the VLP production assay (Overby *et al*., 2007b).

The presumptive fusion peptide in the orthobunyavirus Gc protein had been predicted by bioinformatics approaches because of similarities in sequence and structural motifs with the E1 fusion proteins of alphaviruses (Garry and Garry, 2004). Building on work using transient expression of glycoprotein cDNA clones that

confirmed residues 1066–1087 of LACV Gc mediated fusion (Plassmeyer *et al*., 2005, 2007), recombinant viruses carrying mutations in this region were generated (Soldan *et al*., 2010). The mutant viruses grew less well than wild type and displayed a range of fusion activities that were decreased relative to the parental virus. Interestingly, a cell-based assay indicated that the mutant viruses retained their neurotoxicity, leading to the suggestion that the fusion peptide is associated with neuroinvasive properties of LACV but not necessarily its neurovirulence.

Analysis of variant Maguari orthobunyaviruses carrying deletions at the N-terminus of Gc (Pollitt *et al*., 2006) prompted a study of the requirement for the N-terminal region of BUNV Gc via the generation of recombinant viruses. The cleavage site between NSm and Gc was retained, but amino acid residues thereafter in Gc were sequentially deleted. Nearly half of the Gc protein ectodomain (453 out of 909 residues) could be deleted and viable viruses recovered, and although most were attenuated in cell culture to a greater or lesser degree, the results indicated that the N-terminal half of Gc was not required for infection of mammalian cell. Analysis of the behaviour of mutant glycoproteins and viruses in terms of Golgi targeting and low pH-dependent cell fusion allowed a model for the domain structure of Gc to be predicted (Shi *et al*., 2009). Furthermore, this opened up the possibility of inserting foreign sequences at the N-terminus and viable viruses were created where autofluorescent proteins (both GFP and mCherry) were fused in-frame with Gc (Shi *et al*., 2010). Recombinant virus particles displayed bright autofluorescence when visualised under UV-illumination in a conventional microscope (see Plate 4A), as each of the 650 to 720 Gc molecules in a virion (Obijeski *et al*., 1976; Freiberg *et al*., 2008; Overby *et al*., 2008) was tagged with the fluorescent protein. Infectivity was not markedly impaired and the viruses grew to titres of about 5×10^6 pfu/ml. Although the amino acid sequences of GFP and mCherry are highly similar, the stability of the recombinant viruses was quite distinct. The virus expressing the GFP-Gc fusion was less stable and shed the GFP sequence, plus additional Gc and NSm amino acid residues, after six passages. In contrast the mCherry-Gc expressing virus was completely stable for at least ten consecutive passages. The fluorescent viruses thus allow the different stages of the life-cycle to be visualised in real time, including attachment, assembly and budding in the Golgi, trafficking of progeny virus through the cytoplasm and egress at the plasma membrane (Plate 4B, 4C).

7.8 Outlook

Reverse genetics technologies have revolutionised the study of RNA viruses, and bunyaviruses are no exceptions. The foregoing has illustrated that power of this technique and the remarkable plasticity of the bunyavirus genome, and the scope of modification to viruses appears only limited by the imagination of the investigator. While unravelling the complexities of gene function and hitherto unexplored aspects of viral replication, reverse genetics also has the potential to transform our

ability to control or prevent bunyavirus disease through the development of novel vaccines or antiviral screening tools. However, the technology is still limited to a handful of viruses, and is not yet available for the majority of important bunyaviruses, notably the hantaviruses, CCHFV, or the tospoviruses. No doubt there are idiosyncrasies associated with these viruses that we have to understand though the most important prerequisite for success will be confidence that cDNA clones represent functional sequences.

References

Accardi, L., Prehaud, C., Di Bonito, P., Mochi, S., Bouloy, M. and Giorgi, C. 2001. Activity of Toscana and Rift Valley fever virus transcription complexes on heterologous templates. *Journal of General Virology* **82**: 781–785.

Barr, J. N., Elliott, R. M., Dunn, E. F. and Wertz, G. W. 2003. Segment-specific terminal sequences of Bunyamwera bunyavirus regulate genome replication. *Virology* **311**: 326–338.

Bergeron, E., Albarino, C. G., Khristova, M. L. and Nichol, S. T. 2010. Crimean-Congo hemorrhagic fever virus-encoded ovarian tumor protease activity is dispensable for virus RNA polymerase function. *Journal of Virology* **84**: 216–226.

Billecocq, A., Gauliard, N., Le May, N., Elliott, R. M., Flick, R. and Bouloy, M. 2008. RNA polymerase I-mediated expression of viral RNA for the rescue of infectious virulent and avirulent Rift Valley fever viruses. *Virology* **378**: 377–384.

Bird, B. H., Albarino, C. G., Hartman, A. L., Erickson, B. R., Ksiazek, T. G. and Nichol, S. T. 2008. Rift Valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. *Journal of Virology* **82**: 2681–2691.

Bird, B. H., Albarino, C. G. and Nichol, S. T. 2007. Rift Valley fever virus lacking NSm proteins retains high virulence in vivo and may provide a model of human delayed onset neurologic disease. *Virology* **362**: 10–15.

Blakqori, G., Delhaye, S., Habjan, M., Blair, C. D., Sanchez-Vargas, I., Olson, K. E., Attarzadeh-Yazdi, G., *et al*. 2007. La Crosse bunyavirus nonstructural protein NSs serves to suppress the type I interferon system of mammalian hosts. *Journal of Virology* **81**: 4991–4999.

Blakqori, G., Kochs, G., Haller, O. and Weber, F. 2003. Functional L polymerase of La Crosse virus allows in vivo reconstitution of recombinant nucleocapsids. *Journal of General Virology* **84**: 1207–1214.

Blakqori, G. and Weber, F. 2005. Efficient cDNA-based rescue of La Crosse bunyaviruses expressing or lacking the nonstructural protein NSs. *Journal of Virology* **79**: 10420–10428.

Bouloy, M. 2011. Molecular biology of phleboviruses. In Plyusnin, A. and Elliott, R. M. (eds) *Bunyaviridae. Molecular and Cellular Biology.* Caister Academic Press: Norfolk.

Bouloy, M. and Weber, F. 2010. Molecular biology of Rift Valley fever virus. *Open Virology Journal* **4**: 8–14.

Brennan, B., Li, P. and Elliott, R. M. 2011a. Generation and characterisation of a recombinant Rift Valley fever virus expressing a V5 epitope-tagged RNA-dependent RNA polymerase. *Journal of General Virology*, **92**(Pt 12): 2906–2913.

Brennan, B., Welch, S. R., Mclees, A. and Elliott, R. M. 2011b. Creation of a recombinant Rift Valley fever virus with a two-segmented genome. *Journal of Virology*, **85**: 10310–10318.

Bridgen, A. and Elliott, R. M. 1996. Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 15400–15404.

Bridgen, A., Weber, F., Fazakerley, J. K. and Elliott, R. M. 2001. Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 664–669.

Buchholz, U. J., Finke, S. and Conzelmann, K. K. 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *Journal of Virology* **73**: 251–259.

Buchmeier, M., Peters, C. and De La Torre, J. 2007. Arenaviridae: the viruses and their replication. In Knipe, D. and Howley, P. (eds) *Fields Virology*. Lippincott Williams: Philadelphia, PA.

Drake, J. W. and Holland, J. J. 1999. Mutation rates among RNA viruses *Proceedings of the National Academy of Sciences of the United States of America* **96**: 13910–13913.

Dunn, E. F., Pritlove, D. C., Jin, H. and Elliott, R. M. 1995. Transcription of a recombinant bunyavirus RNA template by transiently expressed bunyavirus proteins. *Virology* **211**: 133–143.

Eifan, S. A. and Elliott, R. M. 2009. Mutational analysis of the Bunyamwera orthobunyavirus nucleocapsid protein gene. *Journal of Virology* **83**: 11307–11317.

Elliott, R. M. 1989. Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. *Virology* **173**: 426–436.

Elliott, R. M. 2005. Negative strand RNA virus replication. In Mahy, B. and Meulen, V. T. (eds) *Topley and Wilson's Microbiology and Microbial Infections, Virology*. 10th edition. London: Hodder Arnold.

Flick, K., Hooper, J. W., Schmaljohn, C. S., Pettersson, R. F., Feldmann, H. and Flick, R. 2003a. Rescue of Hantaan virus minigenomes. *Virology* **306**: 219–224.

Flick, R., Flick, K., Feldmann, H. and Elgh, F. 2003b. Reverse genetics for Crimean-Congo hemorrhagic fever virus. *Journal of Virology* **77**: 5997–6006.

Flick, K., Katz, A., Overby, A., Feldmann, H., Pettersson, R. F. and Flick, R. 2004. Functional analysis of the noncoding regions of the Uukuniemi virus (Bunyaviridae) RNA Segments. *Journal of Virology* **78**: 11726–11738.

Flick, R. and Pettersson, R. F. 2001. Reverse genetics system for Uukuniemi virus (Bunyaviridae): RNA polymerase I-catalyzed expression of chimeric viral RNAs. *Journal of Virology* **75**: 1643–1655.

Fontana, J., Lopez-Montero, N., Elliott, R. M., Fernandez, J. J. and Risco, C. 2008. The unique architecture of Bunyamwera virus factories around the Golgi complex. *Cellular Microbiology* **10**: 2012–2028.

Freiberg, A. N., Sherman, M. B., Morais, M. C., Holbrook, M. R. and Watowich, S. J. 2008. Three-dimensional organization of Rift Valley fever virus revealed by cryoelectron tomography. *Journal of Virology* **82**: 10341–10348.

Garry, C. E. and Garry, R. F. 2004. Proteomics computational analyses suggest that the carboxyl terminal glycoproteins of Bunyaviruses are class II viral fusion protein (beta-penetrenes). *Theoretical Biology and Medical Modelling* **1**: 10.

Gauliard, N., Billecocq, A., Flick, R. and Bouloy, M. 2006. Rift Valley fever virus noncoding regions of L, M and S segments regulate RNA synthesis. *Virology* **351**: 170–179.

Gavrilovskaya, I. N., Brown, E. J., Ginsberg, M. H. and Mackow, E. R. 1999. Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins. *Journal of Virology* **73**: 3951–3959.

Gavrilovskaya, I. N., Shepley, M., Shaw, R., Ginsberg, M. H. and Mackow, E. R. 1998. Beta3 integrins mediate the cellular entry of hantaviruses that cause respiratory failure. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 7074–7079.

Gerrard, S. R., Bird, B. H., Albarino, C. G. and Nichol, S. T. 2007a. The NSm proteins of Rift Valley fever virus are dispensable for maturation, replication and infection. *Virology* **359**: 459–465.

Gerrard, S. R. and Nichol, S. T. 2007b. Synthesis, proteolytic processing and complex formation of N-terminally nested precursor proteins of the Rift Valley fever virus glycoproteins. *Virology* **357**: 124–133.

Habjan, M., Penski, N., Spiegel, M. and Weber, F. 2008. T7 RNA polymerase-dependent and -independent systems for cDNA-based rescue of Rift Valley fever virus. *Journal of General Virology* **89**: 2157–2166.

Habjan, M., Penski, N., Wagner, V., Spiegel, M., Verby, A. K., Kochs, G., Huiskonen, J. T. and Weber, F. 2009a. Efficient production of Rift Valley fever virus-like particles: the antiviral protein MxA can inhibit primary transcription of bunyaviruses. *Virology* **385**: 400–408.

Habjan, M., Pichlmair, A., Elliott, R. M., Overby, A. K., Glatter, T., Gstaiger, M., *et al*. 2009b. NSs protein of Rift Valley fever virus induces the specific degradation of the double-stranded RNA-dependent protein kinase. *Journal of Virology* **83**: 4365–4375.

Hart, T. J., Kohl, A. and Elliott, R. M. 2009. Role of the NSs protein in the zoonotic capacity of Orthobunyaviruses. *Zoonoses and Public Health* **56**: 285–296.

Ikegami, T., Narayanan, K., Won, S., Kamitani, W., Peters, C. J. and Makino, S. 2009. Rift Valley fever virus NSs protein promotes post-transcriptional

downregulation of protein kinase PKR and inhibits eIF2alpha phosphorylation. *PLoS Pathogens* **5**, e1000287.

Ikegami, T., Peters, C. J. and Makino, S. 2005. Rift Valley fever virus nonstructural protein NSs promotes viral RNA replication and transcription in a minigenome system. *Journal of Virology* **79**: 5606–5615.

Ikegami, T., Won, S., Peters, C. J. and Makino, S. 2006. Rescue of Infectious Rift Valley fever virus entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene. *Journal of Virology* **80**: 2933–2940.

Jin, H. and Elliott, R. M. 1991. Expression of functional Bunyamwera virus L protein by recombinant vaccinia viruses. *Journal of Virology* **65**: 4182–4189.

Kalveram, B., Lihoradova, O. and Ikegami, T. 2011. NSs protein of Rift Valley fever virus promotes post-translational downregulation of the TFIIH subunit p62. *Journal of Virology* **85:** 6234–6243.

Katz, A., Freiberg, A. N., Backstrom, V., Schulz, A. R., Mateos, A., Holm, L., Pettersson, R. F., Vaheri, A., Flick, R. and Plyusnin, A. 2010. Oligomerization of Uukuniemi virus nucleocapsid protein. *Virology Journal* **7**: 187.

Kohl, A., Clayton, R. F., Weber, F., Bridgen, A., Randall, R. E. and Elliott, R. M. 2003. Bunyamwera virus nonstructural protein NSs counteracts interferon regulatory factor 3-mediated induction of early cell death. *Journal of Virology* **77**: 7999–8008.

Kohl, A., Dunn, E. F., Lowen, A. C. and Elliott, R. M. 2004a. Complementarity, sequence and structural elements within the 3′ and 5′ non-coding regions of the Bunyamwera orthobunyavirus S segment determine promoter strength. *Journal of General Virology* **85**: 3269–3278.

Kohl, A., Hart, T. J., Noonan, C., Royall, E., Roberts, L. O. and Elliott, R. M. 2004b. A Bunyamwera virus minireplicon system in mosquito cells. *Journal of Virology* **78**: 5679–5685.

Kuri, T., Habjan, M., Penski, N. and Weber, F. 2010. Species-independent bioassay for sensitive quantification of antiviral type I interferons. *Virology Journal* **7**: 50.

Lappin, D. F., Nakitare, G. W., Palfreyman, J. W. and Elliott, R. M. 1994. Localisation of Bunyamwera bunyavirus G1 glycoprotein to the Golgi requires association with G2 but not NSm. *Journal of General Virology* **75**: 3441–3451.

Le May, N., Dubaele, S., Proietti De Santis, L., Billecocq, A., Bouloy, M. and Egly, J. M. 2004. TFIIH transcription factor, a target for the Rift Valley hemorrhagic fever virus. *Cell* **116**: 541–550.

Le May, N., Mansuroglu, Z., Leger, P., Josse, T., Blot, G., Billecocq, A., Flick, R., *et al*. 2008. A SAP30 complex inhibits IFN-beta expression in Rift Valley fever virus infected cells. *PLoS Pathogens* **4**, e13.

Leonard, V. H., Kohl, A., Hart, T. J. and Elliott, R. M. 2006. Interaction of Bunyamwera orthobunyavirus NSs protein with mediator protein MED8: a mechanism for inhibiting the interferon response. *Journal of Virology* **80**: 9667–9675.

Lopez, N., Muller, R., Prehaud, C. and Bouloy, M. 1995. The L protein of Rift Valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules. *Journal of Virology* **69**: 3972–3979.

Lowen, A. C., Boyd, A., Fazakerley, J. K. and Elliott, R. M. 2005. Attenuation of bunyavirus replication by rearrangement of viral coding and noncoding sequences. *Journal of Virology* **79**: 6940–6946.

Lowen, A. C. and Elliott, R. M. 2005. Mutational analyses of the nonconserved sequences in the Bunyamwera orthobunyavirus S segment untranslated regions. *Journal of Virology* **79**: 12861–12870.

Lowen, A. C., Noonan, C., Mclees, A. and Elliott, R. M. 2004. Efficient bunyavirus rescue from cloned cDNA. *Virology* **330**: 493–500.

Lozach, P. Y., Kuhbacher, A., Meier, R., Mancini, R., Bitto, D., Bouloy, M. and Helenius, A. 2011. DC-SIGN as a receptor for phleboviruses. *Cell Host and Microbe* **10**: 75–88.

Mandell, R. B., Koukuntla, R., Mogler, L. J., Carzoli, A. K., Freiberg, A. N., Holbrook, M. R., Martin, B. K., *et al*. 2010. A replication-incompetent Rift Valley fever vaccine: chimeric virus-like particles protect mice and rats against lethal challenge. *Virology* **397**: 187–198.

Muller, R., Saluzzo, J., Lopez, N., Dreier, T., Turell, M., Smith, J. and Bouloy, M. 1995. Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. *American Journal of Tropical Medicine and Hygiene* **53**: 405–411.

Nakitare, G. W. and Elliott, R. M. 1993. Expression of the Bunyamwera virus M genome segment and intracellular localisation of NSm. *Virology* **195**: 511–520.

Naslund, J., Lagerqvist, N., Habjan, M., Lundkvist, A., Evander, M., Ahlm, C., Weber, F. and Bucht, G. 2009. Vaccination with virus-like particles protects mice from lethal infection of Rift Valley Fever Virus. *Virology* **385**: 409–415.

Neumann, G. and Kawaoka, Y. 2004. Reverse genetics systems for the generation of segmented negative-sense RNA viruses entirely from cloned cDNA. *Current Topics in Microbiology and Immunology* **283**: 43–60.

Nichol, S. T., Beaty, B. J., Elliott, R. M., Goldbach, R., Plyusnin, A., Schmaljohn, C. S. and Tesh, R. B. 2005. Bunyaviridae. In Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U. and Ball, L. A. (eds) *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses*. Academic Press: Amsterdam.

Novoa, R. R., Calderita, G., Cabezas, P., Elliott, R. M. and Risco, C. 2005. Key Golgi factors for structural and functional maturation of Bunyamwera virus. *Journal of Virology* **79**: 10852–10863.

Obijeski, J. F., Bishop, D. H., Murphy, F. A. and Palmer, E. L. 1976. Structural proteins of La Crosse virus. *Journal of Virology* **19**: 985–997.

Ogawa, Y., Sugiura, K., Kato, K., Tohya, Y. and Akashi, H. 2007. Rescue of Akabane virus (family Bunyaviridae) entirely from cloned cDNAs by using RNA polymerase I. *Journal of General Virology* **88**: 3385–3390.

Overby, A. K., Pettersson, R. F., Grunewald, K. and Huiskonen, J. T. 2008. Insights into bunyavirus architecture from electron cryotomography of Uukuniemi virus. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 2375–2379.

Overby, A. K., Pettersson, R. F. and Neve, E. P. A. 2007a. The glycoprotein cytoplasmic tail of Uukuniemi virus (Bunyaviridae) interacts with ribonucleoproteins and is critical for genome packaging. *Journal of Virology* **81**: 3198–3205.

Overby, A. K., Popov, V., Neve, E. P. and Pettersson, R. F. 2006. Generation and analysis of infectious virus-like particles of Uukuniemi virus (Bunyaviridae): a useful system for studying bunyaviral packaging and budding. *Journal of Virology* **80**: 10428–10435.

Overby, A. K., Popov, V. L., Pettersson, R. F. and Neve, E. P. 2007b. The cytoplasmic tails of Uukuniemi virus (Bunyaviridae) G(N) and G(C) glycoproteins are important for intracellular targeting and the budding of virus-like particles. *Journal of Virology* **81**: 11381–11391.

Patterson, J. L., Holloway, B. and Kolakofsky, D. 1984. La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *Journal of Virology* **52**: 215–222.

Pettersson, R. F. and Melin, L. 1996. Synthesis, assembly and intracellular transport of *Bunyaviridae* membrane proteins. In Elliott, R. M. (ed.) *Bunyaviridae*. Plenum Press: New York and London.

Pichlmair, A., Habjan, M., Unger, H. and Weber, F. 2010. Virus-like particles expressing the nucleocapsid gene as an efficient vaccine against Rift Valley fever virus. *Vector Borne Zoonotic Diseases* **10**: 701–703.

Piper, M. E., Sorenson, D. R. and Gerrard, S. R. 2011. Efficient cellular release of Rift Valley fever virus requires genomic RNA. *PLoS ONE* **6**: e18070.

Plassmeyer, M. L., Soldan, S. S., Stachelek, K. M., Martin-Garcia, J. and Gonzalez-Scarano, F. 2005. California serogroup Gc (G1) glycoprotein is the principal determinant of pH-dependent cell fusion and entry. *Virology* **338**: 121–132.

Plassmeyer, M. L., Soldan, S. S., Stachelek, K. M., Roth, S. M., Martin-Garcia, J. and Gonzalez-Scarano, F. 2007. Mutagenesis of the La Crosse virus glycoprotein supports a role for Gc (1066–1087) as the fusion peptide. *Virology* **358**: 273–282.

Plotch, S. J., Bouloy, M., Ulmanen, I. and Krug, R. M. 1981. A unique cap (m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23**: 847–858.

Plyusnin, A. and Elliott, R. M. (eds) 2011. *Bunyaviridae: Molecular and Cellular Biology* Caister Academic Press: Norfolk.

Pollitt, E., Zhao, J., Muscat, P. and Elliott, R. M. 2006. Characterization of Maguari orthobunyavirus mutants suggests the nonstructural protein NSm is not essential for growth in tissue culture. *Virology* **348**: 224–232.

Porterfield, J. S., Casals, J., Chumakov, M. P., Gaidamovich, S. Y., Hannoun, C., Holmes, I. H., Horzinek, M. C., *et al*. 1975. Bunyaviruses and Bunyaviridae. *Intervirology* **6**: 13–24.

Pringle, C. R. 1996. Genetics and genome segment reassortment. In Elliott, R. M. (ed.) *The Bunyaviridae*. Plenum Press: New York.

Reguera, J., Weber, F. and Cusack, S. 2010. Bunyaviridae RNA polymerases (L-protein) have an N-terminal, influenza-like endonuclease domain, essential for viral cap-dependent transcription. *PLoS Pathogens* **6**, e1001101.

Roberts, K. L. and Smith, G. L. 2008. Vaccinia virus morphogenesis and dissemination. *Trends in Microbiology* **16**: 472–479.

Rose, J. K. 1996. Positive strands to the rescue again: a segmented negative-strand RNA virus derived from cloned cDNAs. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 14998–15000.

Shi, X., Brauburger, K. and Elliott, R. M. 2005. Role of N-linked glycans on bunyamwera virus glycoproteins in intracellular trafficking, protein folding, and virus infectivity. *Journal of Virology* **79**: 13725–13734.

Shi, X. and Elliott, R. M. 2009. Generation and analysis of recombinant Bunyamwera orthobunyaviruses expressing V5 epitope-tagged L proteins. *Journal of General Virology* **90**: 297–306.

Shi, X., Goli, J., Clark, G., Brauburger, K. and Elliott, R. M. 2009. Functional analysis of the Bunyamwera orthobunyavirus Gc glycoprotein. *Journal of General Virology* **90**: 2483–2492.

Shi, X., Kohl, A., Leonard, V. H., Li, P., Mclees, A. and Elliott, R. M. 2006. Requirement of the N-terminal region of orthobunyavirus nonstructural protein NSm for virus assembly and morphogenesis. *Journal of Virology* **80**: 8089–8099.

Shi, X., Kohl, A., Li, P. and Elliott, R. M. 2007. Role of the cytoplasmic tail domains of Bunyamwera orthobunyavirus glycoproteins Gn and Gc in virus assembly and morphogenesis. *Journal of Virology* **81**: 10151–10160.

Shi, X., Lappin, D. F. and Elliott, R. M. 2004. Mapping the Golgi targeting and retention signal of Bunyamwera virus glycoproteins. *Journal of Virology* **78**: 10793–10802.

Shi, X., Van Mierlo, J. T., French, A. and Elliott, R. M. 2010. Visualizing the replication cycle of bunyamwera orthobunyavirus expressing fluorescent protein-tagged Gc glycoprotein. *Journal of Virology* **84**: 8460–8469.

Smithburn, K., Haddow, A. and Mahaffy, A. 1946. A neurotropic virus isolated from Aedes mosquitos caught in the Semliki Forest. *American Journal of Tropical Medicine* **26**, 189–208.

Soldan, S. S., Hollidge, B. S., Wagner, V., Weber, F. and Gonzalez-Scarano, F. 2010. La Crosse virus (LACV) Gc fusion peptide mutants have impaired growth and fusion phenotypes, but remain neurotoxic. *Virology* **404**: 139–147.

Terasaki, K., Murakami, S., Lokugamage, K. G. and Makino, S. 2011. Mechanism of tripartite RNA genome packaging in Rift Valley fever virus. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 804–809.

Thomas, D., Blakqori, G., Wagner, V., Banholzer, M., Kessler, N., Elliott, R. M., Haller, O. and Weber, F. 2004. Inhibition of RNA polymerase II phosphorylation by a viral interferon antagonist. *Journal of Biological Chemistry* **279**: 31471–31477.

Van Knippenberg, I., Carlton-Smith, C. and Elliott, R. M. 2010. The N-terminus of Bunyamwera orthobunyavirus NSs protein is essential for interferon antagonism. *Journal of General Virology* **91**: 2002–2006.

Verbruggen, P., Ruf, M., Blakqori, G., Overby, A. K., Heidemann, M., Eick, D. and Weber, F. 2011. Interferon antagonist NSs of La Crosse virus triggers a DNA

damage response-like degradation of transcribing RNA polymerase II. *Journal of Biological Chemistry* **286**: 3681–3692.

Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Randall, R. E. and Elliott, R. M. 2002. Bunyamwera bunyavirus nonstructural protein NSs counteracts the induction of alpha/beta interferon. *Journal of Virology* **76**: 7949–7955.

Weber, F., Dunn, E. F., Bridgen, A. and Elliott, R. M. 2001. The Bunyamwera virus nonstructural protein NSs inhibits viral RNA synthesis in a minireplicon system. *Virology* **281**: 67–74.

Won, S., Ikegami, T., Peters, C. J. and Makino, S. 2006. NSm and 78-kilodalton proteins of Rift Valley fever virus are nonessential for viral replication in cell culture. *Journal of Virology* **80**: 8274–8278.

Won, S., Ikegami, T., Peters, C. J. and Makino, S. 2007. NSm protein of Rift Valley fever virus suppresses virus-induced apoptosis. *Journal of Virology* **81**: 13335–13345.

Zhang, Y., Li, X. H., Jiang, H., Huang, C. X., Wang, P. Z., Mou, D. L., Sun, L., *et al*. 2008. Expression of L protein of Hantaan virus 84FLi strain and its application for recovery of minigenomes. *APMIS* **116**: 1089–1096.

8

Using reverse genetics to improve influenza vaccines

Ruth A. Elderfield, Lorian C.S. Hartgroves and Wendy S. Barclay
Department of Medicine, Imperial College London

8.1 Introduction

Influenza is a highly contagious, acute respiratory disease, which causes significant mortality and morbidity worldwide. According to the World Health Organization (WHO), it causes between three and five million cases of severe illness and 250,000–500,000 deaths every year around the world (http://www.who.int/mediacentre/factsheets/fs211/en/). Influenza circulates yearly in the winter season. The elderly, young children and those with chronic medical disorders such as asthma, diabetes and the immune-compromised are most at risk following infection during the seasonal outbreaks. It has a range of symptoms including rhinitis, cough, weakness, febrile illness and headache. Most infections are self-limiting; however, influenza can occasionally progress to viral pneumonia or predispose to secondary bacterial infections. Rare complications of the virus infection include encephalopathy, myocarditis, and myositis.

Every few decades, a novel influenza virus emerges from the animal reservoir and adapts for infection and transmission in humans. The initial introduction of the new virus into a human population with no pre-existing immune knowledge of this strain results in widespread circulation in the form of a pandemic. Often the target groups who suffer more severe illness in the pandemic are different from those during seasonal flu outbreaks, with young adults being more likely to be affected (Nitsch-Osuch *et al*., 2010; Sun *et al*., 2011). The zoonotic event that leads to the outbreak can occur at any time of year and influenza-like illness (ILI) often appears out of normal season during the first wave of a pandemic. The animal reservoirs from which the pandemic virus is likely to emerge are the natural wild aquatic bird population, or pigs in which influenza viruses derived from both birds and humans are known to circulate.

Influenza is caused by infection with a single-stranded, negative sense, RNA virus (Figure 8.1). The influenza A virus encodes 12 known proteins, encoded on

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

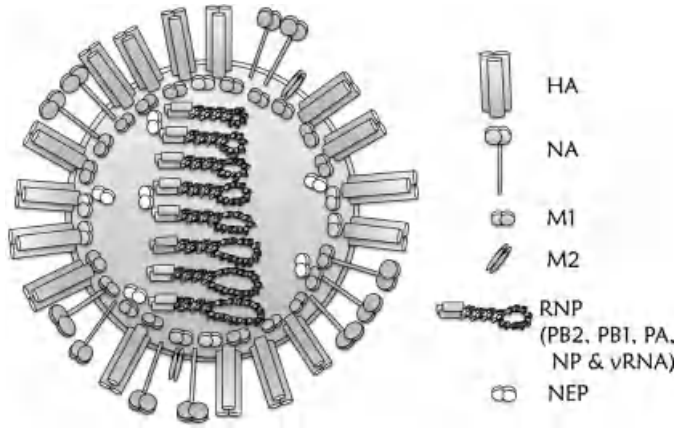

Figure 8.1 Schematic of the influenza A virus displaying the viral structural proteins and genomic segments.

8 RNA genomic segments. The viral envelope contains the glycoproteins haemagglutinin (HA) and neuraminidase (NA) and the short M2 transmembrane protein in host-derived lipids. Inside the envelope the M1 matrix protein surrounds the virion core, comprising the nuclear export protein (NEP) and each of the 8 RNA segments. Each segment is coated with nucleoprotein (NP) and complexed with the RNA-dependent RNA polymerase (RdRp), composed of a heterotrimer of two polymerase basic and one polymerase acidic subunits (PB1, PB2, and PA). This combination of RNA and protein is known as the viral ribonucleoprotein complex or vRNP. NS1 and PB1-F2 are non-structural proteins that help the virus control the host cell immune responses (McAuley *et al*., 2007; Hale *et al*., 2008) and N40 is a recently described product of the PB1 open reading frame which affects replication (Wise *et al*., 2009). None of the three latter proteins is part of the virion.

There are three serologically distinct types of the virus; A, B, and C, of which A and B cause widespread seasonal outbreaks in humans. The influenza B and C viruses have a similar genomic and protein organisation to type A, but there are differences in the RNA segment numbers and length, coding strategies and divergence of the protein sequences. Influenza B has 8 genomic segments, encoding 10 proteins functionally equivalent to those of type A. There is no evidence that influenza B virus encodes either N40 or PB1-F2 proteins, but instead a novel 100 amino acid NB protein is encoded on an alternate reading frame to the NA protein on segment 6 (Shaw *et al*., 1983). The function of NB is currently unknown, but its absence causes attenuation *in vivo* (Hatta and Kawaoka, 2003).

Influenza C virus encodes 9 known viral proteins on only 7 segments. The three polymerase subunits PB1, PB2 and P3 (the latter having a neutral pH, therefore not termed polymerase acidic as with the type A and B viruses), and the NP, M1, CM2, NS1, NS2 proteins provide similar roles as those in influenza A and B, but the precise mechanisms may differ. The coding strategy for the M1 and CM2 protein

generates an additional protein P42, whose functional relevance is undetermined (Pekosz and Lamb 1998; Hongo *et al*., 1999). The roles of entry and exit to and from the cell, usually directed by the HA and NA proteins of type A and B viruses, are for influenza C virus combined in a single haemagglutinin- esterase and fusion (HEF) protein, encoded on segment 4 (Herrler *et al*., 1988).

Influenza A viruses are further classified into subtypes based on the surface glycoproteins; there are 17 HA and 9 NA subtypes and they all exist in wild bird hosts, with the exception of H17 which was recently isolated from bats (Tong *et al*., 2012). Three subtypes have in recent times infected and transmitted within the human population (H1N1, H2N2 and H3N2). Both glycoproteins, but particularly HA, are major antigenic determinants and antibodies directed against HA and NA are crucial in current vaccine strategy.

Influenza B viruses showed a marked antigenic divergence in the late 1980s and are currently broadly divided into two lineages, Yamagata-like and Victoria-like. While both lineages can co-circulate during a season, typically one of them will predominate (Nerome *et al*., 1998; Nakagawa *et al*., 2009). Nonetheless the inability to predict which influenza B virus lineage will cause the burden of disease in any one year and lack of cross-protection have stimulated a debate concerning the modification of the current influenza vaccines to include an influenza B component from each lineage.

Influenza C viruses were initially thought to lack diversity due to the paucity of sequence data available; however, concerted surveillance in Japan has classified the virus into six antigenic groups (reviewed in Muraki and Hongo 2010). However, while influenza B virus has been isolated from seals (Osterhaus *et al*., 2000) and influenza C virus from pigs and dogs (Ohwada *et al*., 1987), humans appear to be the predominant hosts for both these types and a conclusive animal reservoir has not been defined. This means that type B and C viruses do not possess the same pandemic potential as influenza A. For all influenza viruses of humans, new seasonal strains appear from the gradual accumulation of point mutations in the surface glycoproteins. This ‘antigenic drift’ allows the virus to evade immune recognition and forms the basis for the seasonal epidemics which occur each year. The HA proteins of pandemic influenza A viruses are antigenically unrelated to strains circulating in humans. Such viruses may arise rapidly following reassortment between two or more different subtypes of the virus. Alternatively, influenza A viruses of animals may cross the species barrier if a series of mutations throughout their genome combine to enhance their replication in the human host. The introduction of a new subtype of influenza A virus to humans is called antigenic shift and can result in strains to which most adults do not have pre-existing immunity. Antigenic shift can lead to influenza pandemics such as the infamous 1918 ‘Spanish flu’ which killed 40 million people, the Asian flu of 1957, and the Hong Kong flu of 1968, and recently the swine-origin 2009 H1N1 pandemic (Cox and Subbarao, 2000; Garten *et al*., 2009). Despite the devastating effects of a pandemic, the cumulative deaths during intervening years when seasonal influenza circulates have been far greater than those associated with pandemics. Influenza B viruses contribute to the seasonal influenza

burden and are the predominant circulating virus approximately every third season. Although most influenza virus infections are self-limiting, few other diseases have such an effect on absenteeism, hospital admission and economic loss.

8.2 Influenza vaccines

Vaccination is the primary measure available to control influenza. The most widely available vaccines are based on technology over 50 years old and give inadequate protection to the most in need – the young and the elderly. Current vaccine production could be considered somewhat archaic – mostly involving propagation of the vaccine virus in chicken eggs. With the threat of highly pathogenic avian influenza (HPAI) gaining the ability to transmit between humans, the need to develop new vaccine strategies is imperative. Furthermore, depending on the viruses included in the vaccine, egg-based production may be inappropriate. This would apply, for example, to highly pathogenic avian influenza viruses which are pathogenic in eggs, or to circulating human strains for which adaptation for growth in eggs may select changes in the HA gene that affect antigenicity (Minor *et al*., 2009; Hartgroves *et al*., 2010).

8.2.1 Current vaccines

The emergence of new influenza strains by antigenic drift demands the annual reformulation of the vaccines. Retrospective analysis of variants from past major epidemics shows that typically only three or four mutations in the HA molecule are all that is required for a sufficiently drifted epidemic strain to emerge (Smith *et al*., 2004). New variants are identified from epidemiological surveillance studies coordinated by WHO. A panel convenes in February in the northern hemisphere, in September in the south, to predict the circulating strains likely to give the most protection in the coming influenza season. Currently, two influenza A virus strains, representing both subtypes that circulate in humans (H1N1, H3N2) and one influenza B strain are selected for inclusion in the seasonal vaccine. Vaccine manufacturers then have approximately 6 months to produce and register the vaccine. Approximately 50 countries have government-funded immunisation programmes (Nicholson *et al*., 2003) and approximately 350 million doses are available to some 1.2 billion people worldwide who are at risk (http://www.who.int/vaccine_research/diseases/ari/en/index.html).

The emergence of influenza pandemics is unpredictable, making the choice of strain to include as the seed vaccine at the beginning of the outbreak difficult. Several manufacturers have provisional licences to produce pandemic vaccines rapidly based on submission of existing clinical trial data using mock-up or pre-pandemic vaccines. The pandemic vaccine may be required for use in a wider age range, and it is preferable that a single dose of this novel antigen is immunogenic. Thus the formulation may include adjuvants to enhance the immune response allowing

antigen sparing or alternatively may require unadjuvanted higher antigen doses than the usual seasonal vaccine (Girard *et al*., 2010).

8.2.2 Reassortment of influenza viruses to generate inactivated vaccine

The segmented nature of the influenza genome means a vaccine seed can be generated from the process of reassortment in which two influenza viruses infecting the same cell can undergo gene exchange when they package a different combination of RNA segments. Vaccine seeds will ideally be reassortants of the WT epidemic or pandemic strain and a high yield, laboratory-adapted strain such as A/Puerto Rico/8/34 (PR8), that contains the six RNA gene segments encoding the internal proteins from the PR8 virus and the two RNA segments that encode surface antigen genes, HA and NA, from the selected vaccine strains. PR8 virus grows to high titres in embryonated chicken eggs and has been used as the recipient genetic backbone of the influenza A virus vaccine for more than 30 years (Kilbourne, 1969).

No such lab-adapted high growth master strains exist for influenza B viruses and these viruses are simply strain-selected each year. Often yields of influenza B virus are lower than for the influenza A viruses as a result (Vodeiko *et al*., 2003).

Trivalent inactivated influenza vaccine (TIV) is the oldest licensed vaccine in use today. Its effectiveness was first evaluated in 1938 (Francis, 1953). It contains partially purified HA and NA, from formaldehyde or β-propiolactone inactivated virions, detergent treated to give a split product or further purified to make a subunit vaccine that contains only HA and NA proteins. It is administrated by intramuscular injection. When there is a good match between the selected vaccine strains and the circulating strains, the vaccine can confer immunity in up to 80% of healthy adults less than 65 years of age (Nicholson *et al*., 2003; Subbarao and Katz, 2004).

8.2.3 Live attenuated vaccines

Whole, live vaccine, administered intranasally is potentially a better alternative to the split, intramuscular vaccine, as it should induce superior immunity more analogous to a natural virus infection. Live influenza vaccines should prime the cellular immune response as well as the humoral response, and this may be particularly important for vaccination of children (reviewed in Kreijtz *et al*., 2011). To reduce virulence, these vaccines are designed to have limited replication in the upper respiratory tract. As with TIV, a master backbone strain is generated. However, in the case of live attenuated influenza vaccine (LAIV), the backbone confers an attenuated phenotype from internal genes. Vaccines are made from 6:2 reassortants of attenuated backbone and circulating HA and NA. There have been several strategies used to attenuate the backbone strains but the one that is currently used is an adaptation of the virus for growth at sub-optimal temperatures termed cold-adapted (*ca*) with temperature sensitivity (*ts*) included as a selection measure (reviewed in Subbarao and Katz, 2004).

In the US, *ca* A/Ann Arbor/6/60 (H2N2) and *ca* B/Ann Arbor/1/66 are used as master strains (Maassab, 1967). In Russia, the equivalent master strain is *ca* A/Leningrad/134/57 (H2N2) (Ghendon *et al*., 1981). US and Russian strains have similar rates of infectivity in humans (Nicholson *et al*., 1987). A dose of 10^7 $TCID_{50}$ administered intranasally is typically safe and immunogenic in all age groups. The *ca* attenuating mutations are found mostly in NP and the polymerase subunits, PB1, PB2 and PA (Jin *et al*., 2003; Hoffmann *et al*., 2005; Chen *et al*., 2006). LAIVs have been in use in Russia for more than 50 years. MedImmune Vaccines Inc licensed and marketed Flumist in US in 2002.

8.3 The use of reverse genetics to generate recombinant influenza A, B and C viruses

In 1990, the reverse genetics technique to generate recombinant influenza viruses with defined genotypes was first developed in the laboratory of Peter Palese (Luytjes *et al*., 1989; Enami *et al*., 1990). At this time, the 'reverse genetic' technique used helper viruses and exchanged synthetic segments derived from cDNAs one at a time into the influenza genome. RNA was transcribed *in vitro* from cDNA encoding the relevant RNA segment and combined with RNP proteins purified from virus particles. The reconstituted vRNPs were transfected into cells simultaneously infected with the 'helper' virus. Reassortants containing the novel segment were then selected from the progeny which also contained abundant titres of helper virus progeny (Enami *et al*., 1990; Enami and Palese, 1991). Even with the use of tight selection markers such as antigenic differences or *ts*/*ca* mutations, the technique was laborious. Still, some early candidate H5 pandemic vaccines were generated using this technique (Li *et al*., 1999).

Almost 10 years later, two laboratories independently developed a procedure to 'rescue' whole virus genomes entirely from viral cDNAs (Fodor *et al*., 1999; Neumann *et al*., 1999). In its simplest form, the technique required that cells were co-transfected with 8 plasmids, each encoding one of the viral genomic RNAs, and 4 additional 'helper' plasmids directing expression of the RNP proteins (Figure 8.2). The eight viral RNAs were under the control of an RNA polymerase I (pol I) promoter to ensure authentic 5′ termini; in addition, a hepatitis delta virus (HDV) genomic ribozyme or a polymerase I terminator, downstream of the vRNA-coding region, ensured that the 3′ end of the vRNA terminated exactly. The helper plasmids typically expressed proteins from a strong host RNA polymerase II promoter. The helper plasmids encode the three subunits of the viral RNA-dependent RNA polymerase complex (PB1, PB2, and PA) and the nucleoprotein (NP), because these compromise the minimal set of viral proteins required for encapsidation, transcription and replication of the viral genome (Parvin *et al*., 1989). In a further refinement to the system, the production of viral sense vRNAs and mRNAs to direct viral protein expression were combined into single plasmids. To do this, the cDNA of each of the eight influenza virus segments was inserted between the pol-I promoter and the pol-I terminator and the whole pol-I transcription unit was flanked by the pol-II

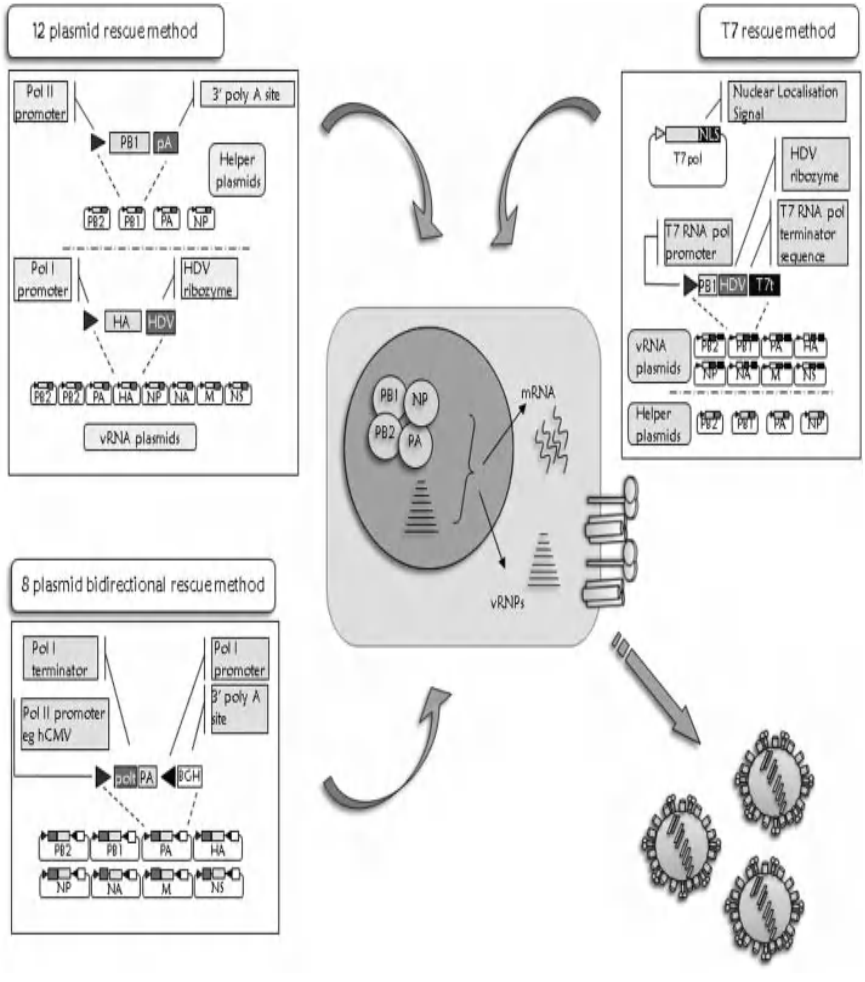

Figure 8.2 Schematic of three strategies used to 'rescue' influenza viruses using reverse genetics. Whereby plasmids encoding the 8 segments of the influenza virus are driven by variable Promoter sequences (Human, canine, avian RNA polymerase I or T7) and terminated by the Hepatitis delta virus (HDV), or T7 terminator. Single direction plasmids are supplemented by RNA polymerase II driven expression plasmids producing the viral polymerases and NP protein. Alternatively Bidirectional plasmids also contain the human cytomegalovirus promoter (hCMV) and the Bovine Growth Hormone (BGH) polyadenylation signal. These plasmids are then transfected into cells in order to generate whole virions.

promoter of the human cytomegalovirus and the polyadenylation signal of the gene encoding bovine growth hormone. The opposite orientation of the two transcription units allowed the synthesis of both negative-sense viral RNA and positive-sense mRNA from one viral cDNA template and reduced the number of plasmids to be co-transfected to eight (Hoffmann *et al*., 2002). More recently still, all the viral cDNAs have been combined into a single plasmid (Neumann *et al*., 2005). However, in a head-to-head comparison, the optimum strategy for efficient virus rescue consisted of eight vRNA pol-I segments on one plasmid and two pol-II plasmids, the first encoding PB1, PB2 and PA as a set and the second directing NP production. In many ways, this combination mimics most closely the components of the virus that first enters the nucleus during an influenza infection. Reverse genetics systems in use today in research labs, as well as in industry or institutes for vaccine generation, tend to use the 8 or 12 plasmid systems with which they are most familiar.

Reverse genetics systems for the rescue of recombinant influenza B viruses were developed in 2002 (Hoffmann *et al*., 2002; Jackson *et al*., 2002). They used 12 and 8 plasmid systems, respectively. Since that time, a total of five systems for influenza B have been generated (Hoffmann *et al*., 2002; Jackson *et al*., 2002; Hatta and Kawaoka, 2003; Imai *et al*., 2004; Wang and Duke *et al*., 2007).

In 2007, two influenza C reverse genetics systems were established using either C/Johannesburg/1/66 or C/Ann Arbor/1/50 as the base strain (Crescenzo-Chaigne and Van Der Werf, 2007; Muraki *et al*., 2007). Both groups found the use of separate pol-I and pol-II plasmids to be advantageous. Recently a third group has generated a 7 plasmid system with bidirectional pol-I/pol-II transcription units (Pachler *et al*., 2010). All three groups incubate the transfected cells at 33°C for 7–10 days rather than the 4–6 days at 37°C as is used for the generation of influenza A viruses, but even with these conditions the titres of recombinant influenza C virus produced are far lower than for influenza A or B viruses.

8.3.1 The use of reverse genetics to generate vaccine seeds for seasonal vaccine

The strains of virus used in the seasonal influenza vaccine are updated every year. The process of generating the appropriate reassortant viruses by combining the chosen epidemic viruses with the backbone strain can be time-consuming and unpredictable. Thus reverse genetics is an obvious method of choice, allowing a prescribed genetic constellation for the influenza vaccine seed. However, the patents for using reverse genetics technology for influenza vaccine are owned by MedImmune and this method is thus used for their product, LAIV but not for generation of TIV for seasonal influenza. To make the FluMist® vaccine seeds, plasmids encoding the six internal gene segments from the influenza A or B cold adapted master strains are thus transfected with novel plasmids that contain the cDNAs for the HA and NA of each of the chosen seasonal vaccine strains. Due to the species specificity of the polymerase I promoter, the earliest reverse genetics systems that

used human promoters in their plasmids were only effective in human cells. This meant that the original transfections were performed into human cells such as the highly transfectable 293T cell line, and then rescued virus was amplified in the more permissive Madin Darby canine kidney (MDCK) cells. However, since 293T cells are not licensed for use in the production of influenza vaccine, this precluded use of the strategy for vaccine purposes. The human polymerase I promoter does function in the African Green monkey cell line, Vero, a licensed vaccine substrate. Recombinant viruses generated in Vero cells could be propagated up to manufacturing capacity either in Vero cells themselves or in eggs (for example, Whiteley *et al*., 2007). Since MDCK cells are suitable for vaccine production, two labs have cloned the canine RNA polymerase I (pol-I) promoter and this has allowed rescue of recombinant influenza A or B viruses directly in a single cell substrate suitable for all stages of vaccine manufacture (Wang and Duke, 2007; Murakami *et al*., 2008b). For virus rescue in avian cells, which may be more efficient for avian viruses, the chicken polymerase I promoter driven reverse genetics system has also been cloned (Massin *et al*., 2005).

An alternative reverse genetics system using the T7 promoter rather than host polymerase I to generate vRNAs has also been described (de Wit *et al*., 2007). The vector contains the T7 RNA polymerase promoter, hepatitis delta virus ribozyme sequence and T7 RNA polymerase terminator sequence. The system is not species-specific since the T7 RNA polymerase is provided in the form of plasmid DNA that is co-transfected with the vRNA and has been used in cell lines from a variety of species including 293T, MDCK and QT6 cells. vRNA is transcribed from the T7 promoter in a negative-sense orientation and efficiency is improved when a T7 RNA polymerase with the inclusion of a nuclear-localisation signal was used.

Finally, without any modification to the plasmids used for reverse genetics, new cell substrates of human origin such as the PER.C6 cell line that are highly transfectable and highly permissive for influenza growth, and licensed for production of human biopharmaceuticals, can be used for the generation of recombinant influenza viruses suitable for vaccine production (Koudstaal *et al*., 2009). Importantly, this allows the generation of vaccine from a clinically relevant strain of human influenza without the need for passage through eggs which is known to select for adapting changes in the HA gene. This indicates that a vaccine strain could be generated rapidly from a synthetic cDNA that matched the sequence of a clinical isolate (Hartgroves *et al*., 2010).

8.4 Using reverse genetics technology for generation of pandemic virus vaccine

Through an agreement between MedImmune and WHO, the reverse genetics technique can be used to generate the vaccine seed in the case of a novel influenza A pandemic. The emergence of the 2009 H1N1 pandemic virus required the swift generation of virus suitable for vaccine production. Reverse genetics recombinant viruses as well as reassortant viruses bearing the HA and NA genes of the pandemic

strain A/California/07/2009 on high growth backbones, were used to generate high yield vaccine seeds for vaccine production in both eggs and cell culture systems. However, during the early phases of vaccine production it became apparent that the growth of the A/Cal/07/09 recombinants was only 30–50% of that typical for vaccines made against seasonal strains (Girard *et al*., 2010). Eventually higher growth variants were obtained. In all, about 30 versions of H1N1/2009 pandemic vaccine, some generated using traditional reassortment methods and some using reverse genetics, were made in multiple countries by different manufacturers, to produce vaccines that were administered as split (just the HA and NA proteins) preparations, whole inactivated virion preparations or live attenuated viruses.

Reverse genetics not only permits the generation of 6:2 reassortants but also has the potential for easy genetic modification of viruses. To date, the most obvious use for the reverse genetic approach for generating influenza vaccine has been for pandemic vaccines in which the HA gene has virulence determinants removed by genetic manipulation.

The replication cycle of the virus requires cleavage of HA by host proteases to activate fusion and infect cells. Natural cleavage of the HA protein is performed by the trypsin-like enzyme Clara tryptase which is secreted from the Clara cells of the respiratory tract. This restricts virus replication to sites in the host where such enzymes are found. HPAI viruses possess multiple, basic amino acids (arginine and lysine) at their cleavage sites and these are cleavable by a ubiquitous protease, furin (Figure 8.3). These viruses are thus able to replicate throughout the host, damaging

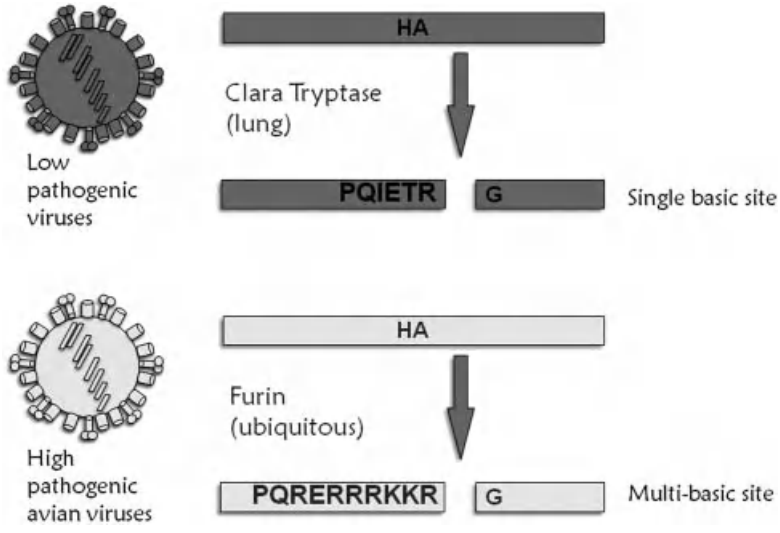

Figure 8.3 Schematic displaying the amino acid variations observed in cleavage sites of haemagglutinin.
High pathogenic avian influenza (HPAI) viruses possess multiple, basic amino acids (arginine and lysine) at their cleavage sites and these are cleavable by a ubiquitous protease, furin. Low pathogenic influenza viruses possess a single cleavage site which is cleaved only by clara tryptases expressed in the respiratory tract, thereby restricting their replication.

vital organs and tissues causing more severe disease and death. Thus highly pathogenic HA genes are unsuitable for inclusion in a vaccine strain since they may confer pathogenicity to the backbone strain, endangering vaccine production workers. Moreover, as part of the genetic make-up of live attenuated viruses, they would pose a risk of recombination with other circulating human strains potentially generating a more dangerous virus than the pandemic agent itself. For H5 and H7 vaccines, designed to protect against HPAI, viruses bearing HAs with the multibasic site deleted have been constructed (Stieneke-Grober *et al*., 1992; Li *et al*., 1999; Subbarao *et al*., 2003a; Subbarao *et al*., 2003b; Horimoto *et al*., 2006; Whiteley *et al*., 2007). Given as inactivated preparations, these vaccines have induced effective immunity against the wild-type HPAI strains, although an adjuvant is needed to induce strong immunity even in adults because they are naïve to the avian HAs. Inactivated vaccines of this type form the basis of most of the pre-pandemic vaccines that have been used to generate data for the mock-up vaccine licences. Overall this approach has been received with such favour that there are now repositories of plasmids and viruses generated in various labs around the world in preparation for the onset of pandemics of several different subtypes (Keawcharoen *et al*., 2010).

However, the success of this approach for LAIVs against avian subtypes has been limited. Although recombinant H5 viruses on the cold adapted backbone have been readily rescued and are immunogenic in mice, ferrets and humans, the vaccine take rate has been extremely poor and consequently little or no immune response has been induced (Karron *et al*., 2009). This is likely because both ferrets and humans have a paucity of avian virus receptors, α2,3 linked sialic acids in their upper respiratory tracts (Shinya *et al*., 2006). In fact, this is one reason why the pandemic threat of H5N1 virus has not yet been realised, because the lack of available receptor in the site of infection limits its transmissibility in humans. Since the cold adapted nature of the Ann Arbor/61 backbone precludes replication of the vaccine virus at the core body temperature, LAIVs are only able to replicate in the cooler temperatures of the nose and throat. Thus, the H5-bearing LAIVs are driven to replicate in a site of the human body where they have no receptors. Attempts have been made to remedy this by engineering mutations into the HA receptor binding site that are predicted to increase its affinity for the α2,6 SA receptors that are present in the nose. In particular, mutations at residues 226 and 228 of glutamine (Q) and glycine (G) to leucine (L), and serine (S) accompanied by the loss of a glycosylation site at residue 160 increased the immune response in ferrets administered this recombinant H5N1 LAIV (Wang *et al*., 2010). Nonetheless, the neutralising antibody titre was still poor in comparison with titres obtained using human adapted HAs on the same *ca* body. The biggest problem for this approach is that the exact mutations required to confer upper respiratory tract tropism are not known, and whether they may affect the antigenicity of the resulting virus is also not certain (Yang *et al*., 2007). Thus, although a LAIV might be the most efficient way to immunise a large population in the face of a pandemic, the approach for pre-pandemic vaccines is less useful at present.

The concept of engineering the cleavage site of the HA protein has been extended to generate a novel method for the attenuation of viruses that might be used

as live attenuated vaccines. Recombinant influenza A and B viruses were generated in which the HA cleavage site was modified to be dependent on elastase for cleavage (Stech *et al*., 2005; Stech *et al*., 2011). The virus was a conditional mutant, attenuated *in vivo* where elastase-like enzymes are not present naturally, but able to grow as well as wild-type virus *in vitro*, in the presence of exogenous elastase. This means an epidemic or pandemic strain can readily be converted to a live attenuated vaccine. The chief difference between this live attenuated vaccine strategy and the cold adapted strains is the self-limiting replication. Cold adapted viruses can cause shedding up to 11 days pi which increases the probability of reversion or recombination, but may also result in a more robust and complete immune response.

8.5 Other strategies for generating live attenuated vaccines based on viruses engineered by reverse genetics

The mutations responsible for the *ts* and *ca* phenotypes of the live attenuated master strains of influenza virus vaccines have been mapped using reverse genetics to generate virus variants with each mutation individually and in combination and then analysing their phenotypes (Jin *et al*., 2003; Chen *et al*., 2006; Chan *et al*., 2008). Mutations that directed the phenotypes resided in several different genes, which is important for the stability of the attenuation. These same mutations have also been introduced into other strains where they have conferred a similar attenuating phenotype (Solorzano *et al*., 2010). This could provide an alternative to the current master strains.

Similarly, other mutations and deletions have been introduced into influenza viruses using reverse genetics to create conditional mutants. These viruses can be propagated to high titres *in vitro* for the production of vaccines, but are attenuated when inoculated into immunocompetent hosts. Perhaps the most advanced of these approaches is the deletion or truncation of the NS1 gene. NS1 is the non-structural protein of influenza A and B virus. It is a multifunctional protein but its major role is arguably to antagonise the induction of the interferon response (reviewed by Hale *et al*., 2008). Thus, viruses that lack NS1 entirely or that encode truncated NS1 genes can only replicate efficiently in interferon deficient cells *in vitro* and are attenuated *in vivo* (Egorov *et al*., 1998; Garcia-Sastre *et al*., 1998; Wacheck *et al*., 2010).This approach has been applied to influenza A and B viruses, including influenza A viruses of swine and horses (Quinlivan *et al*., 2005; Solorzano *et al*., 2005) (reviewed in Richt and Garcia-Sastre, 2009). The higher amount of interferon induced in the initial round of infection with the LAIV that lacks NS1 may well serve as an adjuvant to enhance the adaptive immune response (Phipps-Yonas *et al*., 2008; Romanova *et al*., 2009).

From the volume of published work using this approach, a general consensus that can be drawn is that in most instances complete abrogation of the NS1 protein results in over-attenuation that would be undesirable for vaccine purposes since the

take rate might be too low. This is likely because in addition to its role in interferon control, NS1 has a plethora of other roles in the virus replication cycle and complete loss of the gene affects all of these (reviewed in Hale *et al*., 2008). For example, Hai *et al*. (2008) generated a series of recombinant influenza B viruses with truncated NS1 proteins. All of the mutations truncated the 282 residue B/Yamagata/16/88 NS1 from the C-terminus, resulting in a 16 amino acid protein (effectively del NS1) an 80 amino acid protein (NS1-80) and a 110 residue peptide (NS1-110). As expected, the truncated NS1 proteins were unable to control the induction of interferon to the same degree as the recombinant wild-type virus (rWT) in reporter cell lines and displayed attenuated growth in interferon competent MDCK cells. However, they also displayed attenuation in Vero cells known to be deficient in the interferon receptor (Desmyter *et al*., 1968) which was unexpected and suggested that other critical functions of NS1 in the influenza B virus replication cycle had also been lost. Despite this, using an influenza B permissive transgenic mouse model (C57BL/6) deficient in Protein Kinase R (PKR), they were able to demonstrate decreased viral replication and less weight loss in mice infected with the truncated forms compared to the rWT virus. Moreover, despite modest antibody levels induced by the NS1 truncated viruses, protection was conferred against a rWT virus challenge (Hai *et al*., 2008).

For vaccine production purposes, viruses deficient in the NS1 gene could be propagated in the IFN deficient Vero cell line which is already licensed for such purpose. Alternatively, since it has been shown that the development of the interferon system in the embryonated chicken eggs is incomplete until around 10 days, influenza viruses deficient in NS1 can be propagated in 6–7-day-old eggs (Dauber *et al*., 2004). Finally, cell lines that express interferon antagonists will support the growth of influenza viruses deleted for NS1. These interferon antagonists can be derived from other viruses such as the NPro protein of BVDV that targets IRF-3 or the V protein of PIV5 that targets mda5 and the STAT pathway (Hilton *et al*., 2006; Young *et al*., 2007). Cell lines may also express influenza NS1 protein itself in a direct complementation approach (van Wielink *et al*., 2011).

The success of the delNS1 approach for influenza LAIV is perhaps best illustrated by vaccination and challenge studies in a natural host such as the pig. A recombinant virus based on A/swine/Texas/4199-2/98 with a 126 residue truncated NS1 protein was able to protect swine later infected with the rWT H3N2 virus, resulting in no challenge virus being detected in nasal swabs 2 and 5 dpi and little or no lung damage observed. Interestingly, challenge by a H1N1 A/swine/Minnesota/37866/99 did result in lung lesions in the challenged pigs, but markedly reduced virus load in the nasal swabs, suggesting a partial protection by the delNS1 H3N2 despite a lack of HAI cross-reactivity (Richt *et al*., 2006).

During the course of primary research into the structure and function of influenza virus genes using the reverse genetics approach, it is inevitable that some attenuated viruses will be generated that may have use as LAIVs. Earlier, an M1 mutant generated by reverse genetics was proposed as a novel live attenuated backbone (Xie *et al*., 2009). A more recent example is a description of a series of mutations that

affect the interaction between the NEP protein and viral matrix protein M1. By changing a tryptophan residue W78 or a cluster of glutamate residues E67, E74 and E75 to serines either in combination or independently, virus fitness was compromised *in vivo*, but not to the same degree in cell culture. As the double glutamate mutation E67/E74 grew to wild-type levels in MDCK cells but did not cause weight loss in mice, this was taken forward as a live attenuated candidate. Some 25 days post infection, 100% mice previously infected with this candidate survived a lethal challenge with WT virus. The authors could not exclude that part of the attenuation was due to a concomitant extension of the NS1 C-terminus due to the overlapping reading frames of NS1 and NEP. Nonetheless there was no need for propagation of the recombinant virus in interferon deficient vero cells or in an NS1 protein expressing stable cell line, so in this instance the balance between virus fitness *in vitro* and attenuation *in vivo* was fortuitously achieved (Akarsu *et al*., 2011).

Another way to optimise the balance between attenuation and immunogenicity may lie with increasing the number of gene segments that carry the attenuating mutations. This would also lead to phenotype stability as for the *ca* LAIVs. In one recent description of an experimental recombinant influenza virus vaccine, a panel of LAIV strains were generated for the highly pathogenic avian influenza H5 viruses using a combination of modifications to HA, NS1 and the PB2 gene (Steel *et al*,. 2009). Thus, the HPAI H5N1 virus, A/Vietnam/1203/04 was modified in is polybasic site, thus removing its ability to replicate in the absence of trypsin and also in its ability to encode the full-length NS1 protein, making it less able to control the interferon response. Added to these modifications, a change in the PB2 protein E627K, a polymorphism-enhancing replication in mammalian cells, was also selectively introduced. Reverse genetics allowed the generation of a panel of eight viruses covering a combination of these different modifications. The strains with NS1 truncations showed reduced yield in embryonated chickens eggs. All of the vaccine candidates grew less well than equivalent PR8-based vaccine in human lung epithelial (A549) cells. In mice, high doses ($>10^6{-}10^5$ EID_{50}) of the vaccine strains with the 627K polymorphism and full-length NS1 caused significant weight loss, mice infected with viruses containing two viruses with truncated NS1 and 627K viruses also induced disease but viruses with the third truncation paired with 627K and viruses with the 627E and various NS1 variations were strongly attenuated.

Upon challenge with a virus with the HA and NA genes of A/Vietnam/1203/04, the level of protection appeared to be associated with the dose of the vaccine given and to the level of replication of vaccine. The viruses with the 627K mutation bestowed greater protection than those with the 627E mutation.

Chickens were also vaccinated with the candidate with the single basic site, the 627E polymorphism and a 1-99 aa NS1 truncation. Upon challenge, this strain provided sterilising immunity to homologous H5 challenge strain and partial protection against a lethal dose of a heterologous strain (Steel *et al*., 2009).

Other conditional mutants generated by reverse genetics techniques that have been put forward as novel live attenuated vaccine candidates include a highly

pathogenic H5N1 A/Vietnam/1203/04 virus attenuated by truncations in the cytoplasmic tail region of the M2 protein. The candidate taken forward for *in vivo* studies lacked 11 residues from the C-terminus of the M2 protein and had the multibasic site in the HA protein removed. The virus was able to replicate to the same levels as rWT virus in MDCK cells stably expressing full length M2 protein, but displayed attenuated growth *in vivo*. On challenge with the rWT virus or with a heterologous H5N1 from a different clade (A/Indonesia/7/2005), levels of challenge virus were greatly reduced or even abrogated (Watanabe *et al*., 2002; Watanabe *et al*., 2008). In a variation of this approach, the entire coding capacity for the transmembrane and the cytoplasmic tail of the M2 protein was removed to create an M2 knockout virus. Providing that sufficient M2 knockout virus was used initially (3×10^6–3×10^5 PFU), the mice were protected from subsequent challenge with the homologous rWT virus (Watanabe *et al*., 2009).

More recently, viruses in which the PB2 gene is deleted and replaced by a GFP open reading frame (Ozawa *et al*., 2011), thus dependent on PB2 supplied from an inducible cell line, have been shown to induce protective immunity. It seems likely that conditional mutants could be generated for many of the influenza virus genes and used individually or in combinational approaches in the future.

8.6 Strategies to improve the safety or yield of influenza vaccines

One worry concerning the use of live attenuated influenza vaccines has been the possibility of their reassortment with wild-type influenza viruses in the community and the possibility that this may generate novel viruses with new and undesirable properties. Recently an elegant use of the reverse genetic technique was proposed to prevent this scenario. It is now apparent that in order to assemble eight RNA segments into a virus particle, influenza viruses contain segment-specific packaging signals for each RNA segment that extend across both non-coding and coding regions of each of the eight RNA segments (Fujii *et al*., 2005; Noda *et al*., 2006). By exchanging the packaging signal of the NS and HA RNA segments of a recombinant virus simultaneously, a viable virus was generated in the laboratory that could not undergo reassortment of its NS or HA genes with a wild-type virus, because in doing so it would not achieve the full complement of vRNA packaging information (Gao and Palese, 2009). This strategy could be extended to other segments and make safer the use of live attenuated influenza viruses in the community.

A similar strategy involves the insertion of the HA into a recombinant virus of a different type, for example, Flandorfer *et al*. (2003) generated a recombinant influenza A virus that also expressed the influenza B virus HA gene. The Type B HA was incorporated into virus particles providing its transmembrane domain and cytoplasmic tail were substituted by those of influenza A HA. Conversely, a

recombinant and attenuated influenza B virus (rIBV) with the ectodomain of influenza A virus HA protein incorporated into the influenza B/Yamagata/88 virus segment 4 has been described (Hai *et al*., 2011). The chimeric HA gene retained the signal peptide and non-coding regions of the influenza B segment, preventing any reassortment with circulating influenza A strains. This rIBV also harboured an NS1 truncation for added attenuation. Infection with the recombinant virus protected mice from homologous influenza A virus challenge, though the level of protection varied between the different influenza A virus HAs used, which may be due to the different replication efficiencies of the recombinant viruses, likely affected by the resulting balance of activities of the combination of influenza HA with influenza B NA. The H1 HA of PR8 was able to support replication better than that of the H3 A/Hong Kong/68 HA.

It has often been noted, particularly for reassortant viruses that combine avian virus HA and NA gene segments in the PR8 high growth backbone, that the final yield of HA from the split and subunit preparation of TIV is low. This has been a particular problem for vaccine manufacture of some but not all of the PR8: H5N1 vaccines. Harvey *et al*. (2010) established that the low HA yield was due to low incorporation of the avian virus HA into the recombinant virus particles and hypothesised that this was due to sequence variation between PR8 and the novel HA in either the transmembrane domain or cytoplasmic tail. This suggested that, the avian virus HA proteins were not completely compatible with the internal genes of PR8 such as the M protein that might be involved in the assembly of the virus particle. To overcome this problem, they generated chimeric HA genes in which the transmembrane domain and /or cytoplasmic tail of the avian virus HA were replaced by the equivalent sequences from PR8. The yield of HA was markedly improved; for a H5N1 vaccine candidate the HA antigen yield was increased by 124–245%. Similarly, the yield of the 2009 H1N1 pandemic virus HA could be increased in excess of 200% (Harvey *et al*., 2010). This approach may be more widely used in the future to increase HA yields from a variety of PR8-based vaccines of different subtypes.

8.7 Improvements to the PR8 high growth strain

The PR8 virus has been used as the backbone for influenza A vaccines for more than 50 years. Although, the virus supports high yield growth of influenza viruses in chicken eggs, these adaptive mutations may not confer high growth in cell culture substrates that may be preferred in the future. Webby and colleagues found that replication of reassortant influenza viruses in Vero cells could be enhanced if the recombinant virus contained the NS segment from the epidemic strain rather than that of PR8 (Ozaki *et al*., 2004). Furthermore, genetic manipulation of the standard PR8 genetic backbone by reverse genetics may allow the production of an improved virus that can support enhanced yields. Even for traditional inactivated

vaccine production, this could mean increased doses per egg or batch of cell culture, an improvement that would ultimately reduce costs for seasonal vaccine and be crucial in the face of a pandemic.

There are several different strains of PR8 maintained in laboratories across the world. Certain strains contain amino acids which confer higher growth characteristics in MDCKs which could be important if this cell line were used for the manufacture of vaccines. Reverse genetics has been used to assess growth determinants of the PR8 backbone in eggs compared to MDCK cell culture (Murakami *et al*., 2008a).Two amino acid positions were found to be important for the MDCK high yield phenotype, S360 on PB2 and E55 on NS1. Only the PR8 (Cambridge) strain possesses S360 in PB2, all the others strains possess Y360. E55 NS1 is expressed in several strains and seems to confer improved IFN antagonism.

8.8 Improving the immunogenicity by engineering recombinant viruses that express cytokine genes

Another approach to improve the immunogenicity of LAIV involves the insertion of an additional open reading frame to the genome of the influenza virus that encodes a cytokine gene. Kittel *et al*. (2005) expressed the IL-2 protein from the segment 8 RNA of a recombinant influenza A virus using a bicistronic approach. While all of the mice infected with the IL-2 expressing virus survived, the viral loads were similar to those observed in viruses without the IL-2 expression (Kittel *et al*., 2005). To attenuate the recombinant virus further, they introduced a *ca* mutation that inhibited replication in mice. This now afforded increased protection (7/7) with the IL-2 insertion than with the *ca* alone (4/7). The mice infected with the *ca* IL-2 virus recorded higher levels of $CD8^+$ T Cells and mucosal IgA, the likely cause of the enhanced protection (Ferko *et al*., 2006). However, this approach should be considered carefully before translation into humans following the cautionary tales of the mice in Australia that received the poxvirus with an extra IL-4 gene, because this led to a lethal infection in previously resistant mice (Jackson *et al*., 2001). Similarly, the human clinical trials at Northwick Park whereby administration of the TGN1412 CD28 T cell surface receptor *in vivo* to human volunteers had devastating consequences rightly invokes nervousness about immunomodulatory approaches especially using replicating viruses *in vivo*.

8.9 Novel species-specific attenuation that takes advantage of microRNAs

An elegant and novel approach to generate an influenza virus attenuated in a species specific manner involves the introduction of host micro RNA response sequences into the genome of a recombinant virus. In this instance TenOever and colleagues

engineered two MicroRNA Response Elements (MREs) into the nucleoprotein open reading frame that directed gene silencing of the NP in mice and human cells but not in embryonated eggs, since the equivalent microRNAs are absent from avian hosts. Analysis of a typical infection in representative cell lines saw a decrease in NP protein expression in human cells infected with the MRE containing viruses. Mice infected with a dose $<10^4$ PFU/ML survived both the initial infection and a lethal challenge dose 21 days later (Perez *et al*., 2009).

8.10 Conclusion

During the recent swine-origin H1N1 2009 influenza pandemic, vaccines were manufactured in both eggs and cell culture. Vaccine seeds were produced by classical reassortment and reverse genetics (Robertson *et al*., 2011). In the UK, the government activated two sleeping contracts with Baxter and GSK (http://www.parliament.uk/documents/upload/postpn331.pdf). These were for 72 million doses from Baxter of a whole, inactivated Vero cell line derived vaccine, and 60 million doses from GSK of egg-grown, split vaccine.

The vaccine industry has the tools and techniques available to update the antiquated way in which influenza vaccines are made. Cell culture is a convenient alternative to eggs, and easy scale-up means the number of doses can be controlled as required. Reverse genetics means vaccines can be designed as and when new strains arise. The current pandemic has seen the fast tracking of new vaccines (http://www.emea.europa.eu/pdfs/human/press/pr/46856809en.pdf) and the use of reverse genetics, which is not covered by intellectual property in the event of a pandemic, to generate vaccine seeds (http://whqlibdoc.who.int/hq/2009/WHO_IVB_09.05_eng.pdf). Hopefully, the experience will leave us well prepared in the event of a more pathogenic pandemic in the future.

Meanwhile, there is a huge movement afoot to change entirely the way that influenza vaccines are generated and used. Recent advances in immunological techniques mean that we now have hope that a universal vaccine against influenza may be a possibility (Kreijtz *et al*., 2011). In the short term it is likely that reverse genetics will continue to throw up new ideas about how to improve conventional influenza vaccines. In the longer term, we may see a total shift towards a universal vaccine or the expression of recombinant proteins and the role that influenza virus reverse genetics will play in that development is less apparent.

References

Akarsu, H., Iwatsuki-Horimoto, K., Noda, T., Kawakami, E., Katsura, H., Baudin, F., Horimoto, T. and Kawaoka, Y. 2011. Structure-based design of NS2 mutants for attenuated influenza A virus vaccines. *Virus Research* **155**: 240–248.

Chan, W., Zhou, H., Kemble, G. and Jin, H. 2008. The cold adapted and temperature sensitive influenza A/Ann Arbor/6/60 virus, the master donor virus for live attenuated influenza vaccines, has multiple defects in replication at the restrictive temperature. *Virology* **380**: 304–311.

Chen, Z., Aspelund, A., Kemble, G. and Jin, H. 2006. Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (Flumist). *Virology* **345**: 416–423.

Cox, N. J. and Subbarao, K. 2000. Global epidemiology of influenza: past and present. *Annual Review of Medicine* **51**: 407–421.

Crescenzo-Chaigne, B., and Van Der Werf, S. 2007. Rescue of influenza C virus from recombinant DNA. *Journal of Virology* **81**(20): 11282–11289.

Dauber, B., Heins, G., and Wolff, T. 2004. The influenza B virus nonstructural NS1 protein is essential for efficient viral growth and antagonizes beta interferon induction. *Journal of Virology* **78**(4): 1865–1872.

De Wit, E., Spronken, M.I., Vervaet, G., Rimmelzwaan, G.F., Osterhaus, A.D. and Fouchier, R.A. 2007. A reverse-genetics system for influenza A virus using T7 RNA polymerase. *Journal of General Virology* **88**(Pt 4): 1281–1287.

Desmyter, J., Melnick, J. L., and Rawls, W. E. 1968. Defectiveness of interferon production and of rubella virus interference in a line of African green monkey kidney cells (Vero). *Journal of Virology* **2**(10): 955–961.

Egorov, A., Brandt, S., Sereinig, S., Romanova, J., Ferko, B., Katinger, D., Grassauer, A., *et al*. 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. *Journal of Virology* **72**: 6437–6441.

Enami, M., Luytjes, W., Krystal, M. and Palese, P. 1990. Introduction of site-specific mutations into the genome of influenza virus. *PNAS* **87**: 3802–3805.

Enami, M. and Palese, P. 1991. High-efficiency formation of influenza virus transfectants. *Journal of Virology* **65**: 2711–2713.

Ferko, B., Kittel, C., Romanova, J., Sereinig, S., Katinger, H. and Egorov, A. 2006. Live attenuated influenza virus expressing human interleukin-2 reveals increased immunogenic potential in young and aged hosts. *Journal of Virology* **80**(23): 11621–11627.

Flandorfer, A., García-Sastre, A., Basler, C. F., and Palese, P. 2003. Chimeric influenza A viruses with a functional influenza B virus neuraminidase or hemagglutinin. *Journal of Virology* **77**(17): 9116–9123.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. and Garcia-Sastre, A. 1999. Rescue of influenza A virus from recombinant DNA. *Journal of Virology* **73**: 9679–9682.

Francis, T., JR. 1953. Vaccination against influenza. *Bulletin of the World Health Organization* **8**: 725–741.

Fujii, K., Fujii, Y., Noda, T., Muramoto, Y., Watanabe, T., Takada, A., Goto, H., *et al*. 2005. Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. *Journal of Virology* **79**: 3766–3774.

Gao, Q. and Palese, P. 2009. Rewiring the RNAs of influenza virus to prevent reassortment. *PNAS* **106**: 15891–15896.

Garcia-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P. and Muster, T. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* **252**: 324–330.

Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., Sessions, W. M., Xu, X., *et al*. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **325**: 197–201.

Ghendon, Y. Z., Klimov, A. I., Alexandrova, G. I. and Polezhaev, F. I. 1981. Analysis of genome composition and reactogenicity of recombinants of cold-adapted and virulent virus strains. *Journal of General Virology* **53**: 215–224.

Girard, M. P., Katz, J., Pervikov, Y., Palkonyay, L. and Kieny, M. P. 2010. Report of the 6th meeting on the evaluation of pandemic influenza vaccines in clinical trials World Health Organization, Geneva, Switzerland, 17–18 February 2010. *Vaccine* **28**: 6811–6820.

Hai, R., García-Sastre, A., Swayne, D. E., and Palese, P. 2011. A reassortment-incompetent live attenuated influenza virus vaccine for protection against pandemic virus strains. *Journal of Virology* **85**(14): 6832–6843.

Hai, R., Martínez-Sobrido, L., Fraser, K. A., Ayllon, J., García-Sastre, A., and Palese, P. 2008. Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. *Journal of Virology* **82**(21): 10580–10590.

Hale, B. G., Randall, R. E., Ortin, J. and Jackson, D. 2008. The multifunctional NS1 protein of influenza A viruses. *Journal of General Virology* **89**: 2359–2376.

Hartgroves, L. C., Koudstaal, W., McLeod, C., Moncorge, O., Thompson, C. I., Ellis, J., Bull, C., *et al*. 2010. Rapid generation of a well-matched vaccine seed from a modern influenza A virus primary isolate without recourse to eggs. *Vaccine* **28**: 2973–2979.

Harvey, R., Nicolson, C., Johnson, R. E., Guilfoyle, K. A., Major, D. L., Robertson, J. S. and Engelhardt, O. G. 2010. Improved haemagglutinin antigen content in H5N1 candidate vaccine viruses with chimeric haemagglutinin molecules. *Vaccine* **28**: 8008–8014.

Hatta, M., and Kawaoka, Y. 2003. The NB protein of influenza B virus is not necessary for virus replication in vitro. *Journal of Virology* **77**(10): 6050–6054.

Herrler, G., Dürkop, I., Becht, H and Klenk, HD. 1988. The glycoprotein of influenza C virus is the haemagglutinin, esterase and fusion factor. *The Journal of General Virology* **69**(Pt 4): 839–846.

Hilton, L., Moganeradj, K., Zhang, G., Chen, Y.-H., Randall, R. E., McCauley, J. W., and Goodbourn, S. 2006. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. *Journal of Virology* **80**(23): 11723–11732.

Hoffmann, E., Krauss, S., Perez, D., Webby, R. and Webster, R. G. 2002. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* **20**: 3165.

Hoffmann, E., Mahmood, K., Chen, Z., Yang, C. F., Spaete, J., Greenberg, H. B., Herlocher, M. L., *et al*. 2005. Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66. *Journal of Virology* **79**: 11014–11021.

Hoffmann, E., Mahmood, K., Yang, C.-F., Webster, R. G., Greenberg, H. B., and Kemble, G. 2002. Rescue of influenza B virus from eight plasmids. *PNAS* **99**(17): 11411–11416.

Hongo, S., Sugawara, K., Muraki, Y., Matsuzaki, Y., Takashita, E., Kitame, F., and Nakamura, K. 1999. Influenza C virus CM2 protein is produced from a 374-amino-acid protein (P42) by signal peptidase cleavage. *Journal of Virology* **73**(1): 46–50.

Horimoto, T., Takada, A., Fujii, K., Goto, H., Hatta, M., Watanabe, S., Iwatsuki-Horimoto, K., *et al*. 2006. The development and characterization of H5 influenza virus vaccines derived from a 2003 human isolate. *Vaccine* **24**: 3669–3676.

Imai, M., Watanabe, S., Ninomiya, A., Obuchi, M., and Odagiri, T. 2004. Influenza B virus BM2 protein is a crucial component for incorporation of viral ribonucleoprotein complex into virions during virus assembly. *Journal of Virology* **78**(20): 11007–11015.

Jackson, D., Cadman, A., Zurcher, T., and Barclay, W. S. 2002. A reverse genetics approach for recovery of recombinant influenza B viruses entirely from cDNA. *Journal of Virology* **76**(22): 11744–11747.

Jackson, R. J., Ramsay, A. J., Christensen, C. D., Beaton, S., Hall, D. F., and Ramshaw, I.A. 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *Journal of Virology.* **75**(3): 1205–1210.

Jin, H., Lu, B., Zhou, H., Ma, C., Zhao, J., Yang, C. F., Kemble, G. and Greenberg, H. 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology* **306**(1): 18–24.

Karron, R.A., Talaat, K., Luke, C., Callahan, K., Thumar, B., Dilorenzo, S., McAuliffe, J., *et al*. 2009. Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. *Vaccine*, **27**(36): 4953–4960.

Keawcharoen, J., Spronken, M. I., Vuong, O., Bestebroer, T. M., Munster, V. J., Osterhaus, A. D., Rimmelzwaan, G. F. and Fouchier, R. A. 2010. Repository of Eurasian influenza A virus hemagglutinin and neuraminidase reverse genetics vectors and recombinant viruses. *Vaccine* **28**: 5803–5809.

Kilbourne, E. D. 1969. Future influenza vaccines and the use of genetic recombinants. *Bulletin of the World Health Organization* **41**: 643–645.

Kittel, C., Ferko, B., Kurz, M., Voglauer, R., Sereinig, S., Romanova, J., Stiegler, G. *et al*. 2005. Generation of an influenza A virus vector expressing biologically active human interleukin-2 from the NS gene segment. *Journal of Virology* **79**(16): 10672–10677.

Koudstaal, W., Hartgroves, L., Havenga, M., Legastelois, I., Ophorst, C., Sieuwerts, M., Zuijdgeest, D., *et al*. W. 2009. Suitability of PER.C6 cells to generate

epidemic and pandemic influenza vaccine strains by reverse genetics. *Vaccine* **27**: 2588–93.

Kreijtz, J. H., Fouchier, R. A. and Rimmelzwaan, G. F. 2011. Immune responses to influenza virus infection. *Virus Research* **162**(1–2): 19–30.

Li, S., Liu, C., Klimov, A., Subbarao, K., Perdue, M. L., Mo, D., Ji, Y., *et al*. 1999. Recombinant Influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. *Journal of Infectious Diseases* **179**: 1132–1138.

Luytjes, W., Krystal, M., Enami, M., Parvin, J. D. and Palese, P. 1989. Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**: 1107–1113.

Maassab, H. F. 1967. Adaptation and growth characteristics of influenza virus at 25° C. *Nature* **213**: 612.

Massin, P., Rodrigues, P., Marasescu, M., Van Der Werf, S. and Naffakh, N. 2005. Cloning of the chicken RNA polymerase I promoter and use for reverse genetics of Influenza A viruses in avian cells. *Journal of Virology* **79**(21): 13811–13816.

McAuley, J. L., Hornung, F., Boyd, K. L., Smith, A. M., McKeon, R., Bennink, J., Yewdell, J. W. and McCullers, J. A. 2007. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. *Cell Host Microbe* **2**: 240–249.

Minor, P. D., Engelhardt, O. G., Wood, J. M., Robertson, J. S., Blayer, S., Colegate, T., Fabry, L., *et al*. 2009. Current challenges in implementing cell-derived influenza vaccines: Implications for production and regulation, July 2007, NIBSC, Potters Bar, UK. *Vaccine* **27**: 2907–2913.

Murakami, S., Horimoto, T., Mai Le, Q., Nidom, C. A., Chen, H., Muramoto, Y., Yamada, S., *et al*. 2008a. Growth determinants for H5N1 influenza vaccine seed viruses in MDCK cells. *Journal of Virology* **82**: 10502–10509.

Murakami, S., Horimoto, T., Yamada, S., Kakugawa, S., Goto, H. and Kawaoka, Y. 2008b. Establishment of canine RNA polymerase I-driven reverse genetics for influenza A virus: its application for H5N1 vaccine production. *Journal of Virology* **82**: 1605–1609.

Muraki, Y. and Hongo, S., 2010. The molecular virology and reverse genetics of influenza C virus. *Japanese Journal of Infectious Diseases* **63**(3): 157–165.

Muraki, Y., Murata, T., Takashita, E., Matsuzaki, Y., Sugawara, K. and Hongo, S. 2007. A mutation on influenza C virus M1 protein affects virion morphology by altering the membrane affinity of the protein. *Journal of Virology* **81**(16): 8766–7873.

Nakagawa, N., Natsumi, H., and Toshimasa, N. 2009. Cocirculation of antigenic variants and the vaccine-type virus during the 2004–2005 influenza B virus epidemics in Japan. *Journal of Clinical Microbiology* **47**(2): 352–357.

Nerome, R. Hiromoto, Y., Sugita, S., Tanabe, N., Ishida, M., Matsumoto, M., Lindstrom, S.E., *et al*. 1998. Evolutionary characteristics of influenza B virus since its first isolation in 1940: dynamic circulation of deletion and insertion mechanism. *Archives of Virology* **143**, 8, 1569–1583.

Neumann, G., Fujii, K., Kino, Y. and Kawaoka, Y. 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. *PNAS* **102**: 16825–16829.

Neumann, G., Watanabe, T., Ito, H., *et al*. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *PNAS* **96**: 9345–9350.

Nicholson, K. G., Tyrrell, D. A., Oxford, J. S., *et al*. 1987. Infectivity and reactogenicity of reassortant cold-adapted influenza A/Korea/1/82 vaccines obtained from the USA and USSR. *Bulletin of the World Health Organization* **65**: 295–301.

Nicholson, K. G., Wood, J. M. and Zambon, M. 2003. Influenza. *The Lancet* **362**: 1733.

Nitsch-Osuch, A., Kuchar, E., Gyrczuk, E., *et al*. 2010. Clinical manifestations of influenza caused by A/H1N1v virus among children and teenagers consulted in general practice. *European Journal of Medical Research* **15** Suppl. 2: 105–107.

Noda, T., Sagara, H., Yen, A., Takada, A., *et al*. 2006. Architecture of ribonucleoprotein complexes in influenza A virus particles. *Nature* **439**: 490–492.

Ohwada, K., Kitame, F., Sugawara, K., *et al*. 1987. Distribution of the antibody to influenza C virus in dogs and pigs in Yamagata Prefecture, Japan. *Microbiology and Immunology*, **31**(12): 1173–1180.

Osterhaus, A. D., Rimmelzwaan, G. F., Martina, B. E., Bestebroer, T. M., and Fouchier, R. A. 2000. Influenza B virus in seals. *Science (New York, N.Y.)* **288**(5468): 1051–1053.

Ozaki, H., Govorkova, E.A., Li, C., Xiong, X., Webster, R.G. and Webby, R.J. 2004. Generation of high-yielding influenza A viruses in African green monkey kidney (Vero) cells by reverse genetics. *Journal of Virology* **78**(4): 1851–1857.

Ozawa, M., Victor, S.T., Taft, A.S., Yamada, S., Li, C., Hatta, M., Das, S.C., *et al*. 2011. Replication-incompetent influenza A viruses that stably express a foreign gene. *Journal of General Virology* **92**(Pt 12): 2879–2888.

Pachler, K., Mayr, J., and Vlasak, R. 2010. A seven plasmid-based system for the rescue of influenza C virus. *Journal of Molecular and Genetic Medicine* **4**: 239–246.

Parvin, J. D., Palese, P., Honda, A., Ishihama, A. and Krystal, M. 1989. Promoter analysis of influenza virus RNA polymerase. *Journal of Virology* **63**: 5142–5152.

Pekosz, A., and Lamb, R. A. 1998. Influenza C virus CM2 integral membrane glycoprotein is produced from a polypeptide precursor by cleavage of an internal signal sequence. *PNAS* **95**(22): 13233–13238.

Perez, J.T., Pham, A.M., Lorini, M.H., Chua, M.A., Steel, J. and Tenoever, B.R. 2009. MicroRNA-mediated species-specific attenuation of influenza A virus. *Nature Biotechnology* **27**(6): 572–576.

Phipps-Yonas, H., Seto, J., Sealfon, S. C., Moran, T. M. and Fernandez-Sesma, A. 2008. Interferon-beta pretreatment of conventional and plasmacytoid human dendritic cells enhances their activation by influenza virus. *PLoS Pathogens* **4**: e1000193.

Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T. and Palese, P. 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. *Journal of Virology* **79**: 8431–8439.

Richt, J. A. and Garcia-Sastre, A. 2009. Attenuated influenza virus vaccines with modified NS1 proteins. *Current Topics in Microbiology and Immunology* **333**: 177–195.

Richt, J.A., Lekcharoensuk, P., Lager, K.M., Vincent, A.L., Loiacono, C.M., Janke, B.H., Wu, W.H., *et al*. 2006 Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. *Journal of Virology* **80**(22): 11009–11018.

Robertson, J. S., Nicolson, C., Harvey, R., Johnson, R., Major, D., Guilfoyle, K., Roseby, S., Newman, R., *et al*. 2011. The development of vaccine viruses against pandemic A(H1N1) influenza. *Vaccine* **29**: 1836–1843.

Romanova, J., Krenn, B. M., Wolschek, M., Ferko, B., Romanovskaja-Romanko, E., Morokutti, A., Shurygina, A. P., Nakowitsch, S., *et al*. 2009. Preclinical evaluation of a replication-deficient intranasal DeltaNS1 H5N1 influenza vaccine. *PLoS ONE* **4**: e5984.

Shaw, M.W., Choppin, P.W., and Lamb R.A. 1983. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *PNAS* **80**(16): 4879–4883.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., and Kawaoka, Y. 2006. Avian flu: influenza virus receptors in the human airway. *Nature* **440**(7083): 435–436.

Smith, D. J., Lapedes, A. S., De Jong, J. C., Bestebroer, T. M., Rimmelzwaan, G. F., Osterhaus, A. D. M. E. and Fouchier, R. A. M. 2004. Mapping the antigenic and genetic evolution of influenza virus. *Science* **305**: 371–376.

Solorzano, A., Webby, R. J., Lager, K. M., Janke, B. H., Garcia-Sastre, A. and Richt, J. A. 2005. Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. *Journal of Virology* **79**: 7535–7543.

Solorzano, A., Ye, J. and Perez, D. R. 2010. Alternative live-attenuated influenza vaccines based on modifications in the polymerase genes protect against epidemic and pandemic flu. *Journal of Virology* **84**: 4587–4596.

Stech, J., Garn, H., Wegmann, M., Wagner, R. and Klenk, H. D. 2005. A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. *Nature Medicine* **11**: 683–689.

Stech, J., Garn, H., Herwig, A., Stech, O., Dauber, B., Wolff, T., Mettenleiter, T. C., and Klenk, H. D. 2011. Influenza B virus with modified hemagglutinin cleavage site as a novel attenuated live vaccine. *Journal of Infectious Diseases* **204**(10): 1483–1490.

Steel, J., Lowen, A. C., Pena, L., Angel, M., Solórzano, A., Albrecht, R., Perez, D. R., *et al*. 2009. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. *Journal of Virology* **83**(4): 1742–53.

Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H. D. and Garten, W. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO Journal* **11**: 2407–2414.

Subbarao, K., Chen, H., Swayne, D., Mingay, L., Fodor, E., Brownlee, G., Xu, X., *et al*. 2003. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* **305**: 192–200.

Subbarao, K. and Katz, J. M. 2004. Influenza vaccines generated by reverse genetics. *Current Topics in Microbiology and Immunology* **283**: 313–342.

Sun, S., Zhao, G., Xiao, W., Hu, J., Guo, Y., Yu, H., Wu, X., *et al*. 2011. Age-related sensitivity and pathological differences in infections by 2009 pandemic influenza A (H1N1) virus. *Virology Journal* **8**: 52.

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D. A. A., Chen, L. M., Recuenco, S., Ellison, J. A., Davis. C. T., York, I. A., Turmelle, A. S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M. R., Tang, K., Rowe, L. A. Sammons, S., Xu, X., Frace, M., Lindblade, K. A., Cox, N. J., Anderson, L. J., Rupprecht, C. E. and Donis, R. O. 2012. A distinct lineage of influenza A virus from bats. *PNAS* 2009, 4269–4274.

Van Wielink, R., Harmsen, M. M., Martens, D. E., Peeters, B. P. H., Wijffels, R. H., and Moormann, R. J. M. 2011. MDCK cell line with inducible allele B NS1 expression propagates delNS1 influenza virus to high titres. *Vaccine* **29**(40): 6976–6985.

Vodeiko, G. M., Mcinnis, J., Chizhikov, V., and Levandowski, R. A. 2003. Genetic and phenotypic analysis of reassortants of high growth and low growth strains of influenza B virus. *Vaccine* **21**(25–26): 3867–3874.

Wacheck, V., Egorov, A., Groiss, F., Pfeiffer, A., Fuereder, T., Hoeflmayer, D., Kundi, M., *et al*. 2010. A novel type of influenza vaccine: safety and immunogenicity of replication-deficient influenza virus created by deletion of the interferon antagonist NS1. *Journal of Infectious Diseases* **201**: 354–362.

Wang, Z. and Duke, G. M. 2007. Cloning of the canine RNA polymerase I promoter and establishment of reverse genetics for influenza A and B in MDCK cells. *Virology Journal* **4**: 102.

Wang, W., Lu, B., Zhou, H., Suguitan, A.L.Jr., Cheng, X., Subbarao, K., Kemble, G. and Jin, H. 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. *Journal of Virology* **84**(13): 6570–6577.

Watanabe, S., Watanabe, T. and Kawaoka, Y. 2009. Influenza A virus lacking M2 protein as a live attenuated vaccine. *Journal of Virology* **83**: 5947–5950.

Watanabe, T., Watanabe, S., Kida, H. and Kawaoka, Y. 2002. Influenza A virus with defective M2 ion channel activity as a live vaccine. *Virology* **299**: 266–270.

Watanabe, T., Watanabe, S., Kim, J.H., Hatta, M. and Kawaoka, Y. 2008. Novel approach to the development of effective H5N1 influenza A virus vaccines: use of M2 cytoplasmic tail mutants. *Journal of Virology* **82**(5): 2486–2492.

Whiteley, A., Major, D., Legastelois, I., Campitelli, L., Donatelli, I., Thompson, C., Zambon, M., *et al*. 2007. Generation of candidate human influenza vaccine strains in cell culture: rehearsing the European response to an H7N1 pandemic threat. *Influenza and Other Respiratory Viruses* **1**: 157–166.

Wise, H. M., Foeglein, A., Sun, J., Dalton, R. M., Patel, S., Howard, W., Anderson, E. C., *et al*. 2009. A Complicated Message: Identification of a Novel PB1-Related Protein Translated from Influenza A Virus Segment 2 mRNA. *Journal of Virology* **83**: 8021–8031.

Xie, H., Liu, T. M., Lu, X., Wu, Z., Belser, J. A., Katz, J. M., Tumpey, T. M. and Ye, Z. 2009. A live attenuated H1N1 M1 mutant provides broad cross-protection against influenza A viruses, including highly pathogenic A/Vietnam/1203/2004, in mice. *Journal of Infectious Diseases* **200**: 1874–1883.

Yang, Z.Y., Wei, C. J., Kong, W. P., Wu, L., Xu, L., Smith, D. F., and Nabel, G. J. 2007. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. *Science* **317**(5839): 825–828.

Young, D. F., Carlos, T. S., Hagmaier, K., Fan, L., and Randall, R. E. 2007. AGS and other tissue culture cells can unknowingly be persistently infected with PIV5; a virus that blocks interferon signalling by degrading STAT1. *Virology* **365**(1): 238–240.

Part III

Double-stranded RNA viruses

9

Bluetongue virus reverse genetics

Mark Boyce
Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey

9.1 Introduction to Bluetongue virus

Bluetongue virus (BTV) is the prototype member of the *Orbivirus* genus, belonging to the *Reoviridae* family. BTV is an arboviral pathogen which is the aetiological agent of bluetongue disease in ruminants.

The symptoms of bluetongue disease were first described in detail in 1905 by Spreull who also demonstrated that the aetiological agent was transmissible (Spreull, 1905). Bluetongue disease is now known to be a non-contagious viral infection, affecting a wide range of wild and domesticated ruminant hosts including sheep, goats, cattle, antelope and deer. The virus is vectored between susceptible hosts by biting midges (gnats) belonging to the *Culicoides* genus in which it also replicates (Du Toit, 1944). The mortality and morbidity rates observed vary widely depending both on the virus strain and the infected ruminant species or breed. In susceptible sheep flocks, mortality can be as high as 50–70% in infected animals, although this is rare, and bluetongue disease is generally less severe in cattle (Erasmus, 1975; Elbers *et al*., 2008). The presence of *Culicoides* species capable of vectoring Bluetongue virus is an important limiting factor in the worldwide distribution of BTV. The disease is endemic in many tropical and sub-tropical countries of all continents except Antarctica, making Bluetongue virus one of the most widespread animal pathogens.

Bluetongue was considered to be a disease of ruminants in Africa until 1943 when an outbreak of a particularly virulent strain in Cyprus caused approximately 2500 deaths in sheep, with the mortality rate reaching 70%, although incursions into Cyprus may have been occurring as early as 1924 (Gambles, 1949). Following the severe outbreak in Cyprus, BTV outbreaks were subsequently identified in Israel (Komarov and Goldsmit, 1951), Pakistan (Sarwar, 1962), and India (Sapre, 1964). In California, the condition termed ‘soremuzzle’ was reported in 1952 and its

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

similarity to Bluetongue was recognised. Subsequently BTV was shown to be the cause when it was isolated from the soremuzzle-affected sheep (McKercher *et al*., 1953). The spread of BTV continued through the Middle East, Asia and eventually to Australia as reviewed by Hassan (Hassan, 1992), Doyle (Doyle, 1992) and Daniels *et al*. (Daniels *et al*., 2009). Until 1998, outbreaks in Europe were rare and confined to temporary incursions into southern Europe. Since 1998 there has been a change in the incidence of BTV in Europe, with separate and repeated introductions of BTV into southern and northern Europe. Of the 24 serotypes of BTV, seven have caused outbreaks in mainland Europe since 1998, recently reviewed by Maan *et al*. (Maan *et al*., 2009). The increased northern range of BTV in Europe may be contributed to by climate changes and the secondary effects these have on the distribution of the insect vector populations, their geographic overlap with alternative *Culicoides* vector species which are also capable of transmitting the virus, and the temperature dependence of BTV replication in the insect vector (Wittmann *et al*., 2002; Gloster *et al*., 2007; Carpenter *et al*., 2009; Purse and Rogers, 2009).

Bluetongue disease is largely caused by damage to the small blood vessels, with symptoms including pulmonary and facial oedema, vascular thromboses, coronitis, and when the tongue is affected, it may become cyanotic acquiring a dark blue colour. BTV pathology can produce disease, ranging from subclinical infections to severe fatal disease, as reviewed by MacLachlan and Gard (Maclachlan and Gard, 2009). Transmission to the ruminant host occurs through the bite of an infected blood-feeding *Culicoides*. The virus migrates to the regional lymph node where it replicates, and spreads to the spleen, thymus and other lymph nodes. BTV is disseminated to tissues throughout the host animal where replication occurs in mononuclear phagocytes and endothelial cells, and it is the damage caused to endothelial cells of the small blood vessels which probably leads to the associated lesions, oedema, and thromboses. At the final stage of infection the host develops a long-lasting viraemia, which is highly cell-associated (Barratt-Boyes and MacLachlan, 1995). The association of virions with the plasma membrane of erythrocytes may protect the virus from immune clearance, and prolong the period during which the ruminant is capable of transmitting BTV to the blood-feeding insect vector (Brewer and MacLachlan, 1992, 1994).

9.2 Bluetongue virus replication

9.2.1 Virion structure

The virion has a triple-layered structure, the outer layer of which is lost before the remaining core particle enters the cytoplasm of the host cell (Figure 9.1) (Martin and Zweerink, 1972; Verwoerd and Huismans, 1972; Verwoerd *et al*., 1972; Zhang *et al*., 2010). The viral genome is 19.2kb in total and consists of ten linear double-stranded (ds) RNA molecules that each encode a viral protein; VP1 – VP7 and NS1 – NS3, with alternative initiation codons generating two products of slightly

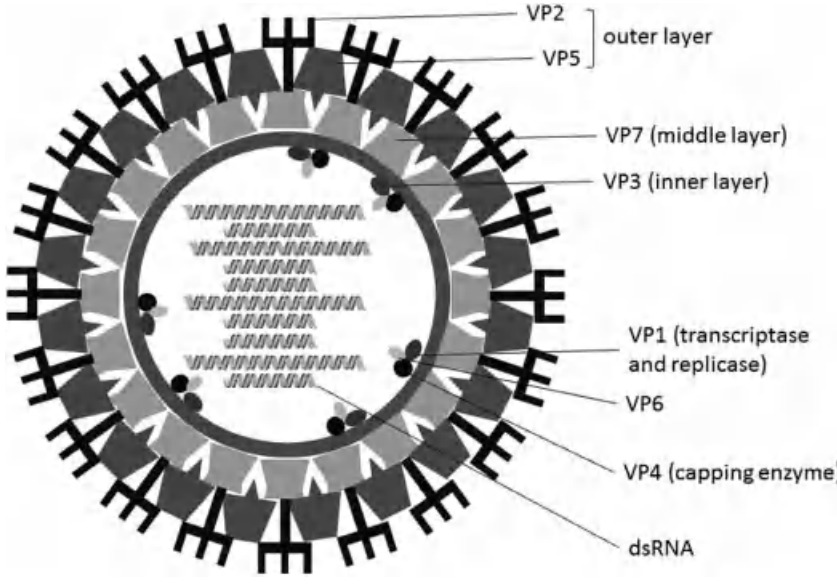

Figure 9.1 Bluetongue virus virion and core. The bluetongue virus virion is 88nm in diameter and consists of a triple-layered capsid surrounding the double-stranded RNA genome. The genome is located in the centre of the particle and is divided into ten linear double-stranded RNA genome segments which each encode a single protein. The inner layer is composed of the VP3 protein, which has the VP4 (capping enzyme), VP1 (RNA-dependent RNA polymerase) and possibly the VP6 protein located at the fivefold symmetry axes to create the transcription complexes. The middle layer is composed of the trimeric VP7 protein. The outermost layer consists of the two trimeric proteins VP2 and VP5 which function in the attachment and entry of mammalian host cells respectively. The core particle lacks the VP2 and VP5 proteins which compose the outer layer and uses the dsRNA genome segments as templates to produce plus sense transcripts which are released into the cytoplasm of the infected cell.

different length (NS3 and NS3A) from segment 10 (Table 9.1) (Verwoerd, 1969; Verwoerd *et al*., 1970). Each segment has the conserved sequence 5′GUUAAA. . . at the 5′ end of the plus sense strand and . . .ACUUAC3′ at the 3′ end (Rao *et al*., 1983; Mertens and Sangar, 1985). The plus sense strand of each genome segment is capped and methylated at the 5′ end, as are the viral transcripts which are identical to the genomic plus sense strands.

9.2.2 Attachment and entry

The outermost protein VP2 is required for attachment to the mammalian host cell by an as yet unidentified receptor (Huismans *et al*., 1983; Hassan and Roy, 1999). Internalisation of the virion is by endocytosis, which can be via a clathrin-mediated pathway or a clathrin-independent pathway, resulting in the virion being located in an endosomal compartment (Forzan *et al*., 2007; Gold *et al*., 2010). The low pH

Table 9.1 Bluetongue virus coding assignments.

Segment No. (length: nt)	Protein	MW (kDa)	Location in virion (VP1 to VP7)	Functions
1 (3944)	VP1	150	Inside core	Viral transcriptase, viral replicase
2 (2940)	VP2	111	Outer shell	Attachment protein, serotype determinant, neutralisation determinant, trimer
3 (2772)	VP3	103	Core inner layer	Innermost capsid layer
4 (1981)	VP4	76	Inside core	5′ capping and methylation of viral transcripts
5 (1635)	VP5	59	Outer shell	Role in low pH dependent release of cores from endosomes into the cytoplasm, trimer
6 (1772)	NS1	64	Non structural	Forms cytoplasmic tubules which are characteristic of orbiviruses, binds viral transcripts, increases the translation of viral transcripts
7 (1156)	VP7	39	Core outer layer	Outer layer of core (middle layer of virion), required for core infectivity in insects, trimer
8 (1125)	NS2	41	Non structural	Forms viral inclusion bodies, site of core assembly, recruits viral transcripts and core proteins, phosphorylated
9 (1049)	VP6	36	Inside core	Binds ssRNA and dsRNA, role in the assembly of cores
10 (822)	NS3, NS3A	26, 24	Non structural	Membrane protein, required for virus egress, glycosylated

dependent penetration of the endosomal membrane is co-ordinated with the removal of the outer layer of the capsid (VP2 and VP5) to release the core into the cytoplasm of the host cell. Entry is believed to be mediated by the VP5 protein which has structural and membrane fusogenic properties in common with the fusion proteins of enveloped viruses (Forzan *et al*., 2004; Zhang *et al*., 2010). In insect cells the core particle itself is infectious with VP7 being sufficient for attachment and entry (Mertens *et al*., 1996; Tan *et al*., 2001).

9.2.3 Transcription

The core particle is the final disassembly stage of the virion, and has the functions of synthesising and extruding capped and methylated plus sense copies (transcripts) of the genome segments into the cytoplasm, while keeping the viral dsRNA separate

from the sensory components of innate immunity. The core is transcriptionally active in the cytoplasm of the host cell, indeed, purified cores will synthesise capped and methylated viral transcripts *in vitro* when provided with nucleoside triphosphates (NTPs) and a methyl donor, demonstrating that no host factors are required for these transcription activities (Verwoerd *et al*., 1972; Verwoerd and Huismans, 1972; Martin and Zweerink, 1972). The viral transcriptase complexes are located inside the VP3 layer of the core at the fivefold symmetry axes (Gouet *et al*., 1999). Single-stranded transcripts are synthesised by the viral RdRp (VP1), which uses the negative sense strands of the dsRNA genome segments as templates. Transcription is co-ordinated with the capping and methylation activities of the VP4 protein, which catalyses a series of reactions resulting in a 5′ cap1 structure identical to that found at the 5′ of cellular messenger RNAs (mRNAs) (Martinez-Costas *et al*., 1998; Ramadevi *et al*., 1998; Ramadevi and Roy, 1998). The completed viral transcripts are extruded through channels at the fivefold symmetry axes of the cores into the cytoplasm (Verwoerd *et al*., 1972; Diprose *et al*., 2001). These viral transcripts have two roles which enable them to initiate the remainder of the replication cycle: (1) serving as mRNAs which are translated by the host cell ribosomes to produce the viral proteins; (2) functioning as templates for the synthesis of the new viral dsRNA genome segments present in progeny virions (Figure 9.2).

9.2.4 Translation

Translation of viral transcripts makes up the majority of active translation in infected mammalian cells by 8 hours post-infection (Huismans, 1979). The 5′ cap1 structure of the BTV transcripts allows the efficient recognition of mRNA by the host cell translation initiation factor eIF4F (Sachs *et al*., 1997). At the 3′ end the viral transcripts lack a 3′ poly(A) tail which is a feature common to almost all cellular mRNAs. Poly(A) binding protein (PABP) which binds to the poly(A) sequence of cellular mRNAs, increasing both the stability of mRNA and the rate at which translation is initiated has no target sequence in BTV mRNAs (Sachs *et al*., 1997). In Bluetongue virus-infected mammalian cells the efficient replacement of cellular translation with the translation of the viral genes is mediated by the NS1 protein (Boyce and Roy, unpublished observations). NS1 binds to the conserved ACUUAC3′ sequence which is located at the extreme 3′ ends of the all BTV mRNAs, enabling the translation of viral mRNA to be specifically increased compared to cellular mRNA (Boyce and Roy, unpublished observations). NS1-regulated translation requires the 5′ cap1 structure to increase translation, demonstrating that the 3′ and 5′ ends of the mRNA communicate (Boyce and Roy, unpublished observations).

9.2.5 Assembly and genome replication

Much of assembly occurs in modified regions of the cytoplasm variously termed ‘virus assembly factories’ or ‘viral inclusion bodies’ (VIBs), with progeny core particles being created at these sites. The major component of VIBs is the viral

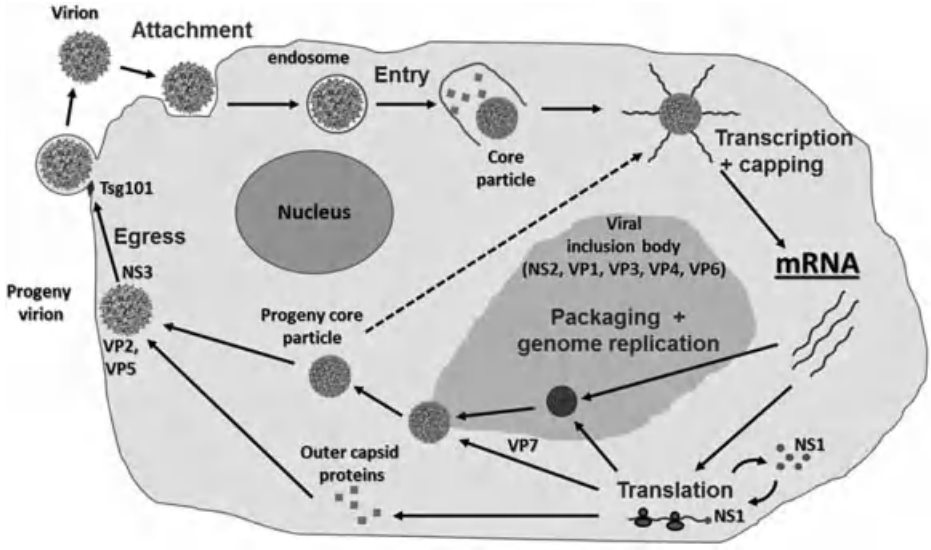

Figure 9.2 BTV replication cycle. Attachment to the host cell is mediated by the trimeric VP2 protein, followed by endocytosis of the virion, which can occur by both clathrin dependent or independent pathways. Acidification of the endosome results in structural changes in the outer capsid of the virion which deliver the core across the membrane into the cytoplasm. The core contains multiple transcription complexes which continuously synthesise and release plus sense copies (transcripts) of the viral genome segments into the cytoplasm. The viral transcripts determine both the synthesis of the viral proteins through translation, and are packaged and replicated to generate progeny dsRNA genome segments. Each transcript is recognised by the NS1 protein which specifically binds to viral transcripts through the conserved sequence present at the 3′ end, allowing their translation to be specifically up-regulated. Core particle assembly takes place in viral inclusion bodies consisting largely of NS2, where the assembly of the core proteins (VP1, VP3, VP4, VP6, and VP7) is coordinated with the packaging and replication of one copy of each transcript. The completed core particles are released into the cytoplasm where they contribute to viral transcription and are converted to virions by the addition of the outer capsid proteins, VP2 and VP5. The trafficking and assembly of the outer capsid involves the subversion of proteins (p11 and Tsg101) from cellular protein trafficking pathways by NS3, in addition to the association of VP2 with the vimentin cytoskeleton and the targeting of VP5 to lipid raft domains of the plasma membrane. Active egress from the infected cell occurs by budding of the virion at the plasma membrane, but how this envelope is lost to generate the naked virion is not known.

non-structural phosphoprotein NS2, which is sufficient to form inclusion bodies with the same morphology as VIBs, when expressed alone, and also self-assembles into multimeric complexes (Thomas *et al*., 1990; Uitenweerde *et al*., 1995; Modrof *et al*., 2005). NS2 also interacts with the viral core proteins VP1, VP3, VP4, VP6 and with viral transcripts, consistent with a role in recruiting the viral components needed to assemble single layered particles (Lymperopoulos *et al*., 2003; Modrof *et al*., 2005; Lymperopoulos *et al*., 2006; Kar *et al*., 2007). The synthesis of new dsRNA genome segments is performed by VP1, which has the catalytic activities

necessary to initiate negative strand synthesis *de novo* using the viral transcripts as templates (Urakawa *et al*., 1989; Boyce *et al*., 2004). Using the viral transcripts VP1 produces complete negative sense strands which remain annealed to the plus sense template to generate dsRNA genome segments (Boyce *et al*., 2004). How the recognition of one copy of each segment is achieved for the coordinated packaging and replication of a complete genomic set remains to be resolved. The co-expression of VP3 is sufficient to target the VP7 protein to NS2 inclusion bodies, indicating that the VIB is where the core particle is completed (Kar *et al*., 2007). NS2 has a nucleotidyl phosphatase activity which hydrolyses the alpha, beta and gamma phosphate groups of NTPs (Taraporewala *et al*., 2001), but how this activity contributes to its functions in core assembly is not yet known. The nascent core particles have two potential roles; to synthesise additional viral transcripts, leading to an increase in the production of viral transcripts and viral proteins, or the completion of virion assembly by the addition of the outer capsid proteins VP2 and VP5. How the balance between these two processes is regulated is not known, but the addition of the outer capsid proteins (VP2 and VP5) prevents further transcription and completes the assembly of infectious virion (Mertens *et al*., 1987).

9.2.6 Egress

The viral transmembrane proteins NS3, and the shorter NS3A are expressed at low level in mammalian cells where cell lysis occurs, and at high level in persistently infected insect cells (French *et al*., 1989, Guirakhoo *et al*., 1995). This correlation and studies of virus release using intracellular virus like particles (VLPs) implicated NS3/NS3A in the egress of BTV (Hyatt *et al*., 1993). How BTV ensures its efficient egress from infected cells is incompletely understood, but it is clear that NS3 engages with cellular proteins involved in protein trafficking and sorting pathways and with VP2 to enable virus release. Although BTV is a non-enveloped virus ultrastructural studies of BTV infected cells show the virion budding through the plasma membrane during egress, but how this envelope is removed to generate the non-enveloped virion is not known.

Through its amino terminus NS3 interacts with the cellular S100A10/p11 protein which is involved in the trafficking of proteins to the plasma membrane (Beaton *et al*., 2002; Rescher and Gerke, 2008; Celma and Roy, 2011). The shorter NS3A (lacking the amino terminal 13 residues) does not interact with S100A10/p11, and BTV which only expresses the NS3A is defective in the trafficking of particles to the plasma membrane in mammalian cells (Celma and Roy, 2011). While NS3 is sufficient for virus replication in mammalian cells, both NS3 and NS3A are required for efficient replication in insect cells, suggesting a role for the shorter variant in the insect vector (Celma and Roy, 2011). The outer capsid protein VP2 associates with the vimentin component of the cytoskeleton, and disruption of the vimentin filaments prevents the efficient release of BTV, indicating that vimentin is required for the trafficking of VP2 or BTV (Eaton and Hyatt, 1989; Bhattacharya *et al*., 2007). The

lipid raft domains of the plasma membrane are essential for the efficient replication of BTV, and NS3 and VP5 both co-purify with these raft domains (Bhattacharya and Roy, 2008). A membrane docking motif in VP5 is required for the association with lipid rafts, consistent with VP5 possessing an independent signal for targeting it to the plasma membrane (Bhattacharya and Roy, 2008).

In BTV-infected mammalian cells, infectious virions are actively released by utilising components of the cellular ESCRT-I (endosomal sorting complexes required for transport) pathway, which mediates vacuolar protein sorting, and later by lysis of the cell (Beaton *et al*., 2002; Wirblich *et al*., 2006). The NS3 protein has been shown to act as a bridge between VP2 and the cellular Tsg101 protein, which is involved in the late stages of membrane fission in the ESCRT-I pathway (Williams and Urbe, 2007). When NS3 is mutated at the Tsg101 interaction motif efficient egress no longer occurs, with virions remaining tethered at the plasma membrane (Celma and Roy, 2009). The mutation of the VP2-interacting domain of NS3 prevents the bridging of VP2 to the cellular trafficking machinery and virions are no longer located at the plasma membrane, with particles remaining in the cytoplasm (Celma and Roy, 2009).

9.3 Reverse genetics

9.3.1 The reverse genetics challenge

The reverse genetics approach to investigating biology requires that the genome of the organism can be modified at the sequence level as determined by the investigator. Reverse genetics is used to discover the phenotype(s) associated with a specific sequence change made in the genome of the organism, rather than beginning with a phenotype and searching for the sequence change which causes it, as in classical genetics. As an approach to understanding how a virus or other organism functions, this is very powerful.

The generation of reverse genetics systems for RNA viruses began with positive strand viruses which have infectious genomes (Racaniello and Baltimore, 1981), and proceeded to negative strand viruses and then to segmented negative strand viruses which require more complicated strategies to alter their genomes, as reviewed by Neumann *et al*. (Neumann *et al*., 2002). Compared to other groups of RNA viruses, the dsRNA viruses have been particularly resistant to attempts to modify their genomes using the techniques of molecular biology, with routine modification still not achieved for some intensively studied members of the *Reoviridae*.

The first evidence of how a member of the *Reoviridae* might be modified in a defined way was the discovery that the transcripts of one serotype of mammalian orthoreovirus could be rescued by a second serotype in a helper virus dependent system (Roner *et al*., 1990). In this case transfection with the transcripts from serotype 3 were recovered into infectious virus when the cells were infected with a serotype 2 helper virus. This initial finding demonstrated that the purified transcripts of

orthoreoviruses can be recovered into the genome of infectious virus, providing that helper functions were supplied *in trans* by another orthoreovirus. From this discovery the replacement of a viral transcript with a transcript derived from a cDNA plasmid clone was the breakthrough that was needed to manipulate the genome of mammalian orthoreoviruses, and allowed the alteration of individual genome segments (Roner and Joklik, 2001). Recently a plasmid-based system where the entire genome of reovirus is derived from plasmid clones has been developed (Kobayashi *et al*., 2007). This approach does not rely on the functions of a helper virus, but generates each viral transcript within the cell from plasmid clones.

The first modifications of the rotavirus VP4 protein were recently made using a helper virus dependent system, where selective pressure against the helper virus is provided by a neutralising antibody (Komoto *et al*., 2006). Using a dual selection with siRNA and temperature sensitivity in the target gene of the helper virus the range of viral genes which could be manipulated has been broadened from surface exposed proteins, which are susceptible to neutralising antibody, to any viral gene for which a temperature sensitive mutant can be derived (Trask *et al*., 2010).

9.3.2 Reassortment of genome segments

As found for other viruses with segmented genomes, reassortment of BTV genome segments can occur when a cell is simultaneously infected with two strains (Gorman, 1990). The result of reassortment is the generation of progeny viruses which contain genome segments from both parental viruses (Figure 9.3). The observation that individual genome segments can be replaced by the corresponding segment of another strain in the context of a dual infection suggests that it should be possible to replace a viral genome segment if an appropriate substitute is provided in the infected cell in sufficient quantity.

9.3.3 Viral ssRNA is infectious

In all members of the *Reoviridae* the viral transcripts generated by the infecting virion act as both mRNAs in translation and as replication intermediates (Figure 9.2). A logical conclusion from this is that a replication cycle could be initiated by introducing viral transcripts into the host cell by a method such as transfection. This approach assumes that sufficient RNA can be introduced to synthesise viral proteins up to the point where progeny core particles are synthesised, and a second round of transcription begins, generating further viral transcripts and proteins. The recovery of virus from RNA has been shown to be possible for BTV and the related African Horse Sickness Virus (AHSV) where the transfection of BSR cells with transcripts produced *in vitro* from purified cores initiated the replication cycle in a minority of cells (Boyce and Roy, 2007; Matsuo *et al*., 2010). In these experiments the transcripts were produced *in vitro* using core particles purified from infected

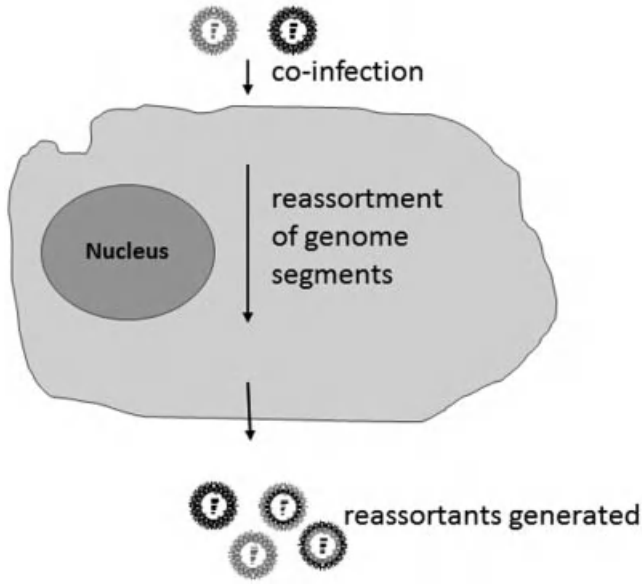

Figure 9.3 Reassortment of genome segments. Co-infection of a single cell with two strains of BTV produces progeny viruses with genome segments derived from both parental strains in addition to the viruses containing all the viral genes from one parent. Some combinations of genome segments may not produce viable genomes.

cells, proving that transcripts generated by cores *in vitro* are functionally equivalent to those produced in infected cells (Boyce and Roy, 2007). The proof that the transcripts themselves initiate the viral replication cycle and not contamination with residual cores is provided by digestion with RNAse A which destroys the infectivity of the purified transcripts but has no effect on the infectivity of cores introduced into cells by transfection (Boyce and Roy, 2007). The crucial finding from these experiments is that transfection with viral ssRNA is sufficient to produce infectious virus, and suggests that the replacement of a viral transcript with an alternative transcript from another source would allow a virus containing the new genome segment to be generated.

The 'proof of principal' of replacing viral genome segments was provided by the introduction of genome segments from a second serotype of BTV. In these experiments BSR cells were transfected with transcripts produced from serotype 1 cores mixed with transcripts produced by serotype 9 cores. Individual plaques were isolated from the transfected monolayer by overlaying the transfected cells with agarose, and after propagation reassortants containing genes from both parents were found to be present among the progeny (Boyce *et al*., 2008) (Figure 9.4). These experiments also showed that reassortants were recovered if the two sets of viral transcripts were synthesised separately and combined, rather than requiring co-synthesis as occurs within a cell infected with two BTV isolates. This result

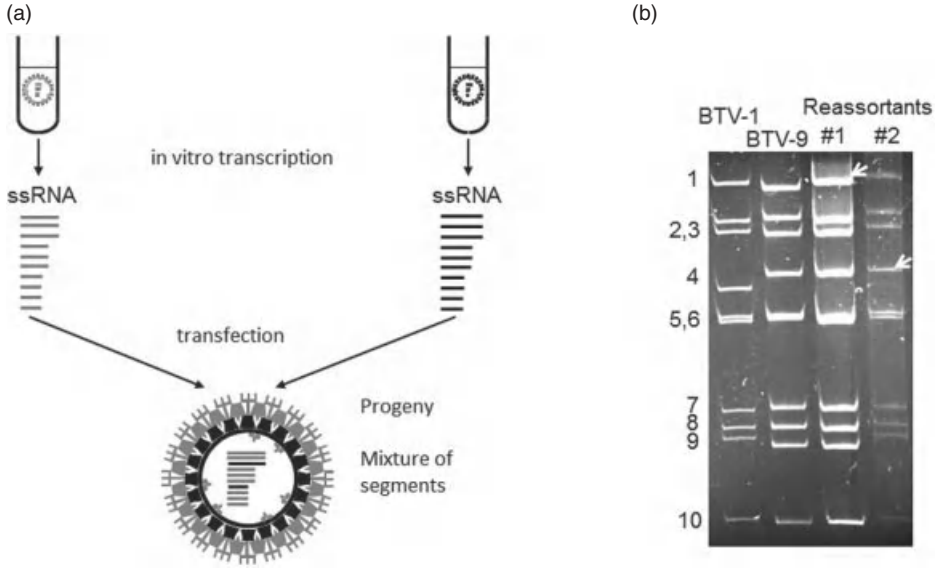

Figure 9.4 Reassortment of genome segments using mixtures of transcripts derived from two types of cores**.** (a) Viral transcripts synthesised using cores derived from two strains of BTV can be mixed after synthesis and used to generate reassortants by the transfection of BSR cells. (b) Polyacrylamide gel profiles of the ten dsRNA genome segments of BTV-1, BTV-9 and reassortants generated by transfection of BSR with a mixture of transcripts synthesised from BTV-1 cores and BTV-9 cores. The migration rate of several dsRNA genome segments differs between the two serotypes, allowing the source of these segments to be rapidly determined. Reassortant #1 contains segment 1 (arrowed) of BTV-1 in a BTV-9-like profile. Reassortant #2 contains segment 4 (arrowed) of BTV-9 in a BTV-1-like profile.

suggested that mixing of a cDNA clone-derived transcript with core-derived transcripts would also allow incorporation of the cloned gene into the viral genome.

9.3.4 The synthesis of viral transcripts from T7 promoter cDNA clones

To make a BTV reverse genetics system from these observations one or more genome segments must be derived from a plasmid clone so that the sequence of the genome segment may be modified. Like other members of the *Reoviridae* the genome segments of BTV have precisely defined 5′ and 3′ end sequences. The most straightforward approach for reverse genetics systems for the *Revoiridae* is to make transcripts which have the exact 5′ and 3′ ends found in the genomic RNA, and if translation is required a 5′ cap should also be present. Cellular mRNAs are capped following transcription by the cellular RNA polymerase II, but capped transcripts generated by intracellular transcription from highly expressing RNA polymerase II

driven plasmids do not have the precisely defined termini which are characteristic of the *Reoviridae*, and are extended at the 3′ end extended by a poly (A) tail. While viral protein expression is high using polII constructs the difference in sequence between the polII transcripts and viral transcripts do not make this an attractive approach. The intracellular synthesis of transcripts with a defined sequence at the 5′ end and 3′ end has been accomplished using RNA polymerase I promoters and terminators or phage promoters and ribozymes as reviewed by Neumann *et al* (Neumann *et al*., 2002). Both these approaches have been successfully used to produce precisely defined transcripts intracellularly and have been successful approaches to creating reverse genetics systems for many negative strand RNA viruses. However, these methods of producing single stranded RNA with defined ends do not add the 5′cap structure essential for efficient translation. Further elaboration using the promiscuous cross-capping activity of poxviruses can be used to ensure the capping of a minority of the transcripts (Fuerst and Moss, 1989). In the case of BTV, transfection with viral transcripts was already shown to generate infectious virus, so the transcripts were synthesised extracellularly using run-off transcription from a linearised plasmid to enable the addition of the plasmid-derived transcript to the infectious viral RNA (Boyce *et al*., 2004; Boyce *et al*., 2008). The system used was the well-characterised phage T7 promoter which allows T7 RNA polymerase to selectively initiate transcription at a precisely defined G nucleotide (the first base of every BTV genome segment) (Rosenberg *et al*., 1987). Transcription terminates at the 3′ base of a linear DNA template in a 'run-off' transcription reaction. Restriction enzymes which cleave asymmetrically and outside of their recognition sequence can be used to generate a defined 3′ end in the plasmid template (Figure 9.5a). Capping of these transcripts can be achieved through the incorporation of a 5′ cap analogue in the place of GTP during the initiation of transcription. The recently designed 'anti-reverse cap analogue' (Stepinski *et al*., 2001) has allowed the *in vitro* synthesis of BTV transcripts which are efficiently translated. Using this approach it is possible to routinely synthesise highly translatable transcripts *in vitro* which also have exactly defined 5′ and 3′ end sequences, as found in the *Reoviridae*.

9.3.5 Reassortment of a T7 promoter plasmid transcript into the genome of BTV

The replacement of a viral transcript with an equivalent T7 transcript requires that the T7 transcript is selected during packaging. The discovery that viral transcripts from two BTV serotypes could be mixed after synthesis and yield reassortants suggested that the simultaneous synthesis of the viral transcripts and the T7 transcript in a single reaction is not necessary to preserve putative RNA-RNA interactions, which may be required for the packaging of one copy of each segment (Boyce *et al*., 2008). This approach was extended to the mixing of core-derived viral transcripts and a T7 transcript with a 5′ cap. Although the length of any genome segments does not vary much between serotypes the migration rate of the dsRNA segments in

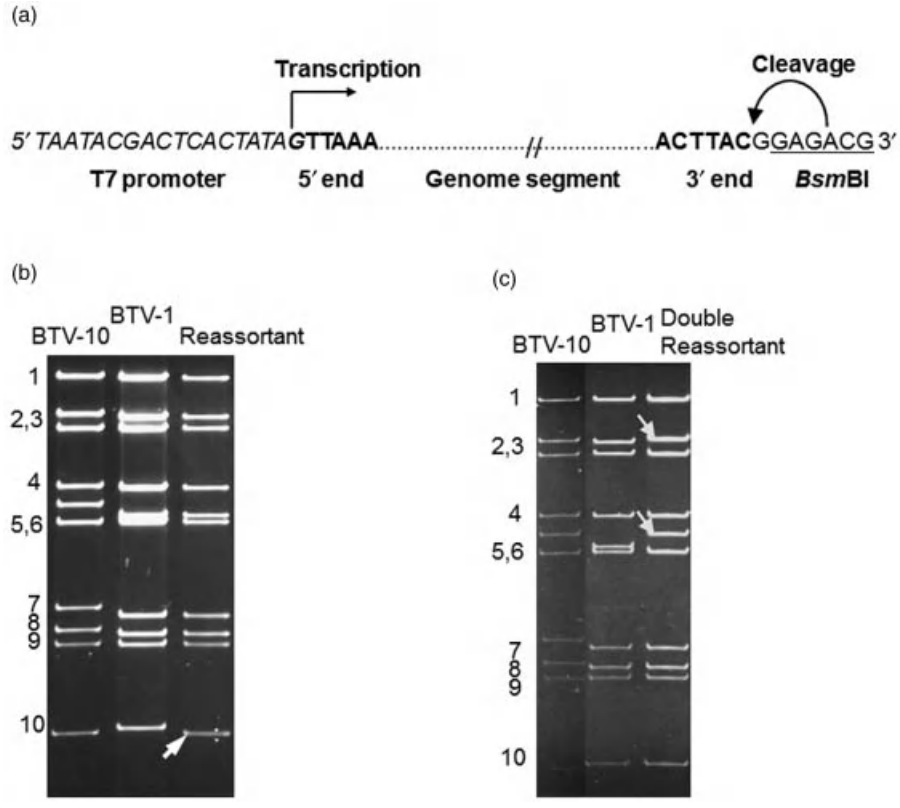

Figure 9.5 Reassortment of T7 transcripts into the genome of infectious BTV. (a) Schematic diagram of the expression cassette in T7 constructs used in the synthesis of BTV transcripts. Each plasmid contains a full length genome segment flanked by a T7 promoter and a restriction enzyme site which cleaves outside its recognition sequence. The conserved 5′ and 3′ ends of the BTV genome segment are shown (bold). A T7 promoter (italics) defines the initiation site of transcription (5′ end), and the *Bsm*BI site (underlined) determines the termination site (3′ end). (b) Single segment reassortment. Polyacrylamide gel profiles of BTV-10, BTV-1 and a reassortant generated by the transfection of BSR with a mixture of transcripts synthesised from BTV-1 cores + BTV-10 segment10 transcript derived from a plasmid clone. Reassorted genome segment 10 is arrowed. (c) Two segment reassortment. Polyacrylamide gel profiles of BTV-10, BTV-1 and a reassortant generated by the transfection of BSR with a mixture of transcripts synthesised from BTV-1 cores + BTV-10 segment 2 and segment 5 transcripts derived from a plasmid clones. Reassorted genome segments 2 and 5 are arrowed.

non-denaturing polyacrylamide gels often varies sufficiently to distinguish between serotypes. This screening approach allowed T7 derived segments from BTV-10 to be detected in reassortment experiments with BTV-1 core transcripts. Using this approach the ratio of the T7 transcript to the corresponding viral transcript was varied to determine the ratio which produced the maximum proportion of reassortant plaques compared to background (all BTV-1 segments) (Boyce *et al*., 2008). A 5:1

ratio of a clone-derived BTV-10 segment 10 transcript to the corresponding cored-derived BTV-1 transcript produces reassortants at a high enough frequency (15% or greater) that detection by screening the dsRNA profile is a practical approach to identifying reassortants among the background of BTV-1 plaques (Boyce *et al*., 2008) (Figure 9.5b). To confirm that the reassortants being generated were derived from the cloned BTV-10 segment a non-coding change was introduced into the clone and shown to be present in the genomic dsRNA of the recovered reassortants (Boyce *et al*., 2008). These experiments described the first targeted alterations of the BTV genome, and proved that the orbiviruses recognise a capped T7 transcript for packaging and negative strand synthesis when it is present during ongoing replication (Boyce *et al*., 2008). A similar approach of reassorting one transcript derived from a T7 promoter plasmid with an infecting virus has also been used recently (van Gennip *et al*., 2010). In this helper virus system, reassortment occurs in cells which are simultaneously infected with the helper virus and transfected with the T7 transcript. The advantage of these approaches is that they only require the construction of a single clone to modify the gene of interest.

The reassortment of T7 transcripts by mixing with core-derived transcripts was extended by simultaneously reassorting segments 2 and 5 encoding the outer capsid proteins VP2 and VP5. The cloned genome segments were from BTV-10 and could be reassorted into the genetic background of BTV-1 using a single transfection to produce a virus which contained both genome segments derived from the cloned BTV-10 genome segments (Boyce *et al*., 2008) (Figure 9.5c). The segment 2+5 reassortant virus generated has the outer capsid of BTV-10 assembled on the core of BTV-1 (Figure 9.1), proving that the variable outer capsid proteins of one serotype can assemble on the core particle of another serotype and retain their functions during infection. Due to the replacement of the outer capsid proteins the serotype of the reassortant has been switched from type 1 to type 10, as determined by neutralisation assays (Boyce and Roy, unpublished observations). This double reassortment approach can be used to generate strains of the desired serotype in the development of live vaccines. The single segment reassortants of BTV-10 VP2 or VP5 could not be recovered in the BTV-1 genetic background, showing that these viruses are not viable or replicate much more slowly than BTV-1. VP2 and VP5 are known to be highly variable between serotypes due to continuing immune selective pressure in the host, and are closely associated in the virion (Zhang *et al*., 2010). The failure of VP2 and VP5 from two different serotypes to produce infectious virions is consistent with the outer capsid proteins co-evolving under immune selective pressure and being incompatible either during assembly or in their roles in attachment and entry in the next round of infection.

The RNA reassortment-based approach to orbivirus reverse genetics is equivalent to the helper virus reassortment systems used for rotaviruses and orthoreoviruses (Roner and Joklik, 2001; Komoto *et al*., 2006; Trask *et al*., 2010). BTV can be recovered completely from RNA transcripts including one or more plasmid-derived transcripts, but the genetic background is provided by transcripts produced by viral cores and therefore of viral origin.

9.3.6 Cloning the BTV genome

The equivalence of T7 transcripts to core-derived transcripts in reassortment experiments suggests that BTV could be recovered completely from T7 transcripts. The reassortment approach to BTV reverse genetics leads to a reduction in the efficiency of plaque recovery from RNA compared to using core-derived transcripts only, and the recovery of virus entirely from T7 transcripts was expected to lead to a further reduction in efficiency. To increase the efficiency of the system priority was given to using a combination of virus isolate and cell line which allows replication of the virus to a high titre, and BTV-1 (South African reference strain) and BSR cells were chosen for this reason. It is important and not trivial to avoid introducing potential mutations into the 19.2kb BTV genome during cloning. To clone the ten genome segments of BTV-1 without defining the 5′ and 3′ ends of each segment during PCR, the sequence-independent amplification strategy for dsRNA recently improved by Potgieter *et al*. and Maan *et al*. was used (Potgieter *et al*., 2002, Maan *et al*., 2007). The method used involves the ligation of hairpin DNA primers to the 3′ ends of the viral dsRNA and cDNA synthesis of the primer-ligated genome segments by reverse transcription. This is followed by PCR amplification to generate complete copies with the terminal sequences unaltered which are subsequently cloned (Maan *et al*., 2007). Each PCR product was sequenced prior to cloning to provide a consensus sequence for the segment (Boyce *et al*., 2008). This was done using conventional dye terminator sequencing and no examples of highly represented variations were discovered in the PCR products. Plasmid clones were generated from this set of PCR products and their sequence compared to the PCR products. The screening of the clones was done by discarding any clone which had coding changes, or had alterations in the terminal ∼200nt at each end of the segment, where regulatory sequences essential to packaging or replication had been identified in orthoreoviruses (Roner *et al*., 2004; Roner and Roehr, 2006). A set of T7 promoter clones (Figure 9.5a) were generated from these clones and used to make a genomic set of T7 transcripts equivalent to the infectious core-derived viral transcripts.

9.3.7 Recovery of BTV entirely from T7 transcripts

The recovery of BTV from T7 transcripts was performed by mixing 300–400ng of each capped T7 transcript and transfecting BSR monolayers (Boyce *et al*., 2008). Individual rescue events can be isolated by overlaying the transfected monolayer with agarose to create a plaque in the cell monolayer for each infectious virus recovered (Figure 9.6). The BTV-1 virus rescued from plasmid-derived T7 transcripts replicates robustly in mammalian and *Culicoides* cells, being indistinguishable from BTV derived from infection. These experiments described the first recovery of an orbivirus completely from cloned viral genome segments, creating a system in which mutants can be generated in a consistent genetic background.

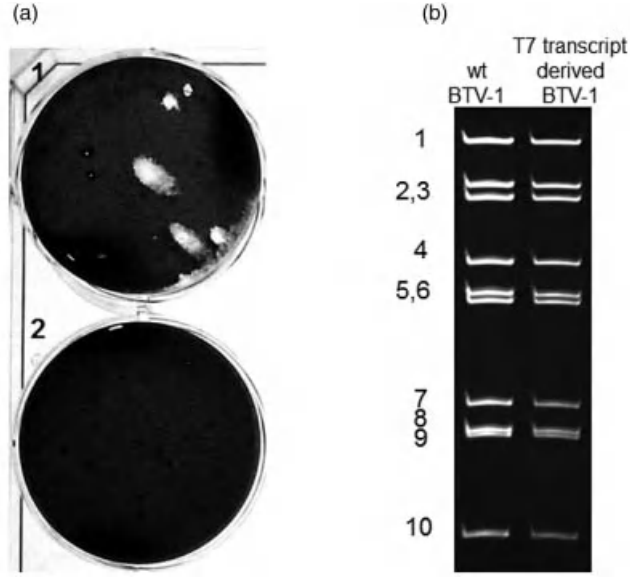

Figure 9.6 The recovery of infectious BTV by transfection with 10 T7 transcripts. (a) Transfected BSR monolayers overlaid with agarose. Well 1, BSR transfected with BTV-1 T7 transcripts. Well 2, BSR not transfected. (b) Genomic dsRNA extracted from BTV recovered from the transfection of BSR monolayers as described in panel A.

The recovery of infectious BTV from T7 transcripts alone demonstrates that T7 transcripts synthesised with a 5′ cap analogue are functionally equivalent to authentic viral transcripts at all stages of the replication cycle (Figure 9.2). The T7 transcripts must be translated to produce the viral proteins and selected during genome packaging and also must act as templates for negative-strand synthesis to produce progeny dsRNA genome segments. Following negative-strand synthesis, the resulting dsRNA genome segments must be competent for transcription in the next infectious cycle.

9.3.8 Increased efficiency of recovery

The efficiency of recovery of BTV from T7 transcripts is lower than for core-derived transcripts. Several modifications to the transfection protocol can be made which increase the recovery of virus, and the successful modifications are informative about the mechanisms of BTV gene expression and genome packaging/replication. The amount of each viral transcript produced by cores *in vitro* is approximately equal in terms of mass, except for segment 6 encoding NS1, which is more highly transcribed than the other segments (Verwoerd and Huismans, 1972). The NS1 protein encoded by segment 6 has recently been shown to bind viral transcripts through the conserved 3′ end sequence and functions as a positive regulator of translation of

viral transcripts (Boyce and Roy, unpublished observations). Increasing the amount of segment 6 transcript used so that its abundance is 2 fold higher than the other transcripts produces a ~2 fold increase in the recovery of virus, which is consistent with its role in specifically upregulating the translation of viral transcripts (Boyce and Roy, unpublished observations).

Further increases in the recovery of virus can be achieved by separating the two roles of the T7 transcripts: (1) mRNA for translation; and (2) replication intermediate for packaging and negative strand synthesis. At early times transcripts can only be used as templates in translation as there are no packaging/replication complexes yet assembled in the infected cell. At later times these complexes have been assembled from viral proteins and the packaging and replication of the transcripts becomes the next step in the replication cycle. By transfecting twice separated by 18 hours viral transcripts are supplied again when they can be incorporated into assembling packaging/replication complexes, producing ~10x increase in the number of plaques recovered (Boyce and Roy unpublished observations). This can be demonstrated by using a marker transcript in the second transfection (Figure 9.7). Using

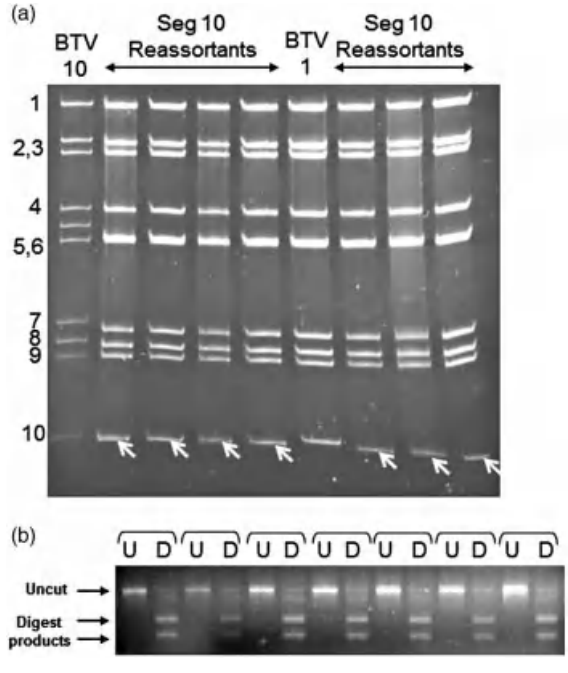

Figure 9.7 Transcripts from a second transfection are packaged. (a) BTV genomic dsRNA profiles recovered using BTV-1 T7 transcripts in the first transfection and BTV-10 segment 10 + BTV-1 segments 1-9 in the second transfection. Polyacrylamide gel profiles of BTV-10, BTV-1 and the reassortants generated. Lower migrating BTV-10 segment 10 indicated. (b) RT-PCR and *Hae*II digestion digestion of segment 10 RT-PCR products from reassortants generated using the double transfection approach in panel A, where the BTV-10 segment 10 contains a *Hae*II marker site. Digests run on agarose gels. U = undigested RT-PCR product, D = *Hae*II digested RT-PCR product.

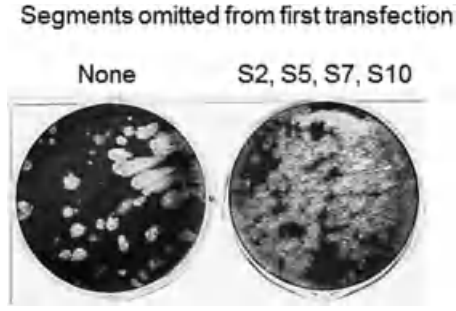

Figure 9.8 An incomplete set of transcripts in the first transfection increases virus recovery. Transfected BSR monolayers overlaid with agarose, so that each plaque is a single rescue event. Well 1) All ten transcripts were used in the first transfection, well 2) Segments 2, 5, 7, and 10 were omitted from the first transfection. All ten transcripts were used in the second transfection in both wells. 200ng each transcript per transfection, except segment 6 (NS1) where 400ng was used.

S10 from BTV-10 with a restriction enzyme site in the second transfection showed that the genome of the recovered BTV is derived from the second transfection in these double transfection experiments (Boyce and Roy, unpublished observations) and has also been shown for AHSV using core-derived transcripts (Matsuo *et al*., 2010). A further increase in the efficiency can be produced by providing only those transcripts required for the assembly of the packaging/replication complex in the first transfection. The omission of the outer capsid proteins (VP2 and VP5) and NS3 will prevent the formation of empty (no genome) virions, which are known to form when the four major structural proteins (VP3, VP7, VP2 and VP5) are expressed in heterologous systems such as the Baculovirus expression system (French *et al*., 1990). The VP7 encoding transcript can also be omitted, suggesting that VP7 is added to the particle at a stage after packaging and replication. A complete set of genome segments are provided in a second transfection and the increase in the recovery of virus is ~5x (Figure 9.8), consistent with the hypothesis that packaging is more efficient when assembly is stalled at this point by omitting VP7, VP2, VP5 and NS3 (Boyce and Roy, unpublished observations; Matsuo and Roy, 2009).

9.3.9 Advantages of the complete T7 system versus reassortment of one T7 transcript

The complete T7 transcript system is equivalent to the plasmid-based transfection approaches successfully used with many RNA viruses, except that the RNA is produced from the plasmids *in vitro* rather than intracellularly. One advantage of plasmid-based reverse genetics over helper virus systems is that every plaque produced from plasmid-based systems is derived from the plasmid clones, so there is no background of wild-type virus from which the desired mutant must be

identified and purified. Furthermore the genetic background of the mutants being generated is consistent between experiments, as no part of the genome is supplied by a helper virus. The absence of helper virus makes the complete T7 system preferable to the reassortment system for rescuing mutants which replicate slowly and which would therefore be difficult to isolate from a background of more rapidly replicating wild-type virus. The disadvantages are the need to construct ten clones containing ~19.2kb in total, and the necessary sequencing involved to ensure that there are no unwanted mutants which would render the virus non-viable. In the case of research projects requiring the investigation of one or two proteins, the reassortment of one or two transcripts into the strain of choice could be a more practical approach.

9.3.10 Complementing cell lines

The recovery of viral mutants using any reverse genetics system can be problematic as the mutants that an investigator would wish to construct will often be lethal, and therefore not recoverable. The use of a complementing cell line often overcomes this limitation by providing the functional protein *in trans* in the host cell, so that the protein is present even when the viral copy of the gene is non-functional (Figure 9.9). In the *Reoviridae* this was first demonstrated with mammalian orthoreoviruses by the recovery of a chloramphenicol acetyl transferase (CAT) reporter in the place of the viral sigma 2 protein, using a cell line expressing sigma 2 (Roner and Joklik, 2001). This approach has been used to generate a number of lethal or slowly replicating BTV mutants in VP6 and NS3 at high titre (Boyce and Roy, unpublished observations; Celma and Roy, 2009, 2011; Matsuo and Roy, 2009). The scope of using complementing cell lines to generate lethal mutants is very broad, with the mutants in the protein coding region of any gene being recoverable in theory, although the possibility of dominant negative mutants being less well complemented remains.

9.4 Uses of reverse genetics in orbivirus research

The introduction of specific mutations into the genome of BTV by reverse genetics can be used to study the virus and its interaction with the host or as a method of altering the phenotype of a virus for a specific application, such as producing a live attenuated vaccine, or screening for antiviral drugs. In the case of investigations into the biology of the virus any process in the infected cell or host organism which is more easily studied using replicating virus than individual viral genes is a good candidate for study using reverse genetics. Some examples are: (1) the dissection of the functions of the viral proteins in the context of replicating virus; (2) the mapping of *cis*-acting sequences in the genome segments; (3) investigation of immune evasion and immunomodulation in the host organism; (4) the identification of pathogenicity

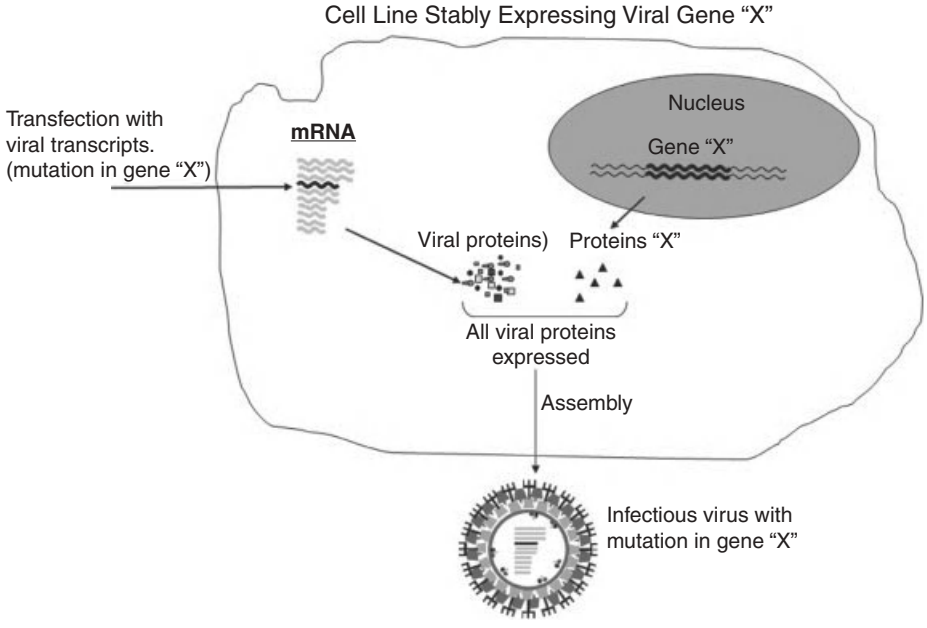

Figure 9.9 Rescue of lethal mutants using a complementing cell line. The viral gene 'X' is stably expressed from the nucleus of the cell. The cell line is generated by transfection of the cell with a plasmid clone expressing the viral gene from a cellular promoter and containing a selectable marker. The gene 'X' expressing cell line is used to recover viral mutants of protein 'X' using the reverse genetics system. Lethal mutants of protein 'X' can be rescued into the genome of infectious virus as the wildtype protein 'X' is provided *in trans* within the cell.

determinants; and (5) the identification and characterisation of the functions of viral proteins which differ between the mammalian host versus and the insect vector.

9.4.1 Egress

The egress of BTV from infected cells has been studied by identifying cellular proteins which interact with NS3 and determining the effect of mutagenesis on these interactions using biochemical assays where one protein has been expressed from a plasmid construct (Beaton *et al*., 2002; Wirblich *et al*., 2006). Using reverse genetics the investigation of egress has been accelerated by the generation of specific mutants which affect the interaction of NS3 with cellular protein trafficking pathways (Celma and Roy, 2009, 2011). The NS3 mutants generated and their effects on the egress of replicating virus have been studied using virus release assays and ultrastructural studies of infected cells (Celma and Roy, 2009, 2011). In each case, the NS3 mutants were recovered and propagated in an NS3 expressing cell line, where the NS3 expressed by the cell allows NS3 defective viruses to replicate to high titre.

The phenotype of these NS3 mutants was then determined in wild-type BSR cells or *Culicoides* cells, where the effect of the mutations is apparent. The carboxy terminus of NS3 was shown to interact with the outer capsid protein VP2 using a series of NS3 mutants, and mutations which destroyed this interaction also prevented the trafficking of virions to the plasma membrane, with particles remaining in the cytoplasm (Celma and Roy, 2009). Mutants were also made in the NS3 domain which was predicted to interact with the cellular partner Tsg101, which is involved in the late stages of membrane fission in the ESCRT-I pathway. Preventing the Tsg101 interaction in infected cells produced a phenotype of virus accumulation at the inner surface of the plasma membrane with very inefficient release (Figure 9.10). These data provided striking evidence that NS3 is required for the release of BTV from infected cells, and does indeed act as a bridge between Tsg101 and VP2, results that would be technically very challenging to clearly demonstrate without using viruses mutated at the interaction sites of NS3 with viral and cellular targets. NS3 has also been shown to interact with S100A10/p11 which is involved in the trafficking of proteins to the plasma membrane, and this interaction domain has been mapped to the extreme amino terminus of NS3 which is absent in the amino terminally truncated NS3A form (Beaton *et al*., 2002). Mutants in the extreme amino terminus of NS3 are defective in virus trafficking in infected mammalian cells (Celma and Roy, 2011). Interestingly the mutational analysis revealed a difference in the requirements of NS3 and NS3A in mammalian cells compared to insect cells, with NS3 being sufficient for virus replication in mammalian cells, but both NS3 and NS3A being required in insect cells. BTV is released largely by lysis from infected mammalian cells, but establishes a persistent infection of *Culicoides* cells. BTV clearly interacts differently with the cells from its two hosts, while being able to

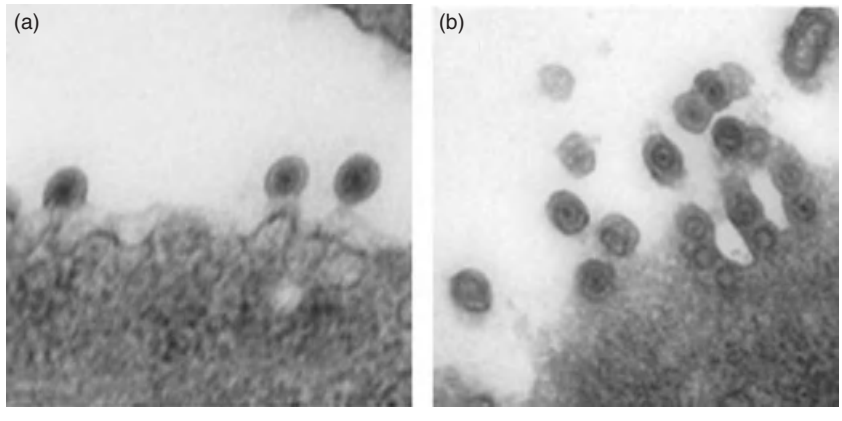

Figure 9.10 BTV NS3 mutant in Tsg101 interaction domain is not efficiently released. Ultrastructural analysis of virus release from BSR cells infected with wildtype BTV-1 (a) or BTV-1 containing the PSAP to GAAP mutation in the Tsg101 interaction domain of NS3 (b). Without interaction with Tsg101 the virions are not released leading to their accumulation in rows at the plasma membrane. Images kindly provided by Cristina Celma and Maria McCrossan.

replicate in both, and mutants generated by reverse genetics can be used to further investigate the molecular basis of the different fates of infected mammalian and *Culicoides* cells.

9.4.2 Viral proteins

The roles of two partially characterised orbivirus proteins, VP6 and NS1, have also been investigated using reverse genetics. VP6 was initially characterised by *in vitro* studies as an RNA binding protein with nucleoside triphosphatase and helicase activities (Stauber *et al*., 1997; Kar and Roy, 2003), although its role in replication was undefined. Using reverse genetics a VP6 defective mutant was generated to determine the stage at which virus replication became blocked without functional VP6 (Matsuo and Roy, 2009). Using transfection with combinations of T7 transcripts the set of viral genes necessary to generate a packaging/replication complex were defined, and VP6 was shown to be essential at this stage of the replication cycle. The NS1 protein is characteristic of the orbiviruses and is the most highly expressed BTV protein (Huismans, 1979). Recently NS1 has been characterised *in vitro* as an RNA binding protein with specificity for the conserved 3′ ends of viral transcripts enabling the translation of viral transcripts to be specifically upregulated (Boyce and Roy, unpublished observations). Reporter assays showed that NS1 increased the translation of single viral transcripts in transfected cells. Using reverse genetics a reporter virus was generated by inserting a luciferase reporter gene into segment 10 of BTV, and the NS1-dependent increase in expression from this reporter was demonstrated to occur in infected mammalian cells (Figure 9.11).

9.4.3 Mapping *cis*-acting sequences

The presence of *cis*-acting sequences which are essential for the packaging or replication of viral RNA have been identified using complementing cell lines both in orthoreoviruses and BTV (Boyce and Roy, unpublished observations; Roner and Roehr, 2006; Roner and Mutsoli, 2007; Matsuo and Roy, 2009). The mapping of the BTV sequences which are sufficient for the packaging and replication of a genome segment has been achieved using cell lines which provide the viral protein encoded by the segment *in trans* (Boyce and Roy, unpublished observations; Matsuo and Roy, 2009). Under these conditions mutants which destroy the function of the protein can be rescued, but mutants that prevent the genome segment from being packaged or replicated are not recoverable. This approach is successful because genomes which lack a segment are not viable, which indicates that a complete set of transcripts is packaged/replicated, and suggests that the ten RNAs interact to form a complex which is packaged. Deletion mutants of the segment are tested for their ability to be recovered in the complementing cell line. This process can be applied iteratively to define the sequences in the segment which are necessary for packaging

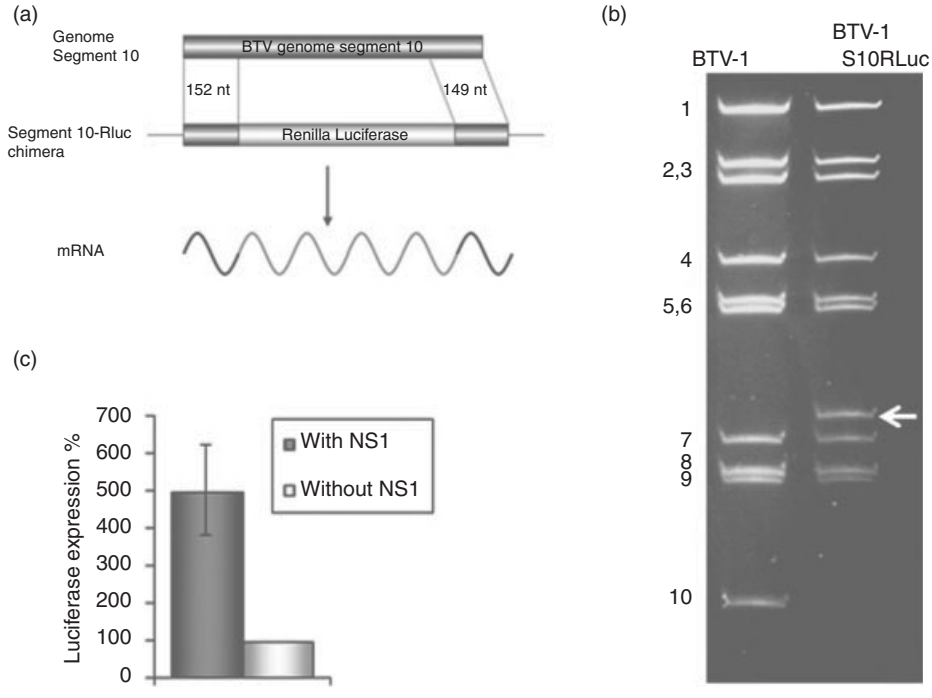

Figure 9.11 BTV containing a quantifiable reporter protein fused with segment 10 is upregulated by NS1. (a) Chimeric genome segment consisting of segment 10 and Renilla luciferase. (b) Polyacrylamide gel dsRNA profiles of BTV-1, and the BTV1 S10 RLuc reporter virus generated by the transfection of the BSR NS3 cell line with transcripts derived from a plasmid clones. The chimeric S10-*Renilla* luciferase segment (arrowed) replaces genome segment 10. (c) The relative expression of *Renilla* luciferase in BSR cells at 1 hour post-infection with BTV1 S10 RLuc when NS1 has been expressed by transient transfection of BSR cells with an NS1 expressing plasmid.

and replication (Figure 9.12). The mapping demonstrates that for segments 9 and 10 the sequences which identify the segment for packaging and replication reside entirely in the termini of the segments. The importance of the cap to packaging and replication has been investigated using uncapped transcripts in the second transfection (Matsuo and Roy, 2009). Uncapped transcripts are recovered into infectious virus, showing that the 5′ cap is not an essential determinant in the recognition of the transcripts during packaging or replication.

9.4.4 Reverse genetics in the development of orbivirus vaccines

The alteration of the BTV genome can be applied to creating live vaccines which have been attenuated by design rather than by repeated passaging. Currently both

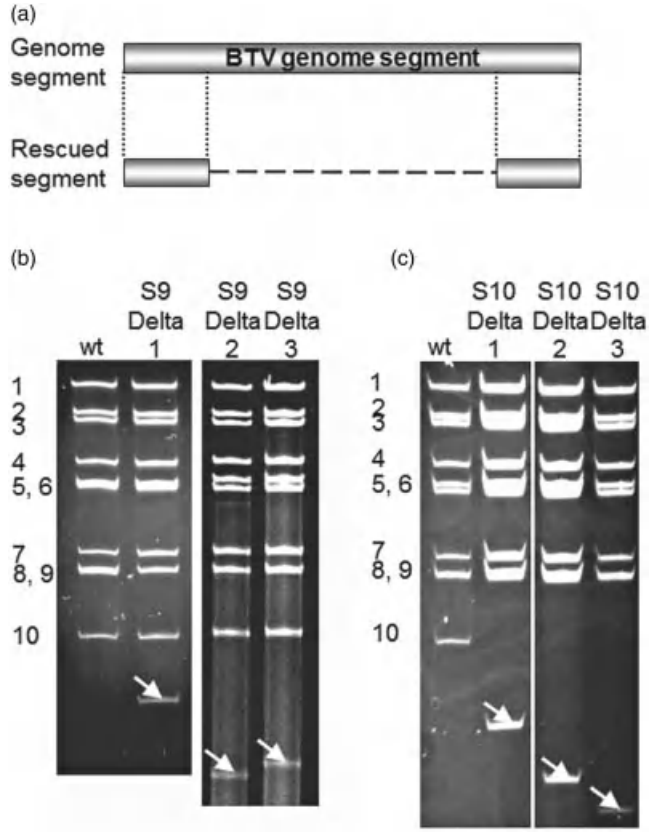

Figure 9.12 BTV segments 9 and 10 contain the sequences required for packaging and replication in their terminal regions. (a) Schematic diagram showing the organisation of a rescued genome segment with the central region deleted. The deletion mutants were generated by the transfection of the BSR VP6 cell line (b) or BSR NS3 cell line (c) with transcripts derived from T7 plasmid clones. (b and c) Polyacrylamide gel genomic dsRNA profiles of BTV-1 with deletions retaining both termini of segment 9 (b) or segment 10 (c). The shorter segments (arrowed) replace the corresponding full-length genome segment.

live attenuated and inactivated vaccines have been used to protect livestock from BTV infection (Roy *et al*., 2009). Both types of vaccine have drawbacks, with live vaccines being associated with teratogenic effects in the offspring of vaccinated mothers, reversion to virulence, and sufficient viraemia to allow transmission and reassortment with field strains producing novel genotypes. Inactivated vaccines have the disadvantage of requiring a larger dose in order to deliver sufficient antigen to raise a protective immune response. Reverse genetics has the potential to allow the design of vaccine strains which are attenuated by making specific mutations in the genome of the virus, and because these changes are designed they can be

made sufficiently extensive to prevent simple reversion to virulence. Any strategy can be employed which attenuates the virulence of the virus while permitting sufficient antigen expression to produce immunity in the host. Additionally the ability to manipulate the genome allows the possibility of creating vaccines which do not express a viral protein, enabling the generation of DIVA (differentiating infected from vaccinate animals) vaccines where antibodies to a viral protein are absent in the vaccinated animal, but present in animals which have been infected. Such information would allow an assessment to be made of whether a region or country is free from BTV. In the case of BTV, the variable outer capsid proteins (VP2 and VP5) of phylogenetically diverse serotypes have been shown to assemble on the core particle of BTV-1 to generate infectious virus (Boyce *et al*., 2008; Matsuo *et al*., 2011). This use of a defined genetic background can be exploited to rapidly produce strains of BTV by combining the VP2 and VP5 clones from the serotype of interest with a standard set of eight clones. One class of vaccine strains which can be produced using reverse genetics is a DISC (disabled infectious single cycle) strain, which expresses viral antigens but cannot complete an infectious cycle in the host. To generate such a strain an inactivating mutation is made in an essential gene using the reverse genetics clone encoding that protein, and the virus is recovered using a complementing cell line which compensates for the defective gene. The gene chosen should be one that is not required for expression of the viral proteins, but is essential for the production of infectious virus. Using this approach a virus which expresses viral antigens but does not replicate in normal cells is rescued and propagated in the complementing cell line. If the mutation chosen prevents expression of the mutated gene, then this approach can be used to make a DIVA vaccine. Due to the lethal mutation the DISC strain cannot replicate in any cell type except the complementing cell line, including the cells of the host animal, making transmission or reassortment with field strains highly improbable. Inoculation of sheep with such BTV DISC strains containing extensive deletions of segment 9 allows no detectable replication by quantitative PCR analysis, but induces neutralising antibodies and protective immunity against challenge with infectious virus of the same serotype (Matsuo *et al*., 2011).

A less extreme attenuation could be achieved using the altered codon bias pioneered with poliovirus (Burns *et al*., 2006; Coleman *et al*., 2008). The replicative fitness of the virus can be compromised by altering the codon bias of the genome, such that viral protein expression or viral RNA synthesis and consequently virus replication are reduced. This approach would be feasible for BTV with a genome size of 19.2kb, with the advantage that multiple replication cycles would occur in the host, permitting immunity to be generated from a small dose. The genome-scale nature of the mutation, would prevent reversion to virulence occurring. Although such vaccines would be transmissible, however poorly, they would be at a disadvantage compared to field strains, and any reassortants generated in the field would inherit the poor expression of the genes derived from the vaccine strain.

9.4.5 The definition of pathogenicity determinants using reverse genetics

The pathogenicity of BTV isolates varies in sheep and cattle, but is not well understood at the genetic level. To understand the genetic basis of pathogenicity the gene variants which lead to increased pathogenicity in particular host species can be identified. Before reverse genetics was possible in the *Reoviridae*, co-infection with the high virulence and low virulence strains of mammalian orthoreovirus was used to generate reassortant progeny viruses with undetermined genotypes, which were subsequently plaque purified and characterised. This approach was used successfully to makes panels of reassortants which were analysed for their virulence in newborn mice, identifying the gene associated with neurovirulence and other phenotypes (Weiner *et al*., 1977). Using reverse genetics, the generation of reassortants is more rapid with the BTV genes derived from two strains determined by the investigator without the requirement for plaque purification and analysis of the genotype. Genome segments associated with the virulent phenotype can be identified using the complete T7 transcript reverse genetics system by introducing each segment from a virulent strain into a defined low virulence genetic background, generating each possible mono-reassortant. This approach can also be adapted to identify constellations of genes if more than one gene is required for virulence, by determining the minimal set of genes which produce the virulent phenotype. A comparison of the sequence of the gene(s) determining virulence with the same gene from the low virulence strain can then be used to identify the amino acid changes in the protein which are associated with the altered phenotype. Confirmation that the identified amino acid changes are sufficient to create the virulent phenotype is provided by using the reverse genetics system to reintroduce these changes into the low virulence gene and demonstrate that they create the virulent phenotype. This approach can rapidly identify the amino acids associated with virulence, and in combination with the investigation of protein function can be used to generate a molecular understanding of the basis of virulence. In addition to identifying coding changes which alter virulence, non-coding sequences which influence the virulence of the strain may also be identified. *Cis*-acting sequences which regulate the transcription of a particular segment, or the translation of a viral transcript are examples of non-coding variations which might be expected to influence virulence through their effects on the level of expression of the viral protein.

9.5 Future perspectives

9.5.1 Potential alterations to the BTV reverse genetics systems

The complete T7 reverse genetics system allows the reproducible recovery of non-lethal mutants. However, the ratio of packaging/replication complexes to viral ss-RNA may not be optimal. One possible means of improving the recovery of virus is

to express the proteins of the packaging/replication complex from highly expressing RNA polymerase II driven plasmids, rather than from capped T7 transcripts. In this case the T7 transcripts for packaging would still be generated *in vitro* using T7 RNA polymerase and introduced at the second transfection. Although the total number of plasmids used is increased an improved efficiency would enable a scaling down of the quantity of T7 transcripts allowing a significant cost saving. Similarly the high cost of producing transcripts with a 5′ cap could be removed as the transcripts which are packaged do not need to be capped (Matsuo and Roy, 2009).

The main improvement that the T7 transcript reassortment system would benefit from is the reduction in the background of wild-type virus produced from the viral ssRNA, to reduce the screening of plaques to identify the reassortant virus. Two methods used successfully for reducing the background of virus containing the wild-type segment in *Reoviridae* helper virus systems could be applied to BTV. The targeted degradation of a specific viral ssRNA *in vitro* using a DNA oligonucleotide with RNaseH to degrade RNA:DNA hybrids was used with mammalian orthoreovirus (Roner and Joklik, 2001), and the intracellular degradation of the wild-type segment has been carried out using siRNA with rotavirus (Trask *et al*., 2010). Both approaches could be applied to BTV and the initial time taken to identify effective oligonucleotides makes this approach suitable for studies of a single gene where many mutants will be made.

9.5.2 The use of protein structure data to design mutants

BTV has been well studied at the molecular and structural levels, with the atomic structure of several of the viral proteins being known or being actively investigated. A knowledge of the spatial arrangement of amino acid residues in a protein can be used to inform the mutagenesis of the protein. Such information allows the mutations to be targeted to specific regions of interest, such as catalytic sites, interfaces between protein subunits, or binding sites for interacting molecules. The BTV mRNA capping protein, VP4, is interesting in that it possesses all the catalytic activities required to cap and methylate viral mRNA in a single polypeptide, and its crystal structure has been recently solved (Martinez-Costas *et al*., 1998; Ramadevi *et al*., 1998; Ramadevi and Roy, 1998; Sutton *et al*., 2007). The use of reverse genetics can be used to investigate the consequences to viral mRNA and protein synthesis of mutating the individual catalytic sites, and relate these findings to biochemical characterisation of the mutants.

The non-structural protein NS1 regulates the translation of viral mRNA and forms regular hollow tubes composed of a ribbon of dimers (Hewat *et al*., 1992). NS1 has recently been identified as an RNA binding protein which binds to the 3′ end of viral mRNAs (Boyce and Roy, unpublished observations) and current investigations into solving the structure using cryo electron microscopy would enable the generation mutants to answer outstanding questions about the functions of this protein. The specific disruption of the interactions between the subunit interface

surfaces of NS1 could be used to prevent tubule formation without disrupting the structure of the NS1 monomers, allowing investigation into whether tubule formation is essential to virus replication. These findings can be related back to translational regulation assays and *in vitro* RNA binding assays.

9.5.3 Immunomodulation

How BTV and other orbiviruses interact with the host immune response is poorly understood. The identification of the viral gene(s) which downregulate the induction or signalling of type I interferon (IFN) is an area of particular interest. As with most lytic viruses orbiviruses may be expected to moderate this branch of innate immunity, and this view is supported by the finding that BTV causes similar symptoms to those produced in ruminants when used to infect knock-out mice lacking the α/β IFN receptor (Calvo-Pinilla *et al*., 2009). The screening for viral genes which downregulate type I IFN can be investigated in transient expression systems, but reverse genetics is the only way the mutations can be reconstituted into the genome of infectious virus, allowing the characterisation of phenomena which only occur during replication within a host, such as virus titre during the viraemic phase, tropism, and virulence. A variant of BTV which cannot prevent the establishment of an 'antiviral state' in the infected animal would be expected to replicate less efficiently therefore identifying a virulence determinant. An understanding of how orbiviruses moderate host immune responses also permits the design of attenuated vaccine strains which cannot moderate host immunity, such as the immunogenic influenza strains lacking a completely functional IFN antagonist, which have been generated using reverse genetics (Ferko *et al*., 2004).

An area of orbivirus research which has so far received little study at the molecular level is the very different outcome of infection of mammals and *Culicoides*, both in the host organism and in cell culture. The infection of mammalian cells is lytic and induces apoptosis whereas *Culicoides* cells produce virus without lysis (Mortola *et al*., 2004; Stewart and Roy, 2010), demonstrating that there must be interactions with the host cell which differ between the two cell types. Reverse genetics can be used to investigate at the molecular level the underlying causes of the virus-induced cytopathic effect seen in mammalian cells, but absent in *Culicoides*. The interactions between viral factors and host cell proteins which lead eventually to the alternative fates in mammalian or *Culicoides* cells remain to be investigated.

9.5.4 Screening for antiviral compounds using reporter viruses

Orbiviruses encode a number of proteins with functions which have no counterpart in the host cell, and as such are potential targets for intervention with small

molecules. While this is unlikely to be cost-effective for BTV itself, the related AHSV has a mortality rate of up to 90% in horses, and protection of economically valuable animals would be useful in the absence of an effective vaccine. Drugs may be screened for their effect on viral replication by using a virus expressing a quantifiable reporter gene such as luciferase (Figure 9.11) (Boyce and Roy, unpublished observations). The read out directly relates to the amount of gene expression and therefore can be used as a first screen to rapidly assess the effect of small molecules on virus propagation in cell culture without the need to directly measure infectious virus titre.

The recent arrival of reverse genetics in *Orbivirus* research is allowing the field to take advantage of recombinant DNA technology, with research into the biology of infection answering questions which were previously delayed. With a variety of new areas now accessible to investigation in the context of virus replication this technology is proving useful to basic and applied researchers of orbiviruses, as has been the case for other viruses.

References

Barratt-Boyes, S. M. and Maclachlan, N. J. 1995. Pathogenesis of bluetongue virus infection of cattle. *Journal of the American Veterinary Medicine Association* **206**: 1322–1329.

Beaton, A. R., Rodriguez, J., Reddy, Y. K. and Roy, P. 2002. The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 13154–9.

Bhattacharya, B., Noad, R. J. and Roy, P. 2007. Interaction between Bluetongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. *Virology Journal* **4**: 7.

Bhattacharya, B. and Roy, P. 2008. Bluetongue virus outer capsid protein VP5 interacts with membrane lipid rafts via a SNARE domain. *Journal of Virology* **82**: 10600–10612.

Boyce, M., Celma, C. C. and Roy, P. 2008. Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. *Journal of Virology* **82**: 8339–8348.

Boyce, M. and Roy, P. 2007. Recovery of infectious bluetongue virus from RNA. *Journal of Virology* **81**: 2179–2186.

Boyce, M., Wehrfritz, J., Noad, R. and Roy, P. 2004. Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity. *Journal of Virology* **78**: 3994–4002.

Brewer, A. W. and MacLachlan, N. J. 1992. Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in vitro. *Veterinary Pathology* **29**: 356–359.

Brewer, A. W. and MacLachlan, N. J. 1994. The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization. *Archives of Virology* **136**: 287–298.

Burns, C. C., Shaw, J., Campagnoli, R., Jorba, J., Vincent, A., Quay, J. and Kew, O. 2006. Modulation of poliovirus replicative fitness in HeLa cells by deoptimization of synonymous codon usage in the capsid region. *Journal of Virology* **80**: 3259–3272.

Calvo-Pinilla, E., Rodriguez-Calvo, T., Anguita, J., Sevilla, N. and Ortego, J. 2009. Establishment of a bluetongue virus infection model in mice that are deficient in the alpha/beta interferon receptor. *PLoS ONE* **4**, e5171.

Carpenter, S., Wilson, A. and Mellor, P. S. 2009. Culicoides and the emergence of bluetongue virus in northern Europe. *Trends in Microbiology* **17**: 172–178.

Celma, C. C. and Roy, P. 2009. A viral nonstructural protein regulates bluetongue virus trafficking and release. *Journal of Virology* **83**: 6806–6816.

Celma, C. C. and Roy, P. 2011. Interaction of Calpactin Light Chain (S100A10/p11) and a viral NS protein is essential for intracellular trafficking of nonenveloped bluetongue virus. *Journal of Virology* **85**: 4783–4791.

Coleman, J. R., Papamichail, D., Skiena, S., Futcher, B., Wimmer, E. and Mueller, S. 2008. Virus attenuation by genome-scale changes in codon pair bias. *Science* **320**: 1784–177.

Daniels, P., Lunt, R. and Prichard, I. 2009. Bluetongue viruses in Australasia and East Asia. In Mellor, P. S., Baylis., M. and Mertens, P. P. C. (eds) *Bluetongue*. Academic Press: New York.

Diprose, J. M., Burroughs, J. N., Sutton, G. C., Goldsmith, A., Gouet, P., Malby, R., Overton, I., *et al*. 2001. Translocation portals for the substrates and products of a viral transcription complex: the bluetongue virus core. *EMBO Journal* **20**: 7229–7239.

Doyle, K. A. 1992. An overview and perspective on orbivirus disease prevalence and occurrence of vectors in Australia and Oceania. In Walton, T. E. and Osburn, B. I. (eds) *Bluetongue, African Horse Sickness, and Related Orbiviruses*. CRC Press: Boca Raton, FL, pp. 44–57.

Du Toit, R. M. 1944. The transmission of bluetongue and horse sickness by Culicoides Onderstepoort. *Journal of Veterinary Science Animals India* **19**: 7–16.

Eaton, B. T. and Hyatt, A. D. 1989. Association of bluetongue virus with the cytoskeleton. *Subcellular Biochemistry* **15**: 233–273.

Elbers, A. R., Backx, A., Mintiens, K., Gerbier, G., Staubach, C., Hendrickx, G. and Van Der Spek, A. 2008. Field observations during the Bluetongue serotype 8 epidemic in 2006. II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. *Preventive Veterinary Medicine* **87**: 31–40.

Erasmus, B. J. 1975. Bluetongue in sheep and goats. *Australian Veterinary Journal* **51**: 165–170.

Ferko, B., Stasakova, J., Romanova, J., Kittel, C., Sereinig, S., Katinger, H. and Egorov, A. 2004. Immunogenicity and protection efficacy of replication-deficient influenza A viruses with altered NS1 genes. *Journal of Virology* **78**: 13037–13045.

Forzan, M., Marsh, M. and Roy, P. 2007. Bluetongue virus entry into cells. *Journal of Virology* **81**: 4819–4827.

Forzan, M., Wirblich, C. and Roy, P. 2004. A capsid protein of nonenveloped Bluetongue virus exhibits membrane fusion activity. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 2100–2105.

French, T. J., Inumaru, S. and Roy, P. 1989. Expression of two related nonstructural proteins of bluetongue virus (BTV) type 10 in insect cells by a recombinant baculovirus: production of polyclonal ascitic fluid and characterization of the gene product in BTV-infected BHK cells. *Journal of Virology* **63**: 3270–3278.

French, T. J., Marshall, J. J. and Roy, P. 1990. Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. *Journal of Virology* **64**: 5695–5700.

Fuerst, T. R. and Moss, B. 1989. Structure and stability of mRNA synthesized by vaccinia virus-encoded bacteriophage T7 RNA polymerase in mammalian cells. Importance of the 5′ untranslated leader. *Journal of Molecular Biology* **206**: 333–348.

Gambles, R. M. 1949. Bluetongue of sheep in Cyprus. *Journal of Comparative Pathology* **59**: 176–190.

Gloster, J., Mellor, P. S., Manning, A. J., Webster, H. N. and Hort, M. C. 2007. Assessing the risk of windborne spread of bluetongue in the 2006 outbreak of disease in northern Europe. *Veterinary Record* **160**: 54–56.

Gold, S., Monaghan, P., Mertens, P. and Jackson, T. 2010. A clathrin independent macropinocytosis-like entry mechanism used by bluetongue virus-1 during infection of BHK cells. *PLoS ONE* **5**, e11360.

Gorman, B. M. 1990. The Bluetongue viruses. In Roy, P. and Gorman, B. M. (eds) *Bluetongue Viruses*. Springer-Verlag: Berlin.

Gouet, P., Diprose, J. M., Grimes, J. M., Malby, R., Burroughs, J. N., Zientara, S., Stuart, D. I. and Mertens, P. P. 1999. The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. *Cell* **97**: 481–490.

Guirakhoo, F., Catalan, J. A. and Monath, T. P. 1995. Adaptation of bluetongue virus in mosquito cells results in overexpression of NS3 proteins and release of virus particles. *Archives of Virology* **140**: 967–974.

Hassan, A. 1992. Status of bluetongue in the Middle East and Asia. In Walton, T. E. and Osburn, B. I. (eds) *Bluetongue, African Horse Sickness, and Related Orbiviruses*. CRC Press: Boca Raton, FL, pp. 38–43.

Hassan, S. S. and Roy, P. 1999. Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *Journal of Virology* **73**: 9832–9842.

Hewat, E. A., Booth, T. F., Wade, R. H. and Roy, P. 1992. 3-D reconstruction of bluetongue virus tubules using cryoelectron microscopy. *Journal of Structural Biology* **108**: 35–48.

Huismans, H. 1979. Protein synthesis in bluetongue virus-infected cells. *Virology* **92**: 385–396.

Huismans, H., Van Der Walt, N. T. and Cloete, M. 1983. The biochemical and immunological characterization of bluetongue virus outer capsid polypeptides. In: Compans, R. W. and Bishop, D. H. L. (eds) *Double-Stranded RNA Viruses*. Elsevier: New York.

Hyatt, A. D., Zhao, Y. and Roy, P. 1993. Release of bluetongue virus-like particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A. *Virology* **193**: 592–603.

Kar, A. K., Bhattacharya, B. and Roy, P. 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Molecular Biology* **8**: 4.

Kar, A. K. and Roy, P. 2003. Defining the structure-function relationships of bluetongue virus helicase protein VP6. *Journal of Virology* **77**: 11347–11356.

Kobayashi, T., Antar, A. A. R., Boehme, K. W., Danthi, P., Eby, E. A., Guglielmi, K. M., Holm, G. H., *et al*. 2007. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. *Cell Host and Microbe* **1**: 147–157.

Komarov, A. and Goldsmit, L. 1951. A disease similar to bluetongue in cattle and sheep in Israel. *Refuah Veterinarith* **8**: 96–100.

Komoto, S., Sasaki, J. and Taniguchi, K. 2006. Reverse genetics system for introduction of site-specific mutations into the double-stranded RNA genome of infectious rotavirus. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 4646–4651.

Lymperopoulos, K., Noad, R., Tosi, S., Nethisinghe, S., Brierley, I. and Roy, P. 2006. Specific binding of Bluetongue virus NS2 to different viral plus-strand RNAs. *Virology* **353**: 17–26.

Lymperopoulos, K., Wirblich, C., Brierley, I. and Roy, P. 2003. Sequence specificity in the interaction of Bluetongue virus non-structural protein 2 (NS2) with viral RNA. *Journal of Biological Chemistry* **278**: 31722–31730.

Maan, S., Maan, N. S., Nomikou, K., Anthony, S., Ross-Smith, N., Singh, K. P., Samuel, A. R., *et al*. 2009. Molecular epidemiology studies of bluetongue virus. In Mellor, P. S., Baylis., M. and Mertens, P. P. C. (eds) *Bluetongue*. Academic Press: New York.

Maan, S., Rao, S., Maan, N. S., Anthony, S. J., Attoui, H., Samuel, A. R. and Mertens, P. P. 2007. Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. *Journal of Virological Methods* **143**: 132–139.

MacLachlan, N. J. and Gard, G. 2009. Clinical signs and pathology. In Mellor, P. S., Baylis., M. and Mertens, P. P. C. (eds) *Bluetongue*. Academic Press: New York.

Martin, S. A. and Zweerink, H. J. 1972. Isolation and characterization of two types of bluetongue virus particles. *Virology* **50**: 495–506.

Martinez-Costas, J., Sutton, G., Ramadevi, N. and Roy, P. 1998. Guanylyltransferase and RNA 5′-triphosphatase activities of the purified expressed VP4 protein of bluetongue virus. *Journal of Molecular Biology* **280**: 859–866.

Matsuo, E., Celma, C., Boyce, M., Viarouge, C., Sailleau, C., Dubois, E., Bréard, E., Thiéry, R., *et al*. 2011. Manuscript under review.

Matsuo, E., Celma, C. C. and Roy, P. 2010. A reverse genetics system of African horse sickness virus reveals existence of primary replication. *FEBS Letters* **584**: 3386–3391.

Matsuo, E. and Roy, P. 2009. Bluetongue virus VP6 acts early in the replication cycle and can form the basis of chimeric virus formation. *Journal of Virology* **83**: 8842–8848.

McKercher, D. G., McGowan, B., Howarth, J. A. and Saito, J. K. 1953. A preliminary report on the isolation and identification of the bluetongue virus from sheep in California. *Journal of the American Veterinary Medical Association* **122**: 300–301.

Mertens, P. P., Burroughs, J. N. and Anderson, J. 1987. Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. *Virology*, **157**: 375–386.

Mertens, P. P., Burroughs, J. N., Walton, A., Wellby, M. P., Fu, H., O'Hara, R. S., Brookes, S. M. and Mellor, P. S. 1996. Enhanced infectivity of modified bluetongue virus particles for two insect cell lines and for two Culicoides vector species. *Virology* **217**: 582–593.

Mertens, P. P. and Sangar, D. V. 1985. Analysis of the terminal sequences of the genome segments of four orbiviruses. *Virology*, **140**, 55–67.

Modrof, J., Lymperopoulos, K. and Roy, P. 2005. Phosphorylation of bluetongue virus nonstructural protein 2 is essential for formation of viral inclusion bodies. *Journal of Virology* **79**: 10023–10031.

Mortola, E., Noad, R. and Roy, P. 2004. Bluetongue virus outer capsid proteins are sufficient to trigger apoptosis in mammalian cells. *Journal of Virology* **78**: 2875–2883.

Neumann, G., Whitt, M. A. and Kawaoka, Y. 2002. A decade after the generation of a negative-sense RNA virus from cloned cDNA: what have we learned? *Journal of General Virology* **83**: 2635–2362.

Potgieter, A. C., Steele, A. D. and Van Dijk, A. A. 2002. Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. *Journal of General Virology* **83**: 2215–2223.

Purse, B. V. and Rogers, D. J. 2009. Bluetongue virus and climate change. In Mellor, P. S., Baylis., M. and Mertens, P. P. C. (eds) *Bluetongue*. Academic Press: New York.

Racaniello, V. R. and Baltimore, D. 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**: 916–919.

Ramadevi, N., Burroughs, N. J., Mertens, P. P., Jones, I. M. and Roy, P. 1998. Capping and methylation of mRNA by purified recombinant VP4 protein of bluetongue virus. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 13537–13542.

Ramadevi, N. and Roy, P. 1998. Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity. *Journal of General Virology* **79**(Pt 10): 2475–2480.

Rao, C. D., Kiuchi, A. and Roy, P. 1983. Homologous terminal sequences of the genome double-stranded RNAs of bluetongue virus. *Journal of Virology* **46**: 378–383.

Rescher, U. and Gerke, V. 2008. S100A10/p11: family, friends and functions. *Pflugers Archives* **455**: 575–582.

Roner, M. R., Bassett, K. and Roehr, J. 2004. Identification of the 5′ sequences required for incorporation of an engineered ssRNA into the Reovirus genome. *Virology* **329**: 348–360.

Roner, M. R. and Joklik, W. K. 2001. Reovirus reverse genetics: incorporation of the CAT gene into the reovirus genome. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 8036–8041.

Roner, M. R. and Mutsoli, C. 2007. The use of monoreassortants and reverse genetics to map reovirus lysis of a ras-transformed cell line. *Journal of Virological Methods* **139**: 132–142.

Roner, M. R. and Roehr, J. 2006. The 3′ sequences required for incorporation of an engineered ssRNA into the Reovirus genome. *Virology Journal* **3**: 1.

Roner, M. R., Sutphin, L. A. and Joklik, W. K. 1990. Reovirus RNA is infectious. *Virology* **179**: 845–852.

Rosenberg, A. H., Lade, B. N., Chui, D. S., Lin, S. W., Dunn, J. J. and Studier, F. W. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**: 125–135.

Roy, P., Boyce, M. and Noad, R. 2009. Prospects for improved bluetongue vaccines. *Nature Reviews Microbiology* **7**: 120–128.

Sachs, A. B., Sarnow, P. and Hentze, M. W. 1997. Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell* **89**: 831–838.

Sapre, S. N. 1964. An outbreak of bluetongue in goats and sheep in Maharashtra State, India. *Indian Veterinary Review (MandB)* **15**: 69–71.

Sarwar, M. M. 1962. A note on bluetongue in sheep in West Pakistan. *Pakistan Journal of Animal Science* **1**: 1–2.

Spreull, J. 1905. Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapy* **18**: 321–337.

Stauber, N., Martinez-Costas, J., Sutton, G., Monastyrskaya, K. and Roy, P. 1997. Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function and a helicase activity that catalyze the unwinding of double-stranded RNA substrates. *Journal of Virology* **71**: 7220–7226.

Stepinski, J., Waddell, C., Stolarski, R., Darzynkiewicz, E. and Rhoads, R. E. 2001. Synthesis and properties of mRNAs containing the novel 'anti-reverse' cap analogs 7-methyl(3′-O-methyl)GpppG and 7-methyl (3′-deoxy)GpppG. *RNA* **7**: 1486–1495.

Stewart, M. E. and Roy, P. 2010. Role of cellular caspases, nuclear factor-kappa B and interferon regulatory factors in Bluetongue virus infection and cell fate. *Virology Journal* **7**: 362.

Sutton, G., Grimes, J. M., Stuart, D. I. and Roy, P. 2007. Bluetongue virus VP4 is an RNA-capping assembly line. *Nature Structural & Molecular Biology* **14**: 449–451.

Tan, B. H., Nason, E., Staeuber, N., Jiang, W., Monastryrskaya, K. and Roy, P. 2001. RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to Culicoides cells. *Journal of Virology* **75**: 3937–3947.

Taraporewala, Z. F., Chen, D. and Patton, J. T. 2001. Multimers of the bluetongue virus nonstructural protein, NS2, possess nucleotidyl phosphatase activity: similarities between NS2 and rotavirus NSP2. *Virology* **280**: 221–231.

Thomas, C. P., Booth, T. F. and Roy, P. 1990. Synthesis of bluetongue virus-encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. *Journal of General Virology* **71**(Pt 9): 2073–2083.

Trask, S. D., Taraporewala, Z. F., Boehme, K. W., Dermody, T. S. and Patton, J. T. 2010. Dual selection mechanisms drive efficient single-gene reverse genetics for rotavirus. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 18652–18657.

Uitenweerde, J. M., Theron, J., Stoltz, M. A. and Huismans, H. 1995. The multimeric nonstructural NS2 proteins of bluetongue virus, African horsesickness virus, and epizootic hemorrhagic disease virus differ in their single-stranded RNA-binding ability. *Virology* **209**: 624–632.

Urakawa, T., Ritter, D. G. and Roy, P. 1989. Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. *Nucleic Acids Research* **17**: 7395–7401.

Van Gennip, R. G., Veldman, D., Van De Water, S. G. and Van Rijn, P. A. 2010. Genetic modification of Bluetongue virus by uptake of 'synthetic' genome segments. *Virology Journal* **7**: 261.

Verwoerd, D. W. 1969. Purification and characterization of bluetongue virus. *Virology* **38**: 203–212.

Verwoerd, D. W., Els, H. J., De Villiers, E. M. and Huismans, H. 1972. Structure of the bluetongue virus capsid. *Journal of Virology* **10**: 783–794.

Verwoerd, D. W. and Huismans, H. 1972. Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. *Onderstepoort Journal of Veterinary Research* **39**: 185–191.

Verwoerd, D. W., Louw, H. and Oellermann, R. A. 1970. Characterization of bluetongue virus ribonucleic acid. *Journal of Virology* **5**: 1–7.

Weiner, H. L., Drayna, D., Averill, D. R., Jr. and Fields, B. N. 1977. Molecular basis of reovirus virulence: role of the S1 gene. *Proceedings of the National Academy of Sciences of the United States of America* **74**: 5744–5748.

Williams, R. L. and Urbe, S. 2007. The emerging shape of the ESCRT machinery. *Nature Reviews Molecular Cell Biology* **8**: 355–368.

Wirblich, C., Bhattacharya, B. and Roy, P. 2006. Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. *Journal of Virology* **80**: 460–473.

Wittmann, E. J., Mellor, P. S. and Baylis, M. 2002. Effect of temperature on the transmission of orbiviruses by the biting midge, *Culicoides sonorensis*. *Medical and Veterinary Entomology* **16**: 147–156.

Zhang, X., Boyce, M., Bhattacharya, B., Schein, S., Roy, P. and Zhou, Z. H. 2010. Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5 resembles enveloped virus fusion proteins. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 6292–6297.

10

Genetic modification in mammalian orthoreoviruses

Sanne K. van den Hengel,[1,2] Iris J.C. Dautzenberg,[1] Diana J.M. van den Wollenberg,[1] Peter A.E. Sillevis Smitt[2] and Rob C. Hoeben[1]

[1]*Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands*

[2]*Department of Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands*

10.1 Introduction

Mammalian reoviruses are members of the *orthoreovirus* genus of the family of *Reoviridae*. They were first isolated from the gastrointestinal and respiratory tracts of healthy and sick humans in the early 1950s (reviewed by (Joklik, 1981)). The name reovirus is an acronym of *R*espiratory and *E*nteric *O*rphan virus. The term 'orphan' is used to indicate that no serious disease is linked to this virus in humans, while a large proportion of the human population has been exposed to the virus and built neutralising immunity to reoviruses (Minuk *et al*., 1985; Minuk *et al*., 1987). The *orthoreoviruses* have a wide geographic distribution and are isolated from a broad range of mammals, birds and reptiles. Although in humans the mammalian *orthoreoviruses* are non-pathogenic, in newborn mice they can cause severe disease (Tyler and Fields, 2001).

The capsid of the *orthoreovirus* is a non-enveloped icosahedral structure composed of an outer and inner protein shell. In 1963, it was discovered that the genome consists of double-stranded RNA (dsRNA) (Gomatos and Tamm, 1963), and soon afterwards it was found that the genomes of mammalian *orthoreoviruses* consist of 10 distinct genome segments (Shatkin *et al*., 1968).

10.1.1 Taxonomy

To date, three *orthoreovirus* species groups are recognised. In this chapter we will focus on the mammalian *orthoreoviruses*. In this species group three serotype

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

strains of mammalian *orthoreoviruses* have been identified, with the type 1 Lang (T1L), type 2 Jones (T2J), type 3 Abney (T3A) and type 3 Dearing (T3D) as the prototypical representatives. The serotypes are classified according to their neutralisation by specific antibodies and by the classical haemagglutination inhibition assay (Weiner *et al*., 1977; Weiner and Fields, 1977). Genomic variation between the serotypes is found for all segments. The S1 segments, coding for the σ1 attachment protein, has the largest sequence divergence. The sequence variations can have biological consequences (Tyler and Fields, 2001). It was observed by reassortment studies between T1L and T3D that the S1 segments define the pathology. While the serotypes T1L and T3D both infect the central nervous system (CNS) in newborn mice, their routes of infection differ. T1L causes hydrocephalus by spreading hematogenously in the CNS by infecting ependymal cells. In contrast, T3D causes viral encephalitis by spreading via the neural routes in the CNS (Tyler *et al*., 1995).

10.1.2 Genome segments and their proteins

Structural proteins

The genome of the reovirus consists of 10 dsRNA segments with a total size of 23.5 kb. The segments are named according to their sizes (Shatkin *et al*., 1968). There are three large segments (L1, L2 and L3) ranging in size from 3854 to 3916 nt, three medium segments (M1, M2 and M3) with sizes between 2206 to 2304 nt, and four small segments (S1, S2, S3 and S4) varying from 1196 to 1416 nt (Coombs, 2006; Tyler and Fields, 2001). Each segment encodes a single protein, with as a sole exception the S1 segment which encodes two proteins (Jacobs and Samuel, 1985; Sarkar *et al*., 1985). The proteins are named and numbered according to their apparent molecular weight on SDS-PAGE. The names are derived from the Greek character of the segment it was translated from, the L segments code for the λ proteins, the M segments code for the μ proteins, and the S segments encode the σ proteins. The numbering of the proteins does not always correspond to the genome size of the segment from which they are transcribed (Tyler and Fields, 2001). An overview of the segments, and the proteins that they code for is provided in Table 10.1.

The non-enveloped icosahedral outer capsid is about 70 nm in diameter. The inner capsid or core structure is about 52 nm in diameter. Together, these capsids are composed of eight structural proteins (Plate 5a). The five structural proteins forming the inner core structure are λ1, λ2, λ3, μ2 and σ2. The λ1 protein is encoded by L3 segment and is a major structural protein of the inner capsid. This protein is involved in transcription. The λ2 protein is translated from L2 RNA and forms the pentameric turrets of the core structure. During primary transcription this protein adds a methylated cap structure to nascent plus-strand RNA (see below). The

Table 10.1 Overview of the genome segments, proteins and prototypes of the various *ts* mutants.

Segment			Protein			*ts* mutant				
	Total size (nt)	ORF(nt)		Size (aa)	Location	Group	Prototype	ssRNA	Phenotype Protein	dsRNA
L1	3854	19-3819	λ3	1267	Core	D	*tsD357*	+/−	+	−
L2	3916	14-3880	λ2	1289	Core	B	*tsB352*	+	+	+
L3	2901	14-3838	λ1	1275	Core	I	*tsI138*	?	+	−
M1	2304	14-221	μ2	736	Core	H	*tsH11.2*	++	−	−
M2	2203	30-2153	μ1	708	Outer capsid	A	*tsA201*	++	++	++
M3	2241	19-2181	μNS	721	NS	F	*tsF556*[1]	++	++	++
			μNSC	681	NS					
S1	1416	13-1377;	σ1	455	Outer capsid	J	*tsJ128*	?	?	?
		71-430	σ1s	120	NS					
S2	1331	19-1272	σ2	418	Core	C	*tsC447*	?	?	−
S3	1198	28-1125	σNS	366	NS	E	*tsE320*	+/−	+	−
S4	1196	33-1127	σ3	365	Outer capsid	G	*tsG453*	+	+	+

Note: bp - base-pairs; aa – amino acids; [1] Tentative, leaky mutant; ++ (almost) as wild type; + less than 50% of wild type; +/− less than 10% of wild type; − less than 1 % of wild type.

λ3 protein is encoded by L1 segment and functions as the RNA-dependent RNA polymerase (RdRp) and it transcribes the plus and the minus strands (Starnes and Joklik, 1993). The exact function and location of the μ2 protein which is encoded by the M1 segment in the core are still unknown. However, it has been suggested as a co-factor or second subunit of RdRp. The σ2 protein, encoded by the S2 segment, is involved in the assembly of the core particles. This protein decorates the λ1 shell (reviewed by Coombs, 2006 and Tyler and Fields, 2001).

The outer capsid is composed of three structural proteins. The two major outer-capsid constituents are σ3 and μ1 proteins. The third minor outer-capsid protein σ1 forms the spikes at the vertices of the icosahedron and functions as the attachment protein. The σ3 protein is encoded by the S4 segment and provides stability to the capsid and functions as a shield for μ1. Removal of σ3 protein occurs through proteolytic cleavage upon entrance. The μ1 protein is encoded by the M2 segment and provides stability to the outer capsid. This protein is by proteolysis during viral entry to yield the μ1N and μ1C proteins. This is an important process during replication. Removal of σ3 and cleavage of μ1 yield a partially uncoated particle, the so-called intermediate subviral particles (ISVP). These ISVPs have the capacity to disrupt cellular membranes and may function in endosomal escape. Moreover, ISVPs generated *in vitro* by treatment of purified reovirus particles with chymotrypsin or *in-vivo* in the lumen or small intestines by proteases, yields particles that retain their infectivity, and can enter the cell independent of the presence of the canonical reovirus receptor Junction Adhesion Molecule A (JAM-A) (reviewed by Coombs, 2006 and Tyler and Fields, 2001).

Trimers of σ1 proteins encoded by the S1 segment form the spikes on the vertices of the icosahedral capsid (see representative figure at ViralZone, 2011). The spike is anchored in the λ2 protein turret. Through removal of σ1 protein during virus entry, the λ2 turrets undergo a conformational change and become active. The spikes contain a tail and a head domain. The protein spike interacts with cellular receptors. Initially a low-affinity interaction is established with sialic acid residues on the cell surface via interactions with the tail of the spike. Subsequently, high-affinity interactions can establish between the head domain of the spike and JAM-A. Subsequently, Arg-Gly-Asp (RGD) domains in the λ2 protein bind β1-integrins on the cell surface and mediate particle entry by endocytosis (Guglielmi *et al*., 2006; Maginnis *et al*., 2006).

Non-structural proteins

At least three nonstructural proteins are generated during infection. The σ1NS is translated from the second open reading frame of the S1 mRNA. This is a nonessential protein which causes cell cycle arrest during infection (Jacobs and Samuel, 1985; Sarkar *et al*., 1985). The second nonstructural protein, σNS, is encoded by the S3 segment. This protein has a strong affinity with ssRNA (Huismans and Joklik, 1976). It is suggested that it plays a role in the replication and the assembly of

core particles (Antczak and Joklik, 1992). Furthermore it associates with μNS and μ2 to form inclusion domains or viral factories.

The μNS protein is encoded by the M3 segment. It is the third nonstructural protein and associates with the viral mRNA shortly after transcription, and to viral cores, probably to anchor viral components needed for assembly or replication (Becker *et al*., 2003; Broering *et al*., 2000; Kobayashi *et al*., 2009; Mora *et al*., 1987). In addition, by proteolytic cleavage of μNS μNSC is generated. The precise function of this protein is unknown (Tyler and Fields, 2001).

10.1.3 Replication

Entry and uncoating

As for most RNA viruses, genome replication of reoviruses takes place in the cytoplasm, schematically represented in Plate 5b. Reoviruses are internalised into the endosomes. In the endosomes proteolysis facilitates the partial removal of the outer capsid. The uncoating process includes additional cleavage and removal of μ1 and σ3. Subsequently, σ1 molecules are detached from the particles. The activated particles escape from the endosomes by penetrating the endosomal membrane. Through removal of μ1 and σ1 the λ3 RdRp is activated and initiates transcription, yielding full-length plus-strand RNA molecules. The conformational change of λ2 pentamers, through removal of σ1, turns them into channels for the release of plus-strand mRNA into the cytosol. In addition, the λ2 turrets add a methylated cap structure to the 5′ end of the plus-strand RNAs (Miura *et al*., 1974; Chow and Shatkin, 1975; Furuichi *et al*., 1975). On these transcripts the 3′ end remains non-poly-adenylated. The methylated cap structure (7-methyl guanosine triphosphate (m7G(5′)ppp)) is identical to the cap structures of cellular mRNAs and is therefore recognised by the host ribosomes and translated. Initially the four segments L1, M3, S3 and S4 are transcribed and translated which stimulates subsequent transcription and translation of all segments (Watanabe *et al*., 1968; Nonoyama *et al*., 1974; Spandidos *et al*., 1976; Tyler *et al*., 1995). At this stage in the replication cycle, translation of viral and host proteins occurs simultaneously.

The assembly of new particles occurs in newly formed non-membranous structures in the cytoplasm, the so-called viral factories. These may aid in shielding the viral processes from the host cell's components of the innate immune system. The plus-strand transcripts are incorporated into these new cores. Within the core-particles the minus-strand synthesis takes place to yield dsRNA (Antczak and Joklik, 1992; Roner *et al*., 2004). In the newly formed core particles, a secondary round of plus-strand synthesis initiates, yielding uncapped viral transcripts. The uncapped viral transcripts are translated very efficiently since the reoviruses employ a mechanism to modify the ribosomes in such a way that non-capped transcripts are preferentially translated. At this point the host cell protein synthesis

is shut down. This mechanism ensures that the host cell's protein synthesising machinery is used for synthesis of viral proteins rather than for cellular proteins (reviewed by Coombs, 2006).

Lytic infection

Reoviruses normally cause a lytic infection in permissive host cells. The reovirus-induced cell death is independent of productive infection. Exposure of cells to UV-inactivated virus particles can still induce apoptosis. The primary determinants of reovirus induced-apoptosis are associated with the outer capsid protein μ1, although attachment of σ1 to the cell surface strongly enhances the apoptotic signal. The cleavage of the μ1 protein during internalisation is essential for the induction of apoptosis. If the disassembly of the viral particles is blocked by monoclonal antibodies against σ3 or μ1, apoptosis is not initiated. Also monoclonal antibodies against σ1 inhibit apoptosis; however the mechanism here is prevention of binding to the cells surface. Pro-apoptotic signals are generated by binding of the attachment protein to sialic acid and JAM-A. Reovirus mutants lacking the sialic acid binding capacity are still able to induce apoptosis albeit to a lesser extent (Tyler *et al*., 1995; Tyler *et al*., 1996; Connolly and Dermody, 2002; Danthi *et al*., 2008b).

10.1.4 Cis-acting sequences

Knowledge of the replicative cycle of reoviruses has been essential for the development of a reverse genetics system. The essential *cis*-acting sequences that reside in the genome segments need to be identified. Such sequences include the elements required for the initiation of plus and minus-strand synthesis, the sequences in the plus-strand RNA that function as segment-identity labels, and the sequences that are required for incorporating the plus strand RNA's into the newly formed cores. Also it is crucial to identify other constraints that may limit the incorporation of heterologous sequences in reovirus genome segments. In the following paragraphs we will describe the functional elements identified in reovirus genome segments.

Non-translated regions

The particle to infectious unit ratio of reoviruses is low (Spendlove *et al*., 1970), indicating that the assembly of new particles is effective and precise and the infection process is highly efficient. The encapsidation involves assembly of 8 structural proteins with one copy of each of the 10 segments. The precise sorting mechanism which directs one copy of each segment into the particle is not yet well understood. Sequences contained within the 130 nt at the 5′ terminus serve as identity label for each of the segments (Roner and Steele, 2007a; Roner and Steele, 2007b). The

termini of all segments contain relatively short (15–33 nt) non-translated regions (NTRs) with short (4–5 nt) identical sequences at the extreme ends. The 5′ end of the plus-strand RNA starts with the tetranucleotide sequence GCUA- and the sequence at the 3′ end is a pentanucleotide –UCAUC. No other sequence homology exists in the NTR regions of the segment termini (Antczak *et al*., 1982; Gaillard *et al*., 1982; Roner *et al*., 2004).

Assortment of the segments

The first indications that the 5′ and 3′ ends of the segments are important for replication and assembly stem from work by Schlesinger *et al*. (1977), and later by Zou and Brown (1992). In these studies deletion mutants were used. These deletion mutants were formed spontaneously when T3D was passaged at a high multiplicity of infection and contained deletions in the middle of the segments. The deleted segments were replicated and encapsidated in new particles although the presence of helper virus was required. It led to the hypothesis that sequences near the 3′ end are necessary to function as promoter for the RdRp and that the termini are needed for the assembly of new particles (Schlesinger *et al*., 1977; Zou and Brown, 1992).

By employing a reverse genetics system for inserting a chloramphenicol acetyl transferase (CAT) reporter gene in different genome segments (discussed below) more information on the required *cis*-acting sequences at the 5′ and 3′ ends was obtained (Roner and Joklik, 2001). From three segments, for example, S2, M1 and L1, the regions which are important for assembly have been determined. The lengths of these regions were identified through varying the length of the 5′ and 3′ ends and measuring the CAT activity of the progeny virus. The lengths of the elements required for encapsidation correlated with the lengths of the genome segments. L1 required the longest 5′ and 3′ regions (i.e. respectively 129 nt and 139 nt) while S2 needed the shortest regions, at the 5′ end 96 nt and at the 3′ end 98 nt. The M1 segment required at the 5′ end 124 nt and 172 nt at the 3′ end (Roner and Steele, 2007a; Roner and Steele, 2007b). These results indicate that the *cis*-acting sequences do not only encompass the non-translated regions, but extend into the coding regions of the segments. Furthermore, Roner *et al*, in 2004 and 2006, demonstrated that the signals at the 5′ end act independently from the signal at the 3′ end. In the process the signals at the 5′ end function as the segment-identity labels. This became apparent from the observation that chimeric segments, containing the 5′ end of S2 or M1 and the 3′ end of L1 are incorporated as S2 or M1 segments, not as a L1 segment (Roner *et al*., 2004; Roner and Roehr, 2006).

10.1.5 Genome-size constraints

The genome of the mammalian reoviruses is packaged within the 52 nm inner core. In comparison to other RNA viruses, reoviruses have one of the most densely

packaged genomes (Roner and Steele, 2007a). Experiments of the group of Roner and colleagues indicate that the maximum amount of RNA that can be included into the core is almost reached. They showed with their reverse genetics system that upon increasing the length of L1 with 726 nt this segment was still incorporated. However, when creating chimeric segments with the 5′ end of M1 or S2 and the 3′ end of L1, and increasing the size of the resulting segments by 2307 nt or 2500 nt respectively, full incorporation of these segments was inhibited. The modified 5′-L1.CAT.3′-S2, 5′-M1.CAT.3′-S2 and 5′-S2.CAT.3′-L1 segments, in which the sizes were decreased by approximately 980 bp, were still packaged (Roner and Steele, 2007a). These data suggest that there are limitations to the packaging capacity of reoviruses. Such limitations are important if one considers developing heterologous transgene-containing reoviruses.

10.2 Forward-genetics in orthoreoviruses

In forward-genetics studies aberrant phenotypes are isolated followed by the identification of the mutation responsible for this phenotype. Much of today's knowledge on reovirus genes and genome segments has been obtained from studies with this forward-genetics approach. One of the strategies used most commonly was the selection of temperature-sensitive mutants after chemical mutagenesis. This method was often used in combination with the use of reassortants. A third strategy discussed in this chapter is the selection of natural mutants.

10.2.1 Temperature-sensitive mutants

The initial isolation of many reovirus temperature sensitive (*ts*) mutants was after chemical mutagenesis of reovirus stocks. The mutated reoviruses were plated and propagated at a low permissive temperature (usually 32°C), yielding virus which could efficiently replicate at this temperature. Subsequently, clones were isolated that exhibited an impaired growth at a higher temperature (often 39 °C was used).

With this strategy large series of mutants with aberrant phenotypes have been generated, and designated as *ts* mutants. These *ts* mutants were classified in complementation groups on the basis of the absence of complementation between pairs of *ts* mutants. Subsequently these complementation groups could be assigned to the different genome segments of the reovirus (reviewed by Coombs (2006). A summary of the different groups and the segments to which they have been mapped is provided in Table 10.1.

The phenotypes of these mutants have been instrumental in defining the roles of the individual viral proteins in the infectious pathways of the human reoviruses (Coombs, 2006).

10.2.2 Reassortants

In addition to *ts* mutants, reassortment strategies were applied. The reassortants can arise during co-infections of cells with two different *ts* mutants or between different *orthoreovirus* serotypes. Co-infection can lead to exchange of entire genome segments between the viruses. During co-infection, a reassorted genome can be detected in up to 15% of the progeny viruses (Joklik, 1998).

10.2.3 Natural selection/bioselection

A third forward-genetics strategy is the isolation of spontaneous mutants and naturally occurring reoviruses (Chappell *et al*., 1997; Spriggs and Fields, 1982). The absence of proofreading in the reoviral RdRp leads to a high mutation rate (Steinhauer *et al*., 1992). This leads to rapid adaptation of the reoviruses and the selective outgrowth of mutants that have favourably adapted to the host (Domingo and Holland, 1997; Novella *et al*., 1995).

10.3 Reovirus/cell interactions

10.3.1 A key role of σ1 in cell binding

Since the early 1960s it has been well established that Reovirus T3D, but not T1L, could haemagglutinate bovine erythrocytes (Gomatos and Tamm, 1962). Reassortment studies identified σ1 protein as the haemagglutinin. In these studies T1L x T3D reassortants were generated and by using serotype-specific σ1 antisera it was demonstrated that the capacity to haemagglutinate the bovine erythrocytes was strictly correlated with the presence of the σ1 protein of T3D (Weiner *et al*., 1977; Weiner *et al*., 1978; Weiner *et al*., 1980a; Weiner and Fields, 1977). Further studies using these reassortants and serotype specific sera pinpointed σ1 also as the protein responsible for infection of permissive nucleated cells (Lee *et al*., 1981).

10.3.2 Sialic acids

Attachment studies of the T3D virus to different cell types (erythrocytes, L cells, lymphocytes and murine erythroleukaemia (MEL) cells) made clear that haemagglutination (HA) in erythrocytes is caused by binding to terminal sialic acids of glycoproteins on the cell surface (Gomatos and Tamm, 1962; Armstrong *et al*., 1984; Co *et al*., 1985; Gentsch and Pacitti, 1985; Paul and Lee, 1987). Sialic acid is a generic term for the *N*- or *O*-substituted derivatives of neuraminic acid and also the name of the most common neuraminic acid, the terminal N-acetylneuraminic acid

(NeuAc). With S1 reassorted viruses of T3D and T1L (reassortants T1L+S1-T3D (1HA3) and T3D+S1-T1L (3HA1)) it was demonstrated that reovirus attachment to sialylated oligosaccharides on glycoproteins was solely mediated by the σ1 attachment protein (Pacitti and Gentsch, 1987).

The σ1 protein is present as a homo-trimer (Leone *et al*., 1991; Strong *et al*., 1991) at the vertices of the viral particle. It has a distinct 'head' and 'tail' region (Bassel-Duby *et al*., 1985; Nibert *et al*., 1990). The domains that interact with cellular receptors have been identified. The knowledge of the interaction of the σ1 protein with cellular sialic acids was derived from studies employing reovirus T3D field isolates that differ in their capacity to agglutinate human and bovine erythrocytes and to bind sialic acids. Sequence analyses of the S1 genes of the different virus isolates revealed single point mutations that only cause weak haemagglutination and which are unable to bind sialic acids. These mutations all cluster in one region in the σ1 tail, residue 198-204, which demonstrated that not only the head but also the tail region of the spike is exposed on the capsid in such a way that it can interact with cellular receptors (Dermody *et al*., 1990).

The phenomenon that only T3D and 1HA3 and not T1L viruses could infect murine erythroleukemia (MEL) cells was exploited in studies that mapped the sialic-acid binding-domain of σ1. The capacity of the viruses to infect MEL cells is strictly correlated with the viruses' capacity to haemagglutinate erythrocytes. This suggested sialylated proteins to be involved in MEL cell binding (Rubin *et al*., 1992). To further identify the receptor binding region of the σ1 tail, reovirus type 3 field isolates which were unable to bind sialic acids were adapted by serial passaging to grow in MEL cells. Sequence analyses of these MEL adapted (MA) viruses revealed point mutations that were clustered near the residues 198-204 that had previously been identified as the sialic acid binding region of the σ1 tail. These data demonstrated that this part of the tail of the spike is involved in sialic-acids binding (Chappell *et al*., 1997).

Similarly, T3D reoviruses were selected for growth in the presence of a monoclonal antibody that inhibited haemagglutination. This selection yielded mutants that exhibit a strongly reduced neurovirulence upon inoculation of newborn mice (Spriggs and Fields, 1982). These data demonstrate the relative ease with which reovirus mutants can be obtained by conventional forward genetics strategies.

10.3.3 Junction Adhesion Molecule-A

Evidence that not only the fibrous tail of the σ1 spike but also a domain in the head is involved in receptor binding has accumulated over the years (Weiner *et al*., 1980b; Nagata *et al*., 1987; Williams *et al*., 1988; Duncan *et al*., 1991; Rubin *et al*., 1992; Turner *et al*., 1992; Chappell *et al*., 1997). In 2001, Barton and colleagues identified Junction Adhesion Molecule (JAM) as the cellular receptor to which the σ1 protein's head domain can attach. Additional binding-inhibition experiments with antibodies show that JAM directly binds to σ1. Transient expression of JAM

renders reovirus-resistant cells sensitive to infection (Barton *et al*., 2001). Furthermore, Campbell and colleagues defined that only JAM-A (also known as JAM-1) and not JAM-B or JAM-C can serve as a receptor for reovirus type 1, 2 and 3 (Campbell *et al*., 2005).

JAM is a 25kDa type I transmembrane protein with two extracellular Ig domains (D1 and D2) and a short cytoplasmic tail. The membrane distal extracellular domain (D1) forms a homodimer (Severson and Parkos, 2009). The protein is concentrated at the apical region of intercellular tight junctions of epithelial and endothelial cells (for reviews, see Liu *et al*., 2000; Martin-Padura *et al*., 1998; Severson and Parkos, 2009).

Structure-guided mutational analysis revealed three amino acids located in the D1 domain of JAM-A which are individually required to bind σ1. The amino acids Glu^{61} and Lys^{63} participate in salt bridges with opposing amino acids and thereby have a role in stabilisation of the D1 dimer. The amino acid Leu^{72} is part of a hydrophobic interaction with a residue of the opposing dimer (Guglielmi *et al*., 2006). Since all amino acids required for binding σ1 were located in the dimeric interface of the D1 domain, it was reasoned that σ1 first disrupts the JAM-A dimer and then binds to the monomeric form of the D1 domain. Binding studies indeed showed that the binding affinity between σ1 and the monomeric form of the D1 domain is higher than between the JAM-A homodimers. In addition, using cryocrystallography it was clearly demonstrated that only monomers of JAM-A were bound by σ1 (Kirchner *et al*., 2008). Insight in the amino acid residues important for binding JAM-A was obtained by using the helper-free reverse-genetics system (see below) (Kobayashi *et al*., 2007). The engineered reoviruses with mutant forms of σ1 revealed that the JAM-A binding domain is located at the lower part of the head domain. Moreover, one reovirus σ1 molecule can bind three JAM-A monomers (Kirchner *et al*., 2008) (Picture of JAM in (Guglielmi *et al*., 2006) and JAM-σ1 interaction in (Kirchner *et al*., 2008)).

The knowledge of reovirus T3D binding to its cellular receptors culminated in a multi-step binding model in which attachment protein σ1 first engages sialic acids on the cell surface in a low-affinity interaction and subsequently binds JAM-A with high affinity (Danthi *et al*., 2010; Kobayashi *et al*., 2007). The virion is internalised via clathrin-mediated endocytosis upon interaction between β1-integrins on the cell surface with the capsid protein λ2 (Maginnis *et al*., 2006). This has been suggested since λ2 contains the integrin binding sequences Arg-Gly-Asp (RGD) (Maginnis *et al*., 2006). Furthermore, β1 integrins contain a cytoplasmic domain containing two Asn-Pro-any amino acid-Tyr (NPXY) motifs which are required for functional reovirus entry (Maginnis *et al*., 2008).

10.3.4 Persistent infection

Rather than causing a lytic infection, the T3D reovirus can establish a persistent infection. The initial observations stem from experimental infections of

cultured human embryonic cells (Bell and Ross, 1966). Infectious virus could be recovered from the cultures after 9–12 passages without appearance of overt cytopathic effects.

Taber and colleagues (1976) isolated a culture of persistently infected CHO cells. In these cultures a large number of cells were infected, and the cultures produced reoviruses that were cytopathic for the parental CHO cell line (Taber *et al*., 1976). This suggests that the persistently infected cell lines adapted to resist the reovirus-induced cytopathic effects. Ahmed and co-workers (1981) demonstrated in an L-cell system that the persistently infected cells as well as the reoviruses propagated in the persistently infected cultures acquire changes and thus eventually differ from the parental virus and host cells. The L cells cured from the persistent infection had an increased resistance to *wild-type* T3D viruses, suggesting that the cells adapted by genetic or epigenetic mechanisms, leading to increased virus resistance (Ahmed *et al*., 1981). More recently it was demonstrated that persistently infected cells express reduced amounts of the cathepsins B and L, which are known to be involved in reovirus uncoating (Baer *et al*., 1999; Ebert *et al*., 2002; Ebert *et al*., 2004). These data suggest a co-evolution of host and virus to eventually reach a state of a stable but dynamic equilibrium.

Persistently infected L-cell cultures obtained after co-infections with T2J, which does not result in persistent infections, together with T3D, which is able to give persistent infections, yielded various hybrid recombinant mutants containing all the S4 segment of T3D. This suggested that sequences in this segment underlie the capacity of the virus to establish a latent infection (Ahmed and Fields, 1982). Via a similar approach the capacity of human reoviruses to inhibit host cell RNA and protein synthesis was also been mapped to the S4 segment (Sharpe and Fields, 1982). Taken together, these data imply that persistent infection can only exist if the host cells retain the capacity to synthesise proteins. Strong inhibition, as is the case upon infection with T2J, would be incompatible with such persistence. Furthermore, sequence analyses of the persistent infections demonstrated acquisition of additional mutations in the S4 segment and an increased resistance to ammonium chloride, a weak base that inhibits the pH decrease in endosomes and lysosomes. This again suggests that the viruses adapted to selective pressure operating at the level of cell entry.

In addition to alterations in S4, sequence analyses of the viruses causing persistent infection also revealed mutations in the S1 segment, encoding the spike protein (Ahmed and Fields, 1982; Kim *et al*., 2011; Wetzel *et al*., 1997; Wilson *et al*., 1996). The σ1 protein is known to be involved in the induction of apoptosis in reovirus infected cells (Tyler *et al*., 1995). Isolation of an S1 attenuated reovirus mutant form from persistently infected cells, revealed that although virus replication was maintained the apoptotic potential was reduced. Sequence analysis revealed that the most significant mutation was a nonsense mutation which truncated the σ1 protein, however it could not be ruled out that other mutations in S1 and S4 too are involved in this phenomenon (Kim *et al*., 2011). It would therefore be interesting to test whether the mutations found in the S1 segments of the persistently infected viruses affect the

capacity of σ1 to bind sialylated proteins and to induce apoptosis (Connolly *et al*., 2001; Tyler *et al*., 2001).

In the preceding sections the conventional forward-genetics strategies have been discussed as well as some of their applications. Although they have been instrumental for determining the functions of the different segments, these techniques have their limitations. No directed mutations can be introduced. Therefore, reverse genetics systems have been developed, facilitating not only new studies into the functions of individual reovirus proteins, as well as the development of new reoviruses for use as oncolytic agents.

10.4 Reverse-genetics in orthoreoviruses

So far development of reverse genetic systems for reoviruses has been notoriously difficult. Although in 1982 the cDNAs of all the genome segments had been cloned for the purpose of sequencing (Cashdollar *et al*., 1982), the first reverse genetics system was only described in 1990 (Roner *et al*., 1990). Genetically modified particles were generated with the aid of helper reoviruses. In 2007 the first helper-free system was described (Kobayashi *et al*., 2007). To date, three different systems have been developed which have all their merits and weaknesses.

10.4.1 The infectious-RNA system

The first method for reverse genetics in reovirus T3D employs RNA transfections of all the 10 genome segments, a cell-free translation system, and helper reoviruses (Roner *et al*., 1990).

Active core structures, generated by *in vitro* disassembly of reoviral particles, were used for transcription of plus-strand RNAs *in vitro*. The RNA transcripts were translated using a rabbit reticulocyte lysates (RRL) system. While this step was not essential, it increased the efficiency by 2–3 orders of magnitude. The newly translated viral proteins, together with dsRNA, and ssRNA, were introduced into mouse L fibroblasts by lipofection. Usage of only ssRNA or dsRNA in this mixture is possible but less efficient. A few hours later the helper virus, which can be either serotype 1 or serotype 2, is added to the cultures. Between 24 to 48 hours post infection, virus can be harvested. Either plaque purification, or the use of serotype-specific neutralising antisera, was required to eliminate the helper-viruses (Roner *et al*., 1990; Joklik and Roner, 1995).

This technique was used to generate a compound *ts* mutant. Two *ts* mutants were chosen both of which contain a *ts* mutation albeit on different genome segments (genome segment M2 coding for μ1 and segment S2 encoding σ2). These segments were jointly incorporated in progeny virions generating the compound double *ts* mutant. Creating these double *ts* mutant required removal of the corresponding *wild-type* segments from transfected RNA pool. This can be accomplished

by sequence-specific degradation of the *wild-type* segments through addition of complementary oligonucleotides and RNase H treatment. This enzyme degrades the RNA-strand in complementary DNA/RNA duplexes. Analysis of the double mutant showed that the *ts* phenotype was enhanced compared to the parental single *ts* mutants. Furthermore, exposure of mice to virus revealed that the double *ts* mutant was less pathogenic than the parental viruses, while protective neutralising immunity was still induced (Roner *et al*., 1997).

Transgene-containing reoviruses

The first example of the introduction of a heterologous transgene gene in the reovirus genome was described by Roner and Joklik in 2001. To this end, the infectious RNA-system was used. In this experiment the S2 segment was replaced by an S2 segment modified to include the chloramphenicol acetyl transferase (CAT) gene, as a reporter. The coding sequence of the CAT gene is smaller than the coding sequence of σ2 (753 nt versus 1331 nt, respectively), and its activity can be easily monitored in cell lysates (Roner and Joklik, 2001).

The CAT gene was placed in-frame in the S2 open reading frame. The total lengths of the S2 sequences flanking the CAT gene were 198 nt at the 5′ end and 284 nt at the 3′ end. The modification inactivated the σ2 open reading frame. Therefore all experiments were performed in helper cells that expressed an intact copy of S2 to trans-complement the missing σ2 protein. To generate infectious virus with the modified S2 gene, the wild-type S2 was removed from viral RNA preparation by addition of an oligodeoxyribonucleotide complementary to the S2-transcript and RNase-H treatment. To provide the modified S2 transcripts, the capped S2-CAT RNA was generated by transcribing a cloned version of this S2-CAT segment by T7 RNA polymerase. After removal of the wild-type S2 RNA and addition of capped S2-CAT RNA the mixture was lipofected into helper cells, yielding reoviruses carrying the CAT gene in their S2 segment.

This system was used to identify the regions that are essential for replication and packaging of the segments. These regions were mapped at the segment termini. In an experiment in which the CAT gene was inserted between the 5′ terminal end of the L1 segment and 3′ terminal of the S2 segment, the CAT containing segment was found to replace the L1 segment, but not the S2 segment. Similarly, a 5′-S2.CAT.L1-3′ segment and a 5′-M1.CAT.L1-3′ replace the S2 and M1 segments, respectively. These data indicate that the 5′ terminus determines the segment identity in the segment assortment. Moreover the 5′ and 3′ termini act independently (Roner and Steele, 2007a).

In similar studies, the size constraints were established for packaging and replication as discussed in genome-size constraints. These data show that although the size of the segments can be increased by inserting heterologous sequences, the capacity is limited (Roner and Steele, 2007a).

The infectious-RNA method has been effectively used to engineer alterations in the reovirus genome. However, the method is technically demanding. The RNase

H procedure is not fully efficient, resulting in the presence of residual RNA of the targeted segment, and appearance of viruses with wild-type segments. In addition, the requirement of helper viruses during the generation of the modified viruses is undesirable. The helper virus may yield the formation of reassortants between the 'helper' viruses and the generated virus, although the use of T2J as helper reduces the magnitude of the problem (Joklik and Roner, 1995). Despite these weaknesses, the results obtained with this system have been extremely informative.

10.4.2 The segment-replacement technique

Van den Wollenberg *et al*. recently described an alternative approach for generating genetically modified reoviruses. In an effort to modify the σ1 spike, an S1 segment was generated encoding a σ1 that harbours a C-terminal histidine-tag. The presence of this tag would allow particles to infect JAM-A-negative cells that express on their surface a single-chain antibody recognising the His-tag, as an artificial receptor (van den Wollenberg *et al*., 2008).

This strategy was based on experiments by Rouault and Lemay (Rouault and Lemay, 2003). These authors expressed modified versions σ1, μ1, σ3 and used these proteins to recoat reovirus ISVPs and cores *in vitro* (Chandran *et al*., 1999; Chandran *et al*., 2001; Jane-Valbuena *et al*., 1999) to study reovirus capsid protein structures and functions. In a proof of principle study a foreign epitope, viz. a hexahistine tag, was added to the amino terminus of σ3 (Rouault and Lemay, 2003). For recoating, purified wild-type virions were treated with chymotrypsin to generate ISVPs. The ISVPs were incubated with cellular extracts that contain mutant σ3 proteins. Free σ3 proteins were removed from the recoated ISVPs and by immunoblotting procedure the modified σ3 on the recoated ISVPs could be detected. While this strategy can be used to introduce modified proteins in the capsid, the modification will not persist upon replication since the viral genome is not modified.

Van den Wollenberg *et al*. produced particles with modified proteins incorporated in their capsid by propagating reoviruses on helper cell lines that synthesise modified reoviral proteins. These modified proteins would be incorporated in the capsid during replication of the reovirus. In a first series of experiments reoviruses were propagated on modified cells that express a S1 segment that included a His-tag at the carboxyl terminus of the σ1-encoding open reading frame. It was anticipated that the viruses harvested from these cells would carry the σ1-His in their capsid, but would not carry the modified S1-His segment. However, the authors noted that propagation of wild-type reoviruses on cells expressing the σ1 His-encoding segment as a conventional RNA polymerase II transcript led to frequent replacement of the wild-type genome segment with the modified version. The resulting viruses could be serially passaged on JAM-A-deficient U118MG cells that were modified to express the single-chain Fv capable of binding a His-tag. Hence, this technique allowed the generation of reoviruses that are genetically retargeted. It also demonstrated that the C terminus of the σ1 protein is a suitable location for the insertion

of oligopeptide ligands and shows that it is possible to use genetic modification to retarget the infection of reoviruses (van den Wollenberg *et al*., 2008).

The precise mechanism by which the modified segment is incorporated is still unclear. Two mechanisms could be envisaged. In a first mechanism, the RNA-polymerase II transcript that contains the modified S1 segment, associates with newly formed core particle despite the presence of the long 3′ extension and a poly-A tail. In the core particle the minus-strand synthesis would start even with the 3′ extension and poly-A tail. This would yield a partially double-stranded RNA copy of the polymerase II transcript. If this dsRNA copy serves as template for secondary plus-strand synthesis, new plus strands would be generated that harbour the modified σ1 open reading frame, but are otherwise identical to the wild-type S1 segment. Alternatively, one could anticipate a mechanism that would involve RNA recombination or a template switch during replication of the reovirus genomes. Future studies will aim at resolving the mechanism for the replacement of genetic information.

The segment-replacement system is relatively straightforward, as it is based on the selective advantage for the modified σ1 over the wild-type σ1. The selection is essential for selectively expanding the viruses that have the wild-type genome segment replaced by the modified version. Unfortunately, this method is thwarted by the fact that, with a low frequency, mutants arise in these cultures that have the capacity to infect the U118MG cells independent of JAM-A and the His-tag-specific scFV. This leads to the occurrence of replicating reoviruses that do not carry the desired mutation. Therefore, plaque purification and screening are important to characterise and purify the desired mutants.

10.4.3 A helper-free reverse-genetics systems

A fully helper-free system for reverse genetics was described by Kobayashi and colleagues. It employs 10 different plasmids, each containing a single cloned genome segment. The method starts with infection of susceptible cells with an attenuated vaccinia virus expressing the T7 RNA polymerase, and is followed by naked-DNA transfection of the 10 plasmids encoding each of the genome segments. The genome segments are inserted in the plasmid as full-length cDNA clones derived from the wild-type T3D. These cloned segments are inserted downstream of a bacteriophage T7 promoter. The 3′ terminus of each cloned segment was fused to the hepatitis delta virus (HDV) ribozyme, which generates native 3′ ends without poly-A tail by self-cleavage of the RNA transcript. The attenuated vaccinia virus is replication-deficient and serves to express the T7 RNA polymerase at high level. The supernatants of the transfected cultures were plaque assayed on L-cells and replication-competent viruses could be isolated. The method is robust and productive, viral infection could be established in approximately 1 in 10^5–10^6 cells transfected. This system was validated to confirm that a single amino acid change in σ1 conferred

resistance of σ1 to trypsin cleavage, and that a single amino acid change in σ3 accelerates proteolytic disassembly of the reovirus. Furthermore, with this system the functional domains in μ1 responsible for the induction of reovirus apoptosis in host cells could be mapped (Danthi *et al*., 2008a; Danthi *et al*., 2008b). Introduction of the enhanced green fluorescent protein (eGFP) gene into the S4 segment demonstrated that the technology allowed the incorporation of heterologous transgenes into the reovirus genome. Since this virus lacks a normal S4 segment, the σ3 encoded by S4 needs to be provided in *trans*. Therefore the T3D/S4-GFP virus could only be propagated in cells genetically modified to produce the σ3 protein (Kobayashi *et al*., 2007).

Taken together, these data show that this helper-free reverse genetics method can be used to generate viruses with single amino-acid changes as well as for generating viruses carrying heterologous transgenes.

The advantage in comparison of the two other systems is that there is no requirement for helper viruses. The system is easily amendable for the introduction of various kinds of mutations. However, the efficiency may need further improvement since it is estimated that about 1 in 10^5–10^6 transfected cells can establish viral progeny. As a first step in improvement, instead of inserting the genome segments in 10 separated plasmids, the system has been amended to four plasmids including all the 10 segments. The genome segments are still independently flanked by the T7 promoter and the HDV ribozyme. Furthermore, instead of using the attenuated vaccinia virus, BHK cells stably expressing the T7 polymerase under control of cytomegalovirus promoter can be used, further simplifying the technology (Kobayashi *et al*., 2010). With these improvements, the reverse genetics system is more effective in generating progeny virus.

10.4.4 Rotavirus

Rotaviruses are classified in a separate genus of the *Reoviridae*. Unlike the mammalian *orthoreoviruses*, the human rotaviruses are very pathogenic, causing severe diarrhoea, especially in young children (Dennehy, 2000). It is a major health problem, particularly in developing countries (Simpson *et al*., 2007). At this moment, attenuated live virus is used as vaccine (Bernstein, 2009). The development of a reverse genetics system would allow us to gain more knowledge about the biology of rotaviruses.

Rotaviruses are very similar to the mammalian o*rthoreoviruses*. They contain 11 dsRNA genome segments encoding 13 proteins, and they have a non-enveloped three-layer icosahedral capsid structure (Pesavento *et al*., 2006). Although they resemble reovirus, the development of a reverse genetics system is even more difficult.

So far, attempts to generate a plasmid-based reverse genetics system have been unsuccessful. A first reverse-genetics system was described in 2006 by Komoto *et al*. (Komoto *et al*., 2006). These authors managed to replace the spike-encoding

segment of a human rotavirus by that of a simian homologue. The system employed resembles *the segment-replacement technique* described above, except the cDNA of the simian spike-encoding segment was flanked by a T7 promoter and the HDV ribozyme. Infection of a vaccinia virus vector expressing T7 polymerase was required to provide the T7 polymerase. Selection of the modified virus employed neutralising antibodies against the human spike-protein, selectively enriching the virus population for the viruses containing the simian spike-protein (Komoto *et al*., 2006). So far this method relied on a high selective pressure for the modified rotavirus which limits the application of the technique.

More recently, Troupin *et al*. describe a modified version of the Komoto method to generate a recombinant rotavirus carrying an artificially rearranged rotavirus segment 7, coding for non-structural protein 3 (NSP3) (Troupin *et al*., 2010). Their method is based on the observation that rearranged segments 7 and 11 are preferentially packaged (Gault *et al*., 2001). The usefulness of this system is under debate (Trask *et al*., 2010), since it requires extended passaging at a high multiplicity of infection to recover the recombinant virus.

The latest development in rotavirus reverse genetics is based on the method of Komoto *et al*. together with a dual selection mechanism (Trask *et al*., 2010). Trask *et al*. combined a *ts* mutation of non-structural protein 2 (NSP2), with RNAi-mediated degradation of NSP2 transcripts to select for a recombinant rotavirus evading both mechanisms, *ts* and RNAi degradation. Therefore, the recombinant segment 8 cDNA, coding for NSP2, was modified to contain silent mutations in the region targeted by the RNAi to avoid degradation. The combination of the *ts* helper virus with the RNAi-mediated selection significantly improved the recovery of the recombinant rotavirus. An analysis demonstrated that eight of eight rotavirus isolates contained the recombinant segment 8.

This ‘two-hit’ method may enhance the efficiency of recombinant virus recovery. One limitation of this system is the requirement for gene-specific RNAi, which may not be equally effective for all the 11 segments of the rotavirus.

So far, reverse genetic systems for the introduction of a foreign gene in rotaviruses have not been reported.

10.5 Reovirus as an oncolytic agent

The observation that reoviruses preferentially lyse transformed cells was first made in 1977 by Hashiro *et al*. (Hashiro *et al*., 1977). One year later Theiss and colleagues described the suppressing effects of reovirus infection on lung tumours in mice (Theiss *et al*., 1978). There is a strong indication that the preferential lysis of transformed cells depends on the RAS status of these cells. In non-transformed cells, dsRNA activates the cell’s defence mechanism by phosphorylation of the double-stranded RNA-dependent protein kinase (PKR). This inhibits translation of viral transcripts. If the RAS pathway is constitutively active, phosphorylation of PKR is inhibited, facilitating reovirus replication (Norman *et al*., 2004; Strong

et al., 1991). Although that activated RAS status is important for oncolysis, it is not the sole determinant of cellular sensitivity to reovirus. Kranenburg and colleagues showed that the reovirus propagation and yields are not dependent on the RAS status, although the active RAS stimulated cell lysis (Smakman *et al*., 2005; Smakman *et al*., 2006). In other studies, cellular resistances to reovirus infection were correlated with cathepsin B and L activity. These enzymes were identified as important factors for the reovirus proteolytic disassembly (Alain *et al*., 2007; Baer *et al*., 1999; Kim *et al*., 2007).

The preference for transformed cells plus the absence of significant pathology in humans made reoviruses excellent candidates for oncolytic virus therapies. The Canadian company Oncolytics Biotech initiated several clinical trials with their lead product, Reolysin, which was based on the wild-type T3D strain. At this moment at least 24 clinical trials are active or completed in the UK, US, Canada, and Belgium. The indications comprise a range of cancer types, including prostate cancer, malignant gliomas, pancreatic cancer, lung cancer, and head and neck cancer (Oncolytics Biotech Inc, 2011). Initially, the virus was administered as mono-therapy but in more recent clinical trials reoviruses are combined with more conventional treatment modalities. In these studies no dose-limiting toxicity was reached, underscoring the safety of reovirus-based oncolytic virus therapy (Lal *et al*., 2009). Despite anecdotal evidence of anti-tumour activity, the efficacy has been limited. Currently, several factors such as insufficient tumour penetration of the therapeutic virus, limited spread of the virus within the tumour cell mass, insufficient expression of the reovirus receptor JAM-A on tumour cells, and preexisting humoral immunity, have been proffered to explain the limited tumour–cell transduction (van den Wollenberg *et al*., 2009). It is anticipated that technology to modify reovirus genomes by reverse genetics may aid the generation of new mutants that overcome these current limitations.

10.5.1 Optimising reovirus therapy

To overcome some of the bottlenecks, different options are being explored. An interesting approach to improve the delivery of reoviruses to tumours may involve the use of tumour -seeking cells as delivery vehicles. Several cell types (e.g. T-cells, dendritic cells, macrophages, mesenchymal stem cells) have the ability to migrate to tumours. If these cells are loaded with oncolytic viruses, they may release the virus in the tumour mass after migration. In addition, this approach may shield the virus from the neutralising immunity immune system (Power and Bell, 2008).

The scarcity of the reovirus receptor JAM-A on tumour cells may be overcome by modification of the attachment protein $\sigma 1$. Either bioselection of spontaneous mutants or the use of reverse genetics strategies can be employed. In the latter approach the lessons learned from generating Adenovirus-C mutants that by-pass the dependency of the adenovirus receptor, can be very helpful. Adenovirus-C is widely studied as an oncolytic virus. The attachment protein of adenovirus-C, fibre, recognises

the Coxsackie and Adenovirus Receptor (CAR) receptor. Although the adenovirus fibre and the reovirus spike have no sequence similarity, their 3D structures are remarkably similar (Stehle and Dermody, 2003; Campbell *et al*., 2005). In the adenovirus fibre protein a wide variety of ligands have been inserted. Such ligands include single-chain Fv domains, single-chain T-cell receptors, so-called affibody™ molecules, and integrin-binding RGD motifs. This successfully changed the cellular tropism of the adenoviruses (Waehler *et al*., 2007; Lindholm *et al*., 2008; Arnberg, 2009). It remains to be determined whether the same approach is equally effective in modifying the cellular tropism of reoviruses.

Not only the infection efficiency, but also the tumour -cell specificity of infection can be enhanced. To avoid the infection of non-target cells expressing JAM-A, the σ1 protein can be modified to ablate its association with JAM-A. Viruses with reduced JAM-A binding have been isolated (Kirchner *et al*., 2008; Kim *et al*., 2011;), these mutants were still viable and infected cells presumably via binding to sialic acid residues (Kirchner *et al*., 2008). The mutant with the truncated σ1, isolated by Kim and colleagues, still preferentially targeted tumour cells, however, it showed a reduced toxicity *in vivo*. Also exposure of naïve mice to this virus still resulted in the induction of neutralising immune response. Kobayashi and coworkers used their helper-free reverse-genetics system to generate a mutant which harbours a single amino acid change in the σ1 protein which ablates the capacity to bind JAM-A (Kobayashi *et al*., 2007). Such viruses may facilitate the development of more efficacious tumour -cell selective reoviruses for application as an oncolytic agent.

10.6 Conclusion

To date, several reverse-genetics systems have been described for manipulation of mammalian *orthoreovirus* genomes. These systems have already proved their effectiveness and were used to reveal new viral functions and facilitated the introduction of modified genome segments. Nevertheless, the robustness of these techniques should be further improved to make them more widely applicable. Some of these systems require selection methods to enrich the mutant viruses. The developments of genetically modified variants should be accompanied by the parallel development of procedures to make the mutant reoviruses a safe pharmaceutical product. This requires manufacturing processes that prevent reversion of the mutants to *wild-type* viruses. To ensure that the mutations are retained during the prolonged passaging that is required for production of large clinical-grade batches, the production systems should be developed in parallel. Such systems must provide a continued positive-selection pressure for the presence of mutations or modifications. Only if the development of reverse genetics systems goes hand in hand with the development of dedicated production systems, can the new technology be employed to generate improved mammalian orthoreoviruses for clinical application.

References

Ahmed, R., Canning, W. M., Kauffman, R. S., Sharpe, A. H., Hallum, J. V. and Fields, B. N. 1981. Role of the host cell in persistent viral infection: coevolution of L cells and reovirus during persistent infection. *Cell* **25**(2): 325–332.

Ahmed, R. and Fields, B. N. 1982. Role of the S4 gene in the establishment of persistent reovirus infection in L cells. *Cell* **28**(3): 605–612.

Alain, T., Kim, T. S., Lun, X., Liacini, A., Schiff, L. A., Senger, D. L. and Forsyth, P. A. 2007. Proteolytic disassembly is a critical determinant for reovirus oncolysis. *Molecular Therapy* **15**(8): 1512–1521.

Antczak, J. B., Chmelo, R., Pickup, D. J. and Joklik, W. K. 1982. Sequence at both termini of the 10 genes of reovirus serotype 3 (strain Dearing). *Virology* **121**(2): 307–319.

Antczak, J. B. and Joklik, W. K. 1992. Reovirus genome segment assortment into progeny genomes studied by the use of monoclonal antibodies directed against reovirus proteins. *Virology* **187**(2): 760–776.

Armstrong, G. D., Paul, R. W. and Lee, P. W. 1984. Studies on reovirus receptors of L cells: virus binding characteristics and comparison with reovirus receptors of erythrocytes. *Virology* **138**(1): 37–48.

Arnberg, N. 2009. Adenovirus receptors: implications for tropism, treatment and targeting. *Reviews in Medical Virology* **19**(3): 165–178.

Baer, G. S., Ebert, D. H., Chung, C. J., Erickson, A. H. and Dermody, T. S. 1999. Mutant cells selected during persistent reovirus infection do not express mature cathepsin L and do not support reovirus disassembly. *Journal of Virology* **73**(11): 9532–9543.

Barton, E. S., Forrest, J. C., Connolly, J. L., Chappell, J. D., Liu, Y., Schnell, F. J., Nusrat, A., *et al*. 2001. Junction adhesion molecule is a receptor for reovirus. *Cell* **104**(3): 441–451.

Bassel-Duby, R., Jayasuriya, A., Chatterjee, D., Sonenberg, N., Maizel, J. V., Jr. and Fields, B. N. 1985. Sequence of reovirus haemagglutinin predicts a coiled-coil structure. *Nature* **315**(6018): 421–423.

Becker, M. M., Peters, T. R. and Dermody, T. S. 2003. Reovirus sigma NS and mu NS proteins form cytoplasmic inclusion structures in the absence of viral infection. *Journal of Virology* **77**(10): 5948–5963.

Bell, T. M. and Ross, M. G. 1966. Persistent latent infection of human embryonic cells with reovirus type 3. *Nature* **212**(5060): 412–414.

Bernstein, D. I. 2009. Rotavirus overview. *Pediatric Infectious Diseases Journal* **28**(3 Suppl.): S50–S53.

Broering, T. J., McCutcheon, A. M., Centonze, V. E. and Nibert, M. L. 2000. Reovirus nonstructural protein muNS binds to core particles but does not inhibit their transcription and capping activities. *Journal of Virology* **74**(12): 5516–5524.

Campbell, J. A., Schelling, P., Wetzel, J. D., Johnson, E. M., Forrest, J. C., Wilson, G. A., Aurrand-Lions, M., et al., 2005. Junctional adhesion molecule a serves as

a receptor for prototype and field-isolate strains of mammalian reovirus. *Journal of Virology* **79**(13): 7967–7978.

Cashdollar, L. W., Esparza, J., Hudson, G. R., Chmelo, R., Lee, P. W. and Joklik, W. K. 1982. Cloning the double-stranded RNA genes of reovirus: sequence of the cloned S2 gene. *Proceedings of the National Academy of Sciences of the United States of America* **79**(24): 7644–7648.

Chandran, K., Walker, S. B., Chen, Y., Contreras, C. M., Schiff, L. A., Baker, T. S. and Nibert, M. L. 1999. In vitro recoating of reovirus cores with baculovirus-expressed outer-capsid proteins mu1 and sigma3. *Journal of Virology* **73**(5): 3941–3950.

Chandran, K., Zhang, X., Olson, N. H., Walker, S. B., Chappell, J. D., Dermody, T. S., Baker, T. S. and Nibert, M. L. 2001. Complete in vitro assembly of the reovirus outer capsid produces highly infectious particles suitable for genetic studies of the receptor-binding protein. *Journal of Virology* **75**(11): 5335–5342.

Chappell, J. D., Gunn, V. L., Wetzel, J. D., Baer, G. S. and Dermody, T. S. 1997. Mutations in type 3 reovirus that determine binding to sialic acid are contained in the fibrous tail domain of viral attachment protein sigma1. *Journal of Virology* **71**(3): 1834–1841.

Chow, N. L. and Shatkin, A. J. 1975. Blocked and unblocked 5′ termini in reovirus genome RNA. *Journal of Virology* **15**(5): 1057–1064.

Co, M. S., Gaulton, G. N., Fields, B. N. and Greene, M. I. 1985. Isolation and biochemical characterization of the mammalian reovirus type 3 cell-surface receptor. *Proceedings of the National Academy of Sciences of the United States of America* **82**(5): 1494–1498.

Connolly, J. L., Barton, E. S. and Dermody, T. S. 2001. Reovirus binding to cell surface sialic acid potentiates virus-induced apoptosis. *Journal of Virology* **75**(9): 4029–4039.

Connolly, J. L. and Dermody, T. S. 2002. Virion disassembly is required for apoptosis induced by reovirus. *Journal of Virology* **76**(4): 1632–1641.

Coombs, K. M. 2006. Reovirus structure and morphogenesis. *Current Topics in Microbiology and Immunology* **309**: 117–167.

Danthi, P., Coffey, C. M., Parker, J. S., Abel, T. W. and Dermody, T. S. 2008a. Independent regulation of reovirus membrane penetration and apoptosis by the mu1 phi domain. *PLoS Pathogens* **4**(12): e1000248.

Danthi, P., Guglielmi, K. M., Kirchner, E., Mainou, B., Stehle, T. and Dermody, T. S. 2010. From touchdown to transcription: the reovirus cell entry pathway. *Current Topics in Microbiology and Immunology* **343**: 91–119.

Danthi, P., Kobayashi, T., Holm, G. H., Hansberger, M. W., Abel, T. W. and Dermody, T. S. 2008b. Reovirus apoptosis and virulence are regulated by host cell membrane penetration efficiency. *Journal of Virology* **82**(1): 161–172.

Dennehy, P. H. 2000. Transmission of rotavirus and other enteric pathogens in the home. *Pediatric Infectious Diseases Journal* **19**(10 Suppl.): S103–S105.

Dermody, T. S., Nibert, M. L., Bassel-Duby, R. and Fields, B. N. 1990. A sigma 1 region important for hemagglutination by serotype 3 reovirus strains. *Journal of Virology* **64**(10): 5173–5176.

Domingo, E. and Holland, J. J. 1997. RNA virus mutations and fitness for survival. *Annual Review of Microbiology* **51**: 151–178.

Duncan, R., Horne, D., Strong, J. E., Leone, G., Pon, R. T., Yeung, M. C. and Lee, P. W. 1991. Conformational and functional analysis of the C-terminal globular head of the reovirus cell attachment protein. *Virology* **182**(2): 810–819.

Ebert, D. H., Deussing, J., Peters, C. and Dermody, T. S. 2002. Cathepsin L and cathepsin B mediate reovirus disassembly in murine fibroblast cells. *Journal of Biological Chemistry* **277**(27): 24609–24617.

Ebert, D. H., Kopecky-Bromberg, S. A. and Dermody, T. S. 2004. Cathepsin B is inhibited in mutant cells selected during persistent reovirus infection. *Journal of Biological Chemistry* **279**(5): 3837–3851.

Furuichi, Y., Muthukrishnan, S. and Shatkin, A. J. 1975. 5′-Terminal m-7G(5′)ppp(5′)G-m-p in vivo: identification in reovirus genome RNA. *Proceedings of the National Academy of Sciences of the United States of America* **72**(2): 742–745.

Gaillard, R. K., Li, J. K., Keene, J. D. and Joklik, W. K. 1982. The sequence at the termini of four genes of the three reovirus serotypes. *Virology* **121**(2): 320–326.

Gault, E., Schnepf, N., Poncet, D., Servant, A., Teran, S. and Garbarg-Chenon, A. 2001. A human rotavirus with rearranged genes 7 and 11 encodes a modified NSP3 protein and suggests an additional mechanism for gene rearrangement. *Journal of Virology* **75**(16): 7305–7314.

Gentsch, J. R. and Pacitti, A. F. 1985. Effect of neuraminidase treatment of cells and effect of soluble glycoproteins on type 3 reovirus attachment to murine L cells. *Journal of Virology* **56**(2): 356–364.

Gomatos, P. J. and Tamm, I. 1962. Reactive sites of reovirus type 3 and their interaction with receptor substances. *Virology* **17**: 455–461.

Gomatos, P. J. and Tamm, I. 1963. The secondary structure of reovirus RNA. *Proceedings of the National Academy of Sciences of the United States of America* **49**(5): 707–714.

Guglielmi, K. M., Johnson, E. M., Stehle, T. and Dermody, T. S. 2006. Attachment and cell entry of mammalian orthoreovirus. *Current Topics in Microbiology and Immunology* **309**: 1–38.

Hashiro, G., Loh, P. C. and Yau, J. T. 1977. The preferential cytotoxicity of reovirus for certain transformed cell lines. *Archives of Virology* **54**(4): 307–315.

Huismans, H. and Joklik, W. K. 1976. Reovirus-coded polypeptides in infected cells: isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA, respectively. *Virology* **70**(2): 411–424.

Jacobs, B. L. and Samuel, C. E. 1985. Biosynthesis of reovirus-specified polypeptides: the reovirus s1 mRNA encodes two primary translation products. *Virology* **143**(1): 63–74.

Jane-Valbuena, J., Nibert, M. L., Spencer, S. M., Walker, S. B., Baker, T. S., Chen, Y., Centonze, V. E. and Schiff, L. A. 1999. Reovirus virion-like particles obtained by recoating infectious subvirion particles with baculovirus-expressed sigma3 protein: an approach for analyzing sigma3 functions during virus entry. *Journal of Virology* **73**(4): 2963–2973.

Joklik, W. K. 1981. Structure and function of the reovirus genome. *Microbiological Reviews* **45**(4): 483–501.

Joklik, W. K. 1998. Assembly of the reovirus genome. *Current Topics in Microbiology and Immunology* **233**(Pt 1): 57–68.

Joklik, W. K. and Roner, M. R. 1995. What reassorts when reovirus genome segments reassort? *Journal of Biological Chemistry* **270**(9): 4181–4184.

Kim, M., Egan, C., Alain, T., Urbanski, S. J., Lee, P. W., Forsyth, P. A. and Johnston, R. N. 2007. Acquired resistance to reoviral oncolysis in Ras-transformed fibrosarcoma cells. *Oncogene* **26**(28): 4124–4134.

Kim, M., Garant, K. A., zur Nieden, N. I., Alain, T., Loken, S. D., Urbanski, S. J., Forsyth, P. A., Rancourt, D. E., Lee, P. W. and Johnston, R. N. 2011. Attenuated reovirus displays oncolysis with reduced host toxicity. *British Journal of Cancer* **104**(2): 290–299.

Kirchner, E., Guglielmi, K. M., Strauss, H. M., Dermody, T. S. and Stehle, T. 2008. Structure of reovirus sigma1 in complex with its receptor junctional adhesion molecule-A. *PLoS Pathogens* **4**(12): e1000235.

Kobayashi, T., Antar, A. A., Boehme, K. W., Danthi, P., Eby, E. A., Guglielmi, K. M., Holm, G. H., *et al*. 2007. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. *Cell Host Microbe* **1**(2): 147–157.

Kobayashi, T., Ooms, L. S., Chappell, J. D. and Dermody, T. S. 2009. Identification of functional domains in reovirus replication proteins muNS and mu2. *Journal of Virology* **83**(7): 2892–2906.

Kobayashi, T., Ooms, L. S., Ikizler, M., Chappell, J. D. and Dermody, T. S. 2010. An improved reverse genetics system for mammalian orthoreoviruses. *Virology* **398**(2): 194–200.

Komoto, S., Sasaki, J. and Taniguchi, K. 2006. Reverse genetics system for introduction of site-specific mutations into the double-stranded RNA genome of infectious rotavirus. *Proceedings of the National Academy of Sciences of the United States of America* **103**(12): 4646–4651.

Lal, R., Harris, D., Postel-Vinay, S. and de Bono, J. 2009. Reovirus: Rationale and clinical trial update. *Current Opinion in Molecular Therapy* **11**(5): 532–539.

Lee, P. W., Hayes, E. C. and Joklik, W. K. 1981. Protein sigma 1 is the reovirus cell attachment protein. *Virology* **108**(1): 156–163.

Leone, G., Duncan, R., Mah, D. C., Price, A., Cashdollar, L. W. and Lee, P. W. 1991. The N-terminal heptad repeat region of reovirus cell attachment protein sigma 1 is responsible for sigma 1 oligomer stability and possesses intrinsic oligomerization function. *Virology* **182**(1): 336–345.

Lindholm, L., Henning, P. and Magnusson, M. K. 2008. Novel strategies in tailoring human adenoviruses into therapeutic cancer gene-therapy vectors. *Future Virology* **3**, 45–59.

Liu, Y., Nusrat, A., Schnell, F. J., Reaves, T. A., Walsh, S., Pochet, M. and Parkos, C. A. 2000. Human junction adhesion molecule regulates tight junction resealing in epithelia. *Journal of Cell Science* **113**(Pt 13): 2363–2374.

Maginnis, M. S., Forrest, J. C., Kopecky-Bromberg, S. A., Dickeson, S. K., Santoro, S. A., Zutter, M. M., Nemerow, G. R., *et al*. 2006. Beta1 integrin mediates internalization of mammalian reovirus. *Journal of Virology* **80**(6): 2760–2770.

Maginnis, M. S., Mainou, B. A., Derdowski, A., Johnson, E. M., Zent, R. and Dermody, T. S. 2008. NPXY motifs in the beta1 integrin cytoplasmic tail are required for functional reovirus entry. *Journal of Virology* **82**(7): 3181–3191.

Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C. *et al*. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *Journal of Cell Biology* **142**(1): 117–127.

Minuk, G. Y., Paul, R. W. and Lee, P. W. 1985. The prevalence of antibodies to reovirus type 3 in adults with idiopathic cholestatic liver disease. *Journal of Medical Virology* **16**(1): 55–60.

Minuk, G. Y., Rascanin, N., Paul, R. W., Lee, P. W., Buchan, K. and Kelly, J. K. 1987. Reovirus type 3 infection in patients with primary biliary cirrhosis and primary sclerosing cholangitis. *Journal of Hepatology* **5**(1): 8–13.

Miura, K., Watanabe, K., Sugiura, M. and Shatkin, A. J. 1974. The 5′-terminal nucleotide sequences of the double-stranded RNA of human reovirus. *Proceedings of the National Academy of Sciences of the United States of America* **71**(10): 3979–3983.

Mora, M., Partin, K., Bhatia, M., Partin, J. and Carter, C. 1987. Association of reovirus proteins with the structural matrix of infected cells. *Virology* **159**(2): 265–277.

Nagata, L., Masri, S. A., Pon, R. T. and Lee, P. W. 1987. Analysis of functional domains on reovirus cell attachment protein sigma 1 using cloned S1 gene deletion mutants. *Virology* **160**(1): 162–168.

Nibert, M. L., Dermody, T. S. and Fields, B. N. 1990. Structure of the reovirus cell-attachment protein: a model for the domain organization of sigma 1. *Journal of Virology* **64**(6): 2976–2989.

Nonoyama, M., Millward, S. and Graham, A. F. 1974. Control of transcription of the reovirus genome. *Nucleic Acids Research* **1**(3): 373–385.

Norman, K. L., Hirasawa, K., Yang, A. D., Shields, M. A. and Lee, P. W. 2004. Reovirus oncolysis: the Ras/RalGEF/p38 pathway dictates host cell permissiveness to reovirus infection. *Proceedings of the National Academy of Sciences of the United States of America* **101**(30): 11099–11104.

Novella, I. S., Duarte, E. A., Elena, S. F., Moya, A., Domingo, E. and Holland, J. J. 1995. Exponential increases of RNA virus fitness during large population transmissions. *Proceedings of the National Academy of Sciences of the United States of America* **92**(13): 5841–5844.

Oncolytics Biotech Inc. 2011. Clinical trials. http://www.oncolyticsbiotech.com/clinical.html.

Pacitti, A. F. and Gentsch, J. R. 1987. Inhibition of reovirus type 3 binding to host cells by sialylated glycoproteins is mediated through the viral attachment protein. *Journal of Virology* **61**(5): 1407–1415

Paul, R. W. and Lee, P. W. 1987. Glycophorin is the reovirus receptor on human erythrocytes. *Virology* **159**(1): 94–101.

Pesavento, J. B., Crawford, S. E., Estes, M. K. and Prasad, B. V. 2006. Rotavirus proteins: structure and assembly. *Current Topics in Microbiology and Immunology* **309**, 189–219.

Power, A. T. and Bell, J. C. 2008. Taming the Trojan horse: optimizing dynamic carrier cell/oncolytic virus systems for cancer biotherapy. *Gene Therapy* **15**(10): 772–779.

Roner, M. R., Bassett, K. and Roehr, J. 2004. Identification of the 5′ sequences required for incorporation of an engineered ssRNA into the Reovirus genome. *Virology* **329**(2): 348–360.

Roner, M. R. and Joklik, W. K. 2001. Reovirus reverse genetics: Incorporation of the CAT gene into the reovirus genome. *Proceedings of the National Academy of Sciences of the United States of America* **98**(14): 8036–8041.

Roner, M. R., Nepliouev, I., Sherry, B. and Joklik, W. K. 1997. Construction and characterization of a reovirus double temperature-sensitive mutant. *Proceedings of the National Academy of Sciences of the United States of America* **94**(13): 6826–6830.

Roner, M. R. and Roehr, J. 2006. The 3′ sequences required for incorporation of an engineered ssRNA into the Reovirus genome. *Virology Journal* **3**: 1.

Roner, M. R. and Steele, B. G. 2007a. Features of the mammalian orthoreovirus 3 Dearing l1 single-stranded RNA that direct packaging and serotype restriction. *Journal of General Virology* **88**(Pt 12): 3401–3412.

Roner, M. R. and Steele, B. G. 2007b. Localizing the reovirus packaging signals using an engineered m1 and s2 ssRNA. *Virology* **358**(1): 89–97.

Roner, M. R., Sutphin, L. A. and Joklik, W. K. 1990. Reovirus RNA is infectious. *Virology* **179**(2): 845–852.

Rouault, E. and Lemay, G. 2003. Incorporation of epitope-tagged viral sigma3 proteins to reovirus virions. *Canadian Journal of Microbiology* **49**(7): 407–417.

Rubin, D. H., Wetzel, J. D., Williams, W. V., Cohen, J. A., Dworkin, C. and Dermody, T. S. 1992. Binding of type 3 reovirus by a domain of the sigma 1 protein important for hemagglutination leads to infection of murine erythroleukemia cells. *Journal of Clinical Investigations* **90**(6): 2536–2542.

Sarkar, G., Pelletier, J., Bassel-Duby, R., Jayasuriya, A., Fields, B. N. and Sonenberg, N. 1985. Identification of a new polypeptide coded by reovirus gene S1. *Journal of Virology* **54**(3): 720–725.

Schlesinger, R. W., Stollar, V., Guild, G. M., Igarashi, A., Shenk, T. E. and Peleg, J. 1977. The significance and nature of defective interfering viruses. *Bulletin Schweiz.Akad.Med. Wissenschaft* **33**(4–6): 229–242.

Severson, E. A. and Parkos, C. A. 2009. Structural determinants of Junctional Adhesion Molecule A (JAM-A): function and mechanisms of intracellular signaling. *Current Opinion in Cell Biology* **21**(5): 701–707.

Sharpe, A. H. and Fields, B. N. 1982. Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene. *Virology* **122**(2): 381–391.

Shatkin, A. J., Sipe, J. D. and Loh, P. 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. *Journal of Virology* **2**(10): 986–99.1

Simpson, E., Wittet, S., Bonilla, J., Gamazina, K., Cooley, L. and Winkler, J. L. 2007. Use of formative research in developing a knowledge translation approach to rotavirus vaccine introduction in developing countries. *BMC Public Health* **7**: 281.

Smakman, N., van den Wollenberg, D. J., Borel Rinkes, I. H., Hoeben, R. C. and Kranenburg, O. 2005. Sensitization to apoptosis underlies KrasD12-dependent oncolysis of murine C26 colorectal carcinoma cells by reovirus T3D. *Journal of Virology* **79**(23): 14981–14985.

Smakman, N., van den Wollenberg, D. J., Elias, S. G., Sasazuki, T., Shirasawa, S., Hoeben, R. C., Borel Rinkes, I. H. and Kranenburg, O. 2006. KRAS(D13): Promotes apoptosis of human colorectal tumor cells by ReovirusT3D and oxaliplatin but not by tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Research* **66**(10): 5403–5408.

Spandidos, D. A., Krystal, G. and Graham, A. F. 1976. Regulated transcription of the genomes of defective virions and temperature-sensitive mutants of reovirus. *Journal of Virology* **18**(1): 7–19.

Spendlove, R. S., McClain, M. E. and Lennette, E. H. 1970. Enhancement of reovirus infectivity by extracellular removal or alteration of the virus capsid by proteolytic enzymes. *Journal of General Virology* **8**(2): 83–94.

Spriggs, D. R. and Fields, B. N. 1982. Attenuated reovirus type 3 strains generated by selection of haemagglutinin antigenic variants. *Nature* **297**(5861): 68–70.

Starnes, M. C. and Joklik, W. K. 1993. Reovirus protein lambda 3 is a poly(C)-dependent poly(G): polymerase. *Virology* **193**(1): 356–366.

Stehle, T. and Dermody, T. S. 2003. Structural evidence for common functions and ancestry of the reovirus and adenovirus attachment proteins. *Review of Medical Virology* **13**(2): 123–132.

Steinhauer, D. A., Domingo, E. and Holland, J. J. 1992. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* **122**(2): 281–288.

Strong, J. E., Leone, G., Duncan, R., Sharma, R. K. and Lee, P. W. 1991. Biochemical and biophysical characterization of the reovirus cell attachment protein sigma 1: evidence that it is a homotrimer. *Virology* **184**(1): 23–32.

Taber, R., Alexander, V. and Whitford, W. 1976. Persistent reovirus infection of CHO cells resulting in virus resistance. *Journal of Virology* **17**(2): 513–524.

Theiss, J. C., Stoner, G. D. and Kniazeff, A. J. 1978. Effect of reovirus infection on pulmonary tumor response to urethan in strain A mice. *Journal of National Cancer Institute* **61**(1): 131–134.

Trask, S. D., Taraporewala, Z. F., Boehme, K. W., Dermody, T. S. and Patton, J. T. 2010. Dual selection mechanisms drive efficient single-gene reverse genetics for rotavirus. *Proceedings of the National Academy of Sciences of the United States of America* **107**(43): 18652–18657.

Troupin, C., Dehee, A., Schnuriger, A., Vende, P., Poncet, D. and Garbarg-Chenon, A. 2010. Rearranged genomic RNA segments offer a new approach to the reverse genetics of rotaviruses. *Journal of Virology* **84**(13): 6711–6719.

Turner, D. L., Duncan, R. and Lee, P. W. 1992. Site-directed mutagenesis of the C-terminal portion of reovirus protein sigma 1: evidence for a conformation-dependent receptor binding domain. *Virology* **186**(1): 219–227.

Tyler, K. L., Clarke, P., Debiasi, R. L., Kominsky, D. and Poggioli, G. J. 2001. Reoviruses and the host cell. *Trends in Microbiology* **9**(11): 560–564.

Tyler, K. L. and Fields, B. N. 2001. Mammalian reoviruses. In D. M. Knipe and P. M. Howely, eds., *Fields Virology*, 4th edn. Lippincott, Williams and Wilkins: Philadelphia, PA, pp. 1729–1745.

Tyler, K. L., Squier, M. K., Brown, A. L., Pike, B., Willis, D., Oberhaus, S. M., Dermody, T. S. and Cohen, J. J. 1996. Linkage between reovirus-induced apoptosis and inhibition of cellular DNA synthesis: role of the S1 and M2 genes. *Journal of Virology* **70**(11): 7984–7991.

Tyler, K. L., Squier, M. K., Rodgers, S. E., Schneider, B. E., Oberhaus, S. M., Grdina, T. A., Cohen, J. J. and Dermody, T. S. 1995. Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein sigma 1. *Journal of Virology* **69**(11): 6972–6979.

van den Wollenberg, D. J., Van den Hengel, S. K., Dautzenberg, I. J., Cramer, S. J., Kranenburg, O. and Hoeben, R. C. 2008. A strategy for genetic modification of the spike-encoding segment of human reovirus T3D for reovirus targeting. *Gene Therapy* **15**(24): 1567–1578.

van den Wollenberg, D. J., Van den Hengel, S. K., Dautzenberg, I. J., Kranenburg, O. and Hoeben, R. C. 2009. Modification of mammalian reoviruses for use as oncolytic agents. *Expert Opinion on Biological Therapy* **9**(12): 1509–1520.

ViralZone 2011. Orthoreovirus. http://expasy.org/viralzone/all_by_species/105.html.

Waehler, R., Russell, S. J. and Curiel, D. T. 2007. Engineering targeted viral vectors for gene therapy. *Nature Reviw Genetics* **8**(8): 573–587.

Watanabe, Y., Millward, S. and Graham, A. F. 1968. Regulation of transcription of the Reovirus genome. *Journal of Molecular Biology* **36**(1): 107–123.

Weiner, H. L., Ault, K. A. and Fields, B. N. 1980a. Interaction of reovirus with cell surface receptors. I. Murine and human lymphocytes have a receptor for the hemagglutinin of reovirus type 3. *Journal of Immunology* **124**(5): 2143–2148.

Weiner, H. L., Drayna, D., Averill, D. R., Jr. and Fields, B. N. 1977. Molecular basis of reovirus virulence: role of the S1 gene. *Proceedings of the National Academy of Sciences of the United States of America* **74**(12): 5744–5748.

Weiner, H. L. and Fields, B. N. 1977. Neutralization of reovirus: the gene responsible for the neutralization antigen. *Journal of Expiramental Medicine* **146**(5): 1305–1310.

Weiner, H. L., Powers, M. L. and Fields, B. N. 1980b. Absolute linkage of virulence and central nervous system cell tropism of reoviruses to viral hemagglutinin. *Journal of Infectious Diseases* **141**(5): 609–616.

Weiner, H. L., Ramig, R. F., Mustoe, T. A. and Fields, B. N. 1978. Identification of the gene coding for the hemagglutinin of reovirus. *Virology* **86**(2): 581–584.

Wetzel, J. D., Wilson, G. J., Baer, G. S., Dunnigan, L. R., Wright, J. P., Tang, D. S. and Dermody, T. S. 1997. Reovirus variants selected during persistent infections of L cells contain mutations in the viral S1 and S4 genes and are altered in viral disassembly. *Journal of Virology* **71**(2): 1362–1369.

Williams, W. V., Guy, H. R., Rubin, D. H., Robey, F., Myers, J. N., Kieber-Emmons, T., Weiner, D. B. and Greene, M. I. 1988. Sequences of the cell-attachment sites of reovirus type 3 and its anti-idiotypic/antireceptor antibody: modeling of their three-dimensional structures. *Proceedings of the National Academy of Sciences of the United States of America* **85**(17): 6488–6492.

Wilson, G. J., Wetzel, J. D., Puryear, W., Bassel-Duby, R. and Dermody, T. S. 1996. Persistent reovirus infections of L cells select mutations in viral attachment protein sigma1 that alter oligomer stability. *Journal of Virology* **70**(10): 6598–6606.

Zou, S. and Brown, E. G. 1992. Identification of sequence elements containing signals for replication and encapsidation of the reovirus M1 genome segment. *Virology* **186**(2): 377–388.

Part IV

Recent and future developments

11

Reverse genetics and quasispecies

Antonio V. Bordería and Marco Vignuzzi
Institut Pasteur, Paris

11.1 Definition of quasispecies and evidence

11.1.1 The original theory

The quasispecies concept was first introduced into the virology field to describe the hypothesised behaviour of self-replicating macromolecules in the primordial world. The original theory was initially postulated by Manfred Eigen in 1971 (Eigen, 1971), although the term itself was not coined by him until 1977 (Eigen and Schuster, 1977). According to this theory, macromolecules self-replicate to large numbers at very fast rates, but due to the lack of proofreading mechanisms, most of these newly generated molecules contain changes compared to the initial molecule. This results in a population of extreme heterogeneity forming around the original founder molecules (Eigen and Schuster, 1977). These macromolecules are closely related and linked to each other since they can regenerate one another by mutation. In other words, the frequency of one variant within the population is not only determined by its intrinsic replication, but also by the likelihood of being regenerated through replication error from other variants. Therefore, these molecules form a swarm of inseparable and related molecules, termed *quasispecies*. This last point is the most important part of the theory: this inseparable coupling dictates that selection acts at the level of the whole, on the group, rather than on individuals such that the population behaves like (*quasi*-) a single unit (*species*) (Eigen and Biebricher, 1988).

11.1.2 Quasispecies and virology

The theory as we know it in virology, and use with relative and varying degrees of stringency, was adapted soon afterwards from Eigen's original ideas, following some key observations of RNA viruses. It was in 1978 that Domingo *et al*.

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

(Domingo *et al*., 1978), in studying serially passaged QB phage clones, first demonstrated that RNA viruses, once thought to be clonal, generated highly heterogeneous populations. They found that 15% of the clones studied showed deviations from the total RNA population fingerprinting. From this, they calculated that on average, viable phage should differ in one or two mutations from the parental population. For this reason and in satisfying several principals of Eigen's theory such as extreme population sizes, replication rates and heterogeneity, RNA viruses seemed perfectly suited to the quasispecies theory. Importantly, the application of this theory to the field introduced a new complexity to how we viewed RNA viruses, merging more elaborate concepts from population genetics and virology. New ideas and experiments were designed using the theory as a base to test and develop evolutionary concepts such as the Red Queen Hypothesis (Van Valen, 1973; Clarke *et al*., 1994), Muller's ratchet (Müller, 1964), recovery of virus fitness by large population passages (Martínez *et al*., 1991; Clarke *et al*., 1993; Novella, *et al*., 1995a; Escarmis *et al*., 1999; Yuste *et al*., 2005), founder effect and migration (Cuevas *et al*., 2003). Until then, virologists had enjoyed a relatively simplistic and reductionist view of RNA viruses, that continues to a certain extent today: regardless of whether we are speaking of one or one million dengue particles during infection of a host, for example, we still use the singular 'dengue virus'. The term quasispecies, for many virologists, helped experimentalists switch views from molecular studies asking questions related to single particles, to the wider population. Although it is argued that the use of the term brings more confusion than clarity (see below), it is certain that without its introduction into virology, the study of virus evolution and adaptation would not have advanced as rapidly as it has done.

11.1.3 Quasispecies and population genetics

Despite the validity of the quasispecies theory to mathematically explain the physico-chemical behaviour of the first self-replicating macromolecules, population geneticists challenge its necessity to explain RNA virus populations, arguing that classic population genetics theories exist than can already explain their behaviour (Holmes and Moya, 2002; Holmes, 2010). At best, they consider quasispecies as a valid model within population genetics occurring under a particular set of conditions (Wilke, 2005). Several authors have made the valid point that the framework and evolutionary terms with which to describe virus populations already exist in genetics without the need to confer an almost 'mystical' quality to these organisms by transferring to them terms from the other fields (Holmes and Moya, 2002). Indeed, even group or kin selection, as described by population genetics, could cover much of the ground postulated by quasispecies theory. To be fair, it can also be argued that using the quasispecies as a biological analogy is no more forcing a scenario to fit biology than many other concepts of population genetics, such as the Red Queen hypothesis originating from literature, Muller's ratchet originating from engineering. Beyond the personal preferences for one term or another, population geneticists

raise a valid note of caution: if the quasispecies theory is to be used to explain viral population dynamics, then several key aspects need to be clarified beforehand, and although experimental work has been done to tackle this problem, no paper has yet made this observation in an irrefutable way.

11.1.4 Proving the theory: which conditions must be satisfied

For most researchers in the virology field, quasispecies is merely a term or synonym that encompasses mixed virus populations and genomic variability, very frequently used to describe clinical specimens of chronically infected HIV or HCV patients that present virus populations of great heterogeneity. However, in these cases the term is often detached from the theoretical assumptions, irking scientists more cautious of the genetic terms to be used (Eigen, 1996; Holmes and Moya, 2002). One should perhaps be more careful when referring to RNA viruses as quasispecies, to define how strictly or loosely the term is being used. One way to avoid confusion, as pointed out by many population geneticists and virologists, is to refer to these viral populations as heterogeneous populations or mutant spectra, unless a study is directly trying to understand or test the quasispecies theory. Amidst this confusion, efforts continue to try to solve whether quasispecies is applicable to viruses, either theoretically or experimentally. In the next paragraphs we will explain the different conditions that virus populations must fulfil in order to behave as quasispecies, with current examples and clues that argue in favour or against.

Infinite population sizes

In the original formulation of the theory (Eigen, 1971), the macromolecule populations were considered to be of infinite size. The reason being that the theory required the population to occupy all of sequence space, where no genetic drift was possible. In sequence space, a molecule of length n could occupy a sequence space of n dimensions, and each of the 4^n possible variants of that sequence (from the four possible nucleotides and n nucleotide length) occupies a unique point in that space. Although virus populations can reach very large sizes, they arguably remain for the most part of infections, far from infinity (Jenkins *et al*., 2001). As an example, it is estimated that HIV-1 produces 10^{10} particles per day in an infected individual (Rambaut *et al*., 2004), a considerable size, but not even close to 4^{9200} needed to explore the full sequence space of HIV-1. Nevertheless, modern interpretations of the quasispecies theory suggest that infinite population sizes may not be necessary if genetic drift does not have a big effect on the population (Wilke, 2005). *In silico* experiments by Comas *et al*. support this idea (Comas *et al*., 2005). Working with digital organisms with a range of 'genome size' between 54 to 272, they showed that even populations as small as 250 showed evidence of quasispecies behaviour, such as coupling in selection and survival of the flattest (to be discussed later). Thus, according to new interpretations of the theory, viruses could potentially behave as a

quasispecies, at least from the theoretically standpoint although more experimental work, either *in vitro* or *in vivo*, is required to assess this.

High mutation rates

Quasispecies theory states that the different variants within the viral populations should be coupled in selection. This coupling would be the consequence of high mutation rates. Since the Domingo *et al* 1978 paper, it has become quite evident that RNA viruses are highly error prone, exhibiting the highest mutation rates in nature. Several papers have estimated mutation rates in viruses: bacteriophage Qβ (Domingo and Flavell, 1976), poliovirus 1 (PV-1) (la Torre *et al*., 1992; Drake and Holland, 1999), hepatitis C virus (HCV) (José M Cuevas *et al*., 2009), vesicular stomatitis virus (VSV) (Holland *et al*., 1989; Drake and Holland, 1999), Human immunodeficiency virus 1 (HIV-1) (Mansky and Temin, 1995), to highlight a few that have been recently reviewed and synthesised by Rafael Sanjuán (Sanjuán *et al*., 2010), based on previous work by Drake (Drake *et al*., 1998; Drake and Holland, 1999). One can consider that RNA viruses produce on average one mutation/genome/replication. As Drake and Holland pointed out (Drake and Holland, 1999), this is a very large number and could well fall within the requirements of quasispecies theory. Nevertheless, recent *in silico* studies concluded that for the quasispecies dynamic to arise, mutation rates should be even higher than what has been observed, at an average of 2 mutations/genome/replication (Wilke *et al*., 2001; Comas *et al*., 2005).

Unit of selection

One of the key predictions of quasispecies theory and perhaps the most difficult (or impossible?) to test and confirm is the unit of selection. According to theory, the unit of selection should be the group or cluster of related variants, or quasispecies. This is derived from the coupling of the different genomes due to the high mutation rates. Importantly, experimental work by Esteban Domingo (Domingo *et al*., 1978; Clarke *et al*., 1993; Duarte *et al*., 1994) showed that the fitness of individual virus clones from a virus population was usually lower than the average fitness of the whole population. He concluded that the behaviour of the viral quasispecies could not be entirely explained by its individual components, and suggested that selection was thus acting on a larger group. Despite this and other experimental work, classical evolutionary biologists point out that a similar concept under the name of group selection already exists (Holmes and Moya, 2002), without the need to invoke the quasispecies theory for viruses.

11.1.5 Experimental testing of quasispecies concepts

As mentioned, although absolute proof of quasispecies behaviour in RNA viruses has not yet been achieved, its testing has led to very important contributions in

understanding RNA viruses as populations. Early studies sought to comprehend how virus populations evolved in terms of fitness depending on the initial population structure (for example, size). Lin Chao published in 1990 one of the first papers exploring quasispecies dynamics and its correlation with virus fitness, the replicative advantage of one population under a specific environment (Chao, 1990). Usually, growth-competition experiments are used to determine the relative fitness value of one population against a reference, which arbitrarily is set as having fitness of 1. In this paper, phage Φ6 was subjected to repeated bottlenecking events, effectively reducing the population size in each plaque-to-plaque passage to 1 and, after several passages, the fitness was significantly reduced compared to the relatively high fitness of the original population. Under the quasispecies theory, this fitness reduction could be explained by stochastic sampling of the mutant spectrum (Figure 11.1), where most individual mutations are both rare and deleterious, and masked by compensatory action of neighbouring genomes. When the virus is subjected to bottlenecks over several passages, the mutational load of the viral population will increase due to founder effects of these deleterious mutants and result in a decrease in average fitness. This is also known as Muller's ratchet (Müller, 1964) in analogy to a mechanical ratchet – once a notch has been turned, there is no possibility of going back and the only option is to keep on moving the ratchet forward. These initial observations were supported by several other groups in different animal or plant viruses (Duarte *et al*., 1992; Duarte *et al*., 1993; Escarmís *et al*., 1996; Yuste *et al*., 1999; la Iglesia and Elena, 2007).

Despite the debilitation of viruses subjected to bottlenecks, they recovered fitness if allowed to replicate in large population sizes, as demonstrated for phage Φ6 (Burch and Chao, 1999), FMDV (Escarmis *et al*., 1999), VSV (Novella *et al*., 2005) and HIV-1 (Yuste *et al*., 2005; Bordería *et al*., 2010). Early studies focused on fitness-recovery mutations that fixated to consensus, which were in general few and

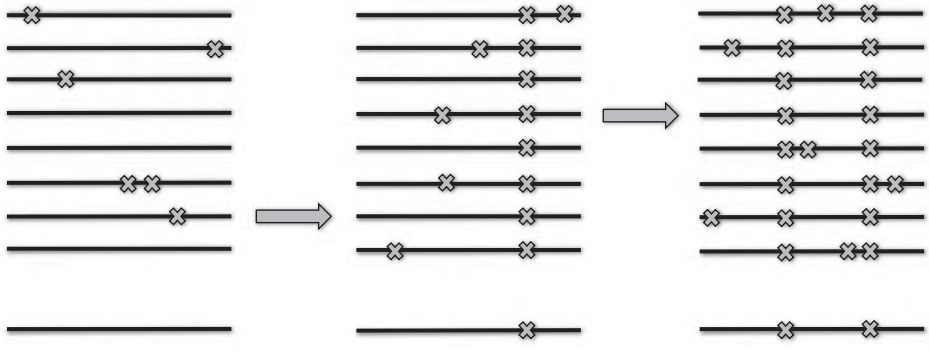

Figure 11.1 Viral mutant spectrum and consensus sequence after consequent bottlenecks of 1 genome. From left to right, if we generate a new population in a new host by randomly selecting one genome from the previous population, we will by chance be selecting a variant that likely carries a deleterious or lower fitness mutation. If we keep passaging in this way, each round will increase the mutational load and produce the effect known as Muller's ratchet.

far between (Yuste *et al*., 2005). More recently, in an HIV-1 study, attention turned to the overall gain in population diversity, noting that fitness increases are often accompanied by sweeping increases in genetic diversity (possible group selection) rather than single, individual genotypes (Bordería *et al*., 2010). Nevertheless this study did not imply that variability is the sole cause of the fitness increase, and did not provide direct evidence of the quasispecies behaviour.

Again, in 2006, two groups (Pfeiffer and Kirkegaard; Vignuzzi and Andino) independently investigated viral quasispecies experimentally using a high fidelity variant of poliovirus, which generated sixfold fewer mutations than wild-type. Both wild-type and high fidelity viruses replicated with similar kinetics, but high fidelity virus generated a significantly less diverse population (a more restricted quasispecies). In mice, high fidelity virus presented a less virulent phenotype (reduced dissemination and lethality) (Pfeiffer and Kirkegaard, 2005; Vignuzzi *et al*., 2006). By restoring the heterogeneity of the high fidelity virus population through chemical mutagenesis to resemble wild-type, dissemination and virulence was also restored (Vignuzzi *et al*., 2006). The authors considered that variability was central to fitness and that undefined sub-populations existing within the wild type population formed a quasispecies that was selected as a unit, due to beneficial cooperation among members. Whether one can consider populations in different compartments, as part of the same quasispecies and therefore under the same selective pressure is uncertain. As pointed out by other authors (Holmes and Moya, 2002; Holmes, 2010), variability is not the only requirement of the quasispecies theory, and most significantly, cooperation is not evidence for it.

One of the most interesting consequences of the quasispecies dynamics in viruses, is that under certain conditions a lower fitness population should outcompete a higher fitness population (la Torre and Holland, 1990). This has been termed *survival of the flattest* in contraposition to the *survival of the fittest* notions of Darwinian evolution (Wilke *et al*., 2001). Selection expectedly acts to push virus populations to increase fitness and replicate faster, displacing in the mutant spectrum those variants that replicate at lower rates and titres. In a situation of low mutation rates this is maintained by the mutation–selection equilibrium, in which selection against deleterious mutations in the population is in a steady state with the rate of generation of those same mutations. In this condition, the virus should select a population composed primarily of very high fitness variants surrounded by lower fitness variants that are strongly selected against, generating a sharp and narrow distribution of variants (Figure 11.2, population A, survival of the fittest). Alternatively, another population, B, could be composed of a wider range of variants with less dramatic differences in fitness with respect to each other, and lower fitness with respect to population A. The population distribution of B would be flatter and wider compared to A (survival of the flattest). In a situation of low mutation rates and mutation–selection equilibrium, deleterious mutations would be under negative selection in both populations and in competition, A would win because of faster replication (high fitness). If the situation changes and mutation rates increase substantially, deleterious mutations would be generated faster than they could be

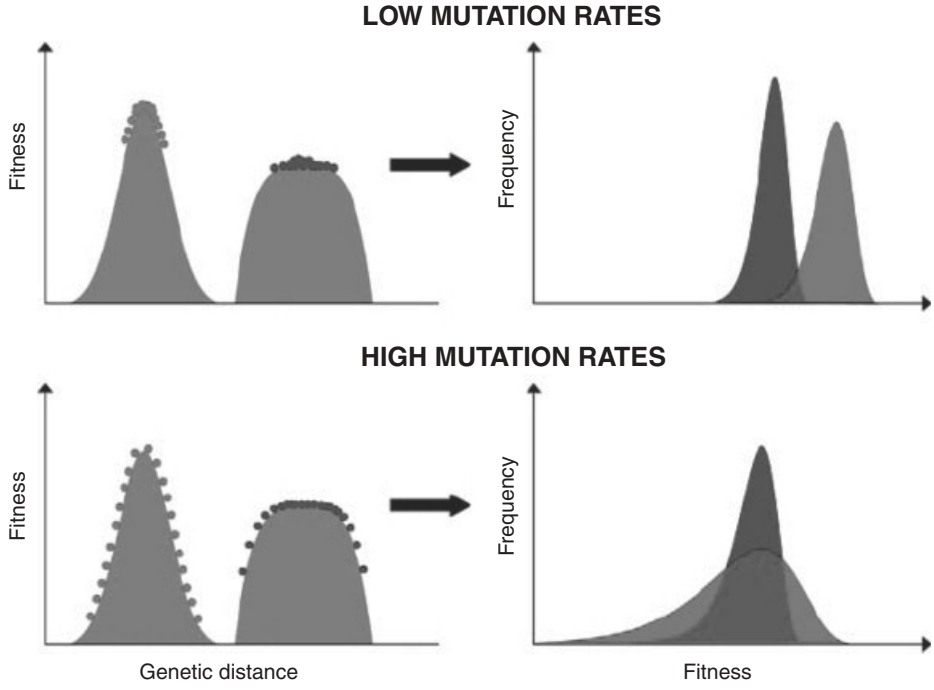

Figure 11.2 Robustness in RNA viruses. Two virus populations are shown in the left panel, one is fittest (on the left), one is robust (on the right). In situations of low mutation rates (upper panels), the fittest population will outcompete the other because of its higher fitness in these growth conditions. In situations of high mutations rates (lower panels), the robust population will outcompete the fitter one, since it can better buffer the effects of increased deleterious mutations. *Source:* Taken from Figure 1 (Sanjuán *et al*. 2007).

cleansed by selection, producing an increase in mutational load in both virus populations. Since B is composed of neighbouring genomes of similar fitness, the mutation of one genome to become its neighbour will have little effect on overall fitness (a population that is genetically 'robust'). Population A on the contrary will be very sensitive to high mutation rates because most members of the population are the same, high fitness genome that when changed to a neighbouring genome, suffer significant fitness decreases. In this scenario, population A would be outcompeted by population B (survival of the flattest).

In support of this, Codoñer *et al*. observed in 2006 evidence of the survival of the flattest in viroids (Codoñer *et al*., 2006). Two different species of viroids were studied, *Chrysanthemum stunt pospiviroid* (CSVd) and *Chrysanthemum chlorotic mottle avsunviroid* (CChMVd). Because CChMVd showed lower replication rates and high genetic variability, it was regarded as a robust viroid when compared to CSVd (similar to the A and B populations above). Two sets of plants infected with the two species of viroids were prepared, one that were exposed to mutagenic, ultraviolet

C light (UVC) and one mock-exposed. Viroids in the UVC-treated plants showed a significant increase in mutation rates. In normal conditions and in competition, the lower fitness CChMVd was displaced by CSVd, in agreement with the survival of the fittest. On the contrary, under high mutation rates (UVC irradiation) CSVd could not out-compete CChMVd, in agreement with the survival of the flattest effect. This paper is an elegant example of the survival of the flattest, as predicted if viruses follow a quasispecies dynamic. In another study of robustness (Sanjuán *et al*., 2007) individual clones were passaged 100 times, that were originally picked from two different VSV populations, one which had never been passaged in cell lines (population A) and another which had been extensively passaged in cell lines (population B). Comparing fitness before and after passage, population A significantly increased its fitness while B did so marginally. When 1000 individual clones from A and B were assayed for fitness, they found that A was the fittest but with a narrow distribution of fitness variants while B had lower fitness but with a wider distribution and variability, possibly indicating higher robustness. When both populations were competed in the presence or absence of a mutagen to increase mutation rates, B fared better in the mutagenic conditions and outcompeted A.

11.2 Reverse genetics and RNA virus population heterogeneity: consensus is always a compromise

Reverse genetics is especially important to virology because viruses are capable of generating many mutations, duplications, deletions and insertions in their genomes. Normally, the technique is used to study mutations that are fixed to the consensus sequence, those that are in the majority of the variants within the population. If a mutation has fixed to consensus, it often confers some kind of fitness advantage and understanding the nature of such mutations is critical to deciphering the observed changes in phenotype. The caveat is that in studying genetic clones, we are negating the natural variability within the virus population. Notwithstanding, mutants taken in isolation can give very significant insights into lost, gained or altered functions, such as alternative cleavage of viral proteins, capsid formation, viral polymerase activity, immune escape and changes in host range tropism. Additionally, other mutations might be deleterious or outright lethal, and others still might be entirely neutral. For some mutations that fall within known functional domains or in proteins with crystal structure, their effects can be more or less deduced. For others, nothing can be presupposed. Regardless, isolation of the mutant is the best means of understanding its contribution to phenotype and reverse genetics is the cleanest method to do so. A prime example of this is the identification of key attenuating determinants for the Sabin strains of poliovirus that constitute the oral polio vaccines. Poliovirus type 1, 2 and 3 were attenuated empirically by lengthy serial passage on non-human substrates, resulting in the accumulation of a dozen

(type 3) to over 50 (type 1) consensus changes. Thanks in large part to reverse genetics, the main determinants of virulence/attenuation were identified, including point mutations in domain V of the viral internal ribosome entry site (IRES) (Wimmer *et al*., 1993) that restore neuropathology when they revert to wild type. Through reverse genetics, these attenuating mutations were shown to decrease viral translation through deficient association of the cellular translation factor eEIF4G to the IRES domain V and impair ribosome interaction (Ochs *et al*., 2003). Similarly, the use of reverse genetics in HIV-1 permitted the understanding of drug resistance to antivirals targeting the Retrotranscriptase (RT) or Protease (PR). In order to verify resistance, site-directed mutagenesis using a genetic clone and assaying for the concentration of drug required to inhibit the virus by 50% or 90% (IC_{50} or IC_{90}, (Shafer, 2002)), provided a ranking by which to organise the potency of the resistance mutation to better diagnose and treat patients (Stanford HIV-1 Drug Database, http://hivdb.stanford.edu/).

11.2.1 Where to start? Generating an infectious clone from a quasispecies

Before generating an infectious cDNA clone with which to perform reverse genetics one has to decide on the starting population (Figure 11.3). Should a clinical isolate or a cell culture stock be used? Should the virus be plaque-purified or grown at large multiplicity of infection? An advantage to cell culture stocks is the high titre attainable for the virus of interest (usually between 10^6–10^{12} infectious units/ml), which is not often obtained in clinical isolates. Moreover, the virus is at least partially adapted to the culture conditions, carrying specific mutations that favour replication and infection of the cell type, thereby increasing the chances of obtaining an infectious and viable virus once reverse genetics is performed. Furthermore, a well-adapted virus in a well-characterised, stable cellular environment is often ideal for testing new mutations because viral proteins will be expressed in high quantities and different phenotypes can be more easily distinguished. In order to obtain a cell culture isolate it is easier to start from a large population passage with high titres, but plaque purified viruses could be equally suitable in certain situations. Large population passages are best to obtain the most representative genome in the mutant spectrum in hopes of obtaining the consensus genome of the viral population. In contrast, plaque purifying virus or obtaining them through limiting dilution will subject them to severe genetic bottlenecks. Although bottlenecks will reset virus variability to zero (which may be a desired effect), the stochastic process will randomly sample mutations within the population. Since most mutations are deleterious and most individual genomes will contain at least one mutation, the selected representative will likely be a debilitated variant from the mutant spectrum and may not present the expected viral sequence. Nevertheless, this approach may be preferred if a thorough sampling of minority variants is desired.

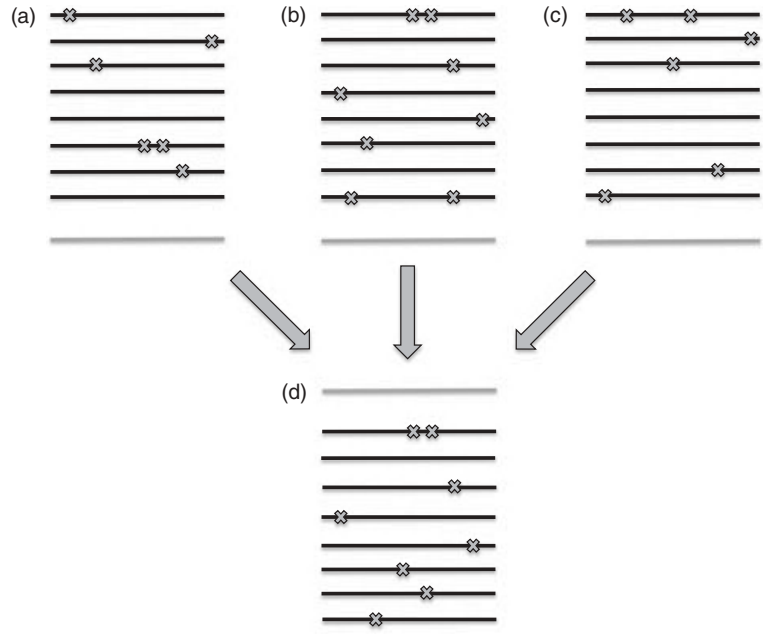

Figure 11.3 Generation of an infectious clone from different sources, to be used in reverse genetics. In the figure, lines represent genomes, X are mutations and the grey lines are the consensus sequences of the population. (a) a viral population obtained *in vitro* from a cell type x (b) a clinical isolate with a different mutant spectrum to A but same consensus and (c) a viral population obtained *in vitro* from a cell type y, with yet another different mutant spectrum to A and B, but same consensus sequence. In generating a new virus population from an infectious clone built on the given consensus sequence of the previous populations (d), yet another mutant spectrum will be obtained, which will likely resemble most the spectrum from the cell type in which it was generated. All of these populations, although sharing the same consensus, might express a different phenotype due to the differences in mutant spectrum.

Although cell-cultured populations have their obvious advantages, the caveat of using a cell-cultured stock is the potential loss of variants that are important for the *in vivo* phenotype that might not be the same ones necessary for cell culture adaptation. Clinical isolates will better represent wild-type virus, although it is true that from the moment one generates variants by reverse genetics in cell culture we are losing much of this information once more. In terms of the clinical or field isolate, it is also important to consider that different compartments within the patient (or ecosystem for that matter) can hold different variants of the virus, adapted to the specific conditions of that compartment. Therefore, obtaining a wild type representative virus of the whole population can prove to be difficult. For example, in HIV-1 the traditional method of obtaining an isolate from the clinic is to co-culture Peripheral Blood Mononuclear Cells (PBMCs) from an infected patient with those

of a naïve patient in order to promote infection and replication of HIV-1 in naïve T-cells to produce sufficient virus for further studies. The question arises as to whether this amplified isolate is representative of the original population of interest.

In some systems, a compromise may be helpful or even necessary. An example is in creating a chimera of sorts between clinical isolates, in order to obtain autologous sequences from patients that are as close as possible to the situation *in vivo*, and inserting them into a more robust infectious clone backbone. This has become common practice in HIV research, where researchers try to study phenotypes of quasispecies or heterogeneous populations whose variants may have evolved and diverged over time. Examples include monitoring the emergence, and effect on phenotype, of cytotoxic T lymphocyte (CTLs) escape variants (Troyer *et al*., 2009) or assessing whether different mutant distributions exist between patient seminal HIV populations (Lawrence *et al*., 2009).

11.2.2 Gain control, lose information

Once the isolate is cloned into a plasmid, virus is usually recovered from infectious RNA either produced *in vitro* and transfected into cell culture, or produced in transfected cells from a eukaryotic transcriptional promoter. For viruses such as influenza, the process is even more complex, since the viral genome is encoded on the negative strand and therefore is not directly infectious but requires co-expression of several viral proteins. Regardless of the method used, the virus stock prepared by reverse genetics from the infectious clone will likely not present the same mutant distribution that would be generated from a natural virus infection and may not reproduce the exact phenotype. An obvious reason is that the phenotype that was seen in clinical conditions is not necessarily represented in cell culture. But beyond this, the conditions in which a virus population is generated will determine, through selection, the frequency at which minority variants will exist within that population. It is possible that certain undetectable variants present in the isolate play a role in the phenotype by contributing to the overall fitness of the virus population, yet may not exist in the newly generated population. Evidence for this is seen in arboviruses, for which infectious clones often grow to lower titres than their corresponding isolates in a cell-dependent manner without any apparent differences in consensus sequence (Kinney *et al*., 1997; Pierro *et al*., 2006; Tsetsarkin *et al*., 2006; Coffey and Vignuzzi, 2011). In this regard, arboviruses highlight the potential influence of passage history, since they alternate between two very different hosts, vertebrates and invertebrates, and are in a compromise to replicate in both. The starting population (isolate) may have a mutant distribution that contains more variants able to replicate in both hosts, although determining what these variants are remains laborious and is generally not done. Indeed, significant changes in phenotype (replication fitness) during adaptation to a new growth conditions often occur without changes to the genotype (consensus sequence), as observed for the arboviruses

(Novella, Clarke, *et al*., 1995b; Novella *et al*., 1999; Weaver *et al*., 1999; Turner and Elena, 2000; Greene *et al*., 2005; Ciota *et al*., 2007; Ciota *et al*., 2008; Coffey *et al*., 2008; Vasilakis *et al*., 2009; Turner *et al*., 2010), and other RNA viruses, including HIV-1 (Yuste *et al*., 2005; Bordería *et al*., 2010). A virus generated by reverse genetics based on the consensus of these higher fitness populations would likely present a phenotype that more closely resembles the original population. In a sense, the genetic information defining the evolving phenotype has been lost. It is important to keep in mind then, that a virus population generated by reverse genetics will likely demonstrate the phenotype of the specific mutation/allele of interest, but will in the process lose additional determinants of the isolate's phenotype that accumulate during the passage history of the virus without necessarily fixating to consensus.

11.2.3 Once the consensus is out . . .

As we commented in the previous section, an infectious cDNA clone can be considered homogeneous, relatively speaking, which can pose a problem in reproducing a desired phenotype; but from the moment it is transfected into a cell line to obtain passage 0 (p0), clonality is already being lost. In fact, even *in vitro* transcribed RNA has mutations randomly introduced by the error prone DNA dependent RNA polymerases used, such a T7 or SP6 RNA polymerase. Additionally, an extra passage is often done in the same or different cell line to obtain a high titre virus stock. Therefore, at least two, if not more, rounds of replication will occur and, with mutation rates of 1/genome/replication, the variability generated is considerable. Thus, cells that most closely resemble the host or maximise viral titre in the fewest cycles should be chosen depending on the desired population, unless population heterogeneity is a wanted outcome. If homogeneity is desired, then purifying the virus by plaque passages or low MOI is an option, but with a risk. These two conditions will promote faster fitness increases and adaptation to the cell line, but the cost will be a drift from the consensus sequence with each round of plaque purification or low MOI passage, and selection of deleterious mutations. For that reason it is important that once the stock is finished, the whole viral genome is sequenced to detect any major changes. Once the infectious clone is obtained and the allele to study is chosen, it is important to verify that there are no minority variants at this site suggesting that the mutation is already in the process of reversion, which can happen if the mutation bears even a moderate fitness cost. Revertants, pseudorevertants and quasi-infectious viruses, viruses that can even generate an infectious population different from the genome defined by the infectious clone cDNA, have been demonstrated in bacteriophages (Licis *et al*., 1998), RNA viruses (Wimmer *et al*., 1993; Agol, 1997; Gromeier *et al*., 1999) and retroviruses (Berkhout *et al*., 1997). A conservative check of at least 20 clones or more to detect minority variants is recommended.

11.3 Examples of the use of the theory to disable or manipulate the quasispecies under controlled environments

11.3.1 From observation to manipulation

In the first decades of studies, pioneered and largely led by J.J. Holland and E. Domingo, it was only possible to describe phenotypes with respect to population heterogeneity, since sequencing techniques were not yet available. Restriction fragment length polymorphism (RFLP) analysis was the best method of measuring a population's relative heterogeneity and restriction enzymes sites or resistance to monoclonal antibodies were used as genetic markers to distinguish among variants in competition experiments. The first viral genome to be sequenced was the bacteriophage MS2, which has a single-stranded RNA genome, by Walter Fiers in 1976 (Fiers *et al*., 1976). The following year, 1977, Sanger and colleagues (Sanger, Nicklen, *et al*., 1977a; Sanger, Air, *et al*., 1977b) sequenced phage ΦX-174 when they developed the new dideoxy chain-reaction sequencing method that would revolutionise the way sequencing was done thereafter. As molecular biology and sequencing technology improved, coupled to the ongoing studies in virus evolutionary biology, it was becoming increasingly evident that viruses were mutating faster than any other organism. These studies brought to light what continues to be one of the biggest problems in virology: the apparent capacity to adapt and evolve rapidly, to escape immune responses, to resist antiviral drugs, to infect new hosts, and to revert from attenuated vaccine strains to pathogenic forms (poliovirus example, stated before).

11.3.2 Lethal mutagenesis of RNA viruses and the extrinsic alteration of the quasispecies

The observation that RNA viruses have extreme mutation rates and generate highly heterogeneous populations that at least resemble, if not form, quasispecies brought forth the notion that they may replicate close to an *error threshold*, or a mutational maximum. Given that on average, an RNA-dependent RNA polymerase (RdRp) produces one mutation per genome per replication (Drake and Holland, 1999; Duffy and Shackelton, 2008) and that most random mutations are detrimental or lethal, it is probable that even a moderate increase in this error frequency would have significant effects on virus population fitness and infectivity, with a loss in maintaining a proper coding sequence and protein function. Proposed theoretically several decades ago (Eigen, 1971; Swetina and Schuster, 1982; Eigen and Biebricher, 1988), it was demonstrated experimentally by using base analogues that 'trick' the viral polymerase into base pairing mistakes in a variety of RNA viruses: arenaviruses (Grande-Pérez *et al*., 2002), picornaviruses (Holland

et al., 1992), retroviruses (Pathak and Temin, 1992), see Table 11.1 for more detailed information.

These initial studies were pivotal in demonstrating that viruses tend to have an intrinsically set mutation frequency, and that these frequencies could be extrinsically modified to the detriment of the virus population. Further characterisation of the mechanisms underlying lethal mutagenesis helped better understand the population dynamics of viruses at very high mutation rates: such as the increased sensitivity to mutagenesis of small versus large populations and the decreased sensitivity to mutagenesis of high versus low fitness populations (Sierra *et al*., 2000). The efficacy of lethal mutagenesis in tissue culture is now well documented. Whether this is a feasible antiviral approach *in vivo* remains to be determined, since doses required in tissue culture may not be attainable in physiologic conditions. Incidentally, ribavirin, a known RNA mutagen, is the therapy en vigueur to treat chronic hepatitis C infection. Studies trying to determine whether mutation rates are affected have been discordant, although evidence suggests that ribavirin accumulates in hepatocytes and that long term treatment increases mutational load (Cuevas *et al*., 2009).

11.3.3 Compounds and conditions that alter virus mutation frequencies

Three compounds regularly used in the laboratory for RNA mutagenesis are ribavirin, FU, and AZC, all of which are base analogues that are directly misincorporated into viral genomes by the viral polymerase. Each compound has a bias for specific types of mutations based on the nucleotide structure for which it is an analogue. AZC is an analogue of cytidine, producing mainly A → G changes and secondarily G → A and C → U. FU is a pyrimidine analogue, producing U → C mutations and less frequently, C → U and G → A. Ribavirin is a purine analogue that can act directly as a chain terminator for viral replication, as well as produce predominantly G → A and secondarily C → U mutations. With increasing interest in lethal mutagenesis, new base analogues are being identified or developed in hopes to maximise efficacy and minimise required doses and cell toxicity. As an example, a ribonucleoside analogue P compound was found to be less mutagenic than ribavirin but better tolerated by the host cell (Graci *et al*., 2008). In all cases, mutagenesis with base analogues relies on the incorrect incorporation of the compound into the virus genome, an error that cannot be corrected since RNA polymerases lack proof reading mechanisms. In the following round of replication, the viral polymerase that encounters such an incorporated base no longer has the normal nucleotide to use as a guide and the base analogues template in a less discriminatory manner. The new attention to altering a virus' natural mutation frequency has led the search for new compounds and conditions that may alter RNA polymerase fidelity through different direct or indirect mechanisms. Recently, other compounds that do not have nucleoside-like structure, such as ion channel inhibitors like amiloride and EIPA have been shown to have mutagenic effects on RNA viruses

Table 11.1 Ribavirin has a broad-spectrum antiviral activity.

Family	Genus	Type species	Effect observed	Reference
Arenaviridae	*Arenavirus*	Junin virus	↓Cytopathic effect	Rodriguez *et al*. (1986)
		Lassa fever virus		Huggins (1989)
		Pichinde virus	↓Virus plaque formation	Smee *et al*. (1992)
		Lymphocytic choriomeningitis virus	↓Virus titer, ↓ infectivity	Ruiz-Jarabo *et al*. (2003)
	Deltavirus	Hepatitis delta virus	↓Replication	Choi *et al*. (1989)
Bornaviridae	*Bornavirus*	Borna disease virus V and He/80	↓Transcription ↓Focus formation	Jordan *et al*. (1999)
Bunyaviridae	*Bunyavirus*	San Angelo	↓Virus plaque formation	Smee *et al*. (1992)
	Hantavirus	Hantaan virus	↓Infectious virus yield, ↑mutation frequency	Severson *et al*. (2003)
		Seoul virus	↓Virus focus formation	Murphy *et al*. (2001)
	Nairovirus	Crimean Congo hemorrhagic fever virus	↓Virus yield	Watts *et al*. (1989)
	Phlebovirus	Rift Valley fever virus	↓Virus detection	Garcia *et al*. (2001)
		Sand y fever Sicilian virus	↓Cytopathic effect	Crance *et al*. (1997)
Flaviviridae	*Flavivirus*	Dengue virus 1, 2 and 4	↓Cytopathic effect	Crance *et al*. (2003)
		Japanese encephalitis virus	↓Cytopathic effect	Crance *et al*. (2003)
		Langat virus	↓Cytopathic effect	Crance *et al*. (2003)
		Usutu virus	↓Cytopathic effect	Crance *et al*. (2003)
		Wesselsbron virus	↓Cytopathic effect	Crance *et al*. (2003)
		West Nile virus	↓Cytopathic effect ↓Replication	Crance *et al*. (2003); Jordan *et al*. (2000)
		Yellow Fever virus 17D and FNV	↓Cytopathic effect	Crance *et al*. (2003)
		Zika virus	↓Cytopathic effect	Crance *et al*., 2003

(*Continued*)

Table 11.1 (*Continued*)

Family	Genus	Type species	Effect observed	Reference
	Hepativirus	GB virus B	↓Virus yield, ↓ infectivity, ↑ error rate	Lanford *et al*. (2001)
		Hepatitis C virus	↑Error rate	Contreras *et al*. (2002); Zhou *et al*. (2003)
Orthomyxoviridae	*Influenzavirus*	Influenza virus A	↓Cytopathic effect	Shigeta *et al*. (1997); Wray *et al*. (1985a)
		In uenza virus B	↓Cytopathic effect	Wray *et al*. (1986)
Paramyxoviridae	*Metapneumovirus*	Human Metapneumovirus	↓Cytopathic effect	Wyde *et al*. (2003)
	Morbillivirus	Measles virus	↓Cytopathic effect	Wyde *et al*. (2000)
	Pneumovirus	Bovine respiratory Syncitial virus	↓Viral proliferation	Bartzatt and Anderson (1989)
		Human Respiratory Syncitial virus	↓Cytopathic effect	Wyde *et al*. (2003)
Picornaviridae	*Aphtovirus*	Foot and mouth disease virus	↓Virus production ↑Mutagenesis	Airaksinen *et al*. (2003)
	Enterovirus	Poliovirus	↓Virus titer, infectivity ↑Error and mutation rate	Crotty *et al*. (2000)
Poxviridae	*Orthopoxvirus*	Camelpox, cowpox, monkeypox, vaccinia virus	↓Plaque formation	Smee *et al*. (2001)
Retroviridae	*Lentivirus*	HIV	↓Virus replication	McCormick *et al*. (1984)
		Maedi Visna virus	↓Virus production	Frank *et al*. (1987)
Togaviridae	*Alphavirus*	Chikungunya virus	↓Cytopathic effect ↓Virus titer	Andrei and De Clercq (1993)
		Semliki Forest virus	↓Virus titer	van Tiel *et al*. (1986)

The references cited show viral inhibition by ribavirin treatment in vitro. Studies demonstrating lethal mutagenesis as a primary antiviral mechanism are highlighted in gray.

Source: Taken from Table 1 in Vignuzzi *et al*. (2005).

(Levi *et al*., 2010). The authors suggested that such compounds alter the intracellular milieu, creating a more hostile environment in which replication fidelity is diminished. They showed that changes in intracellular levels of free Mg^{+} and Mn^{+}, essential co-factors for RNA polymerase activity, result in higher mutation frequencies, suggesting that amiloride is an indirect mutagen by perturbing intracellular ion concentrations (Levi *et al*., 2010). Regardless of the mechanisms involved, studies of lethal mutagenesis demonstrate that virus population heterogeneity is an intrinsic trait that can be readily manipulated and that relatively subtle changes in mutation frequency can have significant impacts on population phenotype. A significant secondary observation made by many of these studies is that these phenotypic changes occur without changes to consensus sequence, since the increase in mutational load, albeit significant, is randomly distributed across the genome such that the genetic average (consensus) of the population is not modified.

11.3.4 Fidelity variants and the intrinsic control of the quasispecies

Lethal mutagenesis was instrumental in showing that RNA viruses exist very close to a maximum mutation frequency and further emphasised the notion that generating extreme diversity must have a selective advantage worth the risk of undergoing mutagenesis. This led to the quest of manipulating the genetic diversity of virus populations in the other direction, by decreasing it, compared to the increases resulting from mutagenic treatment. Based on the fact that RNA viruses can generate escape variants to nearly all antiviral compounds, and that RNA mutagens exert a selective pressure on a virus's ability to faithfully replicate its genome, several teams set out to isolate higher fidelity versions of RNA viruses that would constitute tools to study the role of genetic diversity in a controlled manner. The first to be described was the high fidelity variant of poliovirus, G64S (Pfeiffer and Kirkegaard, 2003), isolated after serial passage in the presence of increasing concentrations of ribavirin. A single point mutation in the polymerase region was identified and its role in ribavirin resistance was demonstrated after virus isolation (Pfeiffer and Kirkegaard, 2003) and by reverse genetics (Vignuzzi *et al*., 2006). The mechanism of resistance was confirmed to be increased RNA polymerase fidelity, despite these enzymes lacking classic proof-reading and repair mechanisms (Arnold *et al*., 2005). Importantly, the higher fidelity variant was shown to generate populations of restricted genetic diversity. The variant was an invaluable tool with which to show a link between the heterogeneity of a virus population and virus fitness/virulence *in vivo*, in the absence of changes in consensus sequence. The studies showed that a less diverse population defined by the same consensus sequence as a wild type population, had reduced capacity to tissue tropism and virulence (Pfeiffer and Kirkegaard, 2005; Vignuzzi *et al*., 2006). These results have important implications for rationalised vaccine development, as higher fidelity versions of RNA viruses may be attenuated by being less adaptable *in vivo*, and may be genetically more stable and less likely to revert to wild type at other attenuating determinants (Vignuzzi *et al*., 2008).

Indeed, when the high fidelity variants and wild-type virus were engineered to carry a target sequence to let-7 microRNA (miRNA) and assayed in cell lines active for the miRNA, wild-type virus was able to escape by numerous mutations in the target sequence in as little as 8 hours, while high fidelity variants were 5X less likely to do so over a 36-hour period.

Since this work, there has been increasing interest on whether fidelity can be modified in other RNA viruses. Recently, a high and low fidelity variant was identified in Coxsackie virus type B3, a closely related picornavirus (CVB3) (Levi *et al*., 2010). The low fidelity variant showed a much higher mutation frequency, and therefore wider mutant spectrum, than wild type or the high fidelity variant, resembling virus populations that are on the verge of lethal mutagenesis. Of note, a high mutation phenotype was observed for a coronavirus (Eckerle *et al*., 2010) with a mutation in nsp14 protein, resulting in a 21-fold increase of the mutation frequency. These differences in the mutation spectrum could have importance in the speed of adaptation in changing environments and different hosts, including the generation of drug resistance mutations or escape variants to evade the immune responses. A high fidelity variant may be less likely to adapt to an altered environment, while a low fidelity variant might increase in deleterious mutations and mutational load beyond a critical threshold. Consequently, it would seem that viruses have evolved to have a specific mutation rate that allows for rapid adaptation while maintaining genomic and proteomic integrity.

11.3.5 Reverse genetics and artificial quasispecies

The coupling of reverse genetics and virus population manipulation is a marriage of elegant simplicity and ordered chaos, which could prove invaluable to fostering new approaches on how RNA viruses are studied. Using reverse genetics, it could be possible to artificially generate a quasispecies specifically designed to better understand virus population dynamics. Following this idea, Pfeiffer and Kirkegaard (Pfeiffer and Kirkegaard, 2006) made use of restriction enzyme site-tagged quasispecies to follow specific variants during infection and dissemination of poliovirus in mice. They tagged four different polioviruses by introducing silent restriction sites into the VP2 and VP3 viral proteins that did not affect growth kinetics. When young mice were injected intramuscularly with a high dose pool of the four viruses, all were detected from a total RNA extraction of muscle tissue. In brains, on the other hand, only one of four viruses was detected indicating the presence of a bottleneck, that seemed to follow a stochastic first-come first-served dynamic. If one of the variants was increased in the initial inoculum, that variant would be the most frequently found in brain and if a 24-hour ‘head start’ was given to one of the tagged-variants in the pool, that variant was predominant in brain. Following the previous work and trying to expand the sensitivity of the assay and the quantity of different tagged variants detected, they developed a new in-situ hybridisation-based assay by generating an artificial quasispecies composed of 10 marked members by

silent mutations in the VP3 capsid protein (Kuss *et al*., 2008), easily recognisable by specific probes used in the hybridisation assay. This reverse-genetics/quasispecies approach confirmed that there are bottlenecks between the peripheral infection sites and the CNS in mice.

In an original twist to study virus population dynamics using controlled populations derived by reverse genetics, Lauring and Andino (Lauring and Andino, 2011) randomly mutagenised a wild-type poliovirus population with one passage in ribavirin. From this mutagenised population, individual viable variants were isolated by limiting dilution and re-cloned into infectious cDNA with unique barcodes. Forty-eight variants were then pooled and passaged in HeLa cells. Relative fitness was estimated from individually tagged variants. They found a significant change in the frequency of 23 out of the 48 variants, due to deleterious mutation, and reduction in fitness (range of 0.09 to 0.73 after 4 passages). The remaining 25 did not dramatically change fitness compared to the wild-type virus, with values ranging from 0.85 to 1. In this way they were able to follow and measure changes in the variant frequencies within the population and correlate it with changes in fitness. After infection either intramuscularly or intravenously with 10^8 PFU of the artificial population, they observed that 6 out of 96 variants accessed the brain, and importantly the tags accessing the brain were different in each mouse, suggesting that it is not an effect of the tag but of specific mutations occurring in the variants that permits them to reach the brain, although they could not define which mutations specifically were implicated.

Yet another example is work by Rafael Sanjuán *et al*. 2004 (Sanjuán *et al*., 2004) on epistasis. Although not a study on viral quasispecies *per se*, it shows how rationalised generation of different mutants can give insights on the epistatic interactions among different variants within a virus population. They generated 47 variants of VSV. Each was assayed for fitness alone or in combination to obtain a map of how interactions were affecting viral fitness, in a beneficial or deleterious way. The work demonstrated a dominance of antagonistic interactions, with a combined effect being smaller than the sum of their individual effect, for both beneficial and deleterious mutations.

11.4 Future prospects of virus population genetics and reverse genetics

11.4.1 Next Generation Sequencing and the search of the minority variant

In recent years, a handful of new ‘deep’ sequencing technologies have emerged with a current market dominance of 454 Roche and Solexa Illumina. 454 Roche, first to be developed, brought about the first full human genome from JD Watson (Wheeler *et al*., 2008). As of 2011, Roche 454 had 1205 scientific publications with

their technology and Solexa Illumina, 1632. The recent development of these Next Generation Sequencing (NGS) technologies will likely revolutionise the study of RNA virus population genetics as well, and its link with reverse genetics. It is becoming increasingly easy to prospect the inherent variability of virus populations and to identify key mutations before they reach frequencies high enough to fix to consensus. Until the advent of NGS, classic Sanger sequencing of RT-PCR products of virus populations could at best identify mutations that either changed consensus sequence, or constituted very significant minority populations (above 25% of total population). Deeper characterisation of the population was laborious, through plaque purification or limiting dilution to isolate individual variants, or subcloning RT-PCR products of the total genomic population into individual sequencing vectors. In most cases, only several dozen viruses, usually only partial sequences, could be obtained. In NGS, thousands to millions of reads can be obtained that cover the whole genome thousands of times over, with each read originating from a different RNA genome, potentially exploring the total sequence space occupied by the virus population. In doing so, one can readily discover minority variants as low as 1% in the population, if not lower when correction for error introduced by the technology is made. Most of these preliminary studies have been performed for HIV-1 primary isolates. NGS technology has been successfully used to identify low frequency HIV variants involved in changing cell tropism, drug resistance and escaping immune responses (C. Wang *et al*., 2007a; G. P. Wang *et al*., 2007b; Eriksson *et al*., 2008; Mitsuya *et al*., 2008; Archer *et al*., 2009; Bimber *et al*., 2009; Le *et al*., 2009; Rozera *et al*., 2009; Rozera, *et al*., 2009b Varghese *et al*., 2009; Archer *et al*., 2010; Bimber *et al*., 2010; Hedskog *et al*., 2010; Ji *et al*., 2010; Love *et al*., 2010; Zagordi *et al*., 2010; Abbate *et al*., 2011a; Abbate *et al*., 2011b; Raymond *et al*., 2011; Saliou *et al*., 2011). But the technology is equally applicable to all RNA viruses, as is evidenced by work in HCV (C. Wang *et al*., 2007a), Hepatitis B virus (HBV) (Solmone *et al*., 2009)), FMDV (Wright *et al*., 2011), human rhinovirus (Cordey *et al*., 2010). Currently, the technology is not perfectly adapted to studying RNA viruses. For quasispecies studies, it is not possible yet to easily link mutations pertaining to the same variant, although with the paired-end reads technology some relations can be determined. Furthermore, numerous amplifications steps in preparing sequencing libraries are required, especially for low titre samples, and several controls should be implemented to better analyse the data: multiple, independent libraries from the same sample to authentic mutation distributions; control populations such as cDNA of the corresponding virus or in vitro transcribed RNA; proper bioinformatic implementation to clean the data set of low quality or non-virus specific reads. Nevertheless, progress is already being made and the benefits of this technology are already evident.

The benefit to reverse genetics on the other hand is already evident. In identifying mutations long before they are at high enough frequency to dominate the population and replace the consensus sequence, NGS permits the researcher to identify mutations of interest early on, which can quickly be regenerated and studied by reverse genetics. For many mutations, that would be of interest to research but whose

fitness cost would be too great to ever dominate a virus population, NGS would permit their identification (for example, minority variants that assist a virus population *in cis* or *in trans*, yet cannot exist on their own).

11.4.2 Understanding sequence space and fitness landscape

One hot topic in the quasispecies debate has been mutational robustness and how it is attained. It seems that robustness can be selected in situations of high mutations rates (Sanjuán *et al*., 2007). High mutation rates and large population sizes are highlights of the RNA virus population dynamics, both of which allow the virus to explore sequence space and move through the fitness landscape. Sequence space can be imagined as multi-dimensional where every point in the space is occupied by a different variant of a genome of a given length. In this sense, the specific and unique genome sequence is the coordinates in this space. If we could simplify sequence space to a two-dimensional map, then the fitness landscape could project outwards from it, in the form of mountains, plains and ravines (high, neutral and low fitness) as determined by the relative fitness of the genome described at each coordinate. Sequence space is a constant for a given genome length, although whether a virus occupies much, or only a little of it, depends on the amount of diversity within the population and the fitness landscape. This landscape, on the other hand, is different for different conditions and environments. In adapting to an environment, the virus population explores and moves throughout this landscape. Some of the peaks in the landscape can be sharp and narrow with high values of fitness while others can be flatter with an average lower fitness. High peaks are good for fast replicating viruses that have maximised fitness for a specific environment, in conditions of low mutation rates; while flatter landscapes are better suited for lower fitness viruses in conditions of high mutation rates. Consequently the same virus can be in one or the other, depending on the environment to which it has been adapting. Once more, this highlights the importance of understanding context, when tackling reverse genetics of an RNA virus population.

11.5 Conclusion

Although there is controversy in the use of the quasispecies theory in the context of virus evolution one cannot negate its influence in shaping modern virology. Without it, our understanding of virus population fluctuations and fitness, adaptability, appearance of resistance mutations to antiviral drug treatments and/or the immune system and even the design of new antiviral therapies such as lethal mutagenesis would not have advanced. For the theory itself, further research is required to prove or disregard it as being applicable to virus evolution. Experiments showing the genome coupling must be developed, trying to shed light on how viral variants in one population relate to each other. Is the replication of one variant also

dependent of the neighbouring variants? Do they really behave as one? Is this dependent on mutation rates and genetic robustness? Regardless of the specific evolutionary mechanism underlying RNA virus selection, for most researchers studying RNA viruses, quasispecies and virus populations are a question of 'You say tomayto, I say tomahto'. What most of us, and very likely the readership of this text are after, is a better appreciation of how vast diversity within virus populations can be and the implications on how to best study them, especially in the context of reverse genetics.

Nowadays, a shift towards understanding the complexity of the viral mutant spectrum rather than the use of clonal populations is on the horizon. The idea that one genotype equals one phenotype is not totally correct, since viruses are composed of multiple variants within the same population of which we can obtain a consensus sequence, but that may not reflect the whole story. Keeping that in mind, one can still undertake the study of 'clonal' populations presenting single mutations, knowing that if unexpected or incomplete phenotypes are observed, then a deeper characterisation of the underlying mutant spectrum may be warranted.

The challenge in the future is to develop new approaches based on modern sequencing technologies and more complex reverse genetics to better map out sequence space and fitness landscapes and define the populations within them. NGS has opened the door to sampling the sequence space of viruses. If we can explore the adaptive landscape, determine the key variants populating it, and assign fitness to the minority and majority variants, we will be closer to understanding how adaptation occurs and why selection will push viruses to one peak or another depending on circumstances. Reconstruction of the populations by reverse genetics will be essential in confirming the data. In this regard, the recent work by Lauring and Andino (Lauring and Andino, 2011) illustrates this effort to move away from the isolated studies of one variant, one genotype, one phenotype. It remains to be seen whether quasispecies theory is the best framework with which to describe RNA virus populations; but it seems likely that the ongoing efforts to determine this, using increasingly powerful technologies, may contribute more to developing modern virology and population genetics than the theory itself.

References

Abbate, I *et al*. 2011a. Analysis of co-receptor usage of circulating viral and proviral HIV genome quasispecies by ultra-deep pyrosequencing in patients who are candidates for CCR5 antagonist treatment. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* **17**(5): 725–731.

Abbate, I. *et al*. 2011b. Detection of quasispecies variants predicted to use CXCR4 by ultra-deep pyrosequencing during early HIV infection. *AIDS (London, England)* **25**(5): 611–617.

Agol, V. 1997. Recombination and other genomic rearrangements in picornaviruses* 1. *Seminars in Virology* **8**(2):77–84.

Archer, J. *et al*. 2009. Detection of low-frequency pretherapy chemokine (CXC motif) receptor 4 (CXCR4)-using HIV-1 with ultra-deep pyrosequencing. *AIDS (London, England)* **23**(10): 1209–1218.

Archer, J. *et al*. 2010. The evolutionary analysis of emerging low frequency HIV-1 CXCR4 using variants through time: an ultra-deep approach. *PLoS Computational Biology* **6**(12), p.e1001022.

Arnold, J.J. *et al*. 2005. Remote site control of an active site fidelity checkpoint in a viral RNA-dependent RNA polymerase. *The Journal of Biological Chemistry* **280**(27): 25706–25716.

Berkhout, B. Klaver, B. and Das, A.T. 1997. Forced evolution of a regulatory RNA helix in the HIV-1 genome. *Nucleic Acids Research* **25**(5): 940–947.

Bimber, B.N. *et al*. 2009. Ultradeep pyrosequencing detects complex patterns of CD8+ T-lymphocyte escape in simian immunodeficiency virus-infected macaques. *Journal of Virology* **83**(16): 8247–8253.

Bimber, B.N. *et al*. 2010. Whole-genome characterization of human and simian immunodeficiency virus intrahost diversity by ultradeep pyrosequencing. *Journal of Virology* **84**(22): 12087–12092.

Bordería, A.V. *et al*. 2010. Initial fitness recovery of HIV-1 is associated with quasispecies heterogeneity and can occur without modifications in the consensus sequence. *PloS One* **5**(4), p.e10319.

Burch, C.L. and Chao, L, 1999. Evolution by small steps and rugged landscapes in the RNA virus phi6. *Genetics* **151**(3): 921–927.

Chao, Lin, 1990. Fitness of RNA virus decreased by Muller's ratchet. *Nature* **348**(6300): 454–455.

Ciota, A.T. *et al*. 2008. Characterization of mosquito-adapted West Nile virus. *The Journal of General Virology* **89**(Pt 7): 1633–1642.

Ciota, A.T. *et al*. 2007. Role of the mutant spectrum in adaptation and replication of West Nile virus. *The Journal of General Virology* **88**(Pt 3): 865–874.

Clarke, D.K. *et al*. 1993. Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. *Journal of Virology* **67**(1): 222–228.

Clarke, D.K. *et al*. 1994. The red queen reigns in the kingdom of RNA viruses. *Proceedings of the National Academy of Sciences of the United States of America* **91**(11): 4821–4824.

Codoñer, F.M. *et al*. 2006. The fittest versus the flattest: experimental confirmation of the quasispecies effect with subviral pathogens. *PLoS Pathogens* **2**(12), p.e136.

Coffey, L.L. and Vignuzzi, M. 2011. Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. *Journal of Virology* **85**(2): 1025–1035.

Coffey, L.L. *et al*. 2008. Arbovirus evolution in vivo is constrained by host alternation. *Proceedings of the National Academy of Sciences of the United States of America* **105**(19): 6970–6975.

Comas, I. Moya, A. and González-Candelas, F. 2005. Validating viral quasispecies with digital organisms: a re-examination of the critical mutation rate. *BMC Evolutionary Biology*, **5**, p.5.

Cordey, S. *et al*. 2010. Rhinovirus genome evolution during experimental human infection. *PloS One* **5**(5), p.e10588.

Cuevas, J. M *et al*. 2009. Effect of ribavirin on the mutation rate and spectrum of hepatitis C virus in vivo. *Journal of Virology* **83**(11): 5760–5764.

Cuevas, J. M. Moya, A. and Elena, S.F. 2003. Evolution of RNA virus in spatially structured heterogeneous environments. *Journal of Evolutionary Biology* **16**(3): 456–466.

Domingo, E. and Flavell, R. 1976. In vitro site-directed mutagenesis: Generation and properties of an infectious extracistronic mutant of bacteriophage Q [beta]* 1. *Gene* **1**(1):3–25.

Domingo, E. *et al*. 1978. Nucleotide sequence heterogeneity of an RNA phage population. *Cell*, **13**(4): 735–744.

Drake, J.W. and Holland, J.J. 1999. Mutation rates among RNA viruses. *Proceedings of the National Academy of Sciences of the United States of America* **96**(24): 13910–13913.

Drake, J.W. *et al*. 1998. Rates of spontaneous mutation. *Genetics* **148**(4): 1667–1686.

Duarte, E. *et al*. 1992. Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. *Proceedings of the National Academy of Sciences of the United States of America* **89**(13): 6015–6019.

Duarte, E.A. *et al*. 1993. Many-trillionfold amplification of single RNA virus particles fails to overcome the Muller's ratchet effect. *Journal of Virology* **67**(6): 3620–3623.

Duarte, E.A. *et al*. 1994. Subclonal components of consensus fitness in an RNA virus clone. *Journal of Virology* **68**(7): 4295–4301.

Duffy, S. and Shackelton, L. 2008. Rates of evolutionary change in viruses: patterns and determinants. *Nature Reviews Genetics* **9**(4):267–276.

Eckerle, L.D. *et al*. 2010. Infidelity of SARS-CoV Nsp14-exonuclease mutant virus replication is revealed by complete genome sequencing. *PLoS Pathogens* **6**(5), p.e1000896.

Eigen, M. 1971. Self-organization of matter and the evolution of biological macromolecules. *Die Naturwissenschaften* **58**(10): 465–523.

Eigen, M. 1996. On the nature of virus quasispecies. *Trends in Microbiology* **4**(6): 216–218.

Eigen, M. and Biebricher, C. 1988. Sequence space and quasispecies distribution, *RNA Genetics* **3**:211–245.

Eigen, M. and Schuster, P. 1977. The hypercycle: a principle of natural self-organization. Part A: Emergence of the hypercycle. *Die Naturwissenschaften* **64**(11): 541–565.

Eriksson, N. *et al*. 2008. Viral population estimation using pyrosequencing. *PLoS Computational Biology* **4**(4), p.e1000074.

Escarmís, C. *et al*. 1996. Genetic lesions associated with Muller's ratchet in an RNA virus. *Journal of Molecular Biology* **264**(2): 255–267.

Escarmís, C. Dávila, M. and Domingo, E. 1999. Multiple molecular pathways for fitness recovery of an RNA virus debilitated by operation of Muller's ratchet. *Journal of Molecular Biology*, **285**(2): 495–505.

Fiers, W. *et al*. 1976. Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. *Nature* **260**(5551): 500–507.

Graci, J.D. *et al*. 2008. Lethal mutagenesis of picornaviruses with N-6-modified purine nucleoside analogues. *Antimicrobial Agents and Chemotherapy* **52**(3): 971–979.

Grande-Pérez, A. *et al*. 2002. Molecular indetermination in the transition to error catastrophe: systematic elimination of lymphocytic choriomeningitis virus through mutagenesis does not correlate linearly with large increases in mutant spectrum complexity. *Proceedings of the National Academy of Sciences of the United States of America* **99**(20): 12938–12943.

Greene, I. P. *et al*. 2005. Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells. *Journal of Virology* **79**(22): 14253–14260.

Gromeier, M. *et al*. 1999. Dual stem loops within the poliovirus internal ribosomal entry site control neurovirulence. *Journal of Virology* **73**(2): 958–964.

Hedskog, C. *et al*. 2010. Dynamics of HIV-1 quasispecies during antiviral treatment dissected using ultra-deep pyrosequencing. *PloS One* **5**(7), p.e11345.

Holland, J. J. *et al*. 1989. Virus mutation frequencies can be greatly underestimated by monoclonal antibody neutralization of virions. *Journal of Virology* **63**(12): 5030–5036.

Holland, J. J. la Torre, de, J. C. and Steinhauer, D. A. 1992. RNA virus populations as quasispecies. *Current Topics in Microbiology and Immunology* **176**: 1–20.

Holmes, E. C. 2010. The RNA virus quasispecies: fact or fiction? *Journal of Molecular Biology* **400**(3): 271–273.

Holmes, E. C. and Moya, A. 2002. Is the quasispecies concept relevant to RNA viruses? *Journal of Virology* **76**(1): 460–465.

Jenkins, G. M. *et al*. 2001. Evidence for the non-quasispecies evolution of RNA viruses [corrected]. *Molecular Biology and Evolution* **18**(6): 987–994.

Ji, H. *et al*. 2010. HIV drug resistance surveillance using pooled pyrosequencing. *PloS One* **5**(2), p.e9263.

Kinney, R. M. *et al*. 1997. Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* **230**(2): 300–308.

Kuss, S. K. Etheredge, C. A. and Pfeiffer, J. K. 2008. Multiple host barriers restrict poliovirus trafficking in mice. *PLoS Pathogens* **4**(6), p.e1000082.

la Iglesia, de, F. and Elena, Santiago F, 2007. Fitness declines in Tobacco etch virus upon serial bottleneck transfers. *Journal of Virology* **81**(10): 4941–4947.

la Torre, de, J. C. and Holland, J.J. 1990. RNA virus quasispecies populations can suppress vastly superior mutant progeny. *Journal of Virology* **64**(12): 6278–6281.

la Torre, de, J. C. *et al*. 1992. High frequency of single-base transitions and extreme frequency of precise multiple-base reversion mutations in poliovirus. *Proceedings of the National Academy of Sciences of the United States of America* **89**(7): 2531–2535.

Lauring, A.S. and Andino, R. 2011. Exploring the fitness landscape of an RNA virus by using a universal barcode microarray. *Journal of Virology* **85**(8): 3780–3791.

Lawrence, P. *et al*. 2009. Construction and tropism characterisation of recombinant viruses exhibiting HIV-1 env gene from seminal strains. *Virology* **386**(2): 373–379.

Le, T. *et al*. 2009. Low-abundance HIV drug-resistant viral variants in treatment-experienced persons correlate with historical antiretroviral use. *PloS One* **4**(6): e6079.

Levi, L. I. *et al*. 2010. Fidelity variants of RNA dependent RNA polymerases uncover an indirect, mutagenic activity of amiloride compounds. *PLoS Pathogens* **6**(10): e1001163.

Licis, N. *et al*. 1998. Long-range translational coupling in single-stranded RNA bacteriophages: an evolutionary analysis. *Nucleic Acids Research* **26**(13): 3242–3246.

Love, T. M. T. *et al*. 2010. Mathematical modeling of ultradeep sequencing data reveals that acute CD8+ T-lymphocyte responses exert strong selective pressure in simian immunodeficiency virus-infected macaques but still fail to clear founder epitope sequences. *Journal of Virology* **84**(11): 5802–5814.

Mansky, L. M. and Temin, H. M. 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *Journal of Virology* **69**(8): 5087–5094.

Martínez, M. A. *et al*. 1991. Fitness alteration of foot-and-mouth disease virus mutants: measurement of adaptability of viral quasispecies. *Journal of Virolog*, **65**(7): 3954–3957.

Mitsuya, Y. *et al*. 2008. Minority human immunodeficiency virus type 1 variants in antiretroviral-naive persons with reverse transcriptase codon 215 revertant mutations. *Journal of Virology* **82**(21): 10747–10755.

Müller, J. H. 1964. The relation of recombination to mutational advance. *Mutation Research* **106**: 2–9.

Novella, I. S. *et al*. 2005. Adaptability costs in immune escape variants of vesicular stomatitis virus. *Virus Research*, **107**(1): 27–34.

Novella, I. S., Clarke, D. K. *et al*. 1995b. Extreme fitness differences in mammalian and insect hosts after continuous replication of vesicular stomatitis virus in sandfly cells. *Journal of Virology* **69**(11): 6805–6809.

Novella, I. S., Duarte, E. A. *et al*. 1995a. Exponential increases of RNA virus fitness during large population transmissions. *Proceedings of the National Academy of Sciences of the United States of America* **92**(13): 5841–5844.

Novella, I. S. *et al*. 1999. Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. *Journal of Molecular Biology* **287**(3): 459–465.

Ochs, K. *et al*. 2003. Impaired binding of standard initiation factors mediates poliovirus translation attenuation. *Journal of Virology* **77**(1): 115–122.

Pathak, V. K. and Temin, H. M. 1992. 5-Azacytidine and RNA secondary structure increase the retrovirus mutation rate. *Journal of Virology* **66**(5): 3093–3100.

Pfeiffer, J. K. and Kirkegaard, K. 2003. A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. *Proceedings of the National Academy of Sciences of the United States of America* **100**(12): 7289–7294.

Pfeiffer, J. K. and Kirkegaard, K. 2005. Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice. *PLoS Pathogens* **1**(2): e11.

Pfeiffer, J. K. and Kirkegaard, K. 2006. Bottleneck-mediated quasispecies restriction during spread of an RNA virus from inoculation site to brain. *Proceedings of the National Academy of Sciences of the United States of America* **103**(14): 5520–5525.

Pierro, D. J. *et al*. 2006. Infectious clone construction of dengue virus type 2, strain Jamaican 1409, and characterization of a conditional E6 mutation. *The Journal of General Virology* **87**(Pt 8): 2263–2268.

Rambaut, A. *et al*. 2004. The causes and consequences of HIV evolution. *Nature Reviews Genetics* **5**(1): 52–61.

Raymond, S. *et al*. 2011. Frequency of CXCR4-using viruses in primary HIV-1 infections using ultra-deep pyrosequencing. *AIDS (London, England)* **25**(13):1668–1670.

Rozera, G., Abbate, I. Bruselles, A., Vlassi, C. *et al*. 2009a. Archived HIV-1 minority variants detected by ultra-deep pyrosequencing in provirus may be fully replication competent. *AIDS (London, England)* **23**(18): 2541–2543.

Rozera, G., Abbate, I. Bruselles, A., Vlassi, C. *et al*. 2009b. Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations. *Retrovirology* **6**: 15.

Saliou, A. *et al*. 2011. Concordance between two phenotypic assays and ultra-deep pyrosequencing for determining HIV-1 tropism. *Antimicrobial Agents and Chemotherapy* **55**(6): 2831–2836.

Sanger, F., Air, G.M. *et al*. 1977b. Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* **265**(5596): 687–695.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977a. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**(12): 5463–5467.

Sanjuán, R., Moya, A. and Elena, S. F. 2004. The contribution of epistasis to the architecture of fitness in an RNA virus. *Proceedings of the National Academy of Sciences of the United States of America* **101**(43): 15376–15379.

Sanjuán, R. *et al*. 2007. Selection for robustness in mutagenized RNA viruses. *PLoS Genetics* **3**(6): e93.

Sanjuán, R. *et al*. 2010. Viral mutation rates. *Journal of Virology* **84**(19): 9733–9748.

Shafer, R. W. 2002. Genotypic testing for human immunodeficiency virus type 1 drug resistance. *Clinical Microbiology Reviews* **15**(2): 247–277.

Sierra, S. *et al*. 2000. Response of foot-and-mouth disease virus to increased mutagenesis: influence of viral load and fitness in loss of infectivity. *Journal of Virology* **74**(18): 8316–8323.

Solmone, M. *et al*. 2009. Use of massively parallel ultradeep pyrosequencing to characterize the genetic diversity of hepatitis B virus in drug-resistant and drug-naive patients and to detect minor variants in reverse transcriptase and hepatitis B S antigen. *Journal of Virology* **83**(4): 1718–1726.

Swetina, J. and Schuster, P. 1982. Self-replication with errors. A model for polynucleotide replication. *Biophysical Chemistry* **16**(4): 329–345.

Troyer, R.M. *et al*. 2009. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLoS Pathogens*, **5**(4), p.e1000365.

Tsetsarkin, K. *et al*. 2006. Infectious clones of Chikungunya virus (La Réunion isolate) for vector competence studies. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)* **6**(4): 325–337.

Turner, P. E. and Elena, S. F. 2000. Cost of host radiation in an RNA virus. *Genetics* **156**(4): 1465–1470.

Turner, P. E. *et al*. 2010. Role of evolved host breadth in the initial emergence of an RNA virus. *Evolution; International Journal of Organic Evolution* **64**(11): 3273–3286.

Van Valen, L. 1973. A new evolutionary law. *Evolutionary Theory* **1**: 1–30.

Varghese, V. *et al*. 2009. Minority variants associated with transmitted and acquired HIV-1 nonnucleoside reverse transcriptase inhibitor resistance: implications for the use of second-generation nonnucleoside reverse transcriptase inhibitors. *Journal of Acquired Immune Deficiency Syndromes (1999)* **52**(3): 309–315.

Vasilakis, N. *et al*. 2009. Mosquitoes put the brake on arbovirus evolution: experimental evolution reveals slower mutation accumulation in mosquito than vertebrate cells. *PLoS Pathogen*, **5**(6): e1000467.

Vignuzzi, M. *et al*. 2006. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* **439**(7074): 344–348.

Vignuzzi, M. Stone, J. K. and Andino, R. 2005. Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications. *Virus Research* **107**(2): 173–181.

Vignuzzi, M., Wendt, E. and Andino, R. 2008. Engineering attenuated virus vaccines by controlling replication fidelity. *Nature Medicine* **14**(2): 154–161.

Wang, C. *et al*. 2007a. Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. *Genome Research* **17**(8): 1195–1201.

Wang, G. P. *et al*. 2007b. HIV integration site selection: analysis by massively parallel pyrosequencing reveals association with epigenetic modifications. *Genome Research* **17**(8): 1186–1194.

Weaver, S. C. *et al*. 1999. Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. *Journal of Virology* **73**(5): 4316–4326.

Wheeler, D. *et al*. 2008. The complete genome of an individual by massively parallel DNA sequencing. *Nature* **452**(7189):872–876.

Wilke, C. 2005. Quasispecies theory in the context of population genetics. *BMC Evolutionary Biology* **5**:44.

Wilke, C. O. *et al*. 2001. Evolution of digital organisms at high mutation rates leads to survival of the flattest. *Nature* **412**(6844): 331–333.

Wimmer, E., Hellen, C. U. and Cao, X. 1993. Genetics of poliovirus. *Annual Review of Genetics* **27**: 353–436.

Wright, C. F. *et al*. 2011. Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing. *Journal of Virology* **85**(5): 2266–2275.

Yuste, E *et al*. 1999. Drastic fitness loss in human immunodeficiency virus type 1 upon serial bottleneck events. *Journal of Virology* **73**(4): 2745–2751.

Yuste, E. *et al*. 2005. Few mutations in the 5′ leader region mediate fitness recovery of debilitated human immunodeficiency type 1 viruses. *Journal of Virology* **79**(9): 5421–5427.

Zagordi, O. *et al*. 2010. Error correction of next-generation sequencing data and reliable estimation of HIV quasispecies. *Nucleic Acids Research* **38**(21): 7400–7409.

12

Summary and perspectives

Anne Bridgen
Croft Dhu, Newtonmore, Inverness-shire, Scotland

12.1 Introduction

The past ten to fifteen years have seen huge advances in research using RNA virus reverse genetic techniques, with application to all families of viruses and many different processes. Technically, the procedures have simplified; for example, the original influenza A virus rescue has been reduced from 12 to 8 plasmids, and now the virus has even been rescued using a single plasmid (Neumann *et al*., 2005). The applications have broadened in line with current scientific interests, with much research in the area of knocking out viral interferon antagonist genes and in analysing host responses in general. Those viruses such as poliovirus, which could already be rescued at the start of this period and which have relatively simple rescue procedures, have lent themselves to thought-provoking experiments to better understand the nature of quasispecies and drug resistance.

In this volume we have looked at nine RNA viruses in depth with examples of research using reverse genetics for these viruses. The specific viruses were chosen to illustrate the ways in which this technique has been applied across a variety of virus families, and also to illustrate as wide a range as possible of the different applications of reverse genetics. These can be broadly categorised as follows (with numbering corresponding to the following sections):

12.2 analysis of the role of specific non-coding sequence motifs involved in replication, transcription, polyadenylation and packaging;

12.3 analysis of the roles of viral proteins;

12.4 analysis of virus–host interactions at a global level;

12.5 understanding the basis of pathogenicity;

12.6 real-time virus imaging *in vitro* and *in vivo*;

12.7 structure-function analysis of viruses and viral domains;

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.

12.8 development of vaccines;

12.9 drug development;

12.10 gene delivery and knock out in plant cells including virus-induced gene silencing (VIGS);

12.11 gene delivery in arthropod and in mammalian cells;

12.12 development of oncolytic viruses and adaptation to this purpose.

Examples of research in these areas will be provided in the following sections.

12.2 Analysis of the role of specific non-coding sequence motifs involved in replication, transcription, polyadenylation and packaging

Since the role of non-coding regions (NCRs) can, to a large extent, be studied using minigenome systems rather than whole virus rescue, this was one of the first areas to be addressed and consequently has yielded a large amount of information. Mutational studies have allowed the sequences involved in the control of replication and transcription to be identified for many viruses; a good example of these experiments is the analysis of transcriptional and replicative signals in the paramyxovirus respiratory syncytial virus (RSV) (Fearns *et al*., 2002; McGivern *et al*., 2005).

Gene rearrangement within the genome of the non-segmented genome virus vesicular stomatitis virus (VSV) indicated that gene expression levels decreased with position down the genome (Ball *et al*., 1999); this has consequently been exploited extensively either to express foreign proteins at prime transcriptional positions or to alter the levels of individual viral proteins and thus enhance the protective immune response (Flanagan *et al*., 2000; Finke *et al*., 2000).

Non-coding sequences are also involved in other processes including packaging, interaction with host proteins, polyadenylation and in the formation of cap structures. The VSV 7mGpppN mRNA capping process is one which has been studied by reverse genetics (Wang *et al*., 2007).

The packaging of segmented viruses has always been an interesting question, that is, how viruses can retain 8 different segments (in the case of influenza A virus) rather than multiple copies of just one. Reverse genetics has helped shed light on this question, as summarised by Hutchinson *et al*. (2010). These authors review the evidence obtained from sequence conservation, and the minimal terminal sequences retained in defective interfering particles or required for the formation of reporter constructs to conclude that there are sequence-specific packaging signals in each segment, located at the genome termini and extending into the coding sequences. Mechanisms of segment selection remain, however, to be elucidated.

12.3 Analysis of the roles of viral proteins

Reverse genetic technology allows the genes for viral proteins to be modified or deleted, and the effects on viral phenotype observed to provide convincing evidence of their functions. Initially most of these studies were performed on non-structural/non-essential genes, as these can be deleted without significant detriment to the virus, but more recently these studies have been extended to the genes of structural proteins. These latter are essential so cannot be deleted from the virus unless they are supplied in trans, but the genes can be mutated to affect specific protein-protein or protein-RNA interactions. Studies on these proteins have, to a large extent, lagged behind those of non-essential genes as the manipulations need to be more complex to retain essential functionality.

Some of the first genes/open reading frames (ORFs) to be abrogated were those coding for virulence factors such as the Bunyamwera virus NSs protein, the influenza A NS1 protein and the paramyxovirus C protein. Others include the ORFs encoding the human metapneumovirus M2, SH and G proteins (Bridgen *et al*., 2001; Garcia-Sastre *et al*., 1998; Radecke and Billeter, 1996; Biacchesi *et al*., 2004 and Buchholz *et al*., 2005, respectively). Some of these proteins have been shown to possess a remarkably wide range of functions, for example, the 26 kDa influenza A virus NS1 protein binds double-stranded RNA, inhibits RIG-I signalling, reduces interferon induction, and inhibits the antiviral actions of PKR and 2′5′-oligoadenylate synthetase (OAS)/ RNase L. It also enhances viral mRNA export and promotes viral mRNA translation by interaction with the eukaryotic initiation factor eIF4GI and the cytoplasmic poly(A)-binding protein PABP1, while simultaneously preventing cellular mRNA maturation, thus reducing expression of cellular proteins, as well as other functions (Hale *et al*., 2008; Schneider and Wolff, 2009). Few of these studies would have been possible without reverse genetics, which has thus promoted a plethora of fruitful work in this area.

An example of reverse genetics applied to the study of structural proteins is the removal of the proteolytic cleavage site in the glycoprotein of the arenavirus Junin virus (Albarino *et al*., 2009). This resulted in the generation of a replication competent virus that could not however infect new cells. There are also numerous examples in which glycoprotein genes have been switched from one virus in a species to another, for example, between several different coronaviruses, or even between species, to affect tropism. Fluorescent tags have been inserted into structural genes and used for imaging, for example, into the Bunyamwera virus Gc protein or the measles virus L protein (see Chapters 6 and 7) or into rabies virus for use in synaptic tracing (Chapter 5). The Bunyamwera virus N protein was studied by sequential mutagenesis of the N protein gene followed by virus rescue of 57 different N variants (Eifan and Elliott, 2009). Other mutants, however, could not be rescued, thus showing the limitations of structural gene mutation.

There are beginning to be some very elegant experiments that use structural information to identify important residues in structural proteins, which are then mutated and the rescued viruses analysed. One such example is the analysis of the VSV

nucleocapsid (N) protein; determination of the 2.9-Å structure of this protein bound to RNA pinpointed specific amino acids which were capable of binding 9 RNA residues by hydrogen bonding (Green and Luo, 2006). These amino acids were then mutated to alanine and the effect on N expression, encapsidation and N-P interactions noted. More recently, additional mutations that would specifically reduce the hydrogen bonding were made and the properties of these mutants investigated by biochemical assays and functionality in a minigenome system (Rainsford *et al*., 2010). This approach, of combining information gleaned from structural studies and genetic manipulation is a powerful combination likely to be used increasingly in future.

12.4 Analysis of virus–host interactions at a global level

The development of high-throughput technologies has transformed the ways in which we can now analyse virus–host interactions. The most commonly used approach has been microarray studies, in which host gene expression is analysed following infection with either wild-type virus or a recombinant virus, in order to compare the impact of a specific mutation or gene deletion/ insertion. In one example, Hartman *et al*. (2008) mutated a single amino acid residue in the IRF-3 inhibitory domain of the Ebola virus VP35 protein and found enormous differences in the innate immune responses of liver cells infected with wild-type virus as compared to a genetically engineered virus containing this mutation, thus confirming the important role of this protein. Global host gene expression of cells infected with the 1918 H1N1 influenza A virus generated from tissue samples of patients was compared with that of cells infected with a recent H1N1 virus containing only a subset of the 1918 genes in order to identify the basis of virulence in the 1918 isolate (Kash *et al*., 2006). The former virus elicited higher host immune responses, and led to more severe disease pathology and quicker deaths in mice. Viral reverse genetics experiments as well as the generation of viral reassortants have been used to identify pathogenic determinants in the 1918 and other pathogenic strains (reviewed by Basler and Aguilar, 2008; see Conenello *et al*., 2011, for good recent examples of the use of reverse genetics for these studies).

More recently, the techniques of siRNA arrays, miRNA arrays, next generation sequencing and proteomic techniques have been applied to the study of virus–host interactions (Peng *et al*., 2009). In siRNA screens, viruses are used to infect cells seeded onto an siRNA array; if host gene products are required for viral growth, then siRNAs directed against these mRNAs will lead to a decrease in reporter activity/viral replication. Conversely, siRNAs against inhibitory cellular proteins will lead to an increase in reporter activity/viral replication. This approach does not require the presence of recombinant virus as wells can be immunostained for the presence of virus, but incorporation of a reporter gene means that the assay is more

direct and, for reporter genes such as luciferase, will also be more quantitative. Interaction partners of influenza A virus have recently been determined this way using a recombinant virus containing a luciferase reporter (Konig *et al*., 2010).

A good recent example of a high-throughput proteomic technique using recombinant virus is that described by Komarova *et al*. (2011). These authors tagged the measles virus accessory V protein with a One-STrEP amino-terminal tag, then used modified tandem affinity chromatography and mass spectrometry analysis to identify 245 cellular interaction partners of the V protein. There are also high-throughput yeast two-hybrid screens, high-throughput drug screening protocols and other methods. These screens can be used in conjunction with reverse genetics and will have enormous impact on our understanding of host pathogen interactions and hence on drug design.

Another elegant way in which recombinant viruses have been used to study host cell interactions is the use of pseudotyped virus, an approach particularly useful for pathogenic viruses. Carette *et al*. (2011) inserted the Ebola virus glycoprotein into VSV and used this recombinant virus to infect haploid cells in which gene disruptions had been introduced using a retroviral gene trap vector. Over a million virus resistant cells were sequenced to identify over 100 mutations that affect virus entry; these mutations disrupted all six members of the homotypic fusion and vacuole protein-sorting (HOPS) multisubunit tethering complex, which is involved in the fusion of endosomes to lysosomes, and the endo/lysosomal cholesterol transporter protein Niemann–Pick C1.

12.5 Understanding the basis of pathogenicity

This section clearly overlaps a number of other sections but we will include some examples of reverse genetics that address this question here. A common approach to studying pathogenesis is to work with related viruses or strains of virus that differ in their pathogenic potential. Segments, genes or even specific mutations can be swapped between viruses in order to probe the basis of the differences. A good example of this approach is demonstrated by Ebihara *et al*. (2006), who worked with a mouse-adapted strain of Zaire Ebola virus that was very pathogenic in mice. This had a number of nucleotide changes from the original wild-type virus, which did not cause disease in mice. These changes were introduced individually into the wild-type virus from the mouse-adapted strain by reverse genetics and those responsible for the differences (mutations in the NP and VP24) were found to play a role in interferon antagonism.

Influenza B virus is another virus in which mutations were identified which confer lethality in mice; for this virus it was a N221S mutation in the M1 protein; this mutation was introduced into a second strain to confirm the role of this mutation in pathogenicity (Jackson *et al*., 2011).

Reverse genetics is commonly used together with high-throughput technologies or imaging to study pathogenesis. Use of fluorescent tags introduced into a viral

protein allows tracking of the virus in the natural host, providing information on the cells infected early in infection, viral passage through the host and the speed and extent of viral spread. Animals can be examined by post mortem tissue section or in mice by *in vivo* imaging (see section below).

12.6 Real-time virus imaging *in vitro* and *in vivo*

Incorporation of fluorescent or bioluminescent tags into viruses allows real-time imaging both in cell culture and also in living animals such as mice. Numerous viruses have been generated expressing such tags either as fusion proteins or expressed from new intergenic regions/promoters, but there are difficulties in retaining both the additional gene and also the pathogenicity of the tagged viruses. For positive sense viruses, the difficulty of tagging viral proteins is that tagging at the termini of proteins generally interferes with proteolytic cleavage of the polyprotein. However, several positive sense RNA viruses including polio, hepatitis C and Sindbis viruses (SINV) have been tagged by a transposon-based insertion mutagenesis method which indicates which sites can be used for gene insertion. These are then used to insert the GFP gene in a recombinant virus (Teterina *et al*., 2010).

There are three excellent examples of incorporation of fluorescent tags into viral proteins of negative sense RNA viruses in this volume. Chapters 6 and 7 describe the tagging of measles virus L protein and Bunyamwera virus Gc glycoprotein; both of these involve fusion of a fluorescent label to a viral protein or, in the case of BUNV Gc, a truncated protein, such that protein-protein interactions of the protein under study remain unaffected (Duprex *et al*., 2002; Shi *et al*., 2010). Tagging of rhabdoviruses is discussed in Chapter 5. Klingen *et al*. (2008) generated a recombinant rabies virus with two fluorescent tags, one expressed as a phosphoprotein-GFP fusion protein and the other using RFP bounded by the N terminal signal sequence and C terminal transmembrane spanning and cytoplasmic domain sequences of the G glycoprotein. This dually labelled virus was able to infect neurones and passage of the virus through them could be observed. In a previous study, a singly-labelled virus was used to study neuronal connections (Wickersham *et al*., 2007). VSV has similarly been tagged with GFP and used to study neuronal movement (van den Pol *et al*,. 2002).

Generation of a pathogenic, fluorescently labelled influenza A virus has only recently been achieved (Manicassamy *et al*., 2010) as most GFP recombinants tend to be attenuated. The virus described in that paper was made by fusing the GFP to the C terminus of the NS1 protein and removing any splice acceptor sites; although attenuated, this virus was pathogenic in mice. The virus was used to show how the drugs amantadine and oseltamivir affected virus spread within infected animals by *ex vivo* imaging of whole organs and also flow cytometry; interestingly there was a marked difference in effectiveness between the two drugs in different cell types.

Luciferase tagged viruses have been used to image viral infection *in vivo* in host species by bioluminescence imaging. Recombinant viruses are used to infect the

host (usually mice), which are transfused with an appropriate substrate, luciferin in the case of *Renilla* luciferase. Two examples of this approach are Cook and Griffin (2003), who imaged both virulent and avirulent Sindbis viruses tagged with luciferase in susceptible and resistant mice, while Tseng *et al*. (2010) tagged a Sindbis vector used in oncolysis in order to look at tumour infiltration. Although mainly used in mice it has also been used in trout with the fish rhabdovirus infectious haematopoietic necrosis virus to determine the point of entry of the virus (Harmache *et al*., 2006). Bioluminescent imaging is very sensitive, has a higher signal to noise ratio than fluorescent imaging and can be used to image two different processes at once using two different luciferase genes. Unfortunately, as with inserting GFP, it can be hard to insert luciferase genes without affecting pathogenicity. To avoid this problem, mice transgenic for various promoters expressing luciferase such as the interferon-β promoter have been developed; this bypasses, to some extent, the need to make recombinant viruses (Luker and Luker, 2010). Transgenic mice are, however, less luminescent than recombinant viruses and they also have a background luminescence in ears, paws and tail (Hutchens and Luker, 2007).

Labelling techniques are thus useful in the study of cellular tropism, cell entry, intracellular movement, confirmation of viral-host protein-protein interactions as well as host neuronal studies. As Manicassamy *et al*. (2010) discuss, they can be applied to 'investigation of strain specific effects in pathogenesis, tissue tropism, and replication kinetics in different hosts *in vivo*'. Non-invasive real-time imaging techniques usually reduce the numbers of host animals required for virus studies. They can also be used in high-throughput experiments, although use of luciferase or chloramphenicol actetyl transferase (CAT) reporters is usually more quantitative for this.

12.7 Structure-function analysis of viruses and viral domains

Huge strides have been made in recent years in determining the structures of wild-type virions and also of specific interacting domains of virus and host proteins using the techniques of cryo electron microscopy, X-ray crystallography and nuclear magnetic resonance (nmr) spectroscopy. As yet, however, most combinations of structural and genetic studies have been performed on individual proteins or domains rather than whole virus. Examples of proteins/domains that have been studied by a mixture of genetic and structural techniques include the SARS coronavirus nonstructural protein 9 (nsp9; SARS is severe acute respiratory syndrome), the nairovirus ovarian tumour (OTU) domain, and the flavivirus nonstructural protein 5 (Davidson, 2009). For example, the SARS nsp9 protein forms a dimer with residues G100 and G104 being located at the helix interface. Determination of the structure of the nsp9 helix at 2.9A resolution showed that the G104E mutant was very different in structure from the G104 wild-type protein, and introduction of this mutation

into the virus by reverse genetics proved lethal (Miknis *et al*., 2009). The nairovirus OTU domain is capable of hydrolysing ubiquitin and ISG-15-peptide linkages as a novel way of evading host cell responses. The structure of the OTU domain in the Crimean-Congo haemorrhagic fever virus (CCHFV) has recently been determined by three different groups; one group has introduced mutations based on the structure of the domain when bound to ubiquitin or ISG-15 in order to selectively impair either ISG-15 or ubiquitin proteolysis and thus to study the relative importance of these two processes in the viral lifestyle (Akutsu *et al*., 2011). A recent example of mutational analysis of a whole virus is the arterivirus equine arteritis virus (EAV) which was mutated in the nsp7 gene to confirm structural predictions made after nmr analysis of the nsp7 protein (Manolaridis *et al*., 2011).

As yet there have been few studies of recombinant virions to parallel those of herpes simplex virus, for which multiple structural determinations have been made for different viral mutants (for example, Zhou *et al*., 1995). An exception is the structural determination of virus-like particles (VLPs) of cowpea chlorotic mottle virus, expressed in *Pichia pastoris* with mutations in the capsid protein (Brumfield *et al*., 2004; Tang *et al*., 2006). Studies have also been performed on another relatively simple virus, Flock House virus, a small RNA virus in the *Nodaviridae* family. This was mutated at the calcium binding sites of the capsid, which affected stability and infectivity without altering the overall architecture of the capsid (Banerjee *et al*., 2010).

Interestingly, a senior virologist commented that structural findings alone do not generally excite when they cannot be confirmed and extended by genomic manipulation of the virus (Johnson, 2008). We might therefore hope that the coming years will see an explosion of work in this area now we can image a greater range of recombinant viruses and with recent improvements to imaging techniques.

12.8 Vaccine generation

Reverse genetics is of enormous importance in the generation of new virus vaccines, either by use of RNA virus vectors in which an appropriate viral gene is inserted, or by modification of viruses in order to attenuate them. Alphaviruses are one of the most commonly used RNA virus vectors; the viral structural proteins are removed from the replicon and those required for packaging are generally supplied *in trans*. The most commonly used vectors are Sindbis virus, Semliki Forest virus (SFV) and Venezuelan equine encephalitis virus (VEE). An example of their use has been to express Lassa and Ebola virus proteins (Pushko *et al*., 2001). In a variation on this theme, the VSV G protein was incorporated into the SFV replicon, which could then be used without the SFV proteins *in trans,* thus eliminating all possibility of regenerating a viable SFV (Rose *et al*., 2008). In that paper, the SFV replicon was either *in vitro* transcribed and transfected into cells or expressed from a transfected plasmid. VEE has been used to express the SARS virus S protein (Baric *et al*., 2006). Other RNA virus vectors include the human parainfluenza virus

HPIV3 vector used for immunisation of children for protection against the virus itself and against SARS (Bukreyev *et al*., 2004). A recombinant VSV has been made with Lassa virus glycoprotein GP instead of the VSV G protein (Geisbert *et al*., 2005).

Virus attenuation generates viruses that can still enter host cells and elicit host responses, but which are limited in their ability to replicate in these cells, making them much safer. Temperature-sensitive mutations are useful for this reason, as the virus can be grown to high titre at the low, permissive temperature, but is limited in its *in vivo* replication; this approach is particularly useful for viruses such as RSV that only grow to low titre *in vitro*. Using reverse genetics, engineered mutations can be used to enhance immunogenicity and generate (multi) attenuated and multivalent viruses (Collins and Murphy, 2005). Most work has been done for those viruses which are serious human pathogens, in particular influenza A virus, but also SARS coronavirus, the respiratory pathogens including RSV, HPIV3 and hMPV (human metapneumovirus), measles and rabies viruses.

Several strategies have been employed to generate attenuated viruses including modification of the viral polymerase, deletion of non-structural genes or even structural genes and modification of replication/ transcription signals. These modifications can be used singly or combined within the same virus to obtain viruses of varying levels of attenuation. Abrogation of genes involved in host defence antagonism, such as the Bunyamwera virus NSs protein (Bridgen *et al*., 2001) or the influenza A virus NS1 protein, are particularly useful in vaccine design as viruses can be grown to high titre in cell lines deficient in interferon signalling but then replication is limited in the *in vivo* setting. Recent advances in using influenza A virus NS1 mutants as potential vaccine candidates are summarised by Richt and Garcia-Sastre (2009).

Deletion or replacement of structural genes is another promising route for viral attenuation. One example of this approach is the deletion of the SARS coronavirus E gene, which is structural but not essential for growth, and leads to a 1000-fold reduction in viral titre in cell culture and attenuation in an animal model (deDiego *et al*., 2007). Another is the replacement of the arenavirus LCMV (lymphocytic choriomeningitis virus) GP gene with the G gene of the unrelated rhabdovirus VSV (serotype Indiana). This resulted in a highly attenuated virus, rLCMV/ INDG, that remained genetically stable and was able to elicit very strong, long-term T cell-mediated immunity against lethal challenge with wild-type LCMV (Bergthaler *et al*., 2006). Ongoing research will widen the ways in which viruses can be attenuated. One group modified the protease cleavage sites in the Junin arenavirus glycoprotein precursor from a SKI-1/S1P peptidase site to a furin cleavage site in an attempt to generate a novel attenuated virus; in this case it was still virulent but similar approaches in the future may succeed (Albarino *et al*., 2009).

Utilising our knowledge of viral quasispecies provides a novel approach to attenuation. Vignuzzi and colleagues selected a poliovirus resistant to the drug ribavirin, which acts by increasing the mutation rate of the polymerase to such an extent that the virus cannot survive; the polymerase from this virus had a single mutation G64S

and had only limited ability to mutate. This virus was found to be attenuated in mice (Vignuzzi *et al*., 2006). Mueller *et al*. (2006) generated a poliovirus by reverse genetics in which the coding sequence had been changed to utilise rare codons; these viruses were found to be attenuated. More recently, this group generated two further viruses in which the P1 capsid region contained all over-represented codons or all under-represented codons (Coleman *et al*., 2008) and found the latter to be greatly attenuated.

One concern of generating attenuated viruses by reverse genetics is that they can then reassort or recombine with circulating wild-type viruses to regain virulence, so a number of different strategies have been investigated to eliminate this risk. The viral gene order of non-segmented viruses can be rearranged to reduce the expression level of the replication proteins, thus attenuating the virus (see the examples of VSV in Flanagan *et al*., 2001 and SARS coronavirus in de Haan *et al*., 2002, though, in this last example, recombinants were made by targetted recombination rather than reverse genetics). The transcription regulatory sequences of coronaviruses have been mutated to prevent recombination (Enjuanes *et al*., 2008). Virus chimaeras, such as a bovine PIV3 vector base that expresses human PIV3 antigens and HPIV1 that expresses HMPV F protein, are less able to revert and also allow use of more robust backbones to display the antigens of viruses that are harder to grow (Collins and Murphy, 2005). For segmented viruses, altering the location of genes between segments, and altering the segment number can also help to prevent reassortment (Gao *et al*., 2010).

Another active area of research is the generation of multivalent vaccines, where viruses can express multiple antigenic variants, or epitopes from more than one virus simultaneously. The recently generated 9 segment influenza A virus (Gao *et al*., 2010) expressed both the H1 and H3 haemagglutinin epitopes from the same virus and protected inoculated mice from both types of wild type virus. Similarly, viruses have been generated by reverse genetics that express most of the influenza B HA protein from an influenza A virus, thus providing protection from two different respiratory pathogens simultaneously (reviewed by Jackson *et al*., 2011). Sato *et al*. (2011) summarise a number of new multivalent viruses generated by reverse genetics including recombinant measles virus expressing epitopes from hepatitis C or Nipah viruses.

12.9 Drug development

Two properties of RNA viruses, their ability to mutate quickly and the existence of viral quasispecies, pose severe difficulties to drug development, so the ability to manipulate the viral genome is particularly important. Reverse genetics approaches have been most useful in drug development for two classes of viruses: high containment viruses and those which do not grow well in cell culture such as hepatitis C virus and noroviruses. Minigenome replication without the generation of infectious virus can be performed at lower biosafety levels than are required for whole

virus, and can be used to test inhibitors of many processes including viral replication and packaging. This is particularly useful for some arenaviruses, filoviruses and nairoviruses that require BSL4 containment. The effectiveness of the nucleoside inhibitor rifampicin against the nairovirus CCHFV has been tested in this way (Bergeron *et al*., 2010) while de la Torre (2008) summarises the impact of minigenome systems in arenavirus drug development. Potential inhibitors of Zaire Ebola virus replication and/or transcription were tested using a minireplicon containing a luciferase gene (Jasenosky *et al*., 2010), while McCarthy *et al*. (2006) performed high-throughput screening for inhibitors of Ebola virus budding using a luciferase assay. De la Torre also mentions the importance of tagged viruses in high-throughput screening of drugs targeting either viral processes or cellular functions required by the virus; in the case of arenaviruses it required the generation of a three-segmented virus rather than a bisegmented one to incorporate an appropriate additional gene (de la Torre, 2008).

The high disease burden caused by some of the flaviviruses such as Dengue virus and West Nile virus has encouraged use of high-throughput studies for this family of viruses. Two main approaches have been used: packaging minigenomes that express a reporter, or using persistently replicating flavivirus genomes tagged with a reporter (Patkar *et al*., 2009). These authors used a yellow fever virus (YFV) replicon that expresses *Renilla* luciferase in a replication dependent manner; this replicon could be packaged using a Sindbis vector to express the viral structural proteins. The authors identified new potential drug targets against YFV that mapped to the ns4b region by high-throughput screening. Recently a full-length Dengue virus containing a *Renilla* luciferase gene in frame with the capsid gene has been generated and used for drug discovery (Zou *et al*., 2011).

Likewise the huge number of people affected by hepatitis C virus (HCV) worldwide (170 million) has also prompted high-throughput drug screening against this virus. Although infectious virus can now be generated from cDNA in Huh-7 cells (Bartenschlager and Pietschmann, 2005), minireplicons continue to provide a convenient way in which to test new drugs that may have been identified by other means, such as NS3 protease cleavage assays, or by rational drug design against the RNA-dependent RNA polymerase enzyme. This is particularly pertinent as the virus replicates in membrane-associated multi-protein complexes, to which potential drugs may not have physical access. One example of *ab initio* high-throughput screening is provided by Huang *et al*., 2008, who identified a compound, R706, in a high-throughput (230,000 chemicals) minigenome assay; this compound was inhibitory in Western blotting and RNA transcription assays but its function was not identified. Further studies identified a related compound, R803, that was more effective than alpha interferon at blocking HCV replication in the minigenome model. Care must, however, be taken that the adaptive mutations introduced to enhance *in vitro* replicon replication do not affect the findings. Uprichard (2010) summarises drug development using minireplicon systems. Additional drug targets to treat HCV infection have been identified using RNAi and proteomic screens to study host-virus interactors. Such studies have led to clinical trials for inhibitors

of cyclophilin, HMG-coA reductase and the liver specific microRNA miR-122 (Uprichard, 2010).

Crowder and Kirkegaard (2005) have used an elegant approach to drug design using poliovirus. Their premise is that drug-sensitive proteins or structures can mask the effect of drug-resistant proteins or structures though oligomerisation. Thus, treating those proteins sensitive to a drug will have a consequently greater effect than treating non-oligomerising components as not only the targeted component will be affected but also the interaction partners. This is called a dominant negative phenotype and various mutations in the capsid and polymerase proteins as well as in the cis-acting replication element and the poliovirus 2A coding region (proteinase-deficient mutations) had this property, as was tested by adding *in vitro* transcribed infectious RNA to wild-type cultures.

12.10 Gene delivery and knock-out in plant cells including virus-induced gene silencing (VIGS)

Reverse genetics of plant viruses has become a very important mechanism of gene delivery and knock-out in plants, which cannot be transfected with the same ease as mammalian cells. Various different viruses have been used for this purpose including Tobacco rattle virus (TRV), a positive sense RNA *Tobravirus* that infects many species including potato and tomato, Tobacco mosaic virus (a *Tobamovirus*), Potato virus X (a *Potexvirus*) and Barley strip mosaic virus (a *Hordeivirus*) (Unver and Budak, 2009). The biggest application is in virus-induced gene silencing (VIGS), whereby viruses are engineered to incorporate sequences derived from the gene to be knocked down. These are introduced into the plant by agro-infiltration or inoculation. The host develops an RNAi defence response against the viral RNA including the additional gene, which then leads to a rapid but transient knock-down of the host mRNA known as post-transcriptional gene silencing. This is useful for those plant species which are not amenable to stable transformation, or for genes that cause embryo lethality. Application of this technique has gone hand-in-hand with increased plant genomic sequencing, and applications will increase as more genomic information becomes available.

The first VIGS vector to be developed in 1995, and still one of the most widely used, is TRV (Kumagai *et al*., 1995; Purkayastha and Dasgupta, 2009). The technique is still being improved and modified; recent work has indicated that silencing via VIGS can last for up to two years using a TRV vector and, moreover, that this silencing can be vertically transmitted to progeny plants by means of seeds, which will greatly enhance the application of this technique (Senthil-Kumar and Mysore, 2011a). Other developments are the continued introduction of new vectors and application to specific tasks such as silencing of root-specific genes or analysis of the role of MAPK cascades in innate immunity. There are now cDNA libraries cloned into VIGS vectors which can be used to study specific sets of genes (Senthil-Kumar

and Mysore, 2011b). Suppression has also been achieved using miRNAs as well as siRNAs. VIGS is thus an effective genomics tool limited only in suitable vectors, viral resistance genes and biosafety issues of VIGS spread (Tang *et al*., 2010; Senthil-Kumar and Mysore, 2011a).

Information derived from VIGS screening can then be used in a positive sense to identify genes responsible for specific traits. These genes can then be used to develop transgenic crop plants as a means of crop improvement (Senthil-Kumar and Mysore, 2011b).

12.11 Gene delivery in arthropod and mammalian cells

A number of RNA viruses have been modified to act as gene delivery vehicles including VSV and several coronaviruses, but by far the most commonly used vectors used *in vitro* derive from alphaviruses such as SINV, SFV and VEE. Alphavirus vectors generally comprise a two component system with, firstly, a vector containing the non-structural genes together with a packaging signal and with the structural genes replaced by the gene(s) of interest and, secondly, a helper RNA encoding the non-structural and structural proteins but with no packaging signal. They have been used extensively for gene delivery *in vitro* into mammalian cells and *in vivo*, as vaccine candidates and for tumour therapy (Rayner *et al*., 2002; Atkins *et al*., 2008). A recent report describes packaging of SINV with 18,000 nucleotides instead of the usual 11,703, with the additional nucleic acid being incorporated into a larger virion with altered morphology (Nanda *et al*., 2009), thus greatly increasing the potential application of this system.

As alphaviruses are mosquito-borne, they can also express heterologous genes in mosquitoes and generation of, for example, GFP-tagged viruses has allowed visualisation of virus spread within the mosquito (Phillips *et al*., 2010). This technique is useful to rapidly assess vector competence of different mosquito vectors. Infection of arthropods is non-cytolytic, with viruses often establishing persistent infection of the vector. Until recently, little was known about mosquito host defence mechanisms other than induction of RNA interference. Other pathways are being probed by the generation of recombinant viruses such as SFV expressing bioluminescent reporter genes. This has shown that SFV reduces cellular expression in mosquito *Aedes albopictus* cells, though to a far lesser extent than happens in eukaryotic cells. In addition, the STAT/IMD pathway was shown to mediate protection against SFV (Fragkoudis *et al*., 2008).

Introduction of, for instance, RNAi directed against genes of viruses such as Dengue, into mosquitoes is likely to be a major application of reverse genetics, since it will be more efficient than other approaches such as direct transfection. A small DNA virus (densovirus) has recently been used for this application (Gu *et al*., 2011), but this has only limited ability to accept additional DNA; larger RNA viruses with a greater insert capability would be tremendously useful for this purpose.

One of the advantages of using viruses as delivery vehicles is that they enter cells via specific virus receptors, and thus many viruses are only capable of

entering a specific cell type. This can be exploited for studies requiring gene delivery to a specific cell type, for example neurones. Neurotropic RNA viruses include the rhabdoviruses rabies virus and VSV, alphaviruses and some coronaviruses. Recent developments with alphaviruses, including altering host preference for specific neuronal cells, have been summarised by Ehrengruber and Lundstrom (2007). Recombinant VSV viruses were developed with the G-gene deleted and with reporter genes for GFP (dG-VSV-GFP) or dsRed (dG-VSV-dsRed) in the strongest transcriptional position. These viruses expressed the reporter genes to high levels and could only replicate in the first brain cell infected, thus eliminating virus spread (van den Pol *et al*., 2009). In this paper they were used to study calcium responses in virus-infected cells. The authors state: 'The complications notwithstanding, viruses, or viral vectors, are the current method of choice for gene transfer into the brain. Viral-mediated gene transfer holds promise for treatment of many brain diseases, including Parkinson's disease, epilepsy, Alzheimer's disease, spinal cord trauma, and stroke.'

12.12 Development of oncolytic virus and adaptation to this purpose

Tumour cells are generally rapidly dividing and, as such, favour viral replication. In addition, many tumour cell lines are deficient in interferon signalling, substantially enhancing virus growth. Thus, many viruses are likely to have a certain propensity for acting as oncolytic agents, and indeed representatives from many viral families have been used for this purpose including measles virus, mumps virus, reovirus, Newcastle disease virus (NDV), VSV and polio (summarised by van den Pol *et al*., 2009). Treatment can be by means of viral replicons, which can be modified to deliver toxic, therapeutic, or disease-modulating genes. Viruses for oncolysis are generally fully replication competent, to enable them to kill the tumour cells in which they are replicating as well as spread within the tumour mass. This does mean that they are associated with a higher risk of complications (van den Pol *et al*., 2009). However, this can be minimised by manipulating them to remove interferon antagonist genes, as this limits their growth in interferon competent, non-tumour cells, as well as other attenuating mutations.

VSV seems to be a particularly good oncolytic agent, as determined by Wollmann *et al*., (2005), who compared the oncolytic potential of 10 different viruses. This is helped by its ability to infect a wide range of cells efficiently. It has been tested on a range of tumours including glioblastoma brain tumours (van den Pol *et al*., 2009) and high risk bladder cancer (Hadaschik *et al*., 2008). Intravenous injections of VSV strain rp30a were found to cross the blood–brain barrier and infect human glioblastoma cells transplanted to the mouse brain (Ozduman *et al*., 2008). A modified VSV with a single chain antibody directed to a specific cell receptor was also used to target mammary tumours (Gao *et al*., 2006). Mutants of VSV exist which are deficient in interferon antagonism and these are thought to be better oncolytic agents (Stojdl *et al*., 2003); indeed, the attenuated AV3 strain is being

used for phase I clinical trials by Hadaschik *et al*. (2008). More recently, Brun *et al*. (2010) assessed a panel of initially 20 rhabdoviruses against different tumour cell lines to identify another potential potent oncolytic agent within this family, Maraba virus. This virus was then cloned and mutated in the G and M genes to increase the maximum tolerable dose in mice by a factor of 100. This modified virus appeared to work well in mouse xenograft tumour models (Brun *et al*., 2010).

Both measles virus and reoviruses have been studied fairly extensively for their application in oncolysis. The efficacy of measles virus was first observed in cancer patients infected naturally. As the vaccine strain of measles has had extensive safety testing, it can be used in human trials. Another advantage is that attenuated vaccine strains of measles virus have adapted to use CD46, a regulator of complement activation that is expressed at high level in tumour cells, whereas the normal SLAM receptor is not expressed so they cannot be infected by wild-type virus (Russell and Peng, 2009). Treatment works well in animal models but is hindered in man because of widespread immunity (Russell and Peng, 2009).

Treatments with reoviruses have reached stage I and II clinical trials as Reolysin (Oncolytics Biotech) (Lal *et al*., 2009; Kelly *et al*., 2009). A full list of clinical trials and tumour types evaluated can be found on the company website: http://www.oncolyticsbiotech.com/clinical.html. Reovirus is apathogenic in man and replicates in tumours aided by Ras signalling being activated, preventing synthesis of the antiviral PKR protein (van den Wollenberg *et al*., 2009). Reovirus mutants have been made to target specific tumours (van den Wollenberg *et al*., 2008). Oncolysis with coronaviruses is less developed but there are still several examples of this approach (Wurdinger *et al*., 2005; Verheije *et al*., 2009).

In summary, several viruses are showing promise in clinical trials as oncolytic agents or vectors. The ability to manipulate the viral genomes means that they can be modified to limit growth to tumours by removing interferon antagonist genes, other toxicity factors can be added into the virus genomes such as the IL-2 gene into NDV, and they can be imaged within the tumours. There are, however, concerns that weakening viruses to restrict them to tumours makes them less efficient to kill the tumours. New research is being done to incorporate tissue-specific miRNAs into the viruses to restrict their infectivity without affecting their pathogenicity in the target tissues (Kelly *et al*., 2008, 2010; Leber *et al*., 2011). We can expect many interesting developments in this field.

12.13 Personal highlights and future directions

Some of my personal highlights of this research have been the generation of viruses with altered segment number, thus challenging our concept of the nature of specific viruses; thus we now have bunyaviruses with 2 segments not 3 (Brennan *et al*., 2011), arenaviruses with 3 segments not 2 (Emonet *et al*., 2009), and influenza A viruses with 9 not 8 segments (Gao *et al*., 2010). The additional segment of the arenavirus LCMV was stabilised by splitting the S segment that

previously expressed in an ambisense fashion into two separate S segments, each containing a structural gene (the nucleoprotein and glycoprotein precursor). The bunyavirus RVFV was made into a bisegmented virus by combining the genetic content of both smaller segments into one, ambisense, segment. Other viruses that were originally negative sense have been generated as ambisense viruses (Finke and Conzelmann, 1997; Flick and Hobom 1999; le Mercier *et al*., 2002). Both these approaches allow additional genetic material, for example, reporter genes useful for imaging, to be introduced into the genome as well as probing the processes of segment packaging.

The regeneration of the 1918 H1N1 influenza A virus by reverse genetics was undeniably a major breakthrough, and has allowed ongoing investigation into the reasons why this virus was so pathogenic. There has been a plethora of really elegant experiments testing the way in which genes are expressed differentially and then exploiting this finding (see Section 12.1). The study of pathogenesis is clearly a major important application.

For the future, I anticipate seeing much more application to the field of structure/function analysis, for example, seeing how modification of a particular domain affects function and structure. Work on arboviruses and mosquitoes, using the former to shorten the life span of mosquitoes such that they are less likely to transmit disease, or make them more sensitive to incoming viruses is another important application (Myles *et al*., 2008). The ds RNA virus families are only just becoming amenable to reverse genetic approaches (see Trask *et al*., 2010; Troupin *et al*., 2010), yet some viruses such as rotavirus are responsible for enormous mortality worldwide. There are vaccines licensed for rotavirus infection but use of reverse genetics techniques could well enhance the safety and effectiveness of these vaccines.

As the techniques become a lot more established, we will begin to see many more applications in, for example, drug and vaccine design and in gene delivery. Neurotropic viruses such as the coronavirus murine hepatitis virus or the rhabdovirus rabies virus are ideally suited to gene delivery into neurones with application to treatment of dementia, Parkinson's disease, Creutzfeld Jacob disease, multiple sclerosis or other conditions. This is an ambitious project, but a recent review (Nilsson *et al*., 2010) summarises several possible gene therapy targets including neurotropic growth factors or beta amyloid degrading enzymes for Alzheimer's disease.

Thus, my overall conclusion is that we have already learned an enormous amount using this technique, demonstrable particularly for those viruses such as influenza A which have a huge number of active researchers. As rescue protocols improve – and even well established rescue protocols such as that for rabies virus are still being improved (Ghanem *et al*., 2012) – we can expect to rescue less amenable viruses to increase our knowledge and understanding.

For other virus families, reverse genetics approaches have only been available for a short period, so there is much to learn. This includes the reovirus family which includes major pathogens such as human rotavirus. For most virus families, it is often only the viral prototype or serious pathogens which have been studied, while

other viruses within the family have not yet been rescued (for instance, very few viruses of fish had been rescued before 2009, Biacchesi, 2011). We can expect to see both a widening and deepening of applications. In conclusion, the future still looks bright for viral reverse genetics experiments.

References

Akutsu, M., Ye, Y., Virdee, S., Chin, J. W. and Komander, D. 2011. Molecular basis for ubiquitin and ISG15 cross-reactivity in viral ovarian tumor domains. *Proceedings of the National Academy of Sciences of the United States of America* **108**(6): 2228–2233.

Albarino, C. G., Bergeron, E., Erickson, B. R., Khristova, M. L., Rollin, P. E. and Nichol, S. T. 2009. Efficient reverse genetics generation of infectious junin viruses differing in glycoprotein processing. *Journal of Virology* **83**(11): 5606–5614.

Atkins, G. J., Fleeton, M. N. and Sheahan, B. J. 2008. Therapeutic and prophylactic applications of alphavirus vectors. *Expert Reviews in Molecular Medicine* **10**: e33.

Ball, L. A., Pringle, C. R., Flanagan, B., Perepelitsa, V. P. and Wertz, G. W. 1999. Phenotypic consequences of rearranging the P, M, and G genes of vesicular stomatitis virus. *Journal of Virology* **73**(6): 4705–4712.

Banerjee, M., Speir, J. A., Kwan, M. H., Huang, R., Aryanpur, P. P., Bothner, B. and Johnson, J. E. 2010. Structure and function of a genetically engineered mimic of a nonenveloped virus entry intermediate. *Journal of Virology* **84**(9): 4737–4746.

Baric, R. S., Sheahan, T., Deming, D., Donaldson, E., Yount, B., Sims, A. C., Roberts, R. S., Frieman, M. and Rockx, B. 2006. SARS coronavirus vaccine development. *Advances in Experimental Medicine and Biology* **581**: 553–560.

Bartenschlager, R. and Pietschmann, T. 2005. Efficient hepatitis C virus cell culture system: what a difference the host cell makes. *Proceedings of the National Academy of Sciences of the United States of America* **102**(28): 9739–9740.

Basler, C. F. and Aguilar, P. V. 2008. Progress in identifying virulence determinants of the 1918 H1N1 and the Southeast Asian H5N1 influenza A viruses. *Antiviral Research* **79**(3): 166–178.

Bergeron, E., Albarino, C. G., Khristova, M. L. and Nichol, S. T. 2010. Crimean-Congo hemorrhagic fever virus-encoded ovarian tumor protease activity is dispensable for virus RNA polymerase function. *Journal of Virology* **84**(1): 216–226.

Bergthaler, A., Gerber, N. U., Merkler, D., Horvath, E., de la Torre, J. C. and Pinschewer, D. D. 2006. Envelope exchange for the generation of live-attenuated arenavirus vaccines. *PLoS Pathogens* **2**(6): E51.

Biacchesi, S. 2011. The reverse genetics applied to fish RNA viruses. *Veterinary Research* **42**(1): 12.

Biacchesi, S., Skiadopoulos, M. H., Yang, L., Lamirande, E. W., Tran, K. C., Murphy, B. R., Collins, P. L. and Buchholz, U. J. 2004. "Recombinant human

Metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate. *Journal of Virology* **78**(23): 12877–12887.

Brennan, B., Welch, S. R., McLees, A. and Elliott, R. M. 2011. Creation of a recombinant Rift Valley fever virus with a two-segmented genome. *Journal of Virology* **85**(19): 10310–10318.

Bridgen, A., Weber, F., Fazakerley, J. K. and Elliott, R. M. 2001. Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **98**(2): 664–669.

Brumfield, S., Willits, D., Tang, L., Johnson, J. E., Douglas, T. and Young, M. 2004. Heterologous expression of the modified coat protein of Cowpea chlorotic mottle bromovirus results in the assembly of protein cages with altered architectures and function. *The Journal of General Virology* **85**(Pt 4): 1049–1053.

Brun, J., McManus, D., Lefebvre, C., Hu, K., Falls, T., Atkinsm, H., Bell, J. C., McCart, J. A., Mahoney, D., and Stojdl, D. F., 2010. Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Molecular Therapeutics* **18**(8): 1440–1449.

Buchholz, U. J., Biacchesi, S., Pham, Q. N., Tran, K., Yang, C. L., Luongo, C. L., Skiadopoulos, M. H., *et al*. 2005. Deletion of M2 gene open reading frames 1 and 2 of human metapneumovirus: effects on RNA synthesis, attenuation, and immunogenicity. *Journal of Virology* **79**(11): 6588–6597.

Bukreyev, A., Lamirande, E. W., Buchholz, U. J., Vogel, L. N., Elkins, W. R., St Claire, M., Murphy, B. R., Subbarao, K. and Collins, P. L. 2004. Mucosal immunisation of African green monkeys (Cercopithecus aethiops) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. *Lancet* **363**(9427): 2122–2127.

Carette, J. E., Raaben, M., Wong, A. C., Herbert, A. S., Obernosterer, G., Mulherkar, N., Kuehne, A. I., *et al*. 2011 Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* **477**(7364): 340–343.

Coleman, J. R., Papamichail, D., Skiena, S., Futcher, B., Wimmer, E. and Mueller, S. 2008. Virus attenuation by genome-scale changes in codon pair bias. *Science (New York, N.Y.)* **320**(5884): 1784–1787.

Collins, P. L. and Murphy, B. R. 2005. New generation live vaccines against human respiratory syncytial virus designed by reverse genetics. *Proceedings of the American Thoracic Society* **2**(2): 166–173.

Conenello, G. M., Tisoncik, J. R., Rosenzweig, E., Varga, Z. T., Palese, P. and Katze, M. G. 2011. A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response in vivo. *Journal of Virology* **85**(2): 652–662.

Cook, S. H. and Griffin, D. E. 2003. Luciferase imaging of a neurotropic viral infection in intact animals. *Journal of Virology* **77**(9): 5333–5338.

Crowder, S. and Kirkegaard, K. 2005. Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. *Nature Genetics* **37**(7): 701–709.

Davidson, A. D. 2009. New insights into flavivirus nonstructural protein 5. *Advances in Virus Research* **74**: 41–101.

DeDiego, M. L., Alvarez, E., Almazan, F., Rejas, M. T., Lamirande, E., Roberts, A., Shieh, W. J., *et al*. 2007. A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. *Journal of Virology* **81**(4): 1701–1713.

de Haan, C. A., Volders, H., Koetzner, C. A., Masters, P. S. and Rottier,P. J. 2002. Coronaviruses maintain viability despite dramatic rearrangements of the strictly conserved genome organization. *Journal of Virology* **76**(24): 12491–12502.

de la Torre, J. C. 2008. Reverse genetics approaches to combat pathogenic arenaviruses. *Antiviral Research* **80**(3): 239–250.

Duprex, W. P., Collins, F. M. and Rima, B. K. 2002. Modulating the function of the measles virus RNA-dependent RNA polymerase by insertion of green fluorescent protein into the open reading frame. *Journal of Virology* **76**(14): 7322–7328.

Ebihara, H., Takada, A., Kobasa, D., Jones, S., Neumann, G., Theriault, S., Bray, M., *et al*. 2006. Molecular determinants of Ebola virus virulence in mice. *PLoS Pathogens* **2**(7): e73.

Ehrengruber, M. U. and Lundstrom, K. 2007. Alphaviruses: Semliki Forest virus and Sindbis virus vectors for gene transfer into neurons. *Current Protocols In Neuroscience /Editorial Board, Jacqueline N.Crawley . . .* [*et al*.] Chapter 4: Unit 4.22.

Eifan, S. A. and Elliott, R. M. 2009. Mutational analysis of the Bunyamwera orthobunyavirus nucleocapsid protein gene. *Journal of Virology* **83**(21): 11307–11317.

Emonet, S. F., Garidou, L., McGavern, D. B. and de la Torre, J. C. 2009. Generation of recombinant lymphocytic choriomeningitis viruses with trisegmented genomes stably expressing two additional genes of interest. *Proceedings of the National Academy of Sciences of the United States of America* **106**(9): 3473–3478.

Enjuanes, L., Dediego, M. L., Alvarez, E., Deming, D., Sheahan, T. and Baric, R. 2008. Vaccines to prevent severe acute respiratory syndrome coronavirus-induced disease. *Virus Research* **133**(1): 45–62.

Fearns, R., Peeples, M. E. and Collins, P. L. 2002. Mapping the transcription and replication promoters of respiratory syncytial virus. *Journal of Virology* **76**(4): 1663–1672.

Finke, S. and Conzelmann, K. K. 1997. Ambisense gene expression from recombinant rabies virus: random packaging of positive- and negative-strand ribonucleoprotein complexes into rabies virions. *Journal of Virology* **71**(10): 7281–7288.

Finke, S., Cox, J. H. and Conzelmann, K. K. 2000. Differential transcription attenuation of rabies virus genes by intergenic regions: generation of recombinant viruses overexpressing the polymerase gene. *Journal of Virology* **74**(16): 7261–7269.

Flanagan, E. B., Ball, L. A. and Wertz, G. W. 2000. Moving the glycoprotein gene of vesicular stomatitis virus to promoter-proximal positions accelerates and

enhances the protective immune response. *Journal of Virology* **74**(17): 7895–7902.

Flanagan, E. B., Zamparo, J. M., Ball, L. A., Rodriguez, L. L. and Wertz, G. W. 2001. Rearrangement of the genes of vesicular stomatitis virus eliminates clinical disease in the natural host: new strategy for vaccine development. *Journal of Virology* **75**(13): 6107–6114.

Flick, R. and Hobom, G. 1999. Transient bicistronic vRNA segments for indirect selection of recombinant influenza viruses. *Virology* **262**(1): 93–103.

Fragkoudis, R., Chi, Y., Siu, R. W., Barry, G., Attarzadeh-Yazdi, G., Merits, A., Nash, A. A., *et al*. 2008. Semliki Forest virus strongly reduces mosquito host defence signaling. *Insect Molecular Biology* **17**(6): 647–656.

Gao, Q., Lowen, A. C., Wang, T. T. and Palese, P. 2010. A nine-segment influenza a virus carrying subtype H1 and H3 hemagglutinins. *Journal of Virology* **84**(16): 8062–8071.

Gao, Y., Whitaker-Dowling, P., Watkins, S. C., Griffin, J. A. and Bergman, I. 2006. Rapid adaptation of a recombinant vesicular stomatitis virus to a targeted cell line. *Journal of Virology* **80**(17): 8603–8612.

Garcia-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P. and Muster, T. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* **252**(2): 324–330.

Geisbert, T. W., Jones, S., Fritz, E. A., Shurtleff, A. C., Geisbert, J. B., Liebscher, R., Grolla, A., Stroher, U., Fernando, L., Daddario, K. M., Guttieri, M. C., Mothe, B. R., Larsen, T., Hensley, L. E., Jahrling, P. B. and Feldmann, H. 2005. Development of a new vaccine for the prevention of Lassa fever. *PLoS Medicine* **2**(6): e183.

Ghanem, A., Kern, A. and Conzelmann, K. K. 2012. Significantly improved rescue of rabies virus from cDNA plasmids. *European Journal of Cell Biology* **91**: 10–16.

Green, T. J. and Luo, M. 2006. Resolution improvement of X-ray diffraction data of crystals of a vesicular stomatitis virus nucleocapsid protein oligomer complexed with RNA. *Acta Crystallographica. Section D, Biological Crystallography* **62**(Pt 5): 498–504.

Gu, J., Liu, M., Deng, Y., Peng, H. and Chen, X., 2011. Development of an efficient recombinant mosquito densovirus-mediated RNA interference system and its preliminary application in mosquito control. *PLoS One*. **6**(6): e21329.

Hadaschik, B. A., Zhang, K., So, A. I., Fazli, L., Jia, W., Bell, J. C., Gleave, M. E. and Rennie, P. S. 2008. Oncolytic vesicular stomatitis viruses are potent agents for intravesical treatment of high-risk bladder cancer. *Cancer Research* **68**(12): 4506–4510.

Hale, B. G., Randall, R. E., Ortin, J. and Jackson, D. 2008. The multifunctional NS1 protein of influenza A viruses. *The Journal of General Virology* **89**(Pt 10): 2359–2376.

Harmache, A., LeBerre, M., Droineau, S., Giovannini, M. and Bremont, M. 2006. Bioluminescence imaging of live infected salmonids reveals that the fin bases

are the major portal of entry for Novirhabdovirus. *Journal of Virology* **80**(7): 3655–3659.

Hartman, A. L., Ling, L., Nichol, S. T. and Hibberd, M. L. 2008. Whole-genome expression profiling reveals that inhibition of host innate immune response pathways by Ebola virus can be reversed by a single amino acid change in the VP35 protein. *Journal of Virology* **82**(11): 5348–5358.

Huang, P., Goff, D. A., Huang, Q., Martinez, A., Xu, X., Crowder, S., Issakani, S. D., Anderson, E., Sheng, N., Achacoso, P., Yen, A., Kinsella, T., Darwish, I. S., Kolluri, R., Hong, H., Qu, K., Stauffer, E., Goldstein, E., Singh, R., Payan, D. G. and Lu, H. H. 2008. Discovery and characterization of substituted diphenyl heterocyclic compounds as potent and selective inhibitors of hepatitis C virus replication. *Antimicrobial Agents and Chemotherapy* **52**(4): 1419–1429.

Hutchens, M. and Luker, G. D. 2007. Applications of bioluminescence imaging to the study of infectious diseases. *Cellular Microbiology* **9**(10): 2315–2322.

Hutchinson, E. C., von Kirchbach, J. C., Gog, J. R. and Digard, P. 2010. Genome packaging in influenza A virus. *The Journal of General Virology* **91**(Pt 2): 313–328.

Jackson, D., Elderfield, R. A. and Barclay, W. S. 2011. Molecular studies of influenza B virus in the reverse genetics era. *The Journal of General Virology* **92**(Pt 1): 1–17.

Jasenosky, L. D., Neumann, G. and Kawaoka, Y. 2010. Minigenome-based reporter system suitable for high-throughput screening of compounds able to inhibit Ebolavirus replication and/or transcription. *Antimicrobial Agents and Chemotherapy* **54**(7): 3007–3010.

Johnson, J. E. 2008. Multi-disciplinary studies of viruses: the role of structure in shaping the questions and answers. *Journal of Structural Biology* **163**(3): 246–253.

Kash, J. C., Tumpey, T. M., Proll, S. C., Carter, V., Perwitasari, O., Thomas, M. J., Basler, C. F., *et al*. 2006. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* **443**(7111): 578–581.

Kelly, E. J., Hadac, E. M., Greiner, S. and Russell, S. J. 2008. Engineering microRNA responsiveness to decrease virus pathogenicity. *Nature Medicine* **14**(11): 1278–1283.

Kelly, E. J., Nace, R., Barber, G. N. and Russell, S. J. 2010. Attenuation of vesicular stomatitis virus encephalitis through microRNA targeting. *Journal of Virology* **84**(3): 1550–1562.

Kelly, K., Nawrocki, S., Mita, A., Coffey, M., Giles, F. J. and Mita, M. 2009. Reovirus-based therapy for cancer. *Expert Opinion on Biological Therapy* **9**(7): 817–830.

Klingen, Y., Conzelmann, K. K. and Finke, S. 2008. Double-labeled rabies virus: live tracking of enveloped virus transport. *Journal of Virology* **82**(1): 237–245.

Komarova, A. V., Combredet, C., Meyniel-Schicklin, L., Chapelle, M., Caignard, G., Camadro, J. M., Lotteau, V., *et al*. 2011. Proteomic analysis of virus-host interactions in an infectious context using recombinant viruses. *Molecular Cell Proteomics* **10**(12): M110.007443.

Konig, R., Stertz, S., Zhou, Y., Inoue, A., Hoffmann, H. H., Bhattacharyya, S., Alamares, J. G., Tscherne, D. M., Ortigoza, M. B., Liang, Y., Gao, Q., Andrews, S. E., Bandyopadhyay, S., De Jesus, P., Tu, B. P., Pache, L., Shih, C., Orth, A., Bonamy, G., Miraglia, L., Ideker, T., Garcia-Sastre, A., Young, J. A., Palese, P., Shaw, M. L. and Chanda, S. K. 2010. Human host factors required for influenza virus replication. *Nature* **463**(7282): 813–817.

Kumagai, M. H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K. and Grill, L. K. 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences of the United States of America* **92**(5): 1679–1683.

Lal, R., Harris, D., Postel-Vinay, S. and de Bono, J. 2009. Reovirus: Rationale and clinical trial update. *Current Opinion in Molecular Therapeutics* **11**(5): 532–539.

Le Mercier, P., Garcin, D., Hausmann, S., and Kolakofsky, D. 2002. Ambisense sendai viruses are inherently unstable but are useful to study viral RNA synthesis. *Journal of Virology* **76**(11): 5492–5502.

Leber, M. F., Bossow, S., Leonard, V. H., Zaoui, K., Grossardt, C., Frenzke, M., Miest, T., *et al*. 2011. MicroRNA-sensitive oncolytic measles viruses for cancer-specific vector tropism. *Molecular Therapeutics* **19**(6): 1097–1106.

Luker, K. E. and Luker, G. D. 2010. Bioluminescence imaging of reporter mice for studies of infection and inflammation. *Antiviral Research* **86**(1): 93–100.

Manicassamy, B., Manicassamy, S., Belicha-Villanueva, A., Pisanelli, G., Pulendran, B. and Garcia-Sastre, A. 2010. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. *Proceedings of the National Academy of Sciences of the United States of America* **107**(25): 11531–11536.

Manolaridis, I., Gaudin, C., Posthuma, C. C., Zevenhoven-Dobbe, J. C., Imbert, I., Canard, B., Kelly G., Tucker P. A., Conte M. R. and Snijder E. J. 2011. Structure and genetic analysis of the arterivirus nonstructural protein 7alpha. *Journal of Virology* **85**(14): 7449–7453.

McCarthy, S. E., Licata, J. M. and Harty, R. N. 2006. A luciferase-based budding assay for Ebola virus. *Journal of Virological Methods* **137**(1): 115–119.

McGivern, D. R., Collins, P. L. and Fearns, R. 2005. Identification of internal sequences in the 3′ leader region of human respiratory syncytial virus that enhance transcription and confer replication processivity. *Journal of Virology* **79**(4): 2449–2460.

Miknis, Z. J., Donaldson, E. F., Umland, T. C., Rimmer, R. A., Baric, R. S. and Schultz, L. W. 2009. Severe acute respiratory syndrome coronavirus nsp9 dimerization is essential for efficient viral growth. *Journal of Virology* **83**(7): 3007–3018.

Mueller, S., Papamichail, D., Coleman, J. R., Skiena, S. and Wimmer, E. 2006. Reduction of the rate of poliovirus protein synthesis through large-scale codon deoptimization causes attenuation of viral virulence by lowering specific infectivity. *Journal of Virology* **80**(19): 9687–9696.

Myles, K. M., Wiley, M. R., Morazzani, E. M. and Adelman, Z. N. 2008. Alphavirus-derived small RNAs modulate pathogenesis in disease vector

mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America* **105**(50): 19938–19943.

Nanda, K., Vancini, R., Ribeiro, M., Brown, D. T. and Hernandez, R. 2009. A high capacity Alphavirus heterologous gene delivery system. *Virology* **390**(2): 368–373.

Neumann, G., Fujii, K., Kino, Y. and Kawaoka, Y. 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. *Proceedings of the National Academy of Sciences of the United States of America* **102**(46): 16825–16829.

Nilsson, P., Iwata, N., Muramatsu, S., Tjernberg, L. O., Winblad, B. and Saido, T. C. 2010. Gene therapy in Alzheimer's disease: potential for disease modification. *Journal of Cellular and Molecular Medicine* **14**(4): 741–757.

Ozduman, K., Wollmann, G., Piepmeier, J. M. and van den Pol, A. N. 2008. Systemic vesicular stomatitis virus selectively destroys multifocal glioma and metastatic carcinoma in brain. *The Journal of Neuroscience* **28**(8): 1882–1893.

Patkar, C. G., Larsen, M., Owston, M., Smith, J. L. and Kuhn, R. J. 2009. Identification of inhibitors of yellow fever virus replication using a replicon-based high-throughput assay. *Antimicrobial Agents and Chemotherapy* **53**(10): 4103–4114.

Peng, X., Chan, E. Y., Li, Y., Diamond, D. L., Korth, M. J. and Katze, M. G. 2009. Virus–host interactions: from systems biology to translational research. *Current Opinion in Microbiology* **12**(4): 432–438.

Phillips, A., Mossel, E., Sanchez-Vargas, I., Foy, B. and Olson, K. 2010. Alphavirus transducing system: tools for visualizing infection in mosquito vectors. *Journal of Visualized Experiments: JoVE* **45**: ii: 2363. doi(45): 10.3791/2363.

Purkayastha, A. and Dasgupta, I. 2009. Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. *Plant Physiology and Biochemistry: PPB/ Société française de physiologie végetale* **47**(11–12): 967–976.

Pushko, P., Geisbert, J., Parker, M., Jahrling, P. and Smith, J. 2001. Individual and bivalent vaccines based on alphavirus replicons protect guinea pigs against infection with Lassa and Ebola viruses. *Journal of Virology* **75**(23): 11677–11685.

Radecke, F. and Billeter, M. A. 1996. The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cells. *Virology* **217**(1): 418–421.

Rainsford, E. W., Harouaka, D. and Wertz, G. W. 2010. Importance of hydrogen bond contacts between the N protein and RNA genome of vesicular stomatitis virus in encapsidation and RNA synthesis. *Journal of Virology* **84**(4): 1741–1751.

Rayner, J. O., Dryga, S. A. and Kamrud, K. I. 2002. Alphavirus vectors and vaccination. *Reviews in Medical Virology* **12**(5): 279–296.

Richt, J. A. and Garcia-Sastre, A. 2009. Attenuated influenza virus vaccines with modified NS1 proteins. *Current Topics in Microbiology and Immunology* **333**: 177–195.

Rose, N. F., Publicover, J., Chattopadhyay, A. and Rose, J. K. 2008. Hybrid alphavirus-rhabdovirus propagating replicon particles are versatile and potent

vaccine vectors. *Proceedings of the National Academy of Sciences of the United States of America* **105**(15): 5839–5843.

Russell, S. J. and Peng, K. W. 2009. Measles virus for cancer therapy. *Current Topics in Microbiology and Immunology* **330**: 213–241.

Sato, H., Yoneda, M., Honda, T. and Kai, C. 2011. Recombinant vaccines against the mononegaviruses: what we have learned from animal disease controls. *Virus Research* **162**(1–2): 63–71.

Schneider, J. and Wolff, T. 2009. Nuclear functions of the influenza A and B viruses NS1 proteins: do they play a role in viral mRNA export? *Vaccine* **27**(45): 6312–6316.

Senthil-Kumar, M. and Mysore, K. S. 2011a. Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in Nicotiana benthamiana and tomato. *Plant Biotechnology Journal* **9**(7): 797–806.

Senthil-Kumar, M. and Mysore, K. S. 2011b. New dimensions for VIGS in plant functional genomics. *Trends in Plant Science* **16**(12): 656–665.

Shi, X., van Mierlo, J. T., French, A. and Elliott, R. M. 2010. Visualizing the replication cycle of bunyamwera orthobunyavirus expressing fluorescent proteintagged Gc glycoprotein. *Journal of Virology* **84**(17): 8460–8469.

Stojdl, D. F., Lichty, B. D., tenOever, B. R., Paterson, J. M., Power, A. T., Knowles, S., Marius, R., Reynard, J., Poliquin, L., Atkins, H., Brown, E. G., Durbin, R. K., Durbin, J. E., Hiscott, J. and Bell, J. C. 2003. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4**(4): 263–275.

Tang, J., Johnson, J. M., Dryden, K. A., Young, M. J., Zlotnick, A. and Johnson, J. E. 2006. The role of subunit hinges and molecular 'switches' in the control of viral capsid polymorphism. *Journal of Structural Biology* **154**(1): 59–67.

Tang, Y., Wang, F., Zhao, J., Xie, K., Hong, Y., and Liu, Y. 2010. Virus-based microRNA expression for gene functional analysis in plants. *Plant Physiology* **153**(2): 632–641.

Teterina, N. L., Levenson, E. A. and Ehrenfeld, E. 2010. Viable polioviruses that encode 2A proteins with fluorescent protein tags. *Journal of Virology* **84**(3): 1477–1488.

Trask, S. D., Taraporewala, Z. F., Boehme, K. W., Dermody, T. S. and Patton, J. T. 2010. Dual selection mechanisms drive efficient single-gene reverse genetics for rotavirus. *Proceedings of the National Academy of Sciences of the United States of America* **107**(43): 18652–18657.

Troupin, C., Dehee, A., Schnuriger, A., Vende, P., Poncet, D. and Garbarg-Chenon, A. 2010. Rearranged genomic RNA segments offer a new approach to the reverse genetics of rotaviruses. *Journal of Virology* **84**(13): 6711–6719.

Tseng, J. C., Granot, T., DiGiacomo, V., Levin, B. and Meruelo, D. 2010. Enhanced specific delivery and targeting of oncolytic Sindbis viral vectors by modulating vascular leakiness in tumor. *Cancer Gene Therapy* **17**(4): 244–255.

Unver, T. and Budak, H. 2009. Virus-induced gene silencing, a post transcriptional gene silencing method. *International Journal of Plant Genomics* **2009**: 198680.

Uprichard, S. L. 2010. Hepatitis C virus experimental model systems and antiviral drug research. *Virologica Sinica* **25**(4): 227–245.

van den Pol, A. N., Dalton, K. P. and Rose, J. K. 2002. Relative neurotropism of a recombinant rhabdovirus expressing a green fluorescent envelope glycoprotein. *Journal of Virology* **76**(3): 1309–1327.

van den Pol, A. N., Ozduman, K., Wollmann, G., Ho, W. S., Simon, I., Yao, Y., Rose, J. K. and Ghosh, P. 2009. Viral strategies for studying the brain, including a replication-restricted self-amplifying delta-G vesicular stomatis virus that rapidly expresses transgenes in brain and can generate a multicolor Golgi-like expression. *The Journal of Comparative Neurology* **516**(6): 456–481.

van den Wollenberg, D. J., van den Hengel, S. K., Dautzenberg, I. J., Cramer, S. J., Kranenburg, O. and Hoeben, R. C. 2008. A strategy for genetic modification of the spike-encoding segment of human reovirus T3D for reovirus targeting. *Gene Therapy* **15**(24): 1567–1578.

van den Wollenberg, D. J., van den Hengel, S. K., Dautzenberg, I. J., Kranenburg, O. and Hoeben, R. C. 2009. Modification of mammalian reoviruses for use as oncolytic agents. *Expert Opinion on Biological Therapy* **9**(12): 1509–1520.

Verheije, M. H., Lamfers, M. L., Wurdinger, T., Grinwis, G. C., Gerritsen, W. R., van Beusechem, V. W. and Rottier, P. J. 2009. Coronavirus genetically redirected to the epidermal growth factor receptor exhibits effective antitumor activity against a malignant glioblastoma. *Journal of Virology* **83**(15): 7507–7516.

Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E. and Andino, R. 2006. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* **439**(7074): 344–348.

Wang, J. T., McElvain, L. E. and Whelan, S. P. 2007. Vesicular stomatitis virus mRNA capping machinery requires specific cis-acting signals in the RNA. *Journal of Virology* **81**(20): 11499–11506.

Wickersham, I. R., Finke, S., Conzelmann, K. K. and Callaway, E. M. 2007. Retrograde neuronal tracing with a deletion-mutant rabies virus. *Nature Methods* **4**(1): 47–49.

Wollmann, G., Tattersall, P. and van den Pol, A. N. 2005. Targeting human glioblastoma cells: comparison of nine viruses with oncolytic potential. *Journal of Virology* **79**(10): 6005–6022.

Wurdinger, T., Verheije, M. H., Raaben, M., Bosch, B. J., de Haan, C. A., van Beusechem, V. W., Rottier, P. J. and Gerritsen, W. R. 2005. Targeting non-human coronaviruses to human cancer cells using a bispecific single-chain antibody. *Gene Therapy* **12**(18): 1394–1404.

Zhou, Z. H., He, J., Jakana, J., Tatman, J. D., Rixon, F. J. and Chiu, W. 1995. Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. *Nature Structural Biology* **2**(11): 1026–1030.

Zou, G,. Xu, H. Y., Qing, M., Wang, Q. Y. and Shi, P. Y. 2011 Development and characterization of a stable luciferase dengue virus for high-throughput screening. *Antiviral Research* **91**(1): 11–19.

Index

Reverse Genetics of RNA Viruses: Applications and Perspectives, First Edition. Edited by Anne Bridgen.