

A decade after the generation of a negative-sense RNA virus from cloned cDNA – what have we learned?

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Since the first generation of a negative-sense RNA virus entirely from cloned cDNA in 1994, similar reverse genetics systems have been established for members of most genera of the *Rhabdo-* and *Paramyxoviridae* families, as well as for Ebola virus (*Filoviridae*). The generation of segmented negative-sense RNA viruses was technically more challenging and has lagged behind the recovery of nonsegmented viruses, primarily because of the difficulty of providing more than one genomic RNA segment. A member of the *Bunyaviridae* family (whose genome is composed of three RNA segments) was first generated from cloned cDNA in 1996, followed in 1999 by the production of influenza virus, which contains eight RNA segments. Thus, reverse genetics, or the *de novo* synthesis of negative-sense RNA viruses from cloned cDNA, has become a reliable laboratory method that can be used to study this large group of medically and economically important viruses. It provides a powerful tool for dissecting the virus life cycle, virus assembly, the role of viral proteins in pathogenicity and the interplay of viral proteins with components of the host cell immune response. Finally, reverse genetics has opened the way to develop live attenuated virus vaccines and vaccine vectors.

Introduction

Negative-strand RNA viruses are classified into seven families (*Rhabdo-*, *Paramyxo-*, *Filo-*, *Borna-*, *Orthomyxo-*, *Bunya-* and *Arenaviridae*). The first four families are characterized by nonsegmented genomes, while the latter three have genomes comprising six-to-eight, three or two negative-sense RNA segments, respectively. The large group of negative-sense RNA viruses includes highly prevalent human pathogens, such as respiratory syncytial virus (RSV), parainfluenza viruses and influenza viruses, and two of the most deadly human pathogens (Ebola and Marburg viruses), as well as viruses with a major economic impact on the poultry and cattle industries [e.g. Newcastle disease virus (NDV) and rinderpest virus (RPV)].

Reverse genetics, as the term is used in molecular virology, describes the generation of viruses possessing a genome

derived from cloned cDNAs. Reverse genetics systems have been established to artificially produce members of the *Rhabdo-*, *Paramyxo-*, *Filo-*, *Bunya-* and *Orthomyxoviridae* families (Table 1) and have revolutionized research on these viruses. In this review, we will describe these experimental systems and discuss their contributions to our understanding of virus replication, the pathogenicity of negative-sense RNA viruses and the development of live attenuated virus vaccines.

An overview of the life cycle of negative-sense RNA viruses

Negative-sense RNA viruses infect their host cells by binding to a host cell receptor via a viral surface glycoprotein. Subsequent fusion of the viral membrane with the plasma membrane (pH-independent pathway) (Fig. 1A) or with endosomal membranes in the acidic environment of late endosomes (pH-dependent pathway) (Fig. 1B) releases viral ribonucleoprotein (RNP) complexes into the cytoplasm. RNP

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Table 1. Negative-sense RNA viruses generated from cloned cDNA

Family	Subfamily	Genus	Species	Abbreviation	Reference
<i>Rhabdoviridae</i>		<i>Vesiculovirus</i>	<i>Vesicular stomatitis virus</i>	VSV	Lawson <i>et al.</i> (1995) Whelan <i>et al.</i> (1995)
<i>Paramyxoviridae</i>	<i>Paramyxovirinae</i>	<i>Lyssavirus</i>	<i>Rabies virus</i>	RV	Schnell <i>et al.</i> (1994)
		<i>Morbillivirus</i>	<i>Measles virus</i>	MV	Radecke <i>et al.</i> (1995) Schneider <i>et al.</i> (1997b) Takeda <i>et al.</i> (2000)
			<i>Rinderpest virus</i>	RPV	Baron & Barrett (1997)
			<i>Canine distemper virus</i>	CDV	Gassen <i>et al.</i> (2000)
		<i>Respirovirus</i>	<i>Sendai virus</i>	SeV	Garcin <i>et al.</i> (1995) Kato <i>et al.</i> (1996)
			<i>Human parainfluenza virus type 3</i>	hPIV3	Durbin <i>et al.</i> (1997a) Hoffman & Banerjee (1997)
			<i>Bovine parainfluenza virus type 3</i>	bPIV3	Haller <i>et al.</i> (2000)
			<i>Simian virus type 5</i>	SV5	He <i>et al.</i> (1997)
			<i>Mumps virus</i>		Clarke <i>et al.</i> (2000)
			<i>Human parainfluenza virus type 2</i>	hPIV2	Kawano <i>et al.</i> (2001)
		<i>Newcastle disease virus</i>	NDV	Peeters <i>et al.</i> (1999) Romer-Oberdorfer <i>et al.</i> (1999) Krishnamurthy <i>et al.</i> (2000)	
	<i>Pneumovirinae</i>	<i>Pneumovirus</i>	<i>Human respiratory syncytial virus</i>	hRSV	Collins <i>et al.</i> (1995)
			<i>Bovine respiratory syncytial virus</i>	bRSV	Buchholz <i>et al.</i> (1999)
<i>Filoviridae</i>		Ebola-like viruses	<i>Ebola virus</i>		Volchkov <i>et al.</i> (2001) Neumann <i>et al.</i> (2002)
<i>Bunyaviridae</i>		<i>Bunyavirus</i>	<i>Bunyamwera virus</i>		Bridgen & Elliott (1996)
<i>Orthomyxoviridae</i>		<i>Influenzavirus A</i>	<i>Influenza A virus</i>		Neumann <i>et al.</i> (1999) Fodor <i>et al.</i> (1999)
		<i>Thogotovirus</i>	<i>Thogoto virus</i>		Wagner <i>et al.</i> (2001)

complexes are composed of the viral RNA (vRNA), the nucleoprotein, which encapsidates the vRNA, and the viral polymerase complex. Individual mRNAs are synthesized during transcription, while replication leads to the synthesis of full-length antigenomic RNAs, which, in turn, serve as templates for genomic vRNA synthesis. Most of the negative-sense RNA viruses replicate in the cytoplasm of infected cells, in contrast to the nuclear replication of orthomyxoviruses and bornaviruses. Thus, for these latter viruses, incoming RNP complexes and newly synthesized NP and polymerase proteins have to be imported into the nucleus, whereas newly assembled RNPs are exported from the nucleus to the cytoplasm. Newly synthesized RNP complexes are assembled with viral structural proteins at the plasma membrane or at membranes of the Golgi apparatus, followed by the release of newly synthesized viruses. For more detailed information on the life cycles of negative-sense RNA viruses, the reader is referred to the relevant chapters in *Fields, Virology* (Knipe & Howley, 2001), and to review articles on this topic (Curran & Kolakofsky, 1999; Buchmeier *et al.*, 2001; de la Torre, 2001; Lamb &

Kolakofsky, 2001a; Lamb & Krug, 2001b; Rose & Whitt, 2001; Sanchez *et al.*, 2001; Schmaljohn & Hooper, 2001).

Reverse genetics systems

The first reverse genetics systems were established with positive-sense RNA viruses (Taniguchi *et al.*, 1978; Racaniello & Baltimore, 1981). Transfection of the full-length RNAs of positive-sense RNA viruses into eukaryotic cells results in viral protein expression which leads to virus replication. In contrast, the genomes of negative-sense RNA viruses alone are noninfectious. Initiation of virus replication and transcription requires coexpression of the viral polymerase complex in addition to vRNA(s); furthermore, the genome has to be complexed with the nucleocapsid protein.

In 1989, Palese and colleagues established the first system for the modification of a negative-sense RNA virus, influenza A virus (Luytjes *et al.*, 1989; Enami *et al.*, 1990; reviewed by Garcia-Sastre & Palese, 1993; Garcia-Sastre *et al.*, 1994b; Palese, 1995; Palese *et al.*, 1996; Neumann & Kawaoka, 1999).

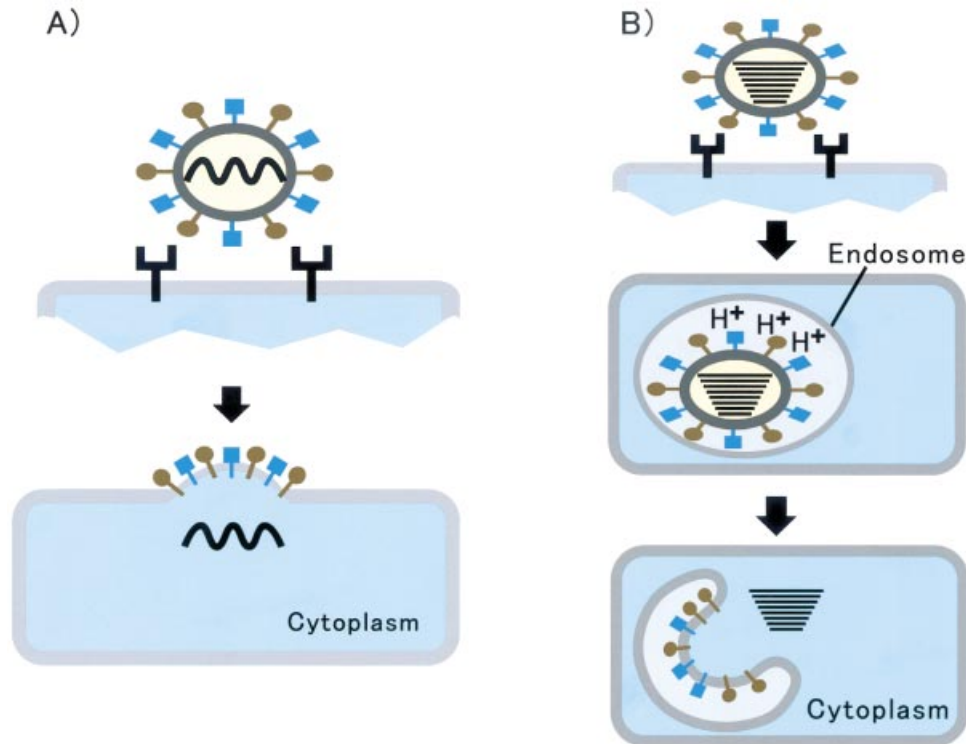


Fig. 1. Schematic diagram of virus entry. Negative-sense RNA viruses bind to host cell receptors via viral surface glycoproteins. (A) pH-independent pathway. Fusion of the viral membrane with the plasma membrane releases RNP complexes into the cytoplasm. (B) pH-dependent pathway. Viruses are internalized by receptor-mediated endocytosis. The low pH in late endosomes triggers conformational changes in the viral glycoproteins that lead to the fusion of the viral and endosomal membranes and the subsequent release of RNP complexes into the cytosol.

A cDNA encoding the reporter protein chloramphenicol acetyltransferase (CAT) was cloned in negative-sense orientation between the 5' and 3' noncoding viral sequences. A T7 RNA polymerase promoter sequence and a recognition sequence for a restriction enzyme that allowed the formation of authentic viral 3' ends flanked this construct. *In vitro* transcription yielded virus-like RNA that was subsequently mixed with purified polymerase and NP proteins to reconstitute RNP complexes. These artificially generated RNPs were transfected into eukaryotic cells infected with helper influenza virus. The viruses that were generated contained the virus-like RNA encoding CAT in addition to the eight influenza vRNAs (Luytjes *et al.*, 1989). This achievement was quickly followed by the first alteration of a viral gene (Enami *et al.*, 1990). Although allowing site-directed mutagenesis of an influenza viral gene for the first time, this system relies on helper-virus infection and strong selection systems are necessary to distinguish the modified virus from the (wild-type) helper virus.

For most negative-sense RNA viruses, the ability to generate infectious negative-sense RNA virus entirely from cloned cDNA was preceded by the establishment of mini-replicon systems. In these systems, T7 RNA polymerase was used to synthesize negative-sense vRNA derived from cDNA

clones of internally deleted viral genomes. Upon transfection of the vRNA into eukaryotic cells that had been infected with helper virus, the artificially generated vRNAs were rescued into viruses (Collins *et al.*, 1991; Park *et al.*, 1991). Pattnaik & Wertz (1991) first established a plasmid-based minireplicon system by providing all five vesicular stomatitis virus (VSV) viral proteins from protein expression plasmids. Experience with these different approaches demonstrated that the RNA-dependent RNA polymerase L, the nucleoprotein N and the phosphoprotein P are essential for the amplification of rhabdo- and paramyxovirus genomes (Pattnaik & Wertz, 1990, 1991; Collins *et al.*, 1991; Conzelmann *et al.*, 1991; Park *et al.*, 1991; Calain *et al.*, 1992; Dimock & Collins, 1993; Sidhu *et al.*, 1995). For Marburg virus (family *Filoviridae*), L, NP and VP35 (which is believed to be the equivalent of the phosphoprotein) constitute the minimal replication unit (Muhlberger *et al.*, 1998), while the VP30 protein is additionally required for replication of the closely related Ebola virus (Muhlberger *et al.*, 1999). In contrast, two proteins, L and NP, are sufficient for the transcription and replication of bunyavirus (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Flick & Pettersson, 2001) or arenavirus (Lee *et al.*, 2000) minigenomes. For influenza viruses, the nucleoprotein and the three polymerase subunits PB2, PB1 and PA are necessary and sufficient for the amplification of vRNAs

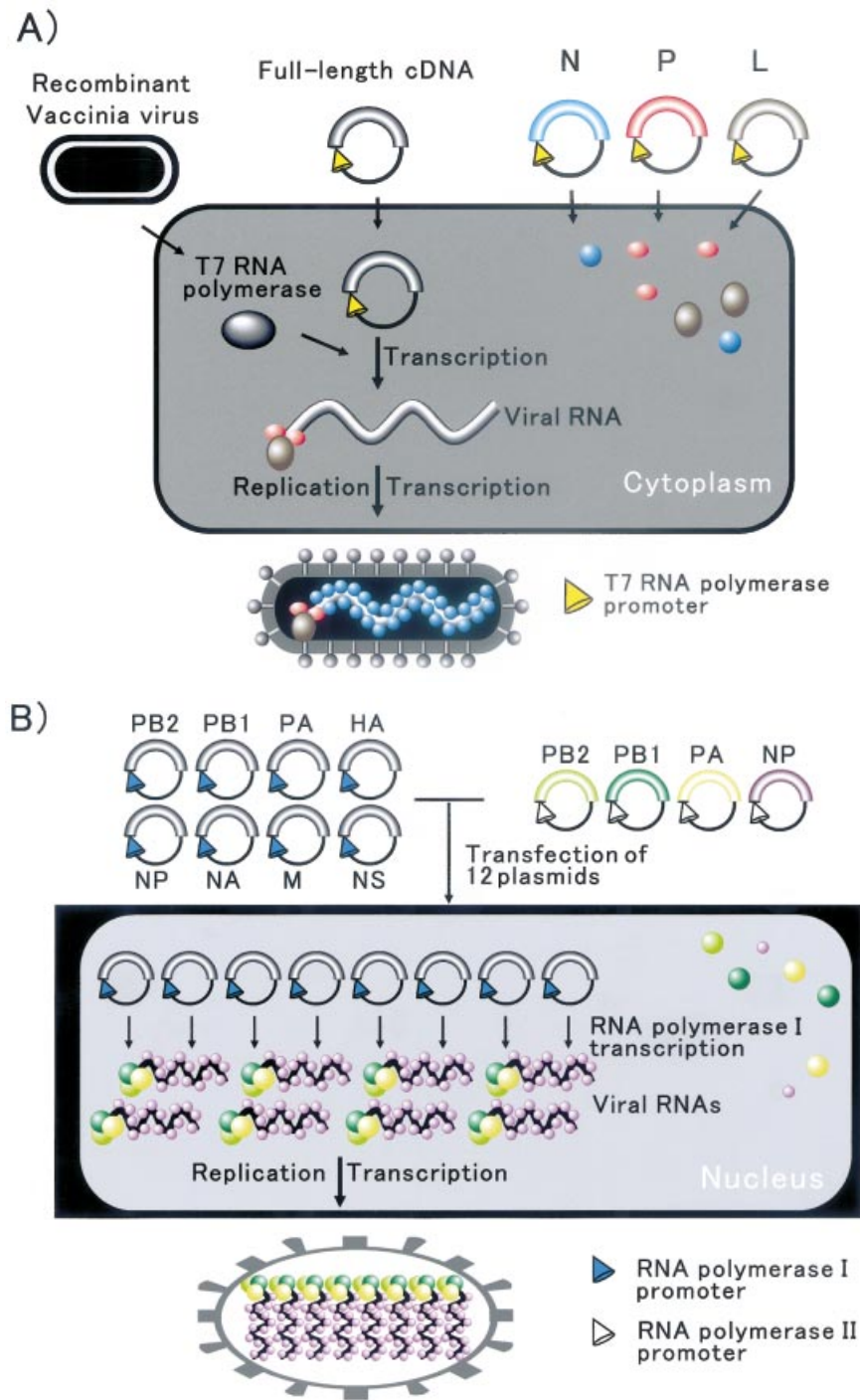


Fig. 2. Systems for the generation of negative-sense RNA viruses from cloned cDNA. **(A)** Schematic diagram for the generation of nonsegmented negative-sense RNA viruses. Cells are cotransfected with protein expression plasmids for the N, P and L proteins and with a plasmid containing a full-length viral cDNA, all under the control of the T7 RNA polymerase promoter. Following infection with recombinant VV encoding T7 RNA polymerase, vRNA is synthesized and the virus replication cycle is initiated. **(B)** Schematic diagram for the generation of influenza A virus. Cells are cotransfected with plasmids that encode all eight vRNAs under the control of the RNA polymerase I promoter. Cellular RNA polymerase I synthesizes vRNAs that are replicated and transcribed by the viral polymerase and NP proteins, all provided by protein expression plasmids.

(Honda *et al.*, 1987, 1988, 1990; Szewczyk *et al.*, 1988; Parvin *et al.*, 1989).

In 1994, Conzelmann and colleagues (Schnell *et al.*, 1994) generated recombinant rabies virus (RV), demonstrating for the first time the feasibility of producing a negative-sense RNA virus entirely from cloned cDNA (Fig. 2A). Cells were cotransfected with protein expression constructs for the L, P and N proteins and with a cDNA construct encoding the full-length RV antigenome, all under control of the T7 RNA polymerase promoter. Infection with recombinant vaccinia virus (VV), which provided T7 RNA polymerase, was the final step needed to produce infectious RV. The key element to this success was the synthesis of a positive-sense antigenomic RNA from cloned DNA. Positive-sense antigenomic RNA, in contrast to negative-sense genomic RNA, cannot hybridize to positive-sense mRNAs encoding the L, P and nucleoproteins and thus does not interfere with virus generation. Moreover, the genomic RNAs of some negative-sense RNA viruses contain stretches of uridine residues followed by hairpin structures that resemble T7 RNA terminator elements, which may cause premature abortion of T7 RNA polymerase transcription (Whelan *et al.*, 1995). Since the initial report by Schnell *et al.* (1994), we have witnessed the generation of an ever-growing number of rhabdo- and paramyxoviruses by reverse genetics (Table 1) (reviewed by Conzelmann, 1996, 1998; Conzelmann & Meyers, 1996; Rose, 1996; Roberts & Rose, 1998, 1999; Marriott & Easton, 1999; Nagai, 1999; Nagai & Kato, 1999; Munoz *et al.*, 2000). Refinements of the original rescue procedure included the expression of T7 RNA polymerase from stably transfected cell lines (Radecke *et al.*, 1995), protein expression plasmids (Lawson *et al.*, 1995) or heat shock procedures to increase rescue efficiencies (Parks *et al.*, 1999). Recently, Ebola virus, a member of the family *Filoviridae*, was also generated from cDNA (Volchkov *et al.*, 2001; Neumann *et al.*, 2002). In contrast to these efforts with positive-sense antigenomic RNA, a number of investigators have also relied on negative-sense genomic RNA to produce Sendai virus (SeV) (Kato *et al.*, 1996), human parainfluenza virus type 3 (hPIV3) (Durbin *et al.*, 1997a) and Ebola virus (Neumann *et al.*, 2002), although with lower efficiencies.

Another breakthrough occurred in 1996 with the first generation of a segmented negative-sense RNA virus from cloned cDNAs (Bridgen & Elliott, 1996). Following the approach outlined by Schnell *et al.* (1994), Bridgen & Elliott (1996) created Bunyamwera virus (family *Bunyaviridae*), thus demonstrating the feasibility of artificially producing more than one vRNA.

The generation of influenza virus is far more complex than that of nonsegmented negative-sense RNA viruses, as it requires eight vRNAs as well as four proteins encoding the three polymerase subunits and NP. Secondly, influenza virus replicates in the nucleus of infected cells, so that the vRNAs and proteins have to be delivered to this cellular compartment. A solution was provided by Hobom and

colleagues (Zobel *et al.*, 1993; Neumann *et al.*, 1994), who established the RNA polymerase I system for the intracellular synthesis of influenza vRNAs. RNA polymerase I, a nucleolar enzyme, synthesizes ribosomal RNA, which, like influenza virus RNA, does not contain 5' cap or 3' poly(A) structures. Hence, RNA polymerase I transcription of a cDNA construct containing an influenza viral cDNA, flanked by RNA polymerase I promoter and terminator sequences, results in influenza vRNA synthesis. This system enabled the generation of influenza virus from plasmids in 1999 (Neumann *et al.*, 1999; Fodor *et al.*, 1999; reviewed by Pekosz *et al.*, 1999; Neumann & Kawaoka, 2001) (Fig. 2B). By transfecting eukaryotic cells with eight RNA polymerase I plasmids encoding all vRNAs, together with protein expression constructs for the polymerase and NP proteins (yielding a total of 12 plasmids), one can now produce more than 10^8 infectious viruses per ml of supernatant derived 2 days after transfection. In a modified RNA polymerase I system, both negative-sense vRNA and positive-sense mRNA can be synthesized from the same template, thus reducing the number of plasmids required (i.e. 8 instead of 12) (Hoffmann *et al.*, 2000a, b; Hoffmann & Webster, 2000).

Thogoto virus, a second member of the family *Orthomyxoviridae*, was recently generated from plasmids (Wagner *et al.*, 2001). The genome of this tick-transmitted orthomyxovirus consists of six segments of negative-sense RNA. The RNA polymerase I system allowed the synthesis of all six vRNAs, while the proteins required for transcription and replication were expressed with the VV T7 RNA polymerase system.

Replication and transcription of nonsegmented negative-sense RNA viruses

The genes of nonsegmented negative-sense RNA viruses are separated by regulatory regions comprising a gene end signal (i.e. transcription stop and polyadenylation), an intergenic region and a gene start signal (i.e. transcription start signal) (Fig. 3). These signals are both sufficient and necessary for gene transcription (Kuo *et al.*, 1996b; Schnell *et al.*, 1996b). The gene transcription units are flanked by so-called leader and trailer regions, which contain the viral promoters for replication and transcription.

Gene end signals

In VSV, the gene end signal is highly conserved among all genes. Alteration of the conserved tetranucleotide transcription stop signal reduced the ability to terminate RNA transcription (Barr *et al.*, 1997a; Hwang *et al.*, 1998) and affected polyadenylation (Barr & Wertz, 2001). Furthermore, the tetranucleotide is functional only when juxtaposed to the uridine stretch that functions as a polyadenylation signal (Barr *et al.*, 1997a) and when positioned at a minimal distance from the gene start sequence (Whelan *et al.*, 2000). In addition to the tetranucleotide, the uridine stretch constituting the poly-

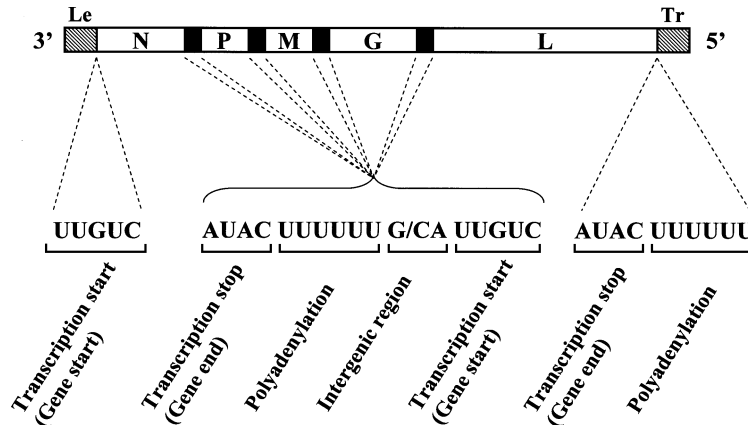


Fig. 3. Genome organization of nonsegmented negative-sense RNA viruses, as exemplified by VSV. The coding regions for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase protein (L) are separated by regulatory sequences that contain a transcription stop (gene end) signal, a polyadenylation signal, a nontranscribed intergenic region and a transcription start (gene start) signal. Transcription units are flanked by a leader (Le) and trailer (Tr) region that contain the genomic and antigenomic viral promoters, respectively.

adenylation signal is critical for termination, since its deletion, shortening or interruption resulted in read-through transcripts (Barr *et al.*, 1997a; Hwang *et al.*, 1998). In contrast to VSV, the polyuridine tracts of simian virus type 5 (SV5) are of variable lengths and nucleotide insertions or deletions did not affect termination but did affect reinitiation at the downstream gene (Rassa *et al.*, 2000).

Intergenic region

The intergenic regions of nonsegmented negative-sense RNA viruses are highly variable, consisting of a conserved dinucleotide (VSV), trinucleotide (morbilli- and respiroviruses) or regions of up to 143 nt (filoviruses). For VSV, alterations of the conserved dinucleotide affected the efficiency of termination of the upstream mRNA as well as the mRNA levels of the downstream gene (Barr *et al.*, 1997b; Stillman & Whitt, 1997, 1998). The various lengths of the intergenic regions of RV correlate with transcriptional attenuation (Finke *et al.*, 2000), whereas the diverse intergenic regions of RSV do not modulate gene expression (Kuo *et al.*, 1996a; Bukreyev *et al.*, 2000a). Similarly, nucleotide insertions into the middle of the SV5 M–F intergenic region did not affect transcription termination and/or initiation; however, replacement of the entire M–F intergenic region with nonviral sequences abolished termination of M gene transcription (Rassa & Parks, 1999).

Gene start signals

The transcription start signals are identical for all VSV genes. Mutational analyses revealed that the first three nucleotides of the 5' start sequence are critical for gene expression (Stillman & Whitt, 1997). Interestingly, mutations

in this sequence did not abolish the initiation of transcription *per se* but affected polymerase processivity as well as capping and/or methylation of mRNA transcripts (Stillman & Whitt, 1999). Further insights into transcription initiation came from RSV minigenomes containing a nucleotide replacement at position 1 of the gene start signal. mRNAs were synthesized with the predicted nucleotide at the 5' end; however, a subpopulation of mRNAs contained the (nontemplated) parental wild-type nucleotide at the 5' end (Kuo *et al.*, 1997). Thus, the polymerase complex may have a preference for the wild-type nucleotide at the start position. For SeV, the start sequence of the F gene was significantly weaker than the P, M and HN start signals (Kato *et al.*, 1999). A recombinant SeV, whose F gene start signal was replaced with the strong P, M or HN gene start signal replicated faster and was more virulent than wild-type virus in mice (Kato *et al.*, 1999).

Replication

The switch from transcription to replication was once thought to be triggered by increasing amounts of soluble N protein, which is required to encapsidate replicating RNA. However, increasing levels of RSV N did not alter the ratio between transcription and replication (Fearnly *et al.*, 1997). Mutational analysis of the genomic and antigenomic promoter elements has resulted in the generation of minireplicons that are replicated poorly but are transcribed well, and vice versa. Rather than switching between transcription and replication, a balance between these two processes may be controlled by the different activities of the genomic and antigenomic promoters (Li & Pattnaik, 1999; Whelan & Wertz, 1999; Hoffman & Banerjee, 2000b). This idea is supported by experiments analysing a copy-back ambisense RV minigenome (Finke & Conzelmann, 1999).

For rhabdo- and paramyxoviruses, the minimal promoter sequences have been mapped to the 12–44 3′-terminal nucleotides of VSV (Li & Pattnaik, 1997, 1999; Whelan & Wertz, 1999), SeV (Tapparel & Roux, 1996), hPIV3 (Hoffman & Banerjee, 2000b) or RSV (Kuo *et al.*, 1996b; Fearn *et al.*, 2000) genomes or antigenomes. For VSV, the extent of complementarity between the 3′ and 5′ ends can affect the level of replication (Wertz *et al.*, 1994; Whelan & Wertz, 1999). However, other reports indicate that the base pairing potential between the 3′ and 5′ ends of the vRNAs does not affect replication efficiency; rather, the primary sequence of the promoter elements determines their strength (Tapparel & Roux, 1996; Hoffman & Banerjee, 2000a). In contrast to rhabdoviruses, the genomic and antigenomic promoters of paramyxoviruses (with the possible exception of RSV) contain second promoter elements (Pelet *et al.*, 1996; Murphy *et al.*, 1998; Tapparel *et al.*, 1998; Murphy & Parks, 1999; Hoffman & Banerjee, 2000b). These elements are composed of three hexamers that contain a repeated motif (Tapparel *et al.*, 1998; Murphy & Parks, 1999; Hoffman & Banerjee, 2000b). Nucleotide deletions or insertions between the two promoter elements were detrimental for their activity, indicating that their relative spacing is critical (Murphy *et al.*, 1998; Tapparel *et al.*, 1998). In structural models, the two elements are positioned on the same face of the RNP helix (Murphy *et al.*, 1998; Tapparel *et al.*, 1998). Thus, upon alteration of their spacing, the interaction of the polymerase complex with the two promoter elements may be abrogated. Recent studies also identified a third promoter element for SV5 (located between the first and the second element) that is required for optimal replication (Keller *et al.*, 2001).

The genomic promoter localizes to the 3′ end of the negative-sense vRNA and hence serves as a promoter for both replication and transcription. Distinct elements have been identified in the genomic promoter that are required for transcription but not for replication, or vice versa (Li & Pattnaik, 1999; Whelan & Wertz, 1999; Peeples & Collins, 2000). In contrast, the antigenomic promoter that localizes to the 3′ end of the positive-sense antigenome functions in replication only.

‘Rule of six’

Efficient replication for most paramyxoviruses was observed only when the total number of nucleotides was divisible by six (dubbed the ‘rule of six’) (reviewed by Kolakofsky *et al.*, 1998). The nucleoprotein interacts with exactly 6 nt, and the rule of six seems to depend on the recognition of nucleotides positioned in the proper *N*-phase context (Vulliamoz & Roux, 2001). Extensive mutagenesis revealed that the rule of six seems to follow taxonomic grouping, being apparently specific to the rubula- (Murphy & Parks, 1997; He & Lamb, 1999; Peeters *et al.*, 2000; Kawano *et al.*, 2001), respiro- (Calain & Roux, 1993, 1995; Harty & Palese, 1995; Hausmann *et al.*,

1996; Durbin *et al.*, 1997b) and morbillivirus (Radecke *et al.*, 1995; Sidhu *et al.*, 1995) genera of the family *Paramyxoviridae*, although with different stringency. The rule of six is not followed by RSV (Samal & Collins, 1996) nor does it apply to VSV (Pattnaik *et al.*, 1995).

Replication and transcription of influenza viruses

Each RNA segment of the segmented negative-sense RNA viruses constitutes a separate transcription unit. The 5′ 13-terminal and the 3′ 12-terminal nucleotides are highly conserved among the eight viral segments of influenza A viruses and form the basic promoter for transcription and replication (Parvin *et al.*, 1989; Yamanaka *et al.*, 1991; Li & Palese, 1992; Seong & Brownlee, 1992a, b; Piccone *et al.*, 1993). Extensive mutational analyses revealed the contributions of individual nucleotides to *in vitro* and *in vivo* promoter activity (Li & Palese, 1992; Piccone *et al.*, 1993; Fodor *et al.*, 1994, 1995; Neumann *et al.*, 1994; Neumann & Hobom, 1995; Pritlove *et al.*, 1995; Flick *et al.*, 1996; Kim *et al.*, 1997). In contrast to the promoter elements of nonsegmented negative-sense RNA viruses, which consist of specific sequence elements at the 3′ ends of the vRNA or cRNA, the genomic and antigenomic promoters of influenza viruses are composed of both the 5′ and the 3′ ends of the vRNA or complementary RNA (cRNA), respectively (Fodor *et al.*, 1993, 1994; Tiley *et al.*, 1994; Neumann & Hobom, 1995; Pritlove *et al.*, 1995). The influenza viral promoters can be divided into two elements: a terminal element (nt 1–9 at the 3′ and 5′ ends of the vRNA or cRNA) and a distal element (nt 10–15 and 11–16 at the 3′ and 5′ ends of the vRNA), which are connected by a flexible joint formed by an unpaired nucleotide (Fodor *et al.*, 1995; Neumann & Hobom, 1995; Flick *et al.*, 1996). Within the terminal element, the nature of the nucleotide sequence is critical, whereas in the distal element, base-pairing between the 3′ and 5′ ends of the vRNA or cRNA is crucial (Fodor *et al.*, 1994, 1995; Neumann & Hobom, 1995; Flick *et al.*, 1996; Kim *et al.*, 1997). Three promoter models, the ‘panhandle’ model (Hsu *et al.*, 1987), the ‘fork’ model (Fodor *et al.*, 1994, 1995; Kim *et al.*, 1997) and the ‘corkscrew’ model (Flick *et al.*, 1996; Flick & Hobom, 1999), have been proposed. The panhandle model suggests formation of a partially double-stranded structure in the entire promoter region, while the fork model proposes double-strand formation for the distal promoter element only. Recent data favour the corkscrew model, which predicts base-pairing between nucleotides at the 5′ or 3′ end rather than base-pairing between the 5′ and 3′ end (Flick & Hobom, 1999; Pritlove *et al.*, 1999; Leahy *et al.*, 2001a, b). The influenza B virus promoter has structural features similar to its influenza A virus counterpart (Lee & Seong, 1996).

During transcription, the polymerase complex proceeds until it encounters the polyadenylation signal, which is formed by a uridine stretch located adjacent to the promoter element.

At the uridine stretch, the polymerase complex 'stutters', resulting in polyadenylation (Zheng *et al.*, 1999; Poon *et al.*, 2000). Efficient polyadenylation requires that an uninterrupted uridine stretch be located 15–22 nt from the 5' end of the vRNA (Luo *et al.*, 1991; Li & Palese, 1994).

The terminal promoter elements contain the information sufficient for replication and transcription; however, signals that modulate these processes seem to be located in the noncoding regions that lie between the promoter and the start or stop codon, respectively. Recombinant viruses with deletions, insertions or mutations in these regions have been generated (Garcia-Sastre *et al.*, 1994b; Barclay & Palese, 1995; Bergmann & Muster, 1996; Zheng *et al.*, 1996). Although these viruses were viable, some had altered amounts of vRNA (Bergmann & Muster, 1996; Zheng *et al.*, 1996).

Phosphoprotein

Although the RNA-dependent RNA polymerase L is believed to contain all catalytic activities, phosphoprotein P is essential for vRNA synthesis. Its interaction with the N protein keeps the latter in a soluble form and imparts specificity to the N protein to encapsidate viral, but not cellular, RNAs. Moreover, binding of P to L allows efficient interaction of L with the N–RNA template (Horikami *et al.*, 1992). Both the amino- and the carboxy-terminal domains of P are critical to execute these functions (De *et al.*, 2000). For VSV P, replacement of single amino acids in the carboxy-terminal region resulted in P mutants that were defective in transcription but supported replication, suggesting that the transcriptase and replicase complexes may not be identical (Das *et al.*, 1997, 1999).

Reverse genetics has also allowed researchers to address the biological significance of P protein phosphorylation in the virus life cycle. The hPIV3 P protein is phosphorylated by the cellular protein kinase C isoform ζ (PKC- ζ) and growth of hPIV3 was abrogated in the presence of a PKC- ζ inhibitor (De *et al.*, 1995). The VSV P protein is phosphorylated at several sites in both the amino- and the carboxy-terminal domains. Replacement of the amino-terminal phosphorylation sites affected transcription (Spadafora *et al.*, 1996; Pattnaik *et al.*, 1997), while the carboxy-terminal phosphorylation sites are necessary for optimal replication (Hwang *et al.*, 1999). In contrast, replacement of the SeV P phosphorylation site did not affect virus replication in cell culture nor did it affect virus pathogenicity in mice (Hu *et al.*, 1999).

Matrix proteins

Matrix proteins constitute the major structural component of the virion shell. Both the VSV and influenza A virus matrix proteins are essential for virion formation and have the ability to induce particle formation (Sakaguchi *et al.*, 1999; Gomez-Puertas *et al.*, 2000; Jayakar *et al.*, 2000; Latham & Galarza,

2001). In contrast, recombinant MV and RV lacking the M gene (ΔM) have been generated (Cathomen *et al.*, 1998a; Spielhofer *et al.*, 1998; Mebatsion *et al.*, 1999). MV ΔM was more efficient in inducing cell fusion than its wild-type counterpart (Cathomen *et al.*, 1998a). Interestingly, MVs isolated from patients with subacute sclerosing panencephalitis are often defective in M gene expression (Baczko *et al.*, 1984).

The SeV M protein is phosphorylated in infected cells but not in mature virions; therefore, M phosphorylation was thought to regulate processes such as virion assembly or budding. However, a recombinant SeV containing a mutation in M that abrogated phosphorylation was indistinguishable from wild-type virus both *in vitro* and *in vivo* (Sakaguchi *et al.*, 1997).

Influenza A virus M2 protein

The influenza virus M2 protein is expressed from a spliced mRNA encoded by segment 7. It is an integral membrane protein that executes functions early and late in the virus replication cycle. In late endosomes, the M2 ion channel activity allows proton influx into the virions, thus causing a pH shift that leads to the dissociation of the M1 protein from RNP complexes. Late in infection, proton influx through the M2 ion channel raises the pH in the trans-Golgi network, thereby preventing acid-induced conformational changes in intracellularly cleaved haemagglutinin (HA). Reverse genetics experiments indicated that the M2 cytoplasmic tail is critical for virus replication, most likely through its interaction with other viral proteins (Castrucci & Kawaoka, 1995); in contrast, replacement of either the serine residue at position 64 (the primary M2 phosphorylation site) or the conserved cysteine residues did not compromise the recombinant viruses *in vitro* or *in vivo* (Castrucci *et al.*, 1997; Thomas *et al.*, 1998).

M2 ion channel activity was long considered essential for virus replication. However, an influenza virus with a deletion in the M2 transmembrane domain was growth-impaired but still replicated in cell culture (Takeda *et al.*, 2002). Another recombinant virus whose M2 transmembrane and cytoplasmic domains were deleted grew in cell culture but not in mice (Watanabe *et al.*, 2001). Thus, M2 ion channel activity is not essential for virus replication *in vitro* but it is required for *in vivo* virus replication.

Influenza A virus NS2 protein

The influenza A virus NS2 protein is translated from a spliced mRNA encoded by segment 8. It contains a classical nuclear export signal (NES) in its amino-terminal region and mediates nuclear export when fused to a reporter construct (O'Neill *et al.*, 1998). Moreover, NS2 interacted with the cellular nuclear export factor CRM1, which mediates export of proteins with classical NESs (Neumann *et al.*, 2000a). In cells infected with virus-like particles (VLPs) that lacked NS2 or

encoded an NS2 protein with an altered NES, viral RNP complexes were retained in the nucleus, thus supporting the role of NS2 as the virus nuclear export factor of viral RNP complexes (Neumann *et al.*, 2000a).

Viral glycoproteins

Rhabdoviruses possess a single glycoprotein (G), which is involved in cell attachment and pH-dependent fusion of the viral and cellular membranes. For orthomyxoviruses, the HA protein binds to cellular receptors and mediates the pH-dependent fusion event, while the sialidase activity of the neuraminidase (NA) protein is required to prevent self-aggregation and to prevent viruses from rebinding to cells from which they were released. In contrast to orthomyxoviruses, different proteins encode the attachment and fusion activities for paramyxoviruses. The G (rhabdo- and pneumoviruses), H (morbilliviruses) or HN (respiro- and rubulaviruses) glycoprotein brings about binding to cellular receptors. The fusion (F) protein is involved in the fusion of the viral membrane with the endosomal or plasma membrane, resulting in the release of RNP complexes into the cytoplasm of infected cells. However, for most paramyxoviruses, coexpression of both F and HN is required for fusion activity, indicating a fusion-promoting activity of the HN protein.

Deletion of glycoproteins

A human RSV (hRSV) strain passaged at progressively lower temperatures contains a deletion of the G gene (as well as of the SH gene), demonstrating that these proteins are not essential for virus replication (Karron *et al.*, 1997a); however, a recombinant hRSV lacking G was attenuated in its replication (Techarpornkul *et al.*, 2001). Similarly, reverse genetics allowed the generation of bovine RSV (bRSV) that lacked the G and/or SH genes (Karger *et al.*, 2001). The artificially generated viruses did not display an altered morphology and grew to titres similar to wild-type virus in cell culture (Karger *et al.*, 2001). Thus, for hRSV and bRSV, the F protein seems to be able to function as an attachment protein, in addition to its critical role in cell fusion (Kahn *et al.*, 1999); however, the G protein is needed for efficient growth of hRSV in mice (Teng *et al.*, 2001).

Proteolytic cleavage of viral glycoproteins determines pathogenicity

Proteolytic cleavage of the viral fusion protein is one of the key factors determining virus pathogenicity. Many of the fusion proteins are synthesized as inactive precursor proteins that are cleaved by host cell proteases to generate two disulfide-linked subunits. It is this activated form that initiates the fusion of the viral and cellular membranes. In avian influenza virus and NDV, fusion proteins (HA for the former

virus) possess multiple basic amino acids at their cleavage site that are recognized by ubiquitous cellular proteases, such as furin (Stieneke-Grober *et al.*, 1992). In contrast, the fusion proteins of avirulent viruses contain single basic residues at this site that are not recognized by the ubiquitous proteases, resulting in local infections. For avian influenza A viruses, researchers established a direct link between HA cleavability and virus pathogenicity and demonstrated that two features were critical for HA cleavage: the length and composition of the basic amino acid stretch as well as the presence or absence of a nearby carbohydrate side chain (Kawaoka & Webster, 1988, 1989; Horimoto & Kawaoka, 1994; Klenk & Garten, 1994). The importance of multiple basic amino acids at the F cleavage site has also been demonstrated for NDV (Peeters *et al.*, 1999). For MV, alteration of the multibasic furin cleavage site of the F protein resulted in recombinant virus that did not induce neural disease, in contrast to wild-type virus (Maisner *et al.*, 2000). Reverse genetics also allowed researchers to replace the multibasic furin-recognition motif of the Ebola virus GP protein with nonbasic amino acids. Interestingly, the recovered virus grew to titres similar to wild-type virus in cell culture (Neumann *et al.*, 2002). Hence, furin-mediated cleavage of the Ebola virus GP protein is not critical for virus replication in cell culture; however, it may be required for virus propagation in animals.

Cross *et al.* (2001) determined the significance of the large hydrophobic amino acids of the influenza A virus fusion peptide, which become exposed after HA cleavage by host cell proteases. Replacement of hydrophobic amino acids with alanine but not with glycine yielded viruses capable of replication. Moreover, cell culture propagation of viruses containing alanine substitutions in the fusion peptide resulted in pseudoreversion to valine, indicating a preference for hydrophobic amino acids with a large side chain in this region (Cross *et al.*, 2001).

Viral glycoproteins determine host cell tropism

Viral glycoproteins are a major factor in determining host cell tropism. To study MV cell tropism, two groups of investigators generated recombinant viruses that contained the H and/or F proteins derived from a lymphotropic wild-type strain (WTF) of MV in the background of the tissue culture-adapted Edmonston vaccine strain (Johnston *et al.*, 1999; Ohgimoto *et al.*, 2001). These studies demonstrated that the WTF H protein can confer the ability to viruses to replicate in secondary lymphoid tissues in cotton rats (Ohgimoto *et al.*, 2001). Furthermore, replacement of the Edmonston strain H gene with that of the wild-type IC-B strain, or vice versa, altered the host cell specificity of the resulting recombinant viruses (Takeuchi *et al.*, 2002).

MV has the potential to cause neurological complications. Substitution of the H gene of the Edmonston B vaccine strain with its counterpart derived from a rodent brain-adapted strain

resulted in a neurovirulent, recombinant virus (Duprex *et al.*, 1999). However, infection progressed more slowly in mice infected with recombinant virus compared to the rodent brain-adapted parental strain. Thus, the H protein is an important (although not the only) factor required for neurovirulence.

Two studies addressed the significance of the RV G protein for neurovirulence by introducing G protein derived from neurovirulent strains into the genetic background of less neurotropic strains. While G is the major determinant of neurotropism, other factors seem to contribute to pathogenicity (Morimoto *et al.*, 2000; Ito *et al.*, 2001). Similarly, reciprocal exchange of the canine distemper virus and MV H proteins demonstrated that they are important determinants of the growth characteristics and the cell tropism of the recombinant viruses (von Messling *et al.*, 2001).

Cytoplasmic tails of glycoproteins

The cytoplasmic tails of the attachment and fusion proteins are thought to be critical for virus assembly, most likely through their interaction(s) with internal viral proteins. The influenza A virus HA protein contains conserved, palmytilated cysteine residues in both its cytoplasmic tail and its transmembrane domain. Replacements of these amino acids yielded conflicting results. While two groups reported the generation of influenza viruses lacking the conserved cysteine residues (Jin *et al.*, 1996; Lin *et al.*, 1997), another group failed to generate such mutants (Zurcher *et al.*, 1994). However, these findings may simply reflect the fact that the different HA subtypes used for these studies differ in their abilities to tolerate amino acid replacements. Analysis of recombinant influenza A viruses whose NA tail was replaced with that of influenza B virus, or whose NA and/or HA cytoplasmic tails were deleted, demonstrated that the HA and NA cytoplasmic tails are not absolutely required for virus propagation (Bilsel *et al.*, 1993; Jin *et al.*, 1994, 1997; Garcia-Sastre & Palese, 1995; Mitnaul *et al.*, 1996). However, deletion of the cytoplasmic tails affected the particle shape, reduced the vRNA:protein ratio and attenuated the recombinant viruses in mice, suggesting that the HA and NA cytoplasmic tails affect virion formation and confer a growth advantage to influenza virus (Garcia-Sastre & Palese, 1995; Mitnaul *et al.*, 1996; Jin *et al.*, 1997; Zhang *et al.*, 2000). Similarly, truncation of the cytoplasmic tails of the SV5 HN (Schmitt *et al.*, 1999) or the SeV HN or F proteins (Fouillot-Coriou & Roux, 2000) resulted in recombinant viruses that were impaired in their growth. Recombinant SV5 or MV with alterations in their HN, F and/or H cytoplasmic tails displayed enhanced cell-to-cell fusion (Cathomen *et al.*, 1998b; Schmitt *et al.*, 1999). In addition, basolateral targeting signals have been identified in the cytoplasmic tails of the MV H and F proteins (Moll *et al.*, 2001). For VSV, replacement of the transmembrane and cytoplasmic domains of G with those of human CD4 protein did not affect virion budding (Schnell *et al.*, 1998). Further analysis demonstrated that a short cytoplasmic tail

without specific sequence requirements is sufficient to promote efficient budding (Schnell *et al.*, 1998). In addition, a short region in the extracellular stem of G was identified that confers efficient virus assembly (Robison & Whitt, 2000).

Influenza A virus NA protein

The influenza A virus NA protein is composed of a stalk of variable length and amino acid composition that connects the transmembrane domain with the globular head, which contains the enzymatic centre. Recombinant viruses with altered stalks demonstrated that viruses with longer stalks replicated to higher titres in eggs (Castrucci *et al.*, 1992, 1994; Castrucci & Kawaoka, 1993; Luo *et al.*, 1993). Stalk-less viruses were not restricted in their growth in tissue culture but failed to grow in eggs and displayed attenuated phenotypes in mice (Castrucci & Kawaoka, 1993). These findings indicate that the NA stalk is not essential for virus replication but modulates the host range, probably by affecting the enzymatic activity of NA.

The NA protein of the neurovirulent influenza A/WSN/33 virus strain differs from all other influenza A virus NAs by virtue of the lack of a glycosylation site at amino acid position 130. To assess the contribution of this glycosylation site for neurovirulence, Li *et al.* (1993c) generated a recombinant virus containing the conserved glycosylation site. The recombinant virus, unlike its parent, did not replicate in mouse brain, demonstrating that this NA glycosylation site is critical for neurovirulence. Further studies demonstrated that influenza A/WSN/33 virus NA binds plasminogen, which, after being activated to plasmin, cleaves HA (Goto & Kawaoka, 1998). *In vitro* and *in vivo* studies revealed that two NA structural features, a carboxy-terminal lysine residue and the lack of the glycosylation site at position 130, were critical for plasminogen binding (Goto & Kawaoka, 1998; Goto *et al.*, 2001). Recombinant viruses in which either one of these features was altered did not replicate in the brain of infected mice, in contrast to wild-type influenza A/WSN/33 virus (Goto *et al.*, 2001).

Ebola virus glycoprotein GP

Expression of the complete, membrane-anchored form of the Ebola virus glycoprotein is achieved through editing of its mRNA, while the unedited mRNA is translated to the secreted glycoprotein (sGP). About 80% of the GP mRNAs remain unedited, resulting in high amounts of sGP in patient sera (Volchkov *et al.*, 1995; Sanchez *et al.*, 1996). In contrast, the closely related Marburg virus translates its GP from an unedited mRNA and does not express sGP. To address the role of transcriptional editing and sGP synthesis in Ebola virus replication, Volchkov *et al.* (2001) generated a virus that expressed GP from an unedited mRNA. The mutant virus expressed higher levels of GP and was more cytotoxic than its wild-type counterpart. This finding suggested that trans-

criptional editing might control the expression levels of GP and hence the cytotoxic effects of this protein.

Accessory proteins

Accessory proteins are not essential for basic levels of virus replication and/or transcription but regulate or modulate a variety of steps in the virus life cycle. For VSV and members of the *Paramyxovirinae* subfamily, accessory proteins are encoded by the P gene and expressed through RNA editing and/or they are expressed from a second reading frame overlapping the P gene. In contrast, accessory proteins of pneumoviruses are expressed from separate, additional transcription units, involving the use of overlapping reading frames.

Influenza A virus NS1 protein

Among negative-sense RNA viruses, the NS1 protein was the first protein shown to counteract the cellular interferon (IFN) response (reviewed by Garcia-Sastre, 2001). In 1998, Garcia-Sastre *et al.* (1998) generated a Δ NS1 influenza A virus that was restricted in its replication in MDCK cells and mice (hence, in systems with a functional IFN response). In contrast, Δ NS1 influenza A virus replicated to titres comparable to wild-type virus in IFN-compromised systems, such as Vero cells or STAT-1^{-/-} mice (Garcia-Sastre *et al.*, 1998; Talon *et al.*, 2000b). NS1 interferes with several cellular pathways to combat the antiviral IFN response. Double-stranded RNA-activated protein kinase (PKR) indirectly stimulates type I IFN, thus playing a critical role in antiviral responses. NS1 binds to RNA (Yoshida *et al.*, 1981), thereby suppressing PKR (Hatada *et al.*, 1999; Bergmann *et al.*, 2000). In line with these findings, Δ NS1 influenza A virus, but not wild-type virus, stimulated the expression of a reporter gene under the control of an IFN-responsive promoter (Garcia-Sastre *et al.*, 1998). Moreover, Δ NS1 influenza A virus activated NF- κ B (Wang *et al.*, 2000), which transactivates IFN- β -regulated promoters and IFN regulatory factor 3 (Talon *et al.*, 2000a), which forms a transcription complex with signal transducer and transcriptional activator-1 (STAT-1) and STAT-2.

In a study to assess the contribution of NS1 to the extreme pathogenicity of the 'Spanish Flu' virus, which killed 20–40 million people in 1918 and 1919, recombinant viruses were generated that contained the NS1 or both the NS1 and the NS2 encoding regions of the Spanish Flu virus in an influenza A/WSN/33 virus genetic background (Basler *et al.*, 2001). The recombinant viruses did not kill mice, in contrast to the parental influenza A/WSN/33 virus, and the results were considered inconclusive because of gene constellation effects.

C proteins

C proteins (reviewed by Nagai, 1999; Nagai & Kato, 1999) are expressed by VSV, morbilli- and respiroviruses from a

second reading frame that overlaps the 5' region of the P gene in the +1 open reading frame (ORF). Morbilliviruses and hPIV3 encode one C protein. For VSV and SeV, ribosomal choice results in the use of different initiation codons (all in the same reading frame), yielding a nested set of two C proteins (designated C' and C) for VSV or a nested set of four proteins (C', C, Y1 and Y2) for SeV (Latorre *et al.*, 1998b). C proteins share a common carboxy-terminal region but differ in their amino termini.

The relative importance of C proteins for the virus life cycle differs among rhabdoviruses and paramyxoviruses. VSV defective in C protein expression is indistinguishable from wild-type virus in cell culture (Kretzschmar *et al.*, 1996), while alteration of the C proteins of hPIV3 (Durbin *et al.*, 1999) and RPV (Baron & Barrett, 2000) restricted virus replication in cell culture and/or experimental animals. The growth characteristics of MV Δ C depended on the cell line tested (Radecke & Billeter, 1996; Escoffier *et al.*, 1999) and MV deficient in C protein expression was attenuated in mice (Patterson *et al.*, 2000). For SeV, C' protein-deficient virus was as virulent as wild-type virus in mice, while C protein-deficient virus was highly attenuated in these animals (Latorre *et al.*, 1998a); this phenotype was attributed to a single amino acid substitution in the SeV C protein (Garcin *et al.*, 1997). Recombinant SeV deficient in the expression of all four C proteins was severely attenuated and grew to titres of 4 log units lower than those observed for the wild-type counterpart (Kurotani *et al.*, 1998). Most importantly, SeV employs all four C proteins to diminish the induction of IFN-stimulated gene expression (Didcock *et al.*, 1999a; Garcin *et al.*, 1999, 2000; Kato *et al.*, 2001), as demonstrated by the experimental requirement to ablate the entire complement of C proteins to fully suppress IFN- α -stimulated gene expression (Gotoh *et al.*, 1999). While all four C proteins interfered with STAT-1 phosphorylation, only C and C' proteins induced its instability (Garcin *et al.*, 2001).

The various C proteins have both positive and negative effects on virus replication and seem to execute their function(s) in a highly coordinated manner (Cadd *et al.*, 1996; Garcin *et al.*, 1997; Tapparel *et al.*, 1997; Kurotani *et al.*, 1998; Latorre *et al.*, 1998a; Baron & Barrett, 2000; Kato *et al.*, 2001; Reutter *et al.*, 2001). Moreover, C proteins may execute functions in assembly, since a recombinant SeV that did not express any of its C proteins differed in size and shape from wild-type virus (Hasan *et al.*, 2000). Thus, although not absolutely required for virus amplification, the C proteins affect several steps in the virus life cycle.

V proteins

With the exception of hPIV1, all morbilli-, rubula- and respiroviruses contain a V gene (reviewed by Nagai, 1999; Nagai & Kato, 1999). For SeV, NDV and morbilliviruses, V protein expression relies on the insertion of one or two nontemplated G residues into the P gene through RNA

editing. In contrast, the rubulavirus V protein is translated from an unedited mRNA. All V proteins share their amino-terminal region with the respective P protein but have a unique carboxy terminus. Suppression of V protein expression has only minor effects on RPV replication (Baron & Barrett, 2000), while NDV Δ V replicated to lower titres in cell culture and was severely attenuated in chickens' eggs (Mebatsion *et al.*, 2001). Recombinant SeV or MV deficient in V protein expression were not restricted in replication in cell culture (Delenda *et al.*, 1997; Schneider *et al.*, 1997a; Escoffier *et al.*, 1999; Patterson *et al.*, 2000) but were attenuated in animals (Kato *et al.*, 1997a, b; Delenda *et al.*, 1998; Tober *et al.*, 1998; Valsamakis *et al.*, 1998; Patterson *et al.*, 2000). hPIV2 Δ V did not grow in CV-1 cells but replicated in Vero cells (Kawano *et al.*, 2001), suggesting a role of the V protein in interplay with the cellular immune response.

The carboxy-terminal domain of V protein (which is not shared by the P protein) contains a cysteine-rich, zinc finger-like domain that is critical for pathogenicity (Kato *et al.*, 1997b; Huang *et al.*, 2000). It interacts with the large subunit of the cellular damage-specific DNA-binding protein (UV-DDB), as shown for SV5, hPIV2, MV and mumps virus (Lin *et al.*, 1998). This interaction is likely responsible for the delay in the division cycle of SV5-infected cells; indeed, the cell cycle progressed normally in cells infected with recombinant SV5 expressing a truncated V protein lacking the unique carboxy terminus (Lin & Lamb, 2000). Most importantly, V proteins interfere with the IFN response. The SV5 V protein targets STAT-1 for proteasome-mediated degradation (Didcock *et al.*, 1999b; Young *et al.*, 2000, 2001). Thus, SV5 and SeV (through its C proteins) target STAT-1, thereby blocking both type I and type II IFN signalling. In contrast, the V protein of hPIV2 induces proteolytic degradation of STAT-2, so that type I but not type II IFN signalling is abolished (Parisien *et al.*, 2001).

RSV NS1 and NS2 proteins

The 3' proximal genes of pneumoviruses (in genome orientation) encode two short, nonstructural proteins (NS1 and NS2) that are missing in other paramyxoviruses. The hRSV NS1 protein was identified as a regulatory protein since it inhibited both transcription and replication in minireplicon systems (Atreya *et al.*, 1998). Deletion of the NS1 gene resulted in virus that was attenuated in chimpanzees (Teng *et al.*, 2000), confirming the role of NS1 in virus growth. Deletion or insertion of a stop codon in the RSV NS2 gene resulted in recombinant viruses that were attenuated in cell culture (Teng & Collins, 1999) and in chimpanzees (Whitehead *et al.*, 1999a). Passage of the NS2 stop mutant in cell culture yielded revertant viruses whose artificial NS2 stop codon had been replaced with a sense codon, thus restoring expression of a (mutant) NS2 protein (Teng & Collins, 1999). This finding demonstrated that, although not essential, NS2 confers a growth advantage to hRSV replication in cell culture. Schlender

et al. (2000) generated bRSV mutants that do not express NS1 and/or NS2. These viruses were highly attenuated in MDBK cells but only moderately affected in IFN-deficient Vero cells, suggesting that NS1 and NS2 antagonize the IFN-mediated antiviral response. For bRSV, both the NS1 and NS2 proteins are required to control the IFN- α/β -mediated cellular response (Schlender *et al.*, 2000). Although hRSV continues to replicate in cells pretreated with type I IFN (Atreya & Kulkarni, 1999), it does not block either type I or type II IFN signalling (Young *et al.*, 2000). These findings suggest that hRSV developed an alternative strategy to counteract the cellular IFN response, one that most likely interferes with events downstream of IFN signalling.

RSV M2 gene

Expression of the hRSV N, P and L proteins, together with a plasmid-encoded minigenome, resulted in premature termination of mRNA synthesis (Collins *et al.*, 1996). Co-expression of low levels of the M2 gene yielded full-length mRNAs and polycistronic read-through transcripts, whereas higher amounts of the M2 gene inhibited replication and transcription (Collins *et al.*, 1996). Similarly, small amounts of the M2 gene enhanced transcription of a bRSV minigenome (Yunus *et al.*, 1998). Further study of the M2 gene, which contains two overlapping ORFs, revealed that M2-1 is required to synthesize full-length mRNAs and read-through transcripts (Collins *et al.*, 1996; Fearn & Collins, 1999) by functioning as an elongation factor and an antitermination factor (Collins *et al.*, 1996; Hardy & Wertz, 1998; Fearn & Collins, 1999; Hardy *et al.*, 1999). In contrast, the M2-2 protein inhibits RNA replication (Collins *et al.*, 1996; Jin *et al.*, 2000a) and may play a role in the switch from transcription to replication (Birmingham & Collins, 1999). Consequently, recombinant RSVs that lost the ability to express M2-2 were restricted in their growth in cell culture and in animal models (Birmingham & Collins, 1999; Jin *et al.*, 2000a; Teng *et al.*, 2000).

SH proteins

The hRSV SH protein is a short transmembrane protein of unknown function. Deletion of SH in a recombinant RSV did not affect RNA replication but slightly increased virus titres in certain cell lines (Bukreyev *et al.*, 1997; Techaarpornkul *et al.*, 2001) and caused moderate attenuation in animal models (Bukreyev *et al.*, 1997; Whitehead *et al.*, 1999a). Deletion of the SV5 SH protein did not significantly alter the growth characteristics of a recombinant virus in cell culture (He *et al.*, 1998). However, SV5 Δ SH induced greater DNA fragmentation and higher caspase-2 and caspase-3 activities than did wild-type virus, indicating that the SH protein interferes with apoptosis (He *et al.*, 2001).

In one study, expression of the RSV M2-2, NS1, NS2 and SH proteins was abrogated in various combinations (Jin *et al.*,

2000b). Virus in which all four genes were deleted could not be generated and deletion of the M2-2 gene together with the NS1 gene also proved detrimental to virus replication. However, recombinant viruses, such as RSV Δ SH, Δ NS1 and Δ NS2, were viable, although they were attenuated in cell culture and in cotton rats. Replication of recombinant viruses was more affected in HEp-2 than in Vero cells, suggesting that one or more of the deleted genes encode proteins that counteract the cellular IFN response. Taken together, these findings indicate that although the RSV M2-2, NS1, NS2 and SH proteins are not essential for virus replication, they encode supporting functions required for efficient virus replication.

Virus vectors

Negative-sense RNA viruses have several biological features that make them promising candidates as vaccine or targeting vectors. Most importantly, they do not replicate through DNA intermediates, so that integration of their genomes into the host cell genome is a remote possibility. Also, most members of this virus group grow to high titres, accommodate additional genetic material and express the foreign peptides or proteins at high levels. Moreover, strong humoral and cellular immune responses have been observed after immunization with negative-sense RNA virus vectors.

Nonsegmented negative-sense RNA viruses

Initial experiments using reporter proteins demonstrated that nonsegmented negative-sense RNA viruses will accommodate foreign genetic material and stably maintain it during serial passages in cell culture (Bukreyev *et al.*, 1996; Mebatsion *et al.*, 1996; Hasan *et al.*, 1997). However, the insertion of additional genetic material decreased virus yield in cell culture (Bukreyev *et al.*, 1996; Hasan *et al.*, 1997; Sakai *et al.*, 1999; Skiadopoulos *et al.*, 2000) and recombinant viruses were attenuated in animal models (Sakai *et al.*, 1999; Skiadopoulos *et al.*, 2000).

Because of the medical importance of human immunodeficiency virus type 1 (HIV-1), a number of studies explored the generation of recombinant viruses expressing retroviral glycoproteins or their cellular receptors. An initial study demonstrated that the human CD4 protein, which serves as the cellular receptor for HIV-1, was expressed from recombinant VSV together with the VSV G protein (Schnell *et al.*, 1996a). This approach was taken one step further with the generation of RV Δ G pseudotyped by transient expression of a chimeric CD4 RV G protein and CXCR4, or by a recombinant VSV Δ G expressing CD4 and CXCR4 from the viral genome (Mebatsion *et al.*, 1997; Schnell *et al.*, 1997). Both the pseudotyped RV and the recombinant VSV selectively infected cells expressing an X4-specific HIV-1 envelope protein, suggesting that gene delivery vectors that specifically recognize HIV-1-infected cells might be useful tools in targeting and eliminating these cells.

To explore the potential of nonsegmented negative-sense RNA viruses as vaccine vectors, a number of investigators generated recombinant RV or VSV expressing the HIV-1 envelope protein and found that they specifically infected CD4⁺ cells (Mebatsion & Conzelmann, 1996; Johnson *et al.*, 1997; Boritz *et al.*, 1999). After a boost with HIV-1 gp120 protein, mice inoculated with the recombinant RV developed a strong humoral response against the HIV-1 protein (Schnell *et al.*, 2000). Also, a recombinant SeV expressing HIV-1 envelope protein was generated and infected natural HIV-1 host cells, such as human primary blood mononuclear cells, macrophages and established T cell lines (Yu *et al.*, 1997).

The HIV-1 Gag protein is another potential target for cell-mediated host immune defence, leading several research groups to generate viruses that expressed this protein. RV expressing HIV-1 Gag induced a strong cytotoxic T lymphocyte response against this protein in mice. A recombinant VSV expressing both Env and Gag demonstrated that VSV can accommodate up to a 40% increase in its genome size (Haglund *et al.*, 2000). More importantly, this virus protected rhesus monkeys from challenge with a pathogenic HIV-1 (Rose *et al.*, 2001), thus demonstrating the potential of recombinant VSV as a vaccine vector.

Since the viral glycoproteins are critical in eliciting an antiviral response, viruses were generated with heterologous surface glycoproteins. Glycoproteins can be exchanged between members of the *Pneumo-* and *Respirovirus* genera, as demonstrated by bRSV, whose G and F proteins were replaced with the HN and F proteins of bovine PIV3 (bPIV3) (Stope *et al.*, 2001). Recombinant bRSV was also recovered after replacement of the attachment glycoprotein only, while replacement of only the fusion protein abrogated virus generation (Stope *et al.*, 2001). Substitution of the MV H and F genes with the VSV G gene in a MV genetic background generated attenuated virus that protected mice against challenge with wild-type VSV (Spielhofer *et al.*, 1998). Furthermore, recombinant VSV expressing influenza A virus HA or NA proteins (Kretzschmar *et al.*, 1997) protected vaccinated mice from lethal challenge with influenza virus (Roberts *et al.*, 1998, 1999). One of the problems associated with VSV vaccine vectors is the potential for induction of neutralizing-antibodies against the VSV G protein, thus preventing reinfection and boosting with the same vector. One study addressed this problem by generating VSV vaccine vectors that were based on the Indiana serotype but contained the glycoprotein genes of the New Jersey or Chandripura subtypes (Rose *et al.*, 2000). The G protein shuttle vectors did not induce cross-neutralizing antibodies and could therefore be useful tools when boosting of immune responses is desirable.

The immune response to virus infections can be enhanced or modulated by the coexpression of cytokines. This strategy was tested by generating MV expressing IL-12 (Singh & Billeter, 1999) or recombinant RSV expressing IFN- γ (Bukreyev *et al.*, 1999) or IL-2 (Bukreyev *et al.*, 2000b). While for the

former virus expression of IL-12 was demonstrated, the latter two viruses were attenuated, ostensibly due to the antiviral activity of IFN- γ or IL-2, respectively.

Influenza virus vectors

Influenza A viruses are promising vector candidates (reviewed by Garcia-Sastre, 2000) because of the availability of 15 HA and 9 NA subtypes, as well as numerous antigenic variants, which would allow repeated immunization. Recombinant viruses expressing foreign polypeptides integrated into the NA stalk or the antigenic sites of HA have been generated (Li *et al.*, 1992, 1993a, b; Castrucci *et al.*, 1994; Muster *et al.*, 1994, 1995; Isobe *et al.*, 1995; Murata *et al.*, 1996; Gilleland *et al.*, 1997; Walker *et al.*, 1997). Results demonstrating immune responses against the foreign peptides include expression of the V3 loop of HIV-1 gp120 protein (Li *et al.*, 1993a), expression of an epitope from the HIV-1 gp41 ectodomain (Muster *et al.*, 1994, 1995) and expression of a cytotoxic T lymphocyte-specific epitope of the lymphocytic choriomeningitis virus nucleoprotein (Castrucci *et al.*, 1994).

Several approaches have been explored to express full-length foreign proteins from influenza A virus vectors. Generation of a bicistronic vRNA with an internal ribosomal entry site (IRES) allowed the expression of a foreign protein via the cap-mediated initiation of translation, while the influenza A virus NA was translated by IRES-mediated internal binding of ribosomes (Garcia-Sastre *et al.*, 1994a). Alternatively, the 17 aa self-cleaving 'protease 2A' sequence from foot-and-mouth disease virus can be inserted between a foreign and a viral protein (Percy *et al.*, 1994). The resulting polyprotein is then cleaved to release the foreign and the viral protein; however, recent findings indicate that the protease 2A sequence may function as an IRES instead of a protease (Donnelly *et al.*, 2001).

In contrast to the generation of bicistronic vRNAs or polyproteins, full-length foreign proteins can also be expressed from additional gene segments. Initial experiments with reporter gene constructs demonstrated that additional gene segments were maintained for only about three passages in cell culture (Luytjes *et al.*, 1989). However, a promoter mutant significantly increased the expression of a reporter construct, most likely because of its preferential replication and/or transcription (Neumann & Hobom, 1995). On the strength of this finding, a recombinant influenza A virus was generated that maintained an additional gene segment with the respective promoter mutations for at least 11 passages in cell culture (Zhou *et al.*, 1998).

Gene delivery particles

The reverse genetics systems established for nonsegmented and segmented negative-sense RNA viruses can also be used to generate VLPs for gene delivery purposes. These particles lack one or more viral genes encoding structural proteins; hence, infectious progeny viruses cannot be generated.

A SeV genome whose F gene was replaced with that of a reporter gene formed particles only when supplemented with F protein (Li *et al.*, 2000). These particles infected fresh cells, resulting in expression of the reporter gene construct. For influenza virus, VLPs were generated by expressing all viral structural proteins and a virus-like RNA that encodes a reporter protein (Mena *et al.*, 1996; Gomez-Puertas *et al.*, 1999; Neumann *et al.*, 2000b). The potential of influenza VLPs as vaccines was demonstrated by Watanabe *et al.* (2002), who generated replication-incompetent particles from cloned cDNAs that lacked the entire NS gene or the NS2 encoding region. Vaccination of mice with the latter protected 94% of mice from challenge with a lethal dose of a homologous influenza virus.

Live attenuated vaccine viruses

Many negative-sense RNA viruses have medical or economical importance (or both), providing a compelling rationale for the development of vaccines that would effectively combat these pathogens. Reverse genetics has opened the door for the development of live attenuated vaccine viruses by introducing attenuating mutations and/or genome rearrangements (Wertz *et al.*, 1998; Ball *et al.*, 1999; Flanagan *et al.*, 2000, 2001), which should induce both humoral and cellular immunity, thus increasing the likelihood of protection from disease. Such vaccines should be more stable and, thus, safer than conventional live attenuated viruses.

RSV

RSV is the leading cause of viral bronchiolitis and pneumonia in infants and young children worldwide and a protective vaccine is greatly needed (reviewed by Collins *et al.*, 1999). Growth of the wild-type RSV A2 strain at progressively lower temperatures yielded a virus (*cp*RSV), which was, however, neither significantly cold-adapted nor temperature-sensitive and still caused upper respiratory disease in seronegative children. In comparison with its parent, *cp*RSV contained five amino acid replacements whose introduction into a wild-type-like RSV background reconstituted the *cp*RSV phenotype (Whitehead *et al.*, 1998b). This mutant was subjected to chemical mutagenesis, followed by biological selection for temperature-sensitivity, in an effort to increase its attenuation. This approach resulted in the temperature-sensitive (*ts*) phenotype *cpts*248 and *cpts*530 RVs; further chemical mutagenesis yielded *cpts*530/1030, *cpts*530/1009 and *cpts*248/404 RSVs. Reverse genetics allowed researchers to determine the relative contributions of individual mutations to the *ts*, attenuated (*att*) and cold-adapted (*ca*) phenotypes of RSV (Juhász *et al.*, 1997, 1999a, b; Whitehead *et al.*, 1998a). Two of these viruses (*cpts*530/1009 and *cpts*248/404 RSVs) were tested in infants and children but neither was sufficiently attenuated (Karron *et al.*, 1997b; Wright *et al.*, 2000). Hence, efforts were made to attenuate *cpts*248/404 RSV even further

by deleting the SH gene (Whitehead *et al.*, 1999a). This modification did not produce the desired results in chimpanzees; however, a more attenuated phenotype might only be apparent in humans. Recombinant viruses that contained combinations of mutations derived from different lineages were also generated. Introduction of an attenuating mutation found in RSV *cpts530/1030* into the *cpts248/404* RSV background yielded a virus that was more temperature-sensitive and attenuated than *cpts248/404* (Whitehead *et al.*, 1999b). Alternatively, RSV Δ NS1 or RSV Δ M2-2 might be useful as vaccines, since their replication in the upper respiratory tract of chimpanzees was restricted 10-fold more than that of RSV *cpts248/404* (Teng *et al.*, 2000).

bRSV is highly restricted for replication in the respiratory tract of primates and therefore has potential as a RSV vector backbone. Substitution of the bRSV G and F genes with their hRSV counterparts generated a virus that did not confer significant protection against challenge with hRSV in chimpanzees, indicating that it was overattenuated (Buchholz *et al.*, 2000).

RSV exists in two antigenic subgroups, A and B, both of which should be included in a vaccine. In order to develop a vaccine candidate, Whitehead *et al.* (1999c) introduced the G and F genes of the B1 strain of RSV subtype B into the RSV subtype A *cpts248/404* backbone. The resultant recombinant virus proved to be highly attenuated and immunogenic in chimpanzees and protected animals against challenge with subtype B RSV (Whitehead *et al.*, 1999c). Similarly, the subtype B glycoproteins were introduced into the subtype A wild-type RSV genome and, to further attenuate the virus, the M2-2 gene was deleted. The resultant virus was highly attenuated in African green monkeys and induced protection against subtype B RSV challenge (Cheng *et al.*, 2001).

hPIV

hPIV1, -2 and -3 are important causes of viral respiratory disease in infants and young children. Growth of the parental JS strain at progressively lower temperatures yielded a live attenuated hPIV3 candidate vaccine. The resultant virus (termed *cp45*) is cold-adapted, temperature-sensitive and attenuated for growth in the respiratory tracts of hamsters, rhesus monkeys, chimpanzees and humans. hPIV *cp45* differs from the parental JS strain by a total of 20 nt replacements and reverse genetics demonstrated that multiple mutations contributed to each of the *ca*, *ts* and *att* phenotypes (Durbin *et al.*, 1997a; Skiadopoulos *et al.*, 1998, 1999a). To attenuate *cp45* hPIV further, Skiadopoulos *et al.* (1999b) introduced an L gene mutation that specifies the *ts* and *att* phenotypes of a live attenuated RSV into the hPIV3 *cp45* background, creating a virus that was more temperature-sensitive and attenuated than its parent. In another study, a bivalent vaccine virus was generated that contained the MV H gene integrated into the background of wild-type or attenuated hPIV3 (Durbin *et al.*,

2000). Attenuated hPIV3 expressing the MV H protein not only protected hamsters against challenge with hPIV3 but also elicited high levels of MV-neutralizing antibodies.

Vaccines against PIV1 and -2 are not available and there is a lack of promising live attenuated vaccine candidates. In an attempt to generate a vaccine candidate, the HN and F glycoproteins of a wild-type hPIV3 isolate were replaced with those of hPIV1 (Tao *et al.*, 1998). Next, *ts* and *att* mutations identified in the L gene of hPIV3 *cp45* were introduced (Tao *et al.*, 1999). Alternatively, the HN and F glycoprotein genes of hPIV1 were directly integrated into the genetic backbone of hPIV3 *cp45* (Skiadopoulos *et al.*, 1999c). A similar approach was pursued to generate an hPIV2 vaccine candidate; however, hPIV3/hPIV2 chimeric viruses were viable only when the HN and F cytoplasmic tails were derived from hPIV3 (Tao *et al.*, 2000b). Chimeric hPIV3/hPIV1 and hPIV3/hPIV2 viruses were attenuated in replication in hamsters and provided protection against challenge with homologous wild-type virus (Skiadopoulos *et al.*, 1999c; Tao *et al.*, 1999, 2000a, b).

A different approach explored the use of bPIV3/hPIV3 chimeric viruses as live attenuated vaccines. Introduction of the hPIV3 HN and F genes into a bPIV3 background created a chimeric virus that was attenuated and protected hamsters from challenge with hPIV3 (Haller *et al.*, 2000). Furthermore, a bivalent vaccine virus was generated that contained the hPIV3 F and HN genes and the RSV G or F gene in an otherwise bPIV3 background (Schmidt *et al.*, 2001). The resultant virus induced protection against both hPIV3 and RSV.

Introduction of the bPIV3 N gene into an hPIV3 genetic background identified the N protein as the determinant for host range restriction of bPIV3 in primates (Bailly *et al.*, 2000). The addition of the MV HA gene to this genetic background yielded a bivalent virus that elicited immune response to both MV and hPIV3 (Skiadopoulos *et al.*, 2001).

Influenza viruses

Growth of influenza A and B viruses at progressively lower temperatures yielded cold-adapted, temperature-sensitive, attenuated master strains with mutations in some of the genes encoding internal proteins. Coinfection of cells with a circulating wild-type virus then allows, through natural reassortment, the selection of viruses that contain the genes encoding the internal proteins from the attenuated master virus, while the genes encoding the surface glycoproteins are derived from the circulating wild-type virus. Currently, live attenuated influenza A and B viruses are in clinical trials (Belshe *et al.*, 1998; Maassab & Bryant, 1999; Boyce & Poland, 2000) but these viruses contain only a limited number of amino acid replacements (Cox *et al.*, 1986, 1988; Herlocher *et al.*, 1996), leaving the risk of reversion to a wild-type sequence. The systems established recently for the generation of influenza viruses from cloned cDNAs could therefore be used to design 'master strains' with multiple attenuating mutations in all viral

proteins. Examples include temperature-sensitive mutations in the polymerase PB2 protein (Subbarao *et al.*, 1995; Parkin *et al.*, 1996, 1997) or deletion of the NS1 gene, which resulted in highly attenuated viruses that protected mice against wild-type infection (Talon *et al.*, 2000b).

RPV

RPV causes severe disease in cattle, leading to appreciable economic losses. An effective live attenuated vaccine is available that provides lifelong protection, but only one RPV serotype exists, so that vaccinated animals cannot be distinguished from those that developed immunity due to a natural infection. To overcome this problem, Walsh *et al.* (2000a, b) generated recombinant RPV that expressed genetic markers, such as green fluorescent protein (GFP) or the influenza virus HA. Cattle immunized with the recombinant viruses expressed anti-GFP or anti-HA antibodies and developed protective immunity against RPV (Walsh *et al.*, 2000b).

Peste-des-petits-ruminant virus (PPRV)

PPRV causes an infection in sheep and goats that clinically resembles RPV infections. Both viruses are members of the *Morbillivirus* genus and their genetic relationship allowed the generation of a recombinant RPV that expressed the PPRV F and H proteins (Das *et al.*, 2000). In contrast, no virus was recovered when only one glycoprotein was replaced. Further studies demonstrated that the RPV/PPRV chimeric virus was attenuated in cell culture and protected goats against infection with wild-type PPRV (Das *et al.*, 2000).

NDV

The severe effects of NDV cause major economic losses within the poultry industry. As with RPV, a live attenuated vaccine is available to combat NDV but vaccinated animals cannot be distinguished from those infected by wild-type virus. Thus, a recombinant virus was generated that expressed the NDV F protein and a chimeric HN protein whose immunogenic globular head was replaced with that of avian paramyxovirus type 4 (APMV4) (Peeters *et al.*, 2001). Neutralizing antibodies are developed against the NDV F protein, while the antibodies developed against the APMV4 HN protein allow a distinction from wild-type NDV isolates (Peeters *et al.*, 2001).

Future perspectives

Less than a decade ago, it was not possible to generate negative-sense RNA viruses. Now, through a series of major scientific advances, investigators have the tools to genetically engineer members of the *Rhabdo-*, *Paramyxo-*, *Filo-*, *Bunya-* and *Orthomyxoviridae* families, while a minireplicon system is available for a member of the *Arenaviridae* (Lee *et al.*, 2000); no

such system has been reported for Borna virus. The insights afforded by these advances not only have the potential to increase understanding of virus life cycles but should also help to clarify the mechanisms by which these viruses cause disease. As exemplified by studies of Hatta *et al.* (2001), reverse genetics can be used to generate single-gene reassortants between highly virulent and avirulent H5N1 influenza viruses, e.g. those infecting humans in Hong Kong in 1997. Two single amino acid substitutions in the PB2 and HA proteins were found to determine the pathogenic potential of these influenza viruses (Hatta *et al.*, 2001). The ability to decipher complex mechanisms of virus pathogenicity will no doubt increase our preparedness for future outbreaks. Finally, reverse genetics will allow researchers to design vectors for therapeutic gene delivery and to produce live attenuated vaccines against the many diseases caused by negative-sense RNA viruses.

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