Infectious rabies viruses from cloned cDNA

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The generation of infectious rabies virus (RV), a non-segmented negative-stranded RNA virus of the Rhabdoviridae family, entirely from cloned cDNA is described. Simultaneous intracellular expression of genetically marked full-length RV antigenome-like T7 RNA polymerase transcripts and RV N, P and L proteins from transfected plasmids resulted in formation of transcriptionally active nucleocapsids and subsequent assembly and budding of infectious rabies virions. In addition to authentic RV, two novel infectious RVs characterized by predicted transcription patterns were recovered from modified cDNA. Deletion of the entire non-translated pseudogene region, which is conserved in all naturally occurring RVs, did not impair propagation of the resulting virus in cell culture. This indicates that non-essential genetic material might be present in the genomes of non-segmented RNA viruses. The introduction of a functional extra cistron border into the genome of another virus resulted in the transcription of an additional polyadenylated mRNA containing pseudogene sequences. The possibility of manipulating the RV genome by recombinant DNA techniques using the described procedure—potentially applicable also for other negative-stranded viruses—greatly facilitates the investigation of RV genetics, virus–host interactions and rabies pathogenesis and provides a tool for the design of new generations of live vaccines.

Key words: genome reconstruction/infectious cDNA/negative-strand RNA/rabies virus/RNA virus

Introduction

Directed genetic manipulation of RNA virus genomes depends on the ability to produce recombinant RNAs which are accepted as a template by the particular viral RNA-dependent RNA polymerases. Transcripts generated by standard DNA-dependent RNA polymerases (e.g. phage T7 RNA polymerase or cellular RNA polymerase II) and mimicking viral genomes are recognized by the polymerases of many positive-stranded RNA viruses. This has allowed the recovery of infectious viruses or replicons from cDNA transcripts and the application of recombinant DNA technology to the analysis of virus genomes and *trans*-acting factors. (for reviews see Bredenbeek and Rice, 1992; Boyer and Haenni, 1994). Since RNAs corresponding to the genomes of positive-stranded RNA viruses may also function as mRNA for translation of the viral polymerases, an infectious cycle may be initiated by introduction of the genome analogues into a cell.

The template of the polymerases of negative-stranded RNA viruses, however, is exclusively a ribonucleoprotein complex (RNP) or 'nucleocapsid', consisting of the genomic RNA tightly encapsidated within nucleoprotein (N or NP) and associated with a phosphoprotein (P). Moreover, and in contrast to positive-stranded RNA viruses, their genomic or antigenomic RNA may not function as mRNA and thus all viral proteins involved in replication and transcription of artificial RNAs have to be provided in *trans*. The lack of appropriate systems for encapsidation of genomic RNA analogues of these viruses in order to provide the appropriate template has hindered the application of recombinant DNA technology to the genetic analysis of these viruses considerably.

Successful generation of functional nucleocapsids of a negative-stranded RNA virus containing artificial RNA was first reported for the segmented influenza A virus. RNA transcripts that contained authentic terminal sequences from influenza genome segments and an internal reporter gene were encapsidated by purified proteins *in vitro*. Upon transfection into cells infected with helper virus they were amplified and expressed (Luytjes *et al.*, 1989). Since re-assortment of transfected recombinant nucleocapsids with RNA segments of the helper virus occurs at high frequencies, re-assortant influenza viruses containing particular engineered genome segments could be isolated (Enami *et al.*, 1990).

Attempts to obtain infectious recombinant negative-stranded RNA viruses with a large, non-segmented genome (order Mononegavirales, e.g. filo-, paramyxo- and rhabdoviruses), which necessitates manipulation of the entire genomes, have failed so far. However, short model genomes could be encapsidated and expressed either by infectious helper viruses (Park *et al.*, 1991; Collins *et al.*, 1991, 1993; De and Banerjee, 1993; Dimock and Collins 1993) or by plasmid-encoded proteins (Calain *et al.*, 1992; Pattnaik *et al.*, 1992; Calain and Roux, 1993; Conzelmann and Schnell, 1994). The latter approach involved co-expression of both genome analogues with predetermined termini (Ball, 1992) and particular viral proteins from transfected plasmids (Pattnaik *et al.*, 1990, 1991) in the transient vaccinia virus/T7 RNA polymerase system (Fuerst *et al.*, 1986) and allowed the determination of both *cis*- and *trans*-acting factors required for the generation of transcriptionally active nucleocapsids.

Here we show for the first time that an infectious non-segmented negative-stranded RNA virus, the neuropathogenic rabies virus of the rhabdovirus family, may be

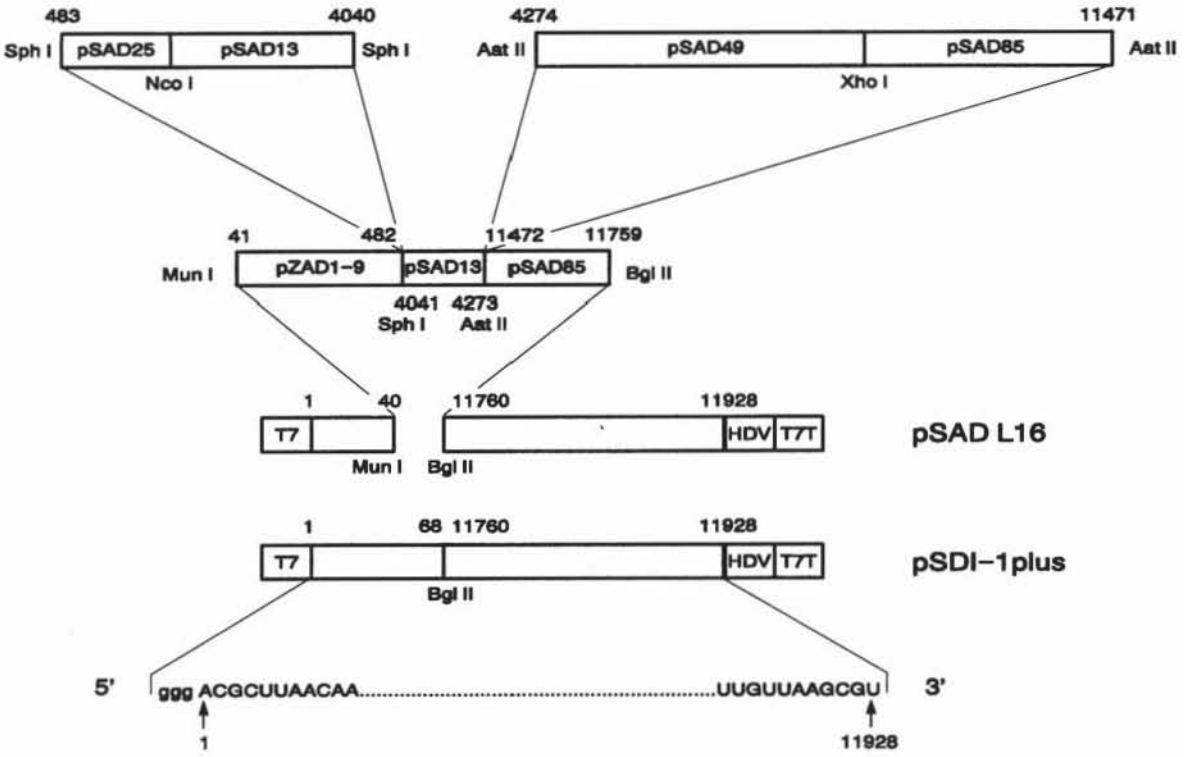

Fig. 1. Simplified scheme for the construction of transcription plasmids containing full-length RV cDNA. Numbers refer to nucleotide positions of the SAD B19 RV antigenome sequence (Conzelmann *et al.*, 1990). The plasmid pSDI-1plus which served as a basis for reconstruction of full-length RV genomic DNA is a counterpart of pSDI-1 (Conzelmann and Schnell, 1994) containing the SDI-1 RV mini-genome that comprises the terminal nucleotides 1–68 and 11 760–11 928 in the opposite direction with respect to the T7 RNA polymerase promoter (T7) and the hepatitis delta virus antigenomic ribozyme sequence (HDV; Perrotta and Been, 1991). The *Mun*I−*Bgl*II fragment of pSDI-1plus was replaced with a 1 kb cDNA construct that was assembled from three SAD B19 cDNA clones as indicated. Insertion of a 3.6 kb *Sph*I and a 7.2 kb *Aat*II fragments which were assembled from two cDNA clones each resulted in the final plasmid pSAD L16 containing full-length SAD B19 cDNA. Transcription of this plasmid by T7 RNA polymerase should yield positive-stranded (antigenomic) RNA possessing three extra non-viral G residues at the 5′ end and a precise 3′ end after autolysis of the ribozyme. T7, T7 promoter; T7T, T7 transcription terminator; HDV, HDV antigenomic ribozyme sequence.

generated entirely from cDNA. We describe the recovery of two novel infectious rabies viruses that exhibit transcription patterns not present in any natural rabies virus and which result from manipulation of the cDNA genome copy. In addition, it is shown that the characteristic pseudogene region of natural rabies viruses is not essential for propagation of the infectious virus in cell culture.

The possibility of manipulating a typical non-segmented negative-stranded RNA virus at the DNA level allows detailed analysis of its replication and transcription mechanisms and provides invaluable tools for the experimental investigation of virus−host interactions and of RV neurotropism, latency and pathogenesis.

Results

Assembly of full-length RV cDNA

The 12 kb negative-stranded rabies virus genome (Tordo *et al.*, 1986; Conzelmann *et al.*, 1990) serves as a template both for replication, yielding encapsidated antigenomic full-length RNA, and for sequential transcription of a short leader RNA and five free monocistronic mRNAs, from which the viral proteins are translated (Coslett *et al.*, 1980; Kurilla *et al.*, 1984; Tordo *et al.*, 1986, 1988; Conzelmann *et al.*, 1990). Recently, we showed that only the N, P and L proteins are needed to form nucleocapsids exhibiting both replication and transcription of mRNAs (Conzelmann and Schnell, 1994). Transcripts generated in cells expressing T7 RNA polymerase from recombinant vaccinia virus (vTF7–3; Fuerst *et al.*, 1986) and which are composed of the 3′ and 5′ terminal regions, including the entire leader sequence and the conditional transcriptional stop/polyadenylation signal of the L cistron, were encapsidated, replicated and gave rise to leader RNA and a short polyadenylated mRNA (Conzelmann and Schnell, 1994). This indicated that for rescue of full-length RNA into infectious virions the envelope proteins M and G do not have to be provided, since they should be expressed autonomously following formation of a biologically active nucleocapsid. In addition, either genome-sense or the complementary antigenome-sense transcripts might be used to initiate a productive infection. The use of antigenomic transcripts, however, seemed more promising, since simultaneous transcription of minus sense genomic RNA and plus sense RNAs from the protein-encoding plasmids and the probably resulting hybridization might interfere with encapsidation of the genomic RNA or translation of proteins. Accordingly, we constructed transcription plasmids giving rise to positive-stranded full-length antigenomic RV RNAs.

The genomic sequence of the attenuated RV vaccine strain SAD B19, comprising 11 928 nucleotides (Conzelmann *et al.*, 1990), provided the basis for the reconstruction of a DNA copy of the full-length RV genome (Figure 1). First, the 237 bp mini-genome sequence contained in the transcription plasmid pSDI-1

(Conzelmann and Schnell, 1994), which consisted of RV 3′ and 5′ terminal non-coding sequences, was cloned in the opposite direction with respect to the T7 promoter and a hepatitis delta ribozyme sequence (pSDI-1-plus; Figure 1). As shown in Figure 1 and detailed in Materials and methods, internal RV cDNA fragments were successively introduced into pSDI-1plus. Only cDNA clones which had been sequenced entirely, and preferably those which had been used for the expression of functional RV proteins (Conzelmann and Schnell, 1994), were assembled by standard cloning techniques (Sambrook *et al.*, 1989). Transcription of the resulting final construct pSAD L16 by T7 RNA polymerase should produce positive-stranded RNA which in sequence is identical to the published RV SAD B19 sequence except for the presence of three extra G residues at the 5′ end. A precise 3′ end of the genome analogue should be generated after transcription, due to the autolytic action of the HDV ribozyme sequence. Using the circular plasmid, *in vitro* transcriptions were performed and the products analysed on denaturing agarose gels. The presence of RNA transcripts co-migrating with 12 kb RV genomic RNA indicated that full-length antigenome RNA is transcribed by T7 polymerase (data not shown).

Recovery of authentic infectious RV

Transfection experiments were carried out with BSR cells which had been infected 1 h previously with the recombinant vaccinia virus vTF7-3 at an m.o.i. of 5. By transfection of pSAD-L16 and subsequent Northern analysis of total RNA it was confirmed that transcripts of RV genome size were also generated intracellularly by the T7 polymerase enzyme expressed from the recombinant vaccinia virus (data not shown). Since only N, P and L proteins are needed for formation of transcriptionally active RV nucleocapsids (Conzelmann and Schnell, 1994), co-expression of this set of proteins along with correct pSAD L16 transcripts should result in the initiation of productive infection. Therefore, 5, 2.5 and 2.5 μg protein-encoding plasmids pT7T-N, pT7T-P and pT7T-L, respectively (Conzelmann and Schnell, 1994) were co-transfected with 2 μg pSAD L16. Possible encapsidation of pSAD L16-derived T7 RNA polymerase transcripts and the resulting expression of RV proteins from the nucleocapsids was checked by indirect fluorescence. A monoclonal antibody directed against RV G protein, which could only be expressed from the recombinant RV genome, was used to screen the cultures. One day after transfection stained cells were present, demonstrating expression of genes from the RV genome. However, only single positive cells were observed in a series of 20 transfection experiments. No fluorescent cell foci indicating the presence of infectious virus were obtained in these experiments. In addition, from cell cultures which were inoculated with the entire supernatant from the transfected cells no infectious virus could be recovered 2 days later. Therefore, in order to isolate the presumed very low number of infectious viruses generated in transfected cells, the experimental procedure was modified. After transfection, cells were grown for 2 days, suspended in the culture medium and then submitted to three cycles of freezing and thawing to release cell-associated RV. Cleared of cell debris by centrifugation, the extracts were used to inoculate fresh cells. These were analysed 2 days later by direct immunofluorescence with

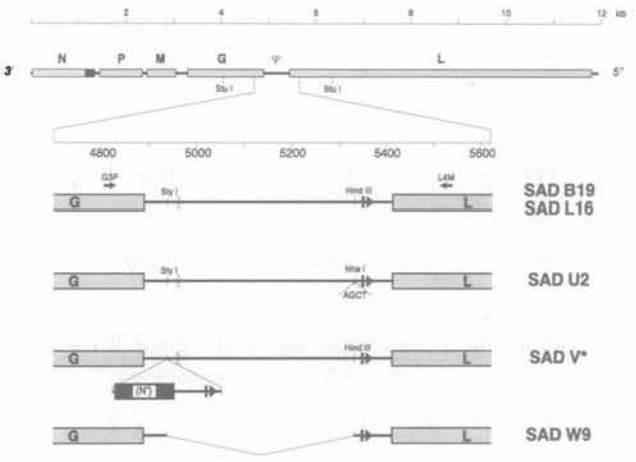

Fig. 2. Organization of the RV pseudogene region (ψ) and construction of recombinant RV genomes (drawn to scale). Numbers indicate the nucleotide positions in the antigenome sequence of SAD B19. At the top, the entire RV genome with its five open reading frames is shown. Mutations were carried out in pPsiX8 containing part of the genome (3823–6668) and reintroduced into the full-length clone pSAD L16 by exchange of the *Stu*I fragment (4014–6364). In the detailed drawing, coding regions are represented by grey boxes, non-coding sequences as lines. Functional transcriptional signal sequences are indicated by the filled bar (stop/polyadenylation) and the arrowhead (mRNA transcription start). The non-functional signal-like sequence defining the start of the ψ region is shown by the open bar. Arrows indicate the position of oligonucleotide primers G3P and L4M used for RT−PCR analysis of the ψ region. In SAD U2, fill-in of *Hin*dIII extensions resulted in insertion of four nucleotides and generation of a unique *Nhe*I site; in SAD V*, a cDNA fragment containing the RV N/P cistron border (SAD B19 nucleotides 1323–1502) was inserted into the *Sty*I site; SAD W9 possesses a deletion of the *StyI/Hin*dIII fragment.

a conjugate directed against RV N protein. In two out of 20 experiments fluorescent foci were observed. The respective supernatants contained infectious virus (SAD L16), which was assumed to represent transfectant virus generated from cDNA transcripts.

Recovery of genetically tagged RV

In order to provide unequivocal evidence that infectious RV can be expressed from DNA, genetic tags were introduced into the genomic cDNA copy. As a target the pseudogene region (ψ), which is characteristic for RV and other members of the lyssavirus genus, was selected. The ψ region is located between the G and L open reading frames and is transcribed as the non-translated 3′ terminal part of the G cistron mRNA (G/ψ mRNA) in all lyssaviruses analysed so far (Figure 2). Since a sequence similar to standard cistron borders is found between the G and ψ sequences, the ψ region is assumed to represent the remnants of a former functional gene (Tordo *et al.*, 1986). A biological function has not as yet been assigned to ψ and we therefore speculated that alterations within the primary sequence of this region might be tolerated. To carry out the manipulations, the plasmid pPsiX8 was constructed that contained part of the pSAD L16 cDNA spanning the entire ψ region as well as flanking parts of the G and L coding regions (Figure 2). The modified ψ sequences were then re-introduced into pSAD L16 by exchange of a *Stu*I fragment (Figure 2).

The first alteration consisted of the addition of four

nucleotides close to the ψ transcriptional stop/polyadenylation signal. To this end, pPsiX8 was cut with *Hin*dIII and religated after fill-in of extensions with Klenow enzyme. By the resulting duplication of nucleotides 5338–5341 (AGCT, SAD B19 positions) a novel unique *Nhe*I site was created at the location of the former *Hin*dIII site (Figure 2). Two independent transfection series of 20 experiments each were carried out with pSAD U2. The generation of infectious viruses was demonstrated after transfer of extracts from transfected cells together with supernatant to fresh cells. In each of the series, focus formation was observed in one experiment. The transfectant viruses (clones SAD U2–13 and SAD U2–32) were passaged by transfer of supernatants to fresh cells two further times, resulting in almost 100% infection of the cells. To demonstrate the presence of the genetic tag in the SAD U2 virus genome, total RNA was isolated from cells infected with SAD U2–13 and reverse transcription−PCR (RT−PCR) of the ψ region was performed. With the primers G3P and L4M (Figure 2), which are specific for the G and L genes, respectively, DNA fragments of ~730 bp were obtained from the genomes of transfectant viruses SAD U2 and SAD L16 and of standard RV SAD B19. However, subsequent digestion with *Hin*dIII was only observed for the PCR DNA obtained from SAD B19 and SAD L16, but not for that from SAD U2. Conversely, only SAD U2-derived DNA was digested with *Nhe*I, giving rise to two fragments of ~530 and 200 bp, respectively (Figure 3). No specific DNA fragments were amplified in the absence of reverse transcriptase, thus contamination with the respective modified plasmids was excluded. Direct RT sequencing of genomic RNA of transfectant virus SAD U2 further confirmed the presence of the expected duplication of four residues at the predicted site, while the rest of the determined sequence corresponded to that of the original SAD B19 genome (data not shown). Thus, it was clear that the SAD U2 virus represented a transfectant virus whose genome originated from engineered cDNA.

The RV ψ region is not essential for propagation in cell culture

The introduction of four additional nucleotides close to the end of the RV ψ region apparently did not affect viability of the transfectant virus SAD U2, nor did it interfere with correct transcription termination of the G mRNA (see below). In order to further evaluate the degree of genetic flexibility of the RV genome in this region, more severe alterations, namely the insertion or deletion of larger sequence stretches, were carried out. The design of the mutations should not only facilitate identification of transfectant viruses, but also allow investigation of the biological functions of the ψ region and of transcriptional signal sequences. With the purpose of investigating (i) whether transcriptional linkage of the ψ sequence to the G mRNA is necessary and (ii) whether the ψ region is essential for propagation of infectious RV in cell culture, two artificial genomic cDNAs (pSAD V* and pSAD W9, respectively; Figure 2) were constructed and utilized in rescue experiments.

In order to unlink ψ sequences from the G cistron, a DNA fragment which should function as a cistron border was introduced between the G and ψ sequences in pSAD

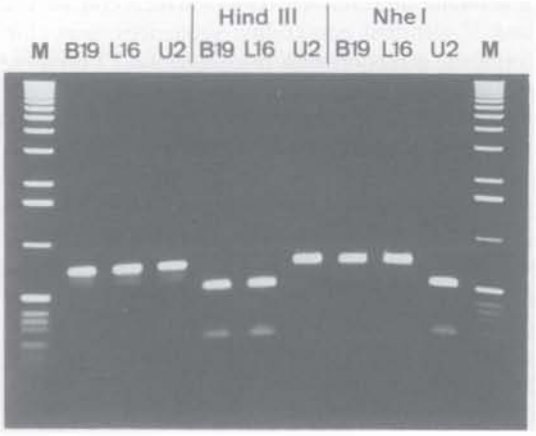

Fig. 3. Demonstration of the genetic tag in the genome of the transfectant virus SAD U2. Total RNA from cells infected with standard RV SAD B19 (B19) and transfectant viruses SAD L16 (L16) and SAD U2 (U2) was isolated 2 days post-infection and used for RT−PCR amplification of the respective ψ regions with primers G3P and L4M (see Figure 2). The amplified DNA was separated on a 1% agarose gel undigested and after digestion with *Hin*dIII and *Nhe*I, respectively. A *Nhe*I restriction site is present only in DNA derived from SAD U2. M, DNA size marker.

V*. The conditional transcription signals of the RV genome defining a cistron border consist of a transcription stop/polyadenylation signal [$^{A}/_{U}G(A)_7$] a non-transcribed intergenic region of 2–24 nucleotides [$C(N)_{1-23}$] and a transcriptional start signal ($AACA^{C}/_{U}{}^{C}/_{U}NCU$, plus sense). A 230 bp DNA fragment containing cDNA derived from the RV N/P cistron border (SAD B19 positions 1323–1502; see Materials and methods for details) was cloned into the filled-in *Sty*I site of pPsiX8, which is located upstream of a non-functional G transcriptional stop signal marking the start of the ψ sequence (Figure 2). By duplication of the *Sty*I overhang and insertion of the 230 bp DNA fragment, the final pSAD V* should express a genome analogue which is 234 nucleotides longer than the standard SAD B19 genome. The other genomic cDNA construct, pSAD W9, lacked almost the entire ψ region. Deletion of the sequence between the *Sty*I and *Hin*dIII sites and subsequent fill-in with Klenow fragment and religation resulted in removal of 396 nucleotides from the standard virus genomic sequence (SAD B19 positions 4942–5337). The genome of SAD W9 should possess a length of 11 532 nucleotides with a predicted 3′ non-translated region of the G cistron of only 73 nucleotides.

As before, pSAD V* and pSAD W9 were used to transfect 20 culture dishes each. In three cultures transfected with SAD V* and in one with SAD W9, rescue was indicated by subsequent isolation of viable virus. After five successive passages, RNA from infected cells and supernatant was isolated and analysed by RT−PCR using the same primers as in the previous experiments. In comparison with standard SAD B19 virus, an enlarged DNA fragment of ~0.9 kb resulted from RNA of cells infected with SAD V*, thus showing that additional sequences were present in the ψ region of this transfectant virus (Figure 4). In contrast, from RNA of cells infected with SAD W9, a DNA fragment of only 0.3 kb was obtained; this size was expected according to the deletion

Fig. 4. PCR analysis of SAD B19 (B19), SAD V* (V*) and SAD W9 (W9) genomes. RT−PCR was performed as described in Figure 3 with primers G3P and L4M. Amplification products were separated on a 1% agarose gel.

made in the cDNA genome copy. No amplification products derived from standard virus genomes were detected in SAD V* or SAD W9 preparations, thus excluding contamination with infectious standard virus. Sequencing of PCR products further confirmed that the original engineered cDNA sequences were rescued into the genomes of SAD V* and SAD W9 transfectant viruses. Accordingly, neither the presence of additional sequences, including 50 vector-derived nucleotides, between the G open reading frame and the ψ region nor the deletion of the entire ψ region interferred with the infectivity and propagation of transfectant rabies viruses.

Transcription analysis of recombinant viruses

The alterations engineered into the genomes of SAD V* and SAD W9 were designed in such a way as to result in phenotypical changes in the transcription pattern and were presumed to affect at least the growth characteristics of the respective transfectant viruses. However, propagation in cell culture, as well as final titres of infectious SAD V* and SAD W9 viruses, were similar to those of standard SAD B19 RV. Three days after infection of cells with vaccinia virus-free stocks (see Materials and methods) at an m.o.i. of 0.01, titres of 10^8 focus-forming units were reached in the supernatants for SAD B19, SAD V* and SAD W9.

In order to determine whether the predicted altered or novel transcription products were synthesized in SAD V*- and SAD W9-infected cells, total cellular RNA was isolated 2 days after infection and analysed by Northern hybridizations. According to the location of transcriptional stop/polyadenylation signals downstream of the G coding region, differently sized G mRNAs should be produced by the particular transfectant viruses. Prominent RNA populations of the expected sizes were readily demonstrated by hybridization with a G-specific probe (Figure 5A, filled arrowheads). Transcription of the standard RV SAD B19 G cistron is terminated at the end of the ψ region and yields the typical polyadenylated G mRNA of 2.3 kb (lane B19). An identical G mRNA was produced by the transfectant virus SAD L16, which corresponds in sequence to the standard virus (lane L16). These two viruses are distinguished in their hybridization pattern

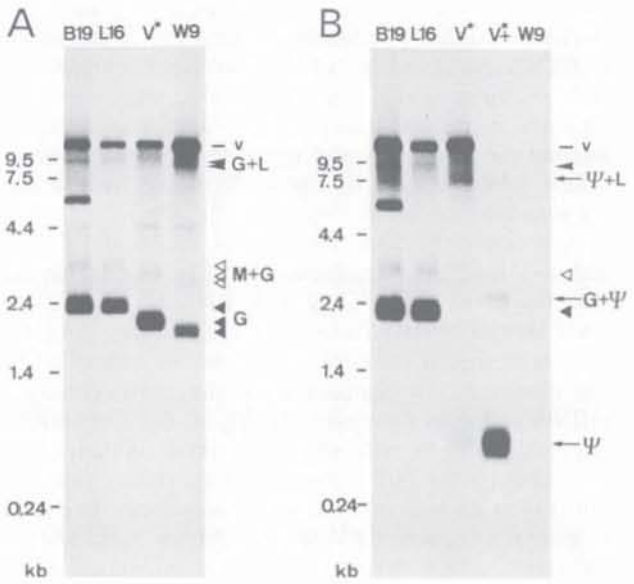

Fig. 5. Northern blot analysis of transcription products from transfectant viruses. Total RNA from cells infected with standard RV SAD B19 (B19) and the transfectant viruses SAD L16 (L16), SAD V* (V*) and SAD W9 (W9) at an m.o.i. of 1 was isolated 2 days post-infection and analysed by Northern hybridization directly or after enrichment of poly(A)$^+$ RNA (V^*_+). (**A**) Demonstration of differently sized G mRNAs. Hybridization was performed with a G-specific cDNA probe. Standard RV SAD B19 is distinguished from the transfectant virus SAD L16 by the presence of a 5.5 kb RNA representing the genome of a defective interfering particle (SAD DI1; Conzelmann *et al.*, 1991). Monocistronic G mRNAs are marked by filled arrowheads, bicistronic M+G RNAs by open arrowheads, bicistronic G+L RNAs by long arrowheads. v, genomic RNA. (**B**) Demonstration of the extra ψ cistron of SAD V*. Hybridization was performed with a ψ region-specific probe (*Sty*I−*Hin*dIII fragment; see Figure 2), monocistronic (ψ) and bicistronic G + ψ and ψ + L RNAs are indicated by arrows; arrowheads are as in (A).

only by the presence of a 5.5 kb RNA in SAD B19-infected cells, which represents the genomic RNA of a defective interfering particle (SAD DI-1; Conzelmann *et al.*, 1991). In contrast, SAD V* synthesized a different G mRNA which possessed the predicted size of 2.1 kb. In SAD W9-infected cells a short 1.9 kb G mRNA, reflecting the 0.4 kb genomic ψ region deletion, was detected. According to these results, correct transcription termination and polyadenylation of the G mRNA was directed by a copy of the N gene transcriptional stop/polyadenylation signal located in a different genome region in SAD V*. In addition, the signal usually terminating ψ region transcription and which, in the genome of SAD W9, is flanked by a different upstream sequence is able to exhibit correct function in the new sequence context.

Using a ψ region-specific probe, no hybridization was detected with RNA from cells infected with the ψ region-deleted SAD W9 virus (Figure 5B, lane W9). While the genomic RNAs of the other viruses and the G mRNAs of SAD B19 and SAD L16 were recognized by this probe, the SAD V* G mRNA did not react. In contrast, a faint band of RNA appeared that corresponded in size to the novel extra ψ mRNA that was predicted by the presence of the extra P gene transcriptional start signal preceding the the SAD V* ψ sequences. As expected, the ψ mRNA could be enriched by oligo(dT)−cellulose chromatography

(lane V^*_+), indicating correct polyadenylation at the former G cistron border. In addition to the short monocistronic ψ mRNA, bicistronic polyadenylated RNAs containing G and ψ sequences (G + ψ) or ψ and L sequences (ψ + L) could be demonstrated. Bicistronic RNAs containing internal poly(A) tracts and resulting most likely from a failure in the release of transcripts occur frequently in RV-infected cells (Conzelmann *et al.*, 1991).

Approximately equal amounts of bicistronic G + ψ and ψ + L RNAs were present in SAD V*-infected cells and, moreover, SAD V* ψ + L RNA (Figure 5B) and SAD B19 or SAD L16 G + L RNA (Figure 5A) were present in similar amounts. Therefore, we concluded that the observed low abundance of the monocistronic ψ mRNA is due to decreased stability of the short mRNA, rather than to a reduced transcription initiation rate. Accordingly, the N/P cistron border exhibits full functionality in the context of G and ψ sequences. In contrast to naturally occurring RV, the transfectant virus SAD V* represents a RV whose genome is composed of six functional cistrons.

Discussion

This report demonstrates for the first time that cloned DNA corresponding to the entire genome of a negative-stranded RNA virus can give rise to a replicating virus. A system completely devoid of infectious helper virus was used to generate transfectant RV '*de novo*' in cell cultures. Intracellularly synthesized T7 RNA polymerase transcripts corresponding to RV antigenomic RNA were incorporated into biologically active nucleocapsids by transiently expressed RV N, P and L proteins. This resulted in regulated expression of all RV proteins, including the envelope proteins M and G, from the new viral genome and subsequent assembly of infectious RV. The authenticity of transfectant viruses was established by the demonstration of genetic tags rescued in viral genomes. Phenotypical features not present in any natural RV were exhibited by transfectant viruses and resulted from the manipulation of viral genomes at the DNA level.

Recently, the basic requirements for the rescue of artificial RNAs have been determined (Conzelmann and Schnell, 1994). Negative-stranded RNA transcripts corresponding to a 237 bp RV mini-genome and essentially comprising only non-coding 5′ and 3′ terminal RV sequences (SDI-1) were efficiently encapsidated into nucleocapsids, amplified and transcribed in cells expressing RV N, P and L proteins. It was shown that foreign reporter gene sequences inserted between the terminal RV sequences are encapsidated and expressed. After additional expression of the RV envelope proteins M and G, infectious virions containing artificial genomes of up to 4 kb were generated (SDI-CAT and SDI-blue). Thus, as demonstrated already for another rhabdovirus, VSV (Pattnaik *et al.*, 1991), in vaccinia virus-infected cells all steps of the RV life cycle are correctly performed. From other experiments with mixed infections of RV and vaccinia virus (unpublished data), it appeared that none of the viruses markedly interfered with propagation of the other, at least during the first 2 days of infection, namely before the cytopathic effect of vaccinia virus resulted in lysis of BSR cells. Although the encapsidation efficiency in the recent rescue experiments appeared to be mainly determined by the size of RNA transcripts, rather than the primary sequence of internal portions, the results suggested that encapsidation of a complete 12 kb RV RNA derived from cDNA might be possible using the same system.

The approach to rescuing full-length RNA, however, had to consider several differences compared with the recent experiments with defective RNA constructs. In addition to the larger size, a major dissimilarity is the presence of RV coding sequences in full-length transcripts. Since large amounts of positive sense N-, P- and L-specific RNAs are produced from the transfected protein-encoding plasmids, hybridization with simultaneously expressed negative-stranded genomic RNA transcripts is probable. It was suspected that possible hybridization, which could affect more than half of the genome analogue, could interfere with the crucial encapsidation step. In addition, translation of N, P and L mRNAs might be affected. In this respect, the use of positive-stranded RNA transcripts corresponding to RV antigenomes was suggested. On the other hand, for initiation of a productive infection a nucleocapsid containing genome sense RNA is required, from which primary transcription of mRNAs and ensuing autonomous and regulated expression of all RV proteins occurs. Accordingly, antigenome transcripts not only have to be encapsidated into a nucleocapsid, but also have to be replicated by the proteins expressed from transfected plasmids. This point, however, was not considered to be a major obstacle, since positive-stranded SDI-CAT RNA transcripts were rescued with approximately the same efficiency as their negative-stranded counterparts (unpublished data). Thus, the initial encapsidation step was regarded as solely determining rescue efficiency and we assembled cDNA to yield T7 transcripts that corresponded to the RV antigenome. With respect to the non-viral G residues at the 5′ end of transcripts which are required for initiation of transcription by T7 RNA polymerase, the pilot experiments with plus stranded SDI-CAT also indicated that trimming of extra nucleotides at the 5′ ends of both genomic (Pattnaik *et al.*, 1992) and antigenomic RNAs by the viral polymerase occurs correctly.

Rescue of SDI-blue RNA, which comprised 3980 bp, had been observed in only one out of 1000 cells expressing both the genome analogue and RV N, P and L proteins (Conzelmann and Schnell, 1994). The initiation of a productive infection from transfected full-length genomic RNA was thus expected to be a very rare event. One series of twenty transfections of 3.2 cm diameter cell cultures, corresponding to a total of ~2×10^7 cells, was sufficient to isolate at least one of each transfectant virus. According to pilot experiments performed to determine virus isolation efficiencies, the presence of one cell infected with SAD B19 rabies virus in a 3.2 cm culture dish of vaccinia virus-infected cells is sufficient for subsequent re-isolation of virus by the employed method of freezing/thawing. Therefore, the rescue efficiency approximates to the observed efficiency of isolation of transfectant virus. This emphasizes the importance—at least in the RV system—of using a system devoid of infectious helper virus in order to isolate transfectant viruses that lack any selective advantage. In contrast, by providing the necessary helper functions by expression from DNA as described

here, it should even be possible to isolate sublethal RV mutants. With this in mind, the severely mutated genomes of SAD W9 and SAD V* were constructed. The respective potential viral progeny was presumed to exhibit diminished viability, due to disturbed gene expression and/or the lack of viral sequences usually present.

Most interestingly, however, all transfectant viruses described in this study did not show obvious differences to standard RV with respect to growth characteristics in cell culture. The design of the mutant genomes allowed us to rule out the presence of contaminating infectious helper virus by PCR analysis (Figures 3 and 4) and Northern hybridization (Figure 5). Thus, each of the transfectant viruses is replicating autonomously. The successful rescue of fully viable transfectant RV with genomes exhibiting the described alterations of the ψ region allows to address both the evolution and function of non-segmented negative-stranded genomes.

The RV ψ region is located in a genomic area that represents the most plastic region of all non-segmented negative-stranded RNA virus genomes and apparently has the disposition to accept insertions of additional genes (for a review see Tordo *et al.*, 1992). Within the rhabdovirus family the extreme examples are VSV and the fish rhabdovirus infectious hematopoetic necrosis virus (IHNV). The VSV genome exhibits a drastic reduction of non-coding sequences and intergenic regions separating the N, P, M, G and L open reading frames (Schubert *et al.*, 1984; Rose and Schubert, 1987). In contrast, the genome of IHNV possesses a complete additional gene (NV) encoding a non-structural protein of unknown function located between the G and L genes (Kurath *et al.*, 1985). The identical location of the RV ψ region in the genome, the presence of motifs similar to transcriptional signal sequences and a size which is similar to the IHNV NV gene led to the assumption that the ψ region represents the remnants of a former functional cistron (Tordo *et al.*, 1986). With respect to the physical genome map, RV can thus be regarded as an intermediate stage compared with VSV and IHNV, in which the additional gene has been shut off by destruction of the transcriptional signals downstream of the G gene. Maintaining, however, the physical space of the former gene, this results in the elongation of the 3′ non-coding region (ψ region) of the G mRNA.

The genetic flexibility of the G/L border in the RV genome has now been demonstrated experimentally. Not only is the insertion of sequences derived from other parts of the RV genome tolerated, but also the introduction of transcriptional signal sequences which maintain their functionality in the ψ sequence context. The resulting physical genome map of the transfectant virus SAD V* thus mimicks that of IHNV by the presence of an extra sixth cistron and the transcription of six monocistronic polyadenylated mRNAs. The SAD W9 genome map, in contrast, approaches that of the 'minimal' rhabdovirus VSV. These findings suggest that insertion of complete genes replacing the ψ region or in addition to the ψ region might be possible and implicate a potential use of RV as a vector.

According to the doctrine that unnecessary genetic material is eliminated rapidly from RNA virus genomes, the presence in all lyssaviruses analysed so far of a ψ region in the form of a 3′ non-coding sequence of the G cistron and its conservation in length had suggested a functional role for ψ sequences in the viral life cycle. Moreover, only 10 nucleotide deviations are found within the entire ψ regions of the American virus isolate SAD B19 ('Street Alabama Dufferin') and of another RV laboratory strain of European origin and totally different passage history (PV, Pasteur Virus). Thus, it was rather surprising that transcriptional unlinkage in SAD V* and, especially, the entire deletion of the ψ sequence in SAD W9 obviously did not interfere with the ability to replicate and to propagate in standard cell culture cells. The conclusion from the results is that either the genome of rabies virus conserves non-essential sequences to an unexpectedly high degree or that the ψ region plays an as yet unknown role in the infected animal. Now, this can be directly addressed. Investigation of the interaction between virus and infected host will certainly be a major application for specifically designed recombinant rabies viruses. Although the RV glycoprotein is one of the best known viral antigens (e.g. Flamand *et al.*, 1993), relatively little is known so far about the molecular mechanisms governing the marked tropism of RV for neuronal cells, rabies latency and rabies neuropathogenesis. The ability to introduce mutations into the genome of RV and to recover clonal virus stocks from a defined sequence will provide invaluable tools for the experimental investigation of rabies. Sequences determining virulence of RV can now be identified and safe, attenuated, live vaccines for immunization of wildlife may be designed according to the proposed application.

Materials and methods

Construction of full-length RV cDNA

The cloning of cDNA spanning the entire genome of RV strain SAD B19 has been described previously (Conzelmann *et al.*, 1990; GenBank accession no. M31046). As the basis for the assembly of an SAD B19 full-length DNA clone, the RV mini-genome sequence contained in the transcription plasmid pSDI-1 (Conzelmann and Schnell, 1994) was used. pSDI-1 contains the SAD B19 genomic 3′ and 5′ ends (SAD B19 nucleotides 1–68 and 11 760–11 928, respectively) inserted between a T7 RNA polymerase promoter and the hepatitis delta virus (HDV) antigenome ribozyme sequence. In order to generate a plasmid to produce positive-stranded SDI-1 transcripts (pSDI-1plus), the RV sequences contained in pSDI-1 were first amplified by PCR using an 11 base primer (5′-ACGCTTAACAA-3′) which, due to the complementary of RV genome ends, corresponds to the 5′ termini of both positive and negative sense viral RNAs. After subsequent partial ligation of a synthetic *Eco*RI/blunt adaptor (T7/3) containing a T7 promoter sequence followed by three G residues (underlined) (5′-AATTCCTGCAGTAATACGACTC-ACTATAGGG-3′) to the amplified RV sequence, the ligation products were cloned in the *Eco*RI/*Sma*I sites of pX8dT. This plasmid is a derivative of pBluescriptII (Stratagene) from which a *Bss*HII–*Cla*I fragment of the multiple cloning site containing the original T7 promoter was deleted. It contains the 84 base HDV antigenomic ribozyme sequence in the *Sma*I site followed immediately by a T7 transcription terminator sequence cloned in the *Bam*HI site (Conzelmann and Schnell, 1994). Constructs that contained a T7 promotor upstream of the plus sense RV sequence were identified by restriction analysis and sequencing. The *Mun*I–*Bgl*II fragment of pSDI-1 (SAD B19 nucleotides 40–68) was then replaced with a 1 kb *Mun*I–*Bgl*II cDNA construct assembled in pBluescriptII from three fragments of different SAD B19 cDNA clones (*Mun*I–*Sph*I, SAD B19 nucleotides 40–482 from pZAD1–9; *Sph*I–*Aat*II, 4041–4273 from pSAD13; *Aat*II–*Bgl*II, 11472–11759 from pSAD85), resulting in pSDI-1170. By insertion of an *Sph*I fragment assembled from the clones pSAD25 and pSAD13 via *Nco*I (SAD B19 nucleotides 482–4041) and an *Aat*II fragment assembled from clones pSAD 49 and pSAD85 via *Xho*I (SAD B19 nucleotides 4273–11472) into the unique

*Sph*I and *Aat*II sites of pSDI-1170, the final basic full-length clone pSAD L16 was completed.

Introduction of genetic tags into the RV genome sequence

Manipulations of the ψ region were carried out in the subclone pPsiX8, containing a 2.8 kb *Xho*I−*Sca*I fragment of pSAD L16, representing SAD B19 nucleotides 3823–6668. The *Stu*I fragments of the modified pPsiX8 plasmids were then isolated and used to replace the corresponding fragment (SAD B19 position 4014–6364) of the full-length clone pSAD L16 (Figure 2). Insertion of four nucleotides into the ψ region and generation of a novel *Nhe*I site was achieved by digestion of pPsiX8 with *Hin*dIII, fill-in of the extensions with Klenow enzyme and religation. The final full-length clone pSAD U2 is distinguished from SAD L16 by the duplication of nucleotides 5338–5341. By double digestion with *Sty*I and *Hin*dIII, Klenow fill-in and religation, 396 bases (SAD B19 nucleotides 4942–5337) were deleted; the final construct was pSAD W9. For the construction of pSAD V*, a 180 bp *Bgl*II−*Asu*II fragment, including the SAD B19 N/P cistron border region, was isolated from pSAD13 (Conzelmann *et al.*, 1990). The fragment contained 97 nucleotides of the N coding region, the entire 3′ non-coding region and the N/P cistron border, consisting of the N transcriptional stop/polyadenylation signal (italics), the intergenic region (lower case) and the first 16 nucleotides of the P cistron, including the transcriptional start signal (underlined) (CAUCAUG*AAAAAAA*cuAACACCCCUCCUUUCGAA, positive sense sequence). The cDNA fragment was first subcloned into the *Eco*RI site of pBluescript after fill-in of the 3′ recessive ends with Klenow enzyme (pNigP-180). After excision with *Hin*dIII/*Xba*I from pNigP and blunt-end generation, the obtained 230 bp fragment, which contained the RV insert flanked by 16 and 34 bp of vector-derived sequences, respectively, was cloned into the filled-in *Sty*I site of pPsiX8. The final full-length construct (pSAD V*) thus possessed a 234 bp insertion compared with pSAD L16.

Analysis of RNA

Total RNA from cells or supernatants was isolated 2 days after infection and analysed as described previously (Conzelmann *et al.*, 1991). After glyoxalation the RNA was separated on 1% agarose gels containing 5% formaldehyde and transferred to Nylon membranes (Duralon-UV; Stratagene). Northern blots were hybridized with ^{32}P-labelled cDNA probes (nick-translation kit; Amersham) in a solution containing 7% SDS, 1 mM EDTA, 0.5 M NaH_2PO_4, pH 7.2, at 68°C for at least 4 h and washed three times for 15 min each in 1% SDS, 1 mM EDTA, 40 mM NaH_2PO_4, pH 7.2.

RT−PCR

Total RNA of infected cells was prepared 2 days after infection with viruses at an m.o.i. of 1. One microgram of RNA was used for reverse transcription with the oligonucleotide primer G3P (5′-GAGGTGTCAGTCACTCC-3′, SAD B19 nucleotides 4811–4827) in a total of 30 µl buffer containing 25 mM KCl, 25 mM Tris−HCl, pH 8.3, 3 mM $MgCl_2$, 5 mM DTT, 2.5 mM dNTPs, 18 U AMV reverse transcriptase (Life Science) and 10 U RNAguard (Pharmacia). After primer annealing for 4 min at 65°C and 10 min at 37°C, the reaction mix was incubated for 45 min at 42°C. One tenth of the reaction was then used directly for DNA amplification with 2.5 U Taq DNA polymerase in 100 µl buffer privided by the supplier (Appligene) [containing 10% DMSO, 0.2 mM each dNTP and 50 ng primers L4M (5′-CAAAGGAGAGTTGAGATTGTAGTC-3′, SAD B19 nucleotides 5516–5539) and G3P]. After 30 cycles (denaturation 30 s, 94°C; annealing 60 s, 45°C; elongation 120 s, 72°C) the PCR products were digested with restriction enzymes or separated directly on 1.0 % agarose gels and visualized using ethidium bromide.

Transfection experiments

Transfection experiments were carried out as described previously (Conzelmann and Schnell, 1994). BHK-21, clone BSR, cells were grown overnight in 3.2 cm diameter dishes in Eagle's medium supplemented with 10% calf serum to 80% confluency and infected at an m.o.i. of 5 with the recombinant vaccinia virus vTF7-3 [kindly provided by T.Fuerst and B.Moss (Fuerst *et al.*, 1986)]. One hour post-infection, cells were washed twice with culture medium lacking calf serum and transfected with a plasmid mixture containing 5 µg pT7T-N, 2.5 µg pT7T-P and 2.5 µg pT7T-L and with 2 µg pSAD-L16, -U2, -V* or W9 plasmid by using the mammalian transfection kit (Stratagene; $CaPO_4$ protocol) according to the supplier's instructions. The precipitate was removed 4 h post-transfection and cells were washed and incubated in Eagle's medium containing 10% calf serum.

Isolation of transfectant viruses and removal of vaccinia virus

Two days post-transfection, cells were suspended in the supernatant by scratching with a rubber policeman. The suspension was submitted to three cycles of freezing and thawing (−70°C/37°C, 5 min each). Cellular debris and excess vaccinia virus, which forms aggregates under these conditions, was pelleted by 10 min centrifugation at 8200–10 000 *g* in a microfuge. The entire supernatant was used to inoculate a culture dish with a confluent monolayer of cells. After incubation for 2 h, the supernatant was replaced by 2 ml fresh culture medium. A cythopathogenic effect (c.p.e.) caused by vaccinia virus was observed 1–2 days post-infection. On average only 10 plaques were observed after centrifugation at 10 000 *g*. RV infection of cells which did not result in detectable c.p.e. was demonstrated 2 days post-infection by direct immunofluorescence staining of the entire monolayer with an anti-N conjugate (Centocore). Half of the supernatants from cultures in which at least one focus (usually 5–50 fluorescing cells) was observed was used for the second passage after centrifugation at 10 000 *g*. For further passaging (2 days each), decreasing aliquots of supernatants were used according to the degree of RV infection.

To get completely rid of vaccinia virus, supernatants from cultures approaching infection of all cells (third or fourth passage) were centrifuged two times for 10 min at 14 000 *g* in a microfuge. The final supernatant was then filtered using a sterile MILLEX-VV 0.1 µm filter unit (Millipore Products, Bedford, MA) and then used to produce high titre stocks of recombinant RVs.

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