Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Genetic engineering of animal RNA viruses

Karl-Klaus Conzelmann and Gregor Meyers

The ability to genetically manipulate viruses has led to extraordinary advances in understanding virus biology and to the establishment of useful vector systems. Initially confined to DNA viruses and retroviruses, RNA viruses have more recently become attractive candidates for expression of heterologous genes and offer promising perspectives for biomedical applications.

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Recombinant DNA technology makes it possible to specifically modify DNA genomes. Because of the small size of their genomes, viruses are particularly amenable to such manipulations. Since the recovery of the first recombinant virus, simian virus 40 (SV40), 20 years ago[1], a variety of DNA viruses and retroviruses has been genetically manipulated to exploit their replication machinery for expression of heterologous genes. When transfected into a cell, the purified DNA of many of these viruses is infectious and gives rise to new viral particles. Alternatively, recombinant DNA can be introduced into virus DNA by homologous recombination with the genome of a helper virus, allowing the manipulation of large DNA viruses, such as herpes[2] or pox viruses[3,4]. In recent years, RNA viruses lacking a DNA phase in their replication cycle have also been manipulated. Since recombinant RNA techniques remain cumbersome, almost all studies with RNA viruses have relied upon cDNA intermediates to produce biologically active RNA molecules. Two major RNA virus groups, the positive strand and the negative strand RNA viruses, are distinguished based on whether their purified RNAs are able to initiate an infectious cycle or not. For the latter group, the minimal infectious unit is not an RNA molecule but a ribonucleoprotein complex (RNP). The reconstitution of functional RNPs from proteins and recombinant RNA has represented a major technical problem and was achieved only two years ago. In this review, basic features of RNA virus gene expression, methods for genetic manipulation of RNA viruses, and perspectives of RNA virus-based expression systems are described.

Gene expression of positive strand RNA viruses

The plus-stranded RNA viruses were the first that were open to direct genetic manipulation[5–7] because their genomic RNA (vRNA) is able to function as an mRNA, directing the production of all viral proteins necessary for the initiation of virus propagation. To provide a template for the synthesis of additional mRNA molecules, replication starts with the polymerization of a minus strand complementary to the genome (cRNA). Thus, for all positive-strand RNA viruses the components of the replicase complex have to be translated directly from the genomic RNA. For the other viral polypeptides, which mainly constitute structural proteins, two principle expression strategies exist, by which positive-strand RNA viruses can be classified into two groups (Fig. 1).

Viruses in the first group generate only one kind of mRNA, which is of genome length and contains one long open reading frame (ORF). Expression of all viral proteins is achieved by translation of this RNA into a polyprotein that is co- and post-translationally

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processed by viral and host cellular proteases. The members of the picornavirus and flavivirus families belong to this first group. The genomes of these viruses are 7–16.5 kb, and are organized such that the structural proteins are encoded in the 5′ region, whereas the 3′ terminal part of the ORF codes for the RNA-dependent RNA polymerase.

The second group comprises the families Togaviridae, Caliciviridae, Coronaviridae and Arteriviridae. These viruses are characterized by the subgenomic RNAs used for gene expression (Fig. 1). In contrast with the first group, the replicase genes of these viruses are located in the 5′ part of the genome upstream of the structural genes. For all of these viruses the subgenomic RNAs are 3′ coterminal with the genomic RNA.

In the case of alphaviruses and rubivirus (family Togaviridae), one subgenomic RNA of about 4 kb is synthesized from a 12 kb genome cRNA after initiation at a specific promoter region. Translation of the subgenomic RNA results in a polyprotein that is processed into the three structural proteins[8]. One species of subgenomic RNA is also found in cells infected with caliciviruses. It is colinear with the 3′ terminal third of the 7.5 kb genomic RNA and is responsible for expression of the capsid protein of these viruses[9,10]. Members of the families Coronaviridae and Arteriviridae have genomes of about 30 and 15 kb, respectively, and express multiple subgenomic mRNAs. The mRNAs form a 3′ coterminal nested set, which implies that all but the smallest subgenomic RNAs contain more than one cistron (Fig. 1). In most cases only the 5′ terminal gene of each mRNA is translated[11–13].

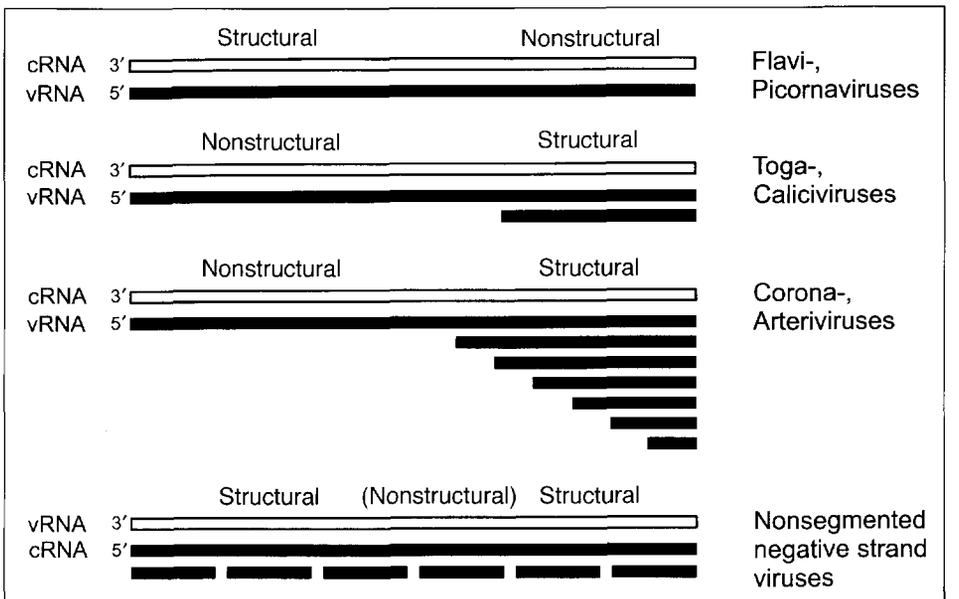

Fig. 1. Basic genome organization and strategies for gene expression in different RNA virus types. Positive strand (mRNA sense) RNAs are drawn as black boxes and negative strand RNAs as open boxes. RNAs present in the virions (vRNA) and the complementary RNAs (replicative intermediates; cRNA) are indicated. High-level expression of proteins that make up virus particle structure (structural) is achieved by transcription of subgenomic RNAs or by an upstream location of the respective genes. Nonstructural proteins include, for example, enzymes for RNA replication (RNA polymerases, helicases) or proteases.

Generation of recombinant positive strand RNA viruses

Usually, the first step towards the generation of a recombinant RNA virus is cDNA cloning of the complete viral genome. This also implies determination of the utmost terminal sequences of the RNA, which contain important signals necessary for replication. These initial steps are followed by attempts to construct full-length cDNA clones encompassing the complete genetic information of the virus. Although merely a technical problem, this part of the work is challenging since the constructs tend to be unstable when amplified in bacteria and deleterious mutations can be acquired during the cloning procedure. In some cases, problems with insert instability can be circumvented by the use of low copy-number plasmids and bacterial hosts bearing mutations that impair DNA recombination and repair[14–16]. For the flaviviruses yellow fever virus and japanese encephalitis virus, however, stable full-length clones have not yet been obtained. Nevertheless, recombinant viruses have been generated after *in vitro* ligation of two fragments that together cover the complete viral genome[17,18].

The picornaviruses poliovirus and coxsackie B3 virus were first recovered after transfection of full-length cDNA constructs into permissive cells[7,19,20]. The efficiency of the respective systems was quite low even when eukaryotic promoters were included. The main difficulties of this approach lie in the generation of the correct RNA termini and, presumably, in problems with nuclear export and unwanted splicing of the *in vivo* generated RNA. Despite these difficulties, the generation of an alphavirus replicon in cells transfected with a cDNA construct has been published recently[21]. Integration of such a construct into the genome of the host cell could lead to inheritable high level expression of a foreign gene. Recombinant nodavirus replicons have been generated after insertion of full-length cDNA constructs into recombinant DNA viruses (vaccinia virus) allowing efficient *in vivo* generation of infectious RNA within the cytoplasm of the infected cells[22].

The strategy of choice for recovery of recombinant viruses is transfection of target cells with genome-like RNA, which is generated *in vitro* by 'runoff' transcription with an RNA polymerase derived from the bacteriophages T3, T7 or SP6 (Fig. 2). The simple structure of the promoters for these enzymes, together with an appropriate restriction site used to linearize the template at the 3′ end of the viral sequence, allow synthesis of an RNA with precise termini. The RNA yields obtained by this method are high and the specific

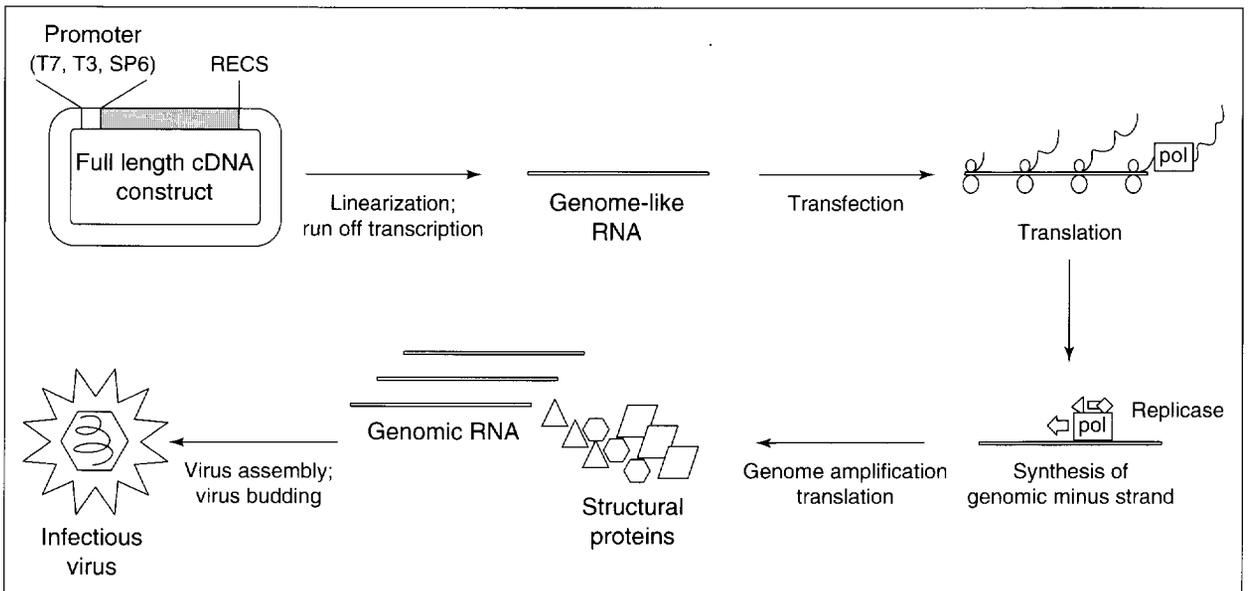

Fig. 2. Schematic presentation of a commonly used strategy for recovery of positive strand RNA viruses from cloned sequences. RECS, restriction endonuclease cleavage site.

infectivity of the transcripts in some cases approaches that of virion RNA[23,24].

The RNA generated *in vitro* can be introduced into cells by various methods. Transfection of RNA, bound to either DEAE dextran or cationic lipids, is most commonly used. Recently, electroporation has also been employed for this purpose and results in transfection efficiencies of almost 100% (Refs 24,25).

Until now, the large genome size of coronaviruses (28–32 kb) has prevented the generation of viruses entirely from cloned sequences. Two different strategies have been pursued to circumvent this problem. One approach is based on the transcription of smaller RNAs that contain deletions and therefore are not able to replicate autonomously. Such RNAs can be a source of defective interfering particles (DIs). DIs are able to replicate with the help of normal viruses, which provide the functions not encoded by the DI itself. Since DI genomes have to contain certain *cis*-acting sequences to be replicated and packaged into infectious particles, construction of synthetic DI genomes can be used for the identification of replication and packaging signals. In addition, recombinant DI-RNAs can serve as replicons designed for the expression of foreign genes[26,27].

The second approach is based on the high frequency of recombination observed for coronaviruses. During one round of replication about 25% of the newly generated RNAs undergo recombination[13]. This extremely high rate of recombination probably resolves the problem of deleterious mutations resulting from replication of the large genome by an error-prone RNA-dependent RNA polymerase. The characteristically efficient homologous recombination can be employed for the genetic manipulation of viruses by transfection of coronavirus-infected cells with, for example, RNA containing a heterologous gene flanked by coronavirus sequences[28,29].

Positive strand RNA virus vector systems

Different positive strand RNA viruses have been used as vectors for the expression of heterologous sequences[24]. Obvious advantages of these systems include the easy and rapid engineering of the DNA constructs and, in contrast to DNA viruses like vaccinia virus, the possibility of avoiding any wild-type virus background by *de novo* generation of viruses entirely from cloned sequences. Among the positive strand RNA viruses that do not possess subgenomic mRNAs, most efforts in vector development have focused on poliovirus. The major aim of these studies was the generation of new vaccines[30]. In this respect, poliovirus is of interest because of the availability of human vaccine strains of defined efficacy and safety, which have the advantages of oral application and the ability to induce mucosal immunity. To this end, poliovirus has been used for the expression of short heterologous peptides integrated into the viral capsid proteins. Detailed knowledge of poliovirus structure and antigenic properties has facilitated the choice of integration sites.

The expression of a foreign protein with defined ends using poliovirus is more difficult. Since the ends of poliovirus proteins are generated by processing at specific protease cleavage sites, defined both by amino acid sequence and protein structure, insertion of a foreign sequence into the genomic RNA usually results in expression of a fusion protein. This problem has been overcome by placing foreign sequences, followed by a cleavage site for the poliovirus 3C protease, at the 5′ end of the open reading frame. Sequences coding for up to 400 amino acid residues have been inserted this way[31].

An elegant alternative method is based on the generation of bicistronic poliovirus RNAs by integration of an internal ribosome entry site (IRES)[32]. IRES elements are usually located at the 5′ end of picornaviral RNAs, and allow translation in a cap-independent

fashion, based on the direct binding of ribosomes to a specific RNA structure[33]. Integration of a second IRES element at an internal position results in translation of downstream sequences starting at a defined initiation site. By positioning the second IRES together with the foreign gene at the end of the viral ORF, a heterologous protein can be expressed with its ends defined by the translation initiation site and the translational stop codon.

For members of the flavivirus family, infectious clones have been described in the past few years[14–18]. In the near future, genetic manipulation of these viruses will be focused by investigations of virus biology and the need to develop vaccines against these pathogens. Construction of chimeras between pathogenic and attenuated viruses will help to identify mutations responsible for attenuation. In the case of pestiviruses, which cause enormous losses to livestock, an additional challenge will be to introduce a specific marker into the vaccine to permit the differentiation of vaccinated animals from those infected with field viruses. Such 'marker' vaccines are prerequisites for vaccination-based eradication of these pathogens.

Flaviviruses could also be vector candiates. One advantage of these viruses is their slow replication, which results in less severe immediate effects on infected cells. Also, naturally occurring pestivirus recombinants have been isolated that contain more than 4 kb of additional sequences in their genome, suggesting that packaging constraints might be less important for these viruses[34].

The most advanced vector systems based on positive strand RNA viruses have been developed for alphaviruses[24,25]. Establishment of these systems has been facilitated by the detailed study of the *cis*-acting elements in the viral genome, which are responsible for replication, packaging and transcription. Because alphaviruses use a subgenomic RNA for gene expression, a foreign protein with defined ends can easily be generated via translation of an additional mRNA.

Three distinct groups of alphavirus expression systems have been described. The first group consists of viruses that have the foreign gene inserted into a non-defective genomic RNA able to replicate and to produce progeny virus. In most cases, the foreign sequence is expressed via a second subgenomic RNA that is transcribed from a duplicated subgenomic promoter. The insert can either be placed upstream or downstream of the structural genes. The system is designed for serial propagation of the recombinant viruses[24].

The second group relies on the generation of synthetic DIs that contain the 5′ and 3′ terminal sequences necessary for replication and packaging. These sequences flank the foreign gene, which again is under the control of the subgenomic promoter. The DI is replicated in the presence of a helper virus that also provides the enzymes for transcription and capping of an mRNA that directs translation of the foreign sequence[24].

For the third type of alphavirus expression system, the heterologous gene is inserted into the genomic RNA and replaces the sequence coding for the structural proteins. The resulting replicon is replication competent but does not generate infectious particles. Efficient expression of foreign genes has been obtained in this way with the polypeptide product representing up to 25% of the total cellular protein[21]. The packaging capacity of these alphavirus vectors is thought to be about 5 kb of foreign sequences[24]. A similar system based on Semliki forest virus is available commercially (GIBCO BRL Life Technologies Ltd, Gaithersburg, MD, USA).

To avoid the need for high efficiency transfection, the replicon can be packaged into infectious virions by providing the structural proteins in *trans*. The easiest way to do this is by the cotransfection of a DI genome that directs the synthesis of the structural proteins[24,25]. An interesting option is to use a DI RNA without packaging signals that results in a 'dead end' or 'suicide' system; after transfection of the recombinant genome together with the engineered DI genome, high titer stocks of recombinant viruses, but no infectious DI particles, are obtained. The viruses can infect new cells with almost 100% efficiency but do not produce progeny virus since the structural genes are missing. A commercially available Sindbis virus expression system relies on this strategy (Invitrogen Corporation, San Diego, USA). However, the 'dead end' strategy for the alphavirus system is somewhat leaky, as infectious viruses can be reconstituted by recombination between the DI RNA and the recombinant genome.

The potential of other positive strand RNA viruses with subgenomic RNAs for expression of foreign sequences has not yet been evaluated. In the near future, genetic manipulation of corona- and caliciviruses will probably be employed mainly for studies on virus biology and vaccine production. There are intrinsic difficulties with the coronavirus system and the first infectious calicivirus clone has been described only recently[35]. However, the simple construction of the calicivirus capsid, which consists of only one major protein that spontaneously assembles into virus-like particles[36], makes this virus group interesting for biotechnological applications. Because of their simple biology, nodaviruses also represent interesting vector candidates. These insect viruses have a bipartite genome; one segment encodes the coat protein precursor and the other segment directs synthesis of the replicase. Expression via vaccinia virus showed that the latter RNA is self replicative and thus can be used for replication and expression of a foreign gene[22].

Gene expression of negative strand RNA viruses

Negative strand RNA viruses represent a diverse group of enveloped viruses. A major distinction lies between viruses whose genomes consist of a single RNA molecule (Mononegavirales order), such as the Rhabdoviridae, Paramyxoviridae and Filoviridae families, and those possessing multipartite (segmented) genomes comprising the Orthomyxoviridae (six to nine segments), the Bunyaviridae (three segments), and the Arenaviridae (two segments)[37]. However, both groups share features characteristic for their typical mode of replication and gene expression.

All negative strand RNA viruses contain a ribonucleoprotein complex (RNP) in which the RNA is

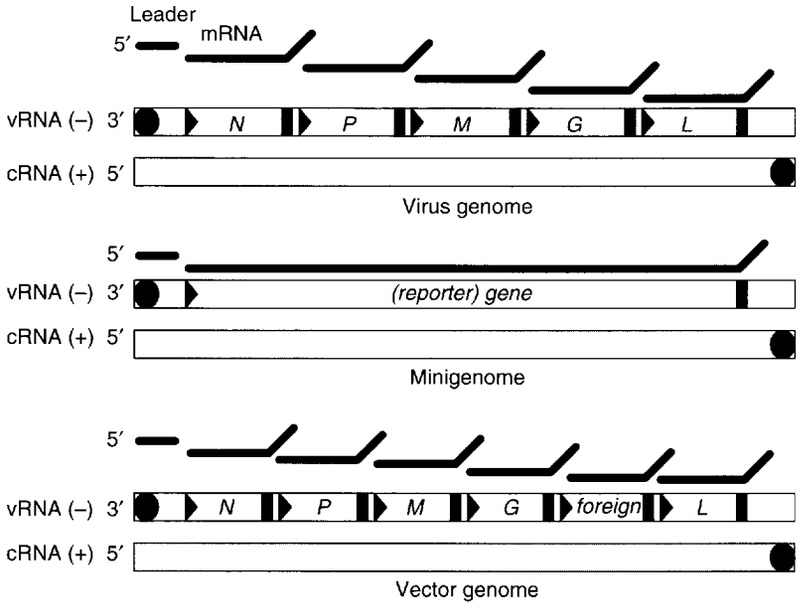

Fig. 3. Organization of recombinant genomes from nonsegmented negative RNA viruses (not drawn to scale). Both vRNA and cRNA are present exclusively in the form of a ribonucleoprotein (RNP) complex and contain a single polymerase entry site at the 3′ end (black sphere). Starting from the genomic (vRNA) promoter either successive transcription of a short leader RNA and free polyadenylated mRNAs or replication of the RNP is possible. Signals for transcriptional start (arrowhead) and stop/polyadenylation (bar) are indicated. The antigenomic (cRNA) promoter directs only replication (for details see text). Virus genome; a simple rhabdovirus genome encoding five proteins is shown, N, P and L are components of the RNP ('core'), M and G are envelope proteins. Minigenome: a typical model genome (1–2 kb) used for rescue experiments, containing the terminal promoters and one gene flanked by transcription start and stop signals. Vector genome: expression of a foreign protein by insertion of the foreign sequences between extra start and stop signals.

tightly encapsidated in a nucleoprotein (N or NP) and associated with the viral RNA-dependent RNA polymerase. In the case of nonsegmented viruses, the polymerase consists of a catalytic subunit (L) and a noncatalytic cofactor, a phosphoprotein (P). In the virions, RNPs that contain the anti-messenger (negative) sense RNA are enclosed into simple envelopes containing an internal matrix protein (M) and one or two transmembrane spike proteins. The initiation of an infectious cycle requires the presence of a complete RNP.

The RNP serves as a template for two distinct RNA synthesis functions, namely the replication of full length RNAs and the transcription of subgenomic mRNAs from internally located cistrons (Fig. 3)[38,39]. Since only one polymerase entry site (promoter) at the 3′ end of the RNAs exists, the polymerase has to act in a processive mode for the synthesis of full length RNAs and in a nonprocessive mode for transcription (Fig. 3). Most simply, this can be explained by the presence of a 'replicase' and 'transcriptase' form of the polymerase complex, with only the latter being able to recognize the internal *cis*-acting stop and restart signals that define the cistron borders. In contrast to transcription, the product of replication is not a free RNA, but an RNP. 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 the RNP is functionally linked. Mechanisms to ensure the synthesis of nonencapsidated mRNAs involve elongation of capped primers originating from cellular mRNAs ('cap-snatching') in segmented viruses[40], or the production of a short leader RNA carrying the encapsidation signal in viruses with a nonsegmented genome. In both segmented and nonsegmented viruses transcription is stopped preterminally at an internal transcription stop-signal whereas during replication, polymerization proceeds to the 5′ terminus of the template. The 3′ terminal region of the resulting antigenomic RNP-RNA is highly similar in its nucleotide composition to that of the genomic RNA and functions as a strong promoter for synthesis of genome sense RNPs, but not for transcription of subgenomic RNAs (Fig. 3).

Owing to this expression strategy, isolated RNA from negative strand RNA viruses cannot be infectious. Proteins can be expressed neither from the genomic RNA, because of its negative polarity, nor from the complementary positive strand, because of the modular organization of the RNA which does not allow translation of (all) viral proteins by the cellular machinery. However, the most severe obstacle to genetic alteration of negative strand RNA viruses and the most striking difference from positive strand RNA viruses is the fact that the RNA must be encapsidated into nucleoprotein in order to function as a template for the polymerase.

Strategies for generation of recombinant viruses

Successful encapsidation of a preformed RNA into nucleoprotein ('illegitimate' encapsidation) and formation of a biologically active RNP was first achieved by Peter Palese and colleagues[41]. Transcripts that contained the terminal promoter sequences from an influenza virus genome segment and an internal chloramphenicol acetyl transferase (CAT) reporter gene were encapsidated *in vitro* by purified influenza virus nucleoprotein (NP) and the viral polymerase proteins PA, PB1 and PB2. After transfection of the reconstituted RNPs into influenza (helper-) virus-infected cells, the RNPS were replicated and transcribed. By reassortment of genome segments, the synthetic segment was incorporated into progeny virus (Fig. 4)[42,43]. Since then, reassortant influenza viruses that possess specific alterations in different genome segments ('transfectant' viruses) have been generated (see Ref. 44 for a recent review).

Probably because of a tighter RNP structure, attempts to encapsidate preformed RNAs of nonsegmented viruses *in vitro* have failed. RNPs of these viruses are only formed inside a cell that provides the required viral proteins, as first demonstrated for a monocistronic Sendai virus minigenome[45] (Fig. 3). By using

similar model genomes, intracellular encapsidation systems were subsequently established for several rhabdo- and paramyxoviruses (reviewed in Ref. 46). The most well-developed approach makes use of the recombinant vaccinia virus–T7 RNA polymerase expression system[47] and involves simultaneous intracellular expression of both viral proteins and RNA from transfected plasmids (Fig. 4). A major breakthrough in optimizing the system was exploitation of the autolytic activity of a hepatitis delta virus ribozyme, which is indiscriminate with regard to sequences 5′ of its cleavage site, to develop plasmid vectors that allowed intracellular generation of RNAs with discrete 3′ termini[22,48]. RNAs ending with the correct 3′ nucleotide can be generated by autolytic cleavage from primary transcripts containing the ribozyme sequence immediately downstream of the virus sequences. The approach appears to be applicable to most of the negative strand RNA viruses, including the segmented Bunyaviruses[49,50] and allowed determination of the particular *trans*-acting factors required for the formation of biologically active RNPs. Accordingly, the N, P and L proteins are necessary and sufficient for providing the basic functions for replication and transcription in apparently all nonsegmented viruses. Supplementary factors may be encoded by paramyxoviruses. While editing of Sendai virus P gene mRNA gives rise to inhibitors of RNA synthesis (V and W proteins)[51], the M2 gene of respiratory syncytial virus (RSV) encodes a factor supporting elongation of mRNA transcription[52]. For rhabdoviruses, formation of complete virus-like particles was achieved after transient coexpression of the full viral protein complement and short model genomes[53,54].

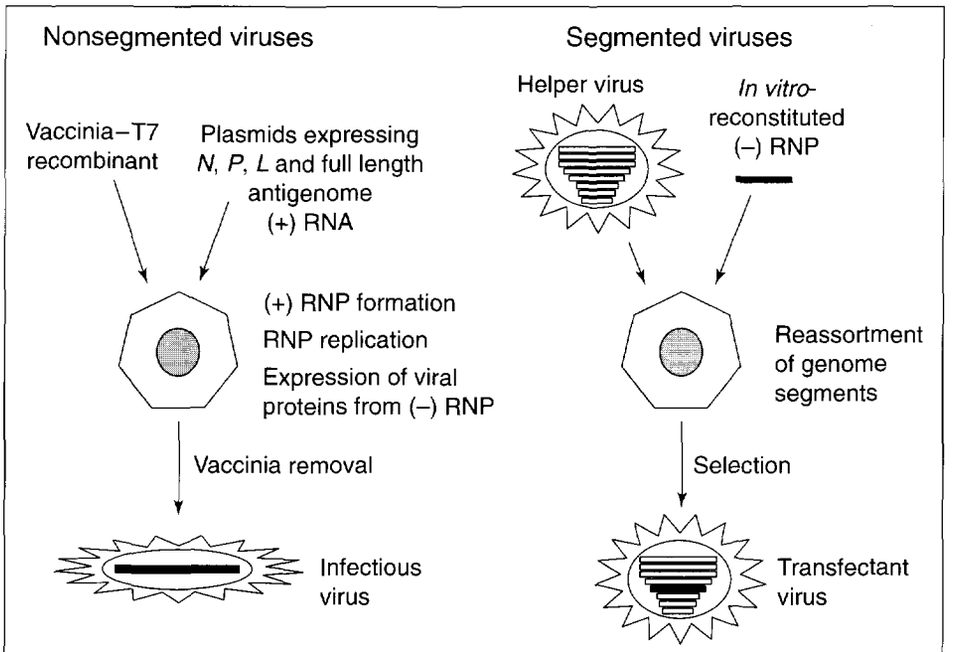

Fig. 4. Rescue of cDNA-derived sequences into negative strand RNA viruses (adapted, with permission, from Ref. 43). The generation of recombinant viruses with a nonsegmented genome involves simultaneous intracellular expression of full length cRNA and the proteins required for formation of an RNP. The expression from transfected plasmids is driven by a recombinant vaccinia–T7 RNA polymerase virus. Recovery of influenza virus possessing a recombinant genome segment is achieved by transfection of an RNP, which is reconstituted *in vitro*, into cells infected with influenza virus. Appropriate selection conditions allow the isolation of a virus (transfectant) containing cDNA-derived RNA (see Ref. 44 for details).

Our lab was the first to show that for the rabies rhabdovirus, the RNA of a negative strand RNA virus can be made infectious by coexpression with the N, P and L proteins[55]. An important feature of the approach was initiation of the infectious cycle by expressing the antigenomic RNA rather than the genomic RNA. This strategy avoided a potentially deleterious antisense problem, in which RNA transcripts encoding the N, P and L proteins would hybridize to the complementary viral genome sequences and interfere with correct encapsidation. As anticipated, rescue of a 12-kb full-length RNA into a functional RNP is inefficient and therefore virus production is observed in only one in 10^6–10^7 transfected cells. The same strategy was used subsequently for successful recovery of the prototype rhabdovirus vesicular stomatitis virus (VSV)[56,57], and also of members from all three genera of the Paramyxoviridae family: recombinant measles (morbilli-) virus[58], Sendai (paramyxo-) virus[59], and human respiratory syncytial (pneumo-) virus[60] were recovered from full-length antigenome RNAs.

Most of the approaches to the recovery of recombinant rhabdo- and paramyxoviruses have been based on vaccinia (vTF7-3 or MVA-T7[47,61]) driven expression of full-length RNA and viral N, P and L proteins. One of the features of this system has become evident, namely its striking potential for homologous DNA recombination. Replacement of a genetic marker in the full-length Sendai virus cDNA with wild-type sequence was observed at high frequency and was triggered by vaccinia-induced recombination of the full-length cDNA with a protein-encoding plasmid. This feature was exploited to create a novel Sendai virus whose genome was derived from the sequences of two independent plasmids[59]. For recovery of measles virus, cell lines constitutively expressing T7 RNA polymerase and the N and P proteins were used, and viral antigenome and L protein were provided transiently from transfected T7-polymerase-driven plasmids[58]. Infectious RSV could be recovered after expression of the elongation factor encoded by the viral M2 gene, in addition to the N, P and L proteins[60].

Applications for recombinant negative strand RNA viruses

The tools to manipulate virus genomes allow investigation of the *cis*-acting sequences that control gene

expression, as well as the function of each viral protein in virus replication, assembly and interaction with the host, and will rapidly advance knowledge in this field of virology. In addition to the feasibility of designing attenuated and effective virus vaccines, the exciting potential of negative strand RNA viruses as vectors to express foreign genes can be exploited. Influenza virus and, more recently, rhabdoviruses have been shown to have the capacity to express additional protein sequences.

Since nonessential genome segments of influenza virus are rapidly lost by reassortment, selective pressure or linkage of foreign sequences to one of the essential segments is required. Because of the monocistronic nature of the influenza virus genome segments, strategies for the construction of vectors are similar to those for picorna- flavi- and pesti-viruses. These include the insertion of foreign epitopes into suitable sites in the viral glycoproteins NA and HA, and the expression of polyproteins that are autolytically cleaved by, for example, at picornaviral 2A protease target sequences[62]. The function of a mammalian IRES element has been exploited to create a bicistronic NA segment expressing two distinct open reading frames[63]. Influenza A and B virus vectors have been obtained that express both B and T cell epitopes from different pathogens[64]. Since influenza virus is one of the few RNA viruses replicating in the nucleus of infected cells, attention has to be paid to potential splicing of mRNAs.

Engineering of additional genes into nonsegmented viruses is considerably facilitated by the modular organization of their genomes (Fig. 3). By insertion of an additional gene end and start signal into the rabies virus genome, a virus was generated containing an extra transcription unit and transcribing an extra mRNA[55]. This approach has allowed successful expression of reporter genes such as CAT from recombinant rabies virus[65] and also from VSV[66]. Most remarkably, the foreign, and nonessential genes are maintained and expressed through, so far, more than 50 cell culture passages. This finding has confirmed the previous observation that genetic material with no apparent function, particularly large noncoding regions in the glycoprotein genes, are maintained stably in rabies and measles virus genomes[55,58]. Continuous streamlining of positive strand RNA virus genomes and elimination of sequences not strictly required is probably achieved through recombination by a copy-choice mechanism, which involves detachment of the polymerase from the template, attachment to another template molecule and resumption of polymerization[67,68]. The tight encapsidation into nucleoprotein, which renders the genomes of rhabdoviruses and paramyxoviruses inaccessible for RNase degradation, will impede this mechanism. The tight RNP structure of nonsegmented negative strand RNA viruses had represented the major obstacle to their genetic manipulation, but now appears to be a most welcome feature of recombinant vectors. By selecting the site for introduction, as well as manipulation of genome and antigenome promoters[59,69], appropriate adjustment of the transcription level of foreign genes appears possible. Moreover, the helical nature of RNPs suggests that minimal constraints on the amount of additional RNA might exist in this virus group.

Perhaps the most exciting potential of rhabdovirus vectors stems from the simple structure of their envelopes and assembly mechanism. The generation of viruses carrying novel proteins in their envelopes and delivering genes only to the target cells of interest now appears realistic. It has already been shown for VSV and rabies virus that the glycoproteins of other strains or serotypes as well as chimeric proteins can replace homologous proteins[56,57,70]. Moreover, earlier studies on the complementation analysis of a naturally occurring temperature-sensitive G mutant have shown that a signal sufficient for directing a foreign glycoprotein (HIV gp160) into the virus envelope is located in the cytoplasmic tail signal of the VSV G protein[71]. Our recent discovery of rabies virion budding at the cell surface in the absence of any viral spike protein[72] has further suggested the possibility that rhabdoviruses with a wide range of surface proteins and the respective receptor specificities can be generated. In a first attempt towards this goal, we have complemented a recombinant G-deficient rabies virus mutant[72] with a chimeric HIV-1 gp160 possessing the cytoplasmic tail signal of the rabies G protein. In contrast to rabies virus, which in cell culture infects all types of cells, the phenotypically complemented pseudotype viruses could infect only cells expressing the HIV receptor proteins $CD4^+$/$fusin^+$ (Ref. 73).

Conclusions

In addition to the established DNA virus vectors, including pox-, herpes-, adeno- and retro-viruses (with both a DNA and an RNA phase), viruses with an 'RNA only' replication strategy have been successfully engineered to produce chimeras. Several RNA virus systems offer advantages in terms of host range, rapid engineering of expression constructs, production of high titer stocks of infectious particles and high levels of expression. Because of the limited genetic stability caused by recombination and error prone replication, positive strand RNA virus vectors might be most useful for transient high-level expression of RNAs and proteins. Although bioengineering of nonsegmented negative strand viruses has just begun, it is obvious that an important gap in the spectrum of viral vectors has been filled. Exciting prospects are offered by the simplicity of their genome organization and mechanism of expression, which should allow regulated expression of foreign genes. Most importantly, they exhibit a high genetic stability that is probably based in the RNP structure. In addition to their use as vaccines, these viruses may therefore have a role to play in somatic gene therapy as nonintegrative vectors. Appropriately attenuated vectors based on parainfluenza viruses or RSV, which exhibit a marked tropism for respiratory epithelia, might be used in the future for therapy of conditions such as cystic fibrosis. Furthermore, much effort will probably go into the development of targetable rhabdovirus-based vectors, which allow delivery of genes into specific cell types, such as cancer cells.

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