Review article

Genetic manipulation of non-segmented negative-strand RNA viruses

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Introduction

Negative-strand RNA viruses are a large and diverse group of enveloped viruses of both medical and economic significance. They are found in hosts from the plant and animal kingdoms, and have a wide range of morphologies, biological properties and genome organizations. A major distinction is made between viruses whose genome consists of a single RNA molecule (order Mononegavirales), including the families Rhabdoviridae, Paramyxoviridae and Filoviridae, and those possessing multipartite (segmented) genomes, comprising the families Orthomyxoviridae (six to nine segments), Bunyaviridae (three segments) and Arenaviridae (two segments) (Pringle, 1991). Particular elements essential for their replication and gene expression have been retained throughout the negative-strand RNA viruses and illustrate that they have originated from a common ancestor (for review see Tordo et al., 1992). Genetic manipulation and analysis of negative-strand RNA virus biology has lagged far behind that of other RNA viruses. In contrast to positive-strand RNA viruses, isolated genome or antigenome RNAs of negative-strand RNA viruses are not infectious. The initiation of an infectious cycle requires the presence of a complete nucleocapsid structure. Only in this form can the RNA function as a template for the virus polymerase. Techniques to introduce recombinant RNA into the genome of a negative-strand RNA virus were first described for the segmented influenza virus (for a recent review see Garcia-Sastre & Palese, 1993). In this article, the most recent developments in the genetic manipulation of non-segmented viruses are briefly summarized. Successful rescue of defective rhabdo- and paramyxovirus model genomes, and the recovery of infectious rabies virus and recently vesicular stomatitis virus (VSV), measles virus and Sendai virus entirely from cDNA, illustrate that negative-strand RNA viruses have come of age in the recombinant DNA world. Genetic engineering of their genomes has become as easy as for positive-strand RNA viruses and will considerably increase our knowledge of virus biology and provide useful biomedical tools.

Replication and transcription of non-segmented negative-strand RNA viruses

Characteristically, the genetic information of negative-strand RNA viruses is exclusively found in the form of a ribonucleoprotein complex (RNP) in which the genomic or antigenomic ssRNA is tightly encapsidated in a nucleoprotein (N or NP) and associated with the virus RNA-dependent RNA polymerase. In the case of non-segmented viruses, the latter consists of a catalytic subunit (L) and a non-catalytic cofactor, a phosphoprotein (P). In the course of virus budding, RNPs that contain the anti-messenger (negative)-sense RNA are wrapped into simple envelopes containing an internal matrix protein (M) and one (G) or two (F and HN) transmembrane spike proteins.

After infection of a cell, the RNP serves as a template for two distinct RNA synthesis functions, transcription of subgenomic, usually non-overlapping mRNAs and the replication of full-length RNAs (for detailed reviews see Banerjee, 1987; Galinski, 1991). The RNP genomes appear to possess only one promoter, at the 3' end of the RNA where the virus RNA-dependent RNA polymerase enters for both mRNA transcription and genome replication. Thus, the polymerase has to act in a processive mode for synthesis of full-length RNA and a non-processive mode for transcription, in which internal genome signals define stop and restart sites. Most simply, this can be explained by the presence of a 'transcriptase' and a 'replicase' form of the polymerase complex. The 'transcriptase', able to recognize the signals, gives rise successively to a short leader RNA and several capped and polyadenylated mRNAs. Conserved signals bordering the cistrons are composed of an oligo(U) stretch which constitutes the template for a 'stuttering' synthesis.

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of the poly(A) tail, a few nucleotides that are scanned by the polymerase but are not transcribed ('intergenic region') and a conserved restart sequence defining the 5' end of the next mRNA. In contrast to transcription, the product of replication is not a free RNA, but an RNP with an encapsidated full-length RNA. Since constant protein synthesis is a prerequisite for replication of all negative-strand RNA viruses, it is assumed that RNA polymerization and encapsidation of the growing RNA chain into nucleoprotein are mechanistically linked. Concurrent encapsidation involves the participation of preformed N–P/NP–P and P–L complexes (Horikami et al., 1992). Since the genomic 3' region would simultaneously represent the promoter and provide the 'encapsidation' signal, mechanisms to prevent encapsidation of mRNAs should exist. In non-segmented viruses this might be achieved through the release of the leader RNA carrying the encapsidation signal. Reinitiation at the junction between the leader and the first protein-coding gene appears to be necessary to convert the polymerase to the non-processive mode, in which elongation is independent of assembly with N protein (Vidal & Kolakofsky, 1989). Other mechanisms to ensure the synthesis of non-encapsidated mRNAs are used by viruses with a segmented genome. Transcription of mRNAs of segmented viruses involves elongation of capped primers originating from cellular mRNAs ('capsnatching'; Plotch et al., 1981; Kolakofsky & Hacker, 1991). In both segmented and non-segmented viruses transcription is stopped pre-terminally at an internal transcription stop signal; during replication polymerization proceeds to the 5' terminus of the template, the last nucleotides of which are complementary to the 3' end. The 3' terminus of the resulting antigenomic RNP–RNA is similar in its nucleotide composition to that of the genomic one and functions as a strong promoter for synthesis of genome-sense RNPs, but not for transcription of subgenomic RNAs (Fig. 1).

With regard to their expression strategy, it is not surprising that isolated RNA from negative-strand RNA viruses is not infectious, a criterion which was originally used to distinguish positive- and negative-strand RNA viruses (Baltimore et al., 1970). Proteins are not expressed from the genomic RNA due to its negative polarity, or from the complementary positive strand due to the modular organization of the RNA, which does not allow translation of (all) virus proteins by the cellular machinery. The most severe obstacle to genetic alteration and the most striking difference from positive-strand RNA viruses, however, is the fact that the polymerases
of negative-strand RNA viruses cannot use nucleic acid directly as a template but only after encapsidation within the nucleoprotein. The hypothesis that encapsidation of RNA occurs only concurrently to elongation was thus a discouraging one. Alteration of a negative-strand RNA virus genome by the application of recombinant DNA technology appeared to be impossible a few years ago.

**Reverse genetics approaches: reconstitution of RNPs**

The situation changed dramatically with the description by Palese and colleagues of a system that allowed successful generation of biologically active RNPs containing artificial RNA (Luytjes et al., 1989). Transcripts that contained authentic terminal sequences from an influenza virus genome segment and an internal chloramphenicol acetyltransferase (CAT) reporter gene were encapsidated in vitro by purified influenza virus nucleoprotein (NP) and the virus polymerase proteins (PA, PB1 and PB2). After transfection of the reconstituted recombiant RNP into influenza virus-infected cells the construct was replicated, transcribed and translated. The helper virus not only provided the proteins needed for further RNA synthesis but, due to the segmented nature of its genome, also allowed packaging of the synthetic genome segment into progeny virus and passage of CAT activity in tissue culture. Thus, reassortant ('trans-') RNPs containing artificial RNA (Luytjes et al., 1989) were successfully generated inside a cell. The synthetic RNA, which was generated in vitro by T7 RNA polymerase transcription from a linearized plasmid in order to create precise ends, corresponded to a Sendai virus minigenome in which the entire coding region was replaced with the coding region of the CAT reporter gene (Fig. 1). This model genome possessed the virus 3'-terminal sequence including the putative promoter for the polymerase and the signal(s) directing leader RNA transcription/release and initiation of mRNA transcription. The 5' end contained the transcription stop/polyadenylation signal derived from the 5'-terminal cistron (L) and encoded the antigenic promoter for replication. In the form of an RNP, this construct thus represents a functional template for replication of RNPs and also, due to the presence of a leader/reinitiation signal, for transcription of positive-stranded leader RNA and a CAT mRNA. Park et al. (1991) demonstrated that after transfection of the in vitro-transcribed RNA construct into cells, CAT activity was observed after subsequent infection of cells with Sendai virus. Control experiments confirmed that CAT expression was Sendai virus-specific. Furthermore, the artificial RNPs were packaged into infectious virus particles, as demonstrated by successful passage of CAT activity by transfer of cell-free supernatants. Thus, it was confirmed that all cis-acting sequences required for encapsidation, initiation of replication and transcription of this parainfluenza virus reside in the terminal sequences of the genome.

Rescue of transfected RNAs by infectious helper virus has also been successful in other parainfluenza virus systems such as respiratory syncytial virus (RSV; Collins et al., 1991), paramyxovirus type 3 (PIV-3; Dimock & Collins, 1993; De & Banerjee, 1993) and measles virus (Sidhu et al., 1995). Assays of reporter gene activity expressed from negative-stranded minigenomes similar to the one described for Sendai virus again indicated that they were amplified, transcribed and incorporated into infectious particles. Thus, the investigation of cis-acting sequences has been possible for a variety of paramyxoviruses. The experiments revealed that the sequence corresponding to the virus 3' terminus has to be the end of the transcript, as replication cannot proceed from an internal site (Collins et al., 1991; De & Banerjee, 1993). In contrast, the 5' end appeared to tolerate additional nucleotides (De & Banerjee, 1993). In addition, it was shown that the nucleotides of the 3'-terminal promoter could be replaced by sequences complementary to the 5' end (i.e. the antigenome promoter; Collins et al., 1991). Not only promoter sequences can be analysed by the reporter gene assay (Harty & Palese, 1995), but also internal transcription signals using bicistronic model genomes that contain two different reporter genes (Kuo et al., 1994). A particular feature of the parainfluenza virus polymerase, RNA editing,
was also found to be template-directed (Park & Krystal, 1992).

Production of RNPs entirely from cDNA-encoded components

A considerable insight into the mechanisms governing transcription of negative-strand RNA viruses has been obtained by the study of naturally occurring defective interfering particles (DIs). Most DIs belong to the non-transcribing copyback type, which possess the parental 5' terminus and a complementary 3' end, therefore allowing replication but not initiation of transcription (Fig. 1). Such DIs have been used extensively to elucidate the trans-acting factors required for their propagation. Proteins expressed from transfected plasmids carrying a T7 promoter in the presence of a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3; Fuerst et al., 1986) were shown to support replication of a VSV DI (Pattnaik & Wertz, 1990) and a Sendai virus DI (Curran et al., 1991). In both cases, the N (NP), P and L proteins were confirmed to be necessary and sufficient for replication. Moreover, in cells co-infected with the VSV DI and recombinant vTF7–3 and expressing all virus proteins including the envelope proteins M and G from transfected plasmids, assembly and budding of infectious particles was observed (Pattnaik & Wertz, 1991). This approach represented a powerful tool for studying the trans-acting factors of VSV and provided a means to replace infectious helper virus in the rescue experiments. This was especially important for the development of rhabdovirus reverse genetics systems since, in contrast to paramyxoviruses, rescue of artificial RNAs in virus-infected cells was not observed for members of this family.

The use of the vaccinia virus/T7 RNA polymerase (VV/T7) expression system allowed the expression of virus proteins from individual plasmids and the intracellular generation of genome analogues from transfected plasmids. A crucial point in this approach was the generation of correct termini of transcripts. Whereas distinct 5' ends were easily determined by the location of the T7 promoter, the generation of discrete 3' ends presented a problem. Transfection of linearized DNA constructs in order to obtain intracellular ‘runoff’ transcripts is not very effective in the presence of vaccinia virus, most likely due to ligation and modification of DNA by vaccinia virus enzymes. Using this approach, rabies virus genome analogues could not be rescued unequivocally (unpublished results). In contrast, a Sendai virus RNA mimicking a natural DI RNA was encapsidated and amplified in cells expressing Sendai virus NP, P and L protein, although this occurred with a relatively low reproducibility (Calain et al., 1992).

A major breakthrough was thus the development of plasmid vectors designed to yield RNAs with discrete 3' termini by the autolytic activity of ribozyme sequences. This was first successfully used for intracellular generation of functional nodavirus RNA (Ball, 1992) and then of an artificial VSV DI RNA in Wertz's laboratory (Pattnaik et al., 1992). Virus cDNA derived from the natural 2 kb VSV DI-T was cloned between the T7 promoter and ribozyme sequences derived from either the genomic strand of tobacco ringspot virus (Prody et al., 1986) or the antigenomic strand of hepatitis delta virus (HDV; Perrotta & Been, 1990, 1991) and followed by a T7 polymerase transcription termination sequence. The latter ribozyme has the advantage that only sequences downstream of the cleavage site are required for autocatalytic activity; it is apparently indiscriminate with regard to upstream sequences. Thus, by autolytic cleavage from primary transcripts containing the HDV ribozyme sequence immediately downstream of the virus sequences, RNAs ending with the correct 3' nucleotide can be generated. According to the ribozyme cleavage mechanism, the 3'-terminal ribose of the (upstream) genome analogue should possess a cyclic 2'-3' phosphate instead of a hydroxyl group (Prody et al., 1986). This modification, however, might contribute to the success of the approach, e.g. in preventing polyadenylation of the RNA by vaccinia virus enzymes (Gershon et al., 1991) or delaying degradation of the RNA 3' terminus. Another modification at the 5' end of the transcripts, the addition of a 5' cap structure by vaccinia virus guanylyltransferase (Davison & Moss, 1989), affects a certain portion of the transcripts. Expression of the DI-T RNA from the transfected plasmid in vTF7-3-infected cells, which also expressed the VSV N, P and L genes from simultaneously transfected plasmids, resulted in efficient encapsidation and replication of the RNA (Pattnaik et al., 1992). It was also confirmed by these authors that a precise 3' end was more crucial for VSV replication than a correct 5' end of the transcript. Constructs with short heterologous 3' extensions or deletions were found to be encapsidated by N protein but were not replicated. Interestingly, extra non-virus G residues at the 5' end, introduced in order to enhance T7 RNA polymerase transcription, were shown to be removed rapidly during replication. As observed previously for the corresponding natural DI RNA, complete infectious DI particles were assembled in cells co-expressing all five VSV proteins from transfected plasmids and thus opened VSV to the analysis of cis-acting sequences involved in replication and trans-acting factors.

The use of the HDV ribozyme sequence for generation of precise 3' ends in combination with vaccinia virus expression proved to enhance encapsidation and replication of the above mentioned Sendai virus DI (Calain...
& Roux, 1993). In both cases, the replication of the copyback type DIs, whose sequences are specialized for efficient replication, could be demonstrated by direct biochemical means.

The first evidence that an RNA construct corresponding to transcriptionally active internal deletion minigenomes can be rescued by plasmid-encoded proteins was reported from our laboratory for the rabies rhabdovirus (Conzelmann & Schnell, 1994). In this case also, a natural DI (Conzelmann et al., 1991) had first served as a template to test the functional expression of virus proteins (see Wertz & Melero, 1993). The efficiency of the system allowed direct demonstration of the presence of rabies virus-specific subgenomic transcripts, a leader RNA and a polyadenylated mini-mRNA in total RNA from cells expressing rabies virus N, P and L protein together with a rabies virus model genome composed only of the authentic virus 3' and 5' ends (SDI-1). In addition, a variety of heterologous sequences between the ends of SDI-1, enlarging the artificial nucleotides is assembled into infectious virion envelopes. The reporter gene model genomes were encapsidated and expressed efficiently. As observed previously for the VSV DI, after additional expression of the envelope proteins M and G, infectious defective virions were assembled. Thus all aspects of replication and transcription as well as protein structure-function relationships of the rabies virus life-cycle are open for experimental analysis.

Subsequently, constructs corresponding to transcribing or non-transcribing model genomes of several non-segmented viruses (VSV, Sendai virus, rabies virus, measles virus, RSV) were expressed in the VV/T7–HDV expression system. This was aimed at the elucidation of cis- and trans-acting transcription factors and involved promoter mutagenesis (Engelhorn et al., 1993; Kälin et al., 1994; Calain & Roux, 1995; Pattnaik et al., 1995; Wertz et al., 1995; Yu et al., 1995) as well as the analysis of particular properties of the virus polymerases (Jacques et al., 1994; Schnell & Conzelmann, 1996) and the virus assembly process (Kaptur et al., 1995; Mebatson et al., 1995; Stillman et al., 1995). The approach appears to be applicable to most of the negative-strand RNA viruses, including the segmented bunyaviruses (Dunn et al., 1995; Lopez et al., 1995). However, virus- and genus-specific peculiarities have been revealed that have to be taken into account in the design of reverse genetics experiments. For example, whereas the entire rhabdovirus life-cycle, including assembly and budding of virions, is correctly performed in the presence of vaccinia virus, Sendai paramyxovirus virion assembly is prevented by vaccinia virus (Calain & Roux, 1993). An intriguing observation has been made by these authors: model genomes of Sendai virus that consist of a multiple of six nucleotides are replicated much more efficiently than those that do not. This is attributed to the structure of the NP protein unit, which is predicted to cover a stretch of six nucleotides in the RNP context. The ‘rule of six’ also applies to a member of the genus Morbillivirus, measles virus (Kälin et al., 1994), whereas corresponding constraints were not observed for the pneumovirus RSV or rhabdoviruses. In addition to the set of N (NP), P and L proteins which is apparently necessary and sufficient for both replication and transcription in all non-segmented viruses (e.g. Yu et al., 1995), paramyxovirus genomes might also encode proteins that act as factors regulating RNA synthesis. While editing of Sendai virus P gene mRNA gives rise to inhibitors of RNA synthesis (V and W proteins; Curran et al., 1991), the RSV M2 gene is predicted to encode a positive factor for mRNA transcription (Grosfeld et al., 1995). Differences in the assembly process apparently exist between rhabdoviruses. Whereas a VSV DI genome as small as 191 nucleotides is assembled into infectious virion envelopes (Pattnaik et al., 1995), the rabies virus SDI-1 genome of 237 nucleotides is not. Only larger constructs (e.g. SDI-CAT, 1032 nucleotides) were found to yield infectious particles (Conzelmann & Schnell, 1994). Reverse genetics approaches using model genomes will provide a versatile and powerful tool for investigation of mechanisms governing negative-strand RNA virus biology.

Recovery of infectious viruses from cDNA

The experiments described above revealed the basic requirements for the rescue of cDNA-derived RNAs of rhabdoviruses and paramyxoviruses and indicated that generation of a complete, non-defective virus should be possible. One difficulty was apparent, the low efficiency of correct encapsidation of long RNAs. In rhabdovirus systems it appeared that each additional kb of RNA in the model genomes resulted in an approximately 10-fold drop in recovery rate (Conzelmann & Schnell, 1994; Stillman et al., 1995). Since for model genomes of 3 and 4 kb, one rescue/encapsidation event was reported to occur per 10° and 10¹ cells, respectively, the estimated values for a full-length rhabdovirus genome of 12 kb appeared frustrating. Most remarkably from this point of view, Collins et al. (1993) described the rescue of a genome analogue that was 50% of full-length. It comprised the entire L gene of RSV and the CAT gene and was rescued after transfection of in vitro-transcribed RNA into RSV-infected cells.

In comparison to rescue experiments involving the previously described model genomes in the VV/T7 system, the approach to recover non-defective virus faced additional problems. A major difference is the presence
of N, P and L coding sequences in full-length transcripts. Therefore, a potentially deleterious antisense problem arises when genome-sense transcripts are used; large amounts of mRNAs encoding the N, P and L proteins, which are required for encapsidation and the initiation of replication and transcription, are predicted to hybridize to the negative-strand RNA. This could seriously affect two important events, proper encapsidation of the genome RNA and synthesis of the necessary proteins. In addition, hybrids might evoke interferon-based antiviral activity in transfected cells. Owing to these considerations, we favoured the use of plus-stranded, antigenic rabies virus RNA transcripts. As with an RNP containing a genome-sense transcript, it should be possible to initiate the infectious cycle using an RNP containing antigenome RNA. In comparison to genomic RNA, only one additional step is needed upon successful encapsidation, namely the replication of the RNP by the plasmid-encoded N, P and L proteins to yield a genome RNP. Transcription of mRNAs from the genome RNA template (initially also by plasmid-encoded proteins) would then prime the autonomous propagation of recombinant virus. To evaluate the suitability of antigenome RNAs, we first tested the rescue efficiency of positive versions of the previously used rabies virus minigenomes. In all cases, they were encapsidated and propagated as efficiently as their negative-strand counterparts (Schnell et al., 1994). Moreover, upon construction of both full-length genome and antigenome plasmids it was revealed that intracellular transcription by the vTF7–3-encoded T7 RNA polymerase consistently yielded approximately fivefold more full-size antigenome RNA than genome RNA, providing an additional argument for using antigenomes in rescue experiments.

Recombinant rabies viruses were reproducibly recovered from cells simultaneously expressing genetically marked antigenome RNA and the N, P and L proteins (Schnell et al., 1994). As anticipated, the observed rescue efficiency was low and was estimated on the average to occur in only one in 10^7 cells. In this respect, the advantages of antigenome RNA over genome RNA seem crucial for the successful recovery of recombinant virus. Owing to the absence of infectious helper virus, the low efficiency does not constitute a difficulty for the recovery of engineered virus variants. Once the bottleneck of proper encapsidation and initiation of an infectious cycle is overcome, variants replicating to titres even orders of magnitudes lower than the parental strain can be isolated. Since the recombinant vaccinia virus which is needed to prime the system can be eliminated easily by filtration or by passage in non-permissive cells, pure clonal virus stocks could be obtained.

We could further confirm the authenticity of the reverse genetics system by the generation of novel viruses exhibiting phenotypic features not present in any natural rabies virus. One variant lacked a 0.4 kb non-transcribed 3′ region (pseudogene) of the G gene. Both in cell culture (Schnell et al., 1994) and in infected mice (unpublished results), the behaviour of the variant was indistinguishable from that of the parental strain, the attenuated SAD B19 live vaccine (Conzelmann et al., 1990), indicating that the pseudogene, which is found in all members of the genus *Lyssavirus*, represents non-essential genetic material. Most interestingly with regard to the use of viruses from the order *Mononegavirales* as vectors for the expression of heterologous genes (see below) is that another variant could be generated whose genome contains an additional functional transcription unit. Insertion of transcriptional stop, polyadenylation and restart sequences into the pseudogene region resulted in transcription of an additional polyadenylated mRNA (Schnell et al., 1994).

Recent successful recovery of other recombinant viruses confirmed that the use of antigenome rather than genome transcripts may generally tip the balance towards success. By using the same experimental approach, the recovery of recombinant VSV (Indiana serotype) from full-length antigenome RNA transcripts was reported from two laboratories. In Rose’s group, an infectious chimaeric VSV possessing the glycoprotein gene of the New Jersey serotype was generated (Lawson et al., 1995). Since these glycoproteins have a sequence identity of approximately 50%, the generation of ‘VSV’ possessing heterologous glycoproteins is foreseeable. Wertz and colleagues recovered a recombinant VSV whose N, P, M and L genes corresponded to the San Juan strain, whereas the G gene was from the Orsay strain (Whelan et al., 1995). In both cases, recovery was not possible when starting with plasmids yielding genome-sense transcripts. As indicated by *in vitro* transcription experiments, one of the reasons for their failure might be premature termination of transcription by T7 RNA polymerase (Whelan et al., 1995).

In addition to rhabdoviruses, the first recovery of recombinant virus of a paramyxovirus, measles virus, has recently been achieved by Billeter’s group (Radecke et al., 1995). In order to avoid possible inhibition of virus assembly in vaccinia virus-infected cells, an alternative system for expression of virus proteins by T7 RNA polymerase has been developed in this case. Cell lines constitutively expressing T7 RNA polymerase and the virus NP and P proteins were transfected with T7 polymerase-driven plasmids encoding the measles virus L protein and a full-length measles virus antigenome RNA. Rescue of infectious virus (as demonstrated by the appearance of syncytia) was observed in up to six out of 10^6 transfected cells (Radecke et al., 1995). The generation of helper cell lines allowing the expression of
appropriate ratios of virus proteins may also prove useful for future rescue of other paramyxoviruses (Willenbrinck & Neubert, 1994).

Despite the inhibition of Sendai virus virion assembly in vaccinia virus-infected cells, Kolakofsky and colleagues recently succeeded in recovering recombinant virus from full-length antigenome transcripts using the VV/T7 system (Garcin et al., 1996). The use of AraC [1-(β-D-arabinofuranosyl)cytosine] to stop DNA synthesis, injection of the transfected culture cells into the allantoic cavity of embryonated chicken eggs and subsequent further passage in eggs resulted in the loss of vaccinia virus and the recovery of recombinant Sendai virus. One of the advantages (or disadvantage, depending on the intention) of the vaccinia virus expression system became evident; its high recombinogenic properties. A genetic marker in the full-length cDNA which confers a selective disadvantage to the recombinant virus was often found to be replaced by the wild-type sequence from a protein-encoding plasmid. Intracellular homologous DNA recombination induced by vaccinia virus (Evans et al., 1988; Ball, 1995) was exploited to create a novel virus whose genome was derived from the sequences of two independent plasmids. The recombinant virus possessed a chimaeric 3′ end composed of sequences from the antigenomic (replication) promoter (3′-terminal) and the genomic (replication and transcription) promoter. This non-defective copyback genome transcribed mRNAs and interfered with standard Sendai virus replication.

Perspectives: novel viruses, vectors, vaccines, chimaeras and cell targeting

Genetic manipulation of negative-strand RNA viruses was subject to constraints experienced with all of the virus groups. The problem was relatively straightforward, but its solution was not trivial. For the segmented influenza virus and non-segmented rhabdo- and paramyxoviruses, ways have been found that allow introduction of cDNA-derived sequences into infectious virus. A large range of diverse viruses can now be engineered as easily as positive-strand viruses (for reviews see Boyer & Haenni, 1994; Bredenbeek & Rice, 1992). It is possible to investigate cleanly the nature of the cis-acting sequences which control virus RNA synthesis, as well as the function of each virus protein in replication, assembly and interaction with the host. This will fundamentally change this field of virology. Tools for the identification of mechanisms involved in pathogenesis of virus infection are provided. It is also now feasible to design approaches to generate attenuated viruses for use as live virus vaccines. No effective vaccines currently exist against RSV or PIV. Similar approaches may lead to improved (or at least updated) measles virus vaccines.

Perhaps even more exciting is the potential to use negative-strand RNA viruses as vectors to express foreign genes. It has been shown for influenza virus and now for rabies virus that both have the capacity to express additional protein sequences (e.g. Garcia-Sastre et al., 1993) or transcription units (Schnell et al., 1994). Obviously, any vector use of RNA viruses depends on the stability of the foreign genetic material during replication of the virus. Genetic material that is not essential for efficient virus propagation was identified in the rabies virus (Schnell et al., 1994) and measles virus genomes (Radecke et al., 1995). Large non-coding regions present in the G gene of rabies virus (3′-terminal) or in the F gene of measles virus (5′-terminal) could be deleted without having an obvious effect on virus replication. This finding was compelling, since in viruses (and particularly in RNA viruses) non-coding regions are usually limited to a minimum. Continuous streamlining of plus-strand RNA virus genomes and elimination of sequences not strictly required is probably achieved through illegitimate recombination by copy-choice (for a recent discussion see Mindich, 1995). As evident from the formation of DI RNAs, recombination also happens in non-segmented negative-strand RNA viruses; however, as expected from the tight RNP structures, it occurs several orders of magnitude less frequently than in most positive-strand RNA viruses. From this point of view, in comparison to vaccine vectors based on various positive-strand RNA viruses such as the one recently proposed for poliovirus (Andino et al., 1994), rhabdo- or paramyxoviruses appear more suitable a priori. Owing to the modular nature of their genomes it is easy to engineer additional genes into the viruses. By selecting the site for introduction as well as manipulation of genome and antigenome promoters (Garcin et al., 1996; Wertz et al., 1995), transcription and replication might be regulated. The linear relationship between the length of the helical rhabdovirus RNP and the particle size suggests that only minimum constraints on the amount of additional RNA might exist in this virus group.

Finally, viruses carrying novel proteins in their envelopes may have a role to play in gene therapy. It is possible that transient expression of genes is therapeutic for certain conditions such as cystic fibrosis and cancer and the use of RNA viruses may have advantages over that involving viruses with a DNA phase. The possibility of manipulating the cell tropism of rhabdoviruses in such a way that they deliver genes only to the target cells of interest appears to be realistic. It has now been shown for VSV that the glycoproteins of other strains or serotypes can replace the homologous proteins (Lawson et al., 1995; Whelan et al., 1995). By using defective viruses and so avoiding handling of hazardous infectious virus, it has been shown that the rabies virus glycoprotein can be
replaced functionally both by a glycoprotein from another lyssavirus (Mokola virus) and by chimaeric proteins composed of rabies virus and Mokola virus sequences (Mebatsion et al., 1995). Moreover, earlier studies confined to complementation analysis of a naturally occurring temperature-sensitive G mutant have shown that a totally unrelated foreign glycoprotein (human immunodeficiency virus gp160) carrying the appropriate cytoplasmic tail signal of the VSV G protein is incorporated into the virus envelope and mediates infection of CD4+ cells (Owens & Rose, 1993). It is predicted that much effort will go into the exploitation of virus vectors for delivery of genes and of other medically useful biological agents.

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