Rescue of Synthetic Genomic RNA Analogs of Rabies Virus by Plasmid-Encoded Proteins

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Proteins entirely expressed from cDNA were used to rescue synthetic RNA genome analogs into infectious defective particles of rabies virus (RV). Synthetic negative-stranded RNAs containing 3′- and 5′-terminal RV sequences and transcriptional signal sequences were transcribed from plasmids transfected into cells expressing T7 RNA polymerase from recombinant vaccinia virus. After simultaneous expression of RV N, P, and L proteins from plasmids containing a T7 RNA polymerase promoter, the synthetic genomes were encapsidated, replicated, and transcribed by the RV polymerase proteins. Insertion of the bacterial chloramphenicol acetyltransferase gene or β-galactosidase (*lacZ*) gene between the 3′ and 5′ termini containing transcriptional signal sequences resulted in transcription of mRNAs and expression of chloramphenicol acetyltransferase and β-galactosidase, respectively. Upon simultaneous expression of N, P, M, G, and L proteins, virions carrying the foreign genes were assembled and released into the supernatant. The possibility of rescuing cDNA into rabies virions by proteins also expressed entirely from cDNA opens the possibility of studying the functions of each RV protein and analyzing *cis*-acting signals of the RV genome.

Successful recovery of infectious RNA viruses and functional RNA replicons from cDNA has greatly facilitated molecular genetic analyses of viral proteins and *cis* regulatory elements. Until recently, this technology was applicable only to positive-stranded RNA viruses, whose RNA genomes may function as mRNA and initiate a complete infectious cycle upon introduction into appropriate host cells (reviewed in reference 2). For negative-stranded RNA viruses, however, primary transcription is a prerequisite for initiation of a productive infection. Both genomic and antigenomic RNAs are encapsidated within the nucleocapsid (N) protein and tightly associated with the viral RNA-dependent RNA polymerase. Only the encapsidated RNAs function as a template for replication and transcription of mRNAs (10). In contrast to deproteinized genomic or antigenomic RNA, the ribonucleoprotein complex (RNP) is infectious after introduction into cells.

For influenza virus, an efficient method was developed to assemble biologically active RNPs by using purified N proteins and synthetic RNAs containing terminal sequences of a genome segment and a reporter gene. Transfection of the RNPs into cells infected with influenza virus resulted in replication and expression and in the incorporation of the synthetic genome segment into infectious virions (15). The nonsegmented paramyxoviruses Sendai virus, respiratory syncytial virus, and parainfluenza virus type 3 were shown to rescue similar constructs after transfection of in vitro-transcribed RNAs into infected cells (5, 9, 16). An efficient system completely devoid of infectious helper virus was used for vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family. Intracellularly transcribed synthetic RNA corresponding to the genome of a nonexpressing natural VSV copyback-type defective interfering (DI) particle was rescued by VSV proteins expressed from plasmids (17).

Within the *Rhabdoviridae* family, rabies virus (RV) is the prototype of the genus *Lyssavirus*. Complete nucleotide sequencing of RV genomes revealed that its genome organization is similar to that of VSV (6, 22). The 11.9-kb genomic RNA contains five open reading frames coding for the N protein, phosphoprotein (P, formerly NS or M1), matrix protein (M), transmembrane glycoprotein (G), and RNA-dependent RNA polymerase (L). The occurrence of a pseudogene region (Ψ) between the G and L genes, as well as the variable lengths of the intergenic regions, distinguishes RV from VSV. General transcription and replication mechanisms of RV seem to be similar to those of VSV; for RV, however, the lack of efficient in vitro transcription systems so far has hindered detailed analyses.

In this study, we used a system for the intracellular synthesis of T7 RNA polymerase transcripts corresponding to RV defective genomes of the internal deletion type. These RNAs are encapsidated and assembled into infectious virions by RV proteins expressed entirely from plasmids. The system allows both analysis of *cis*-acting signals in the RV genome and analyses of structure-function relations of individual RV proteins. In addition, it is shown that large foreign sequences may be incorporated into rabies virions and expressed by RV vectors.

MATERIALS AND METHODS

Construction of protein-encoding plasmids. Plasmid pSKT7T, derived from pBluescript SK II− (Stratagene), contains a 137-bp *Bgl*II fragment spanning the T7 transcriptional terminator sequence (TΦ) from pTF7-5 (11) inserted into the *Bam*HI site. cDNA constructs containing the entire coding regions of the RV N, P, M, G, and L genes (strain SAD B19 positions 58 to 1500, 1499 to 2430, 2485 to 3158, 3311 to 5138, and 5394 to 11883, respectively [6]) were cloned between the T7 promoter and terminator sequence according to standard protocols (20). The resulting constructs were called pT7T-N, pT7T-P, pT7T-M, pT7T-G, and pT7T-L.

Generation of RV genome analogs. An *Eco*RI-*Tth*111I fragment of cDNA clone pZAD5 spanning the SAD B19 genome 3′ end (6) was replaced by synthetic DNA (oligonucleotide

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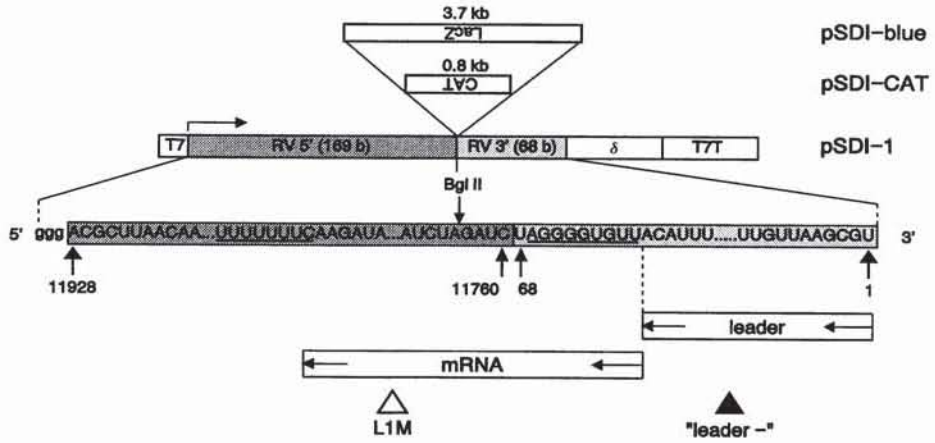


FIG. 1. Schematic representation of plasmids used to generate negative-stranded RV genome analogs. pSDI-1 contains RV 5′ and 3′ noncoding sequences flanked by a T7 RNA polymerase promoter (T7) and the antigenome HDV ribozyme sequence (δ) followed by a T7 transcription terminator sequence (T7T). The negative-stranded transcripts (lower line) possess three extra G residues at the 5′ end and include the putative RV signal sequences for transcription start of the N cistron and transcription stop/polyadenylation of the L cistron (underlined). Numbers indicate positions of nucleotides in the RV SAD B19 sequence (6). Expected RV specific transcripts are indicated by boxes, and locations of oligonucleotides used for hybridization (Fig. 2) are indicated by arrowheads. pSDI-CAT and pSDI-blue contain the coding regions of the CAT and *lacZ* genes, respectively, in an antisense orientation with respect to the T7 promoter. The foreign inserts were cloned into the filled-in *Bgl*II site of pSDI-1. b, bases.

54+ [5′-AATTCGCGGCCGCGAATGCGACGCTTAACAA CCAGATCAAAGAAAAAACAGACA-3′] and the complementary oligonucleotide 51 − [5′-ATGTCTGTTTTTTCTTTG ATCTGGTTGTTAAGCGTCGCATTCGCGGCCGCG-3′]) containing a *Bsm*I restriction site in such a way that linearization and fill-in with Klenow fragment resulted in blunt-ended DNA ending with the 3′-terminal nucleotide of the SAD B19 sequence (underlined). An 84-base oligonucleotide corresponding to the hepatitis delta virus (HDV) antigenome ribozyme sequence (oligonucleotide HDV − AG+) was completed to double-stranded DNA by Klenow polymerase, using a primer complementary to the 3′-terminal 18 nucleotides of HDV − AG+, and cloned directly to the RV 3′-end nucleotide after restriction of the cDNA with *Bsm*I and Klenow fill-in (pSDI-1; Fig. 1). For positioning of a T7 promoter sequence upstream of the RV genome 5′ terminus, genomic first-strand cDNA was primed with the synthetic oligonucleotide L1P (7). The synthetic add-on primer used for second (minus)-strand cDNA synthesis (oligonucleotide 38/2 [5′-GGTACCGTAA TACGACTCACTATAGGGACGCTTAACAA-3′]) contains the T7 promoter sequence, three G residues, and 11 nucleotides complementary to the RV plus-strand sequence (underlined). 5′- and 3′-terminal cDNA fragments were ligated together after *Bgl*II (SAD B19 position 11759) and *Sfa*NI (SAD B19 position 64) restriction, respectively, and Klenow fill-in. The resulting construct possesses a recreated *Bgl*II site at the 3′/5′ junction and contains 237 RV nucleotides (positions 1 to 68 and 11760 to 11928). pSDI-CAT and pSDI-blue were constructed by insertion of the 0.8-kb *Hin*dIII-Klenow fragment from pCM7 (Pharmacia) and the 3.7-kb *Hin*dIII-*Bam*HI-Klenow fragment of pCH110 (Pharmacia), which contain the coding regions of the bacterial chloramphenicol acetyltransferase (CAT) and *lacZ* genes, respectively, into the *Bgl*II site of pSDI-1 after fill-in with Klenow fragment (Fig. 1). The resulting genome analogs comprise 1,032 and 3,980 nucleotides, respectively.

DNA transfections and virus infections. Expression experiments were carried out in BHK-21 (clone BSR) cells after infection with the recombinant vaccinia virus vTF7-3 (multiplicity of infection of 10), which expresses the T7 RNA polymerase (kindly provided by T. Fuerst and B. Moss) (12). One hour postinfection, cells were transfected by using the Stratagene mammalian transfection kit ($CaPO_4$ transfection protocol) according to the supplier's instructions, with 2 μg of pSDI-1, 2 μg of pSDI-CAT, or 3 μg of pSDI-blue and with combinations of 5 μg of pT7T-N, 2.5 μg of pT7T-P, 2.5 μg of pT7T-L, 2 μg of pT7T-M, and 2 μg of pT7T-G per 3.2-cm-diameter culture dish. Cells and supernatants were harvested 48 h posttransfection for further analysis and passaging experiments. Infection of BSR cells with RV SAD B19 was performed as described previously (7).

RNA analyses. Total RNA of infected cells or supernatants was isolated and analyzed by denaturing agarose gel electrophoresis and Northern (RNA) hybridizations as described previously (7). ^{32}P-labelled oligonucleotides L1M (5′-ATCCG GTTCACAGGC-3′; SAD B19 positions 11789 to 11804, minus sense), the complementary oligonucleotide L1P, and oligonucleotide leader − (5′-GATCTGGTTGTTAAGCG-3′; SAD B19 positions 2 to 18, minus sense) were hybridized at 45°C (7). CAT and *lacZ* DNA fragments were labelled with ^{32}P by nick translation (Amersham nick translation kit) and used for hybridization as described previously (7).

CAT assays. CAT assays were done by standard procedures adapted from Gorman et al. (13). Cell extracts were prepared by three cycles of freezing and thawing. Equal amounts of protein were adjusted to a volume of 30 μl with Tris-HCl (pH 7.5) and incubated with 10 μl (0.25 μCi) of [^{14}C]chloramphenicol (53 mCi/mmol; Amersham) and 5 μl of 4 mM acetyl-coenzyme A (Boehringer). Incubation time was 1 h.

β-Galactosidase (β-Gal) assays. Cells were fixed for 5 min in 4% paraformaldehyde (pH 7.6) at 4°C and stained for 4 h at 37°C with phosphate-buffered saline containing 2 mM $MgCl_2$, 5 mM potassium hexacyanoferrate(III), 5 mM potassium hexacyanoferrate(II)trihydrate (Fluka), and 2.45 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fluka).

RESULTS

Expression of RV proteins. In contrast to VSV, RV proteins involved in replication and transcription have not been studied in efficient in vitro systems. To reconstitute intracellular replication and transcription systems, all five RV proteins were expressed from cDNA by using the vaccinia virus/T7 RNA polymerase expression system (11). cDNA constructs containing the entire coding regions for the N, P, M, G, and L proteins of the SAD B19 strain of RV (6) were assembled and cloned in the expression plasmid pSKT7T containing a T7 RNA polymerase promoter and the T7 transcription terminator sequence (pT7T-N, pT7T-P, pT7T-M, pT7T-G, and pT7T-L; see Materials and Methods). Upon transfection of the plasmids into cells infected with the vaccinia virus recombinant expressing T7 RNA polymerase (vTF7-3 [12]), proteins which according to apparent molecular weight and analyses with specific antibodies were indistinguishable from the respective RV proteins were generated (data not shown). The expressed N, P, and L proteins were shown to be functional by their ability to replicate and transcribe the RNA of a naturally occurring RV DI particle, SAD DI-1 (7; unpublished data).

Construction of transcription plasmids directing the synthesis of genome-like RNAs. Whereas most VSV DI RNAs are of the copyback type, RV defective RNAs of the internal deletion type occur frequently (7; unpublished data). They possess 3′ and 5′ termini similar to those of the parental standard genomes and are transcriptionally active. Thus, we made cDNA constructs whose transcription should result in synthetic RNAs corresponding to internal deletion type genomes.

All *cis*-acting signals required for packaging and transcriptase entry are apparently located within the terminal noncoding sequences in the nonsegmented negative-stranded viruses. Therefore, we constructed a cDNA corresponding to a genome analog in which all open reading frames were deleted (pSDI-1; Fig. 1). The fragment derived from the 3′ end contained SAD B19 nucleotides 1 to 68 (6) including the entire 58-nucleotide-long leader sequence, the putative transcriptional start site of the N mRNA (position 59), and, except for two nucleotides (CA) preceding the N transcriptional start codon, the entire 5′ noncoding sequence of the N cistron. The 5′-terminal fragment spanned 169 nucleotides (SAD B19 positions 11760 to 11928) including 38 nucleotides of the L coding region, the L trailer region with the transcriptional stop/polyadenylation signal (position 11858), and the entire nontranscribed terminal sequence. Initially, a T7 RNA polymerase promoter was placed directly upstream of the 5′-end sequences so that RNA transcription would begin at the very terminal nucleotide of the RV genome. However, no detectable amounts of RNAs were transcribed either in vitro or after transfection of the plasmids into cells infected with vTF7-3 (not shown). To provide an efficient initiation sequence for the T7 RNA polymerase, three G residues were inserted between T7 promoter and RV sequences. This modification resulted in transcription of large amounts of RNA. To generate RNA transcripts ending with the authentic RV 3′-terminal nucleotide, the ribozyme sequence from the antigenomic strand of HDV was cloned directly downstream of the 3′ RV sequence (1). Autolytic cleavage should occur at the 5′ end of the ribozyme RNA and generate a 3′ end of the upstream RNA corresponding to the authentic RV 3′ terminus. Accordingly, transcription of plasmid pSDI-1 by T7 RNA polymerase and subsequent autolytic cleavage directed the synthesis of a negative-stranded, defective RV genomic RNA with three extra G residues at the 5′ end and the authentic 3′-terminal nucleotide (Fig. 1).

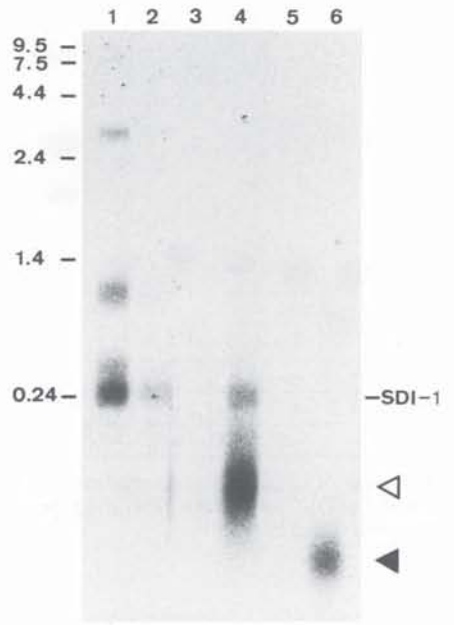
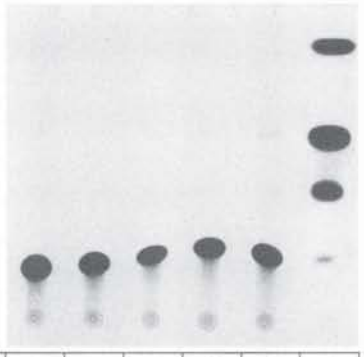


FIG. 2. Demonstration of SDI-1 transcripts by Northern hybridization. Total RNA from cells infected with vTF7-3 and transfected with pSDI-1 and plasmids encoding N and P proteins (lanes 1, 3, and 5) or, in addition, L protein (lanes 2, 4, and 6) was separated in a 2% denaturing agarose gel (one-fourth of the RNA obtained from a transfection experiment per lane). The Northern blot was hybridized with oligonucleotides L1P (lanes 1 and 2), L1M (lanes 3 and 4), and leader− (lanes 5 and 6). Positive-stranded transcripts corresponding in size to the expected SDI genome (SDI-1), mRNA (white arrowhead), and leader RNA (black arrowhead) appeared in cells simultaneously expressing N, P, and L proteins. Sizes are indicated in kilobases.

SDI-1 RNA is packaged into nucleocapsids and functions as a template for RV polymerase. In BSR cells infected with vTF7-3 and transfected 1 h postinfection with pSDI-1, the plasmid directed the synthesis of RNA molecules with the expected size of the minigenome. In addition, larger RNA species probably resulting from incomplete autolysis of the HDV ribozyme were present in substantial amounts. By Northern hybridization with oligonucleotides L1P and L1M, it was confirmed that only negative-sense pSDI-1 RNAs were produced (Fig. 2, lanes 1 and 3). After cotransfection of pSDI-1 with three plasmids encoding RV proteins (pT7T-N, pT7T-P, and pT7T-L), however, positive-sense RNA could be detected with oligonucleotide L1M (Fig. 2, lane 4). This result indicated that T7-derived RNA transcripts had been encapsidated by the RV N protein and were used as a template by the RV polymerase. All three proteins had to be expressed simultaneously; omission of any of the plasmids abrogated plus-strand RNA transcription (Fig. 2, lane 3). Distinct populations of positive-stranded RNAs were observed. In addition to a band corresponding in size to the genome of SDI-1 with a length of 237 bases, smaller RNAs of heterogeneous sizes were prominent. After hybridization with oligonucleotide leader−, derived from the 3′ end of the RV genome, an additional population of small RNAs was detected (Fig. 2, lane 6). This composition of positive-stranded RNAs is consistent with the assumptions that the minus-strand genome was replicated to



N	+	−	−	+	+	+
P	−	+	−	−	+	+
L	−	−	+	+	−	+

FIG. 3. Expression of CAT activity in cells infected with vTF7-3 and transfected with pSDI-CAT and RV N, P, and L protein-encoding plasmids as indicated at the bottom.

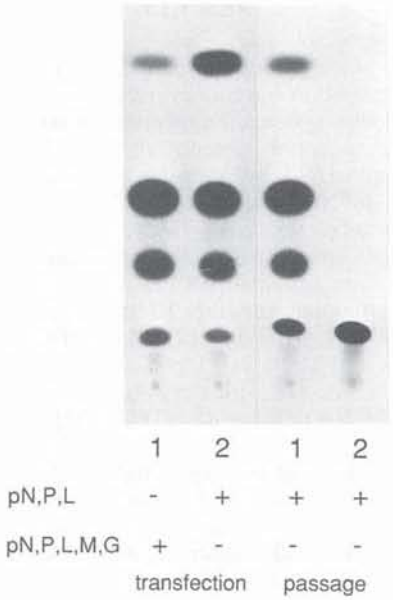


FIG. 4. Transmission of CAT activity. Expression of CAT was analyzed in cells transfected with pSDI-CAT and the indicated plasmids (transfection) and after transfer of the respective supernatants to cells expressing N, P, and L (passage) 48 h after transfection/infection.

yield positive-stranded SDI-1 genomic RNA and that correct transcription of positive-stranded RNAs by the RV polymerase occurred. According to the location of the putative RV transcriptional start and stop/polyadenylation signals within the pSDI-1-derived genome (Fig. 1), transcription should give rise to a 58-base leader RNA (14) and an mRNA of 146 bases which is polyadenylated to a varying extent.

Expression of foreign sequences mediated by RV proteins. To investigate whether packaging into nucleocapsids and transcription by RV polymerase is restricted to RV-derived sequences, transcription plasmids containing foreign sequences were constructed. With regard to versatile monitoring of expression products, the bacterial reporter genes CAT and *lacZ* were selected. pSDI-CAT and pSDI-blue were constructed by insertion of the respective DNA fragments into the *Bgl*II site of pSDI-1. They directed the synthesis of RV genome-like RNAs, in which the RV coding regions were replaced by the coding regions of the CAT or *lacZ* gene (0.8 or 3.7 kb), respectively (Fig. 1). Accordingly, CAT or β-Gal may not be expressed from the resulting negative-stranded T7 transcripts, but only after formation of a functional RV nucleocapsid and subsequent transcription of positive-sense RNAs by the RV polymerase complex.

Extracts from cells infected with vTF7-3 and transfected with pSDI-CAT and RV protein-encoding plasmid pT7T-N, -P, or -L were analyzed in standard CAT assays. Significant CAT activity was observed after cotransfection of all three RV protein-encoding plasmids together with pSDI-CAT, indicating efficient transcription of sense CAT RNA by RV polymerase (Fig. 3). Omission of any of the plasmids resulted in background CAT expression. Thus, encapsidation of the T7 polymerase-derived RNA including the foreign CAT sequence had apparently occurred and led to a nucleocapsid which was used efficiently as a template by the RV transcriptase complex.

In the case of SDI-CAT, expression of CAT activity must be measured from the pool of transfected cells. In contrast, successful expression of β-Gal from SDI-blue by RV proteins should allow monitoring of encapsidation and transcription in single cells by a simple chromogenic assay. It should even be possible to demonstrate single encapsidation events with subsequent transcription of mRNAs from the SDI-blue genome. Two days after infection with vTF7-3 and transfection with pSDI-blue and RV protein-encoding plasmids, cells were incubated with X-Gal as a substrate and analyzed by light microscopy. Blue staining of cells was strictly dependent on transfection with pSDI-blue. After simultaneous transfection of N-, P-, and L-encoding plasmids, an average of 200 stained cells per experiment were observed. In contrast, control transfections in which one of the RV protein-encoding plasmids was omitted usually yielded no blue-staining cells; in each of two of six controls, one stained cell was found. This result indicated successful encapsidation and expression of β-Gal from the synthetic SDI-blue RV genome by the RV polymerase.

Synthetic genomes are rescued into virions and may be passaged. The foregoing results showed that plasmid-derived RNA transcripts may be encapsidated by RV N protein in a way that allows recognition, replication, and transcription by the RV polymerase. To investigate whether the recombinant RNPs may be assembled into virus particles, RV M- and G-encoding plasmids were included in the transfection protocols. Release of complete virions should allow infection of cells by incubation with the resulting cell culture supernatants. Because of the versatility of the CAT system and the assumed high efficiency of encapsidation, pSDI-CAT was used to optimize the transfection protocols. Supernatants from cells transfected with pSDI-CAT and protein-expressing plasmids were harvested 2 days posttransfection and transferred to fresh cells. These were transfected 2 h postinfection with N-, P-, and L-encoding plasmids, and CAT activity was analyzed 2 days later. In cultures incubated with supernatant from cells expressing all five RV proteins, CAT activity was detected (Fig. 4). In contrast, supernatant from experiments in which either an M- or G-encoding plasmid or both (Fig. 4) were not included failed to transmit CAT activity. CAT activity was again dependent on simultaneous expression of N, P, and L proteins; it was abolished by omission of any of the three plasmids (shown for the absence of L in Fig. 5B). The observed CAT activity correlated to SDI-CAT RNA replication. The presence of CAT-specific RNAs in total cellular RNA was

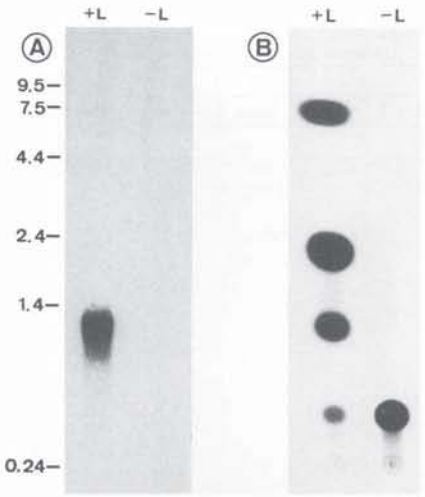


FIG. 5. Transmission of SDI-CAT RNA and CAT expression. Supernatant from cells expressing RV N, P, M, G, and L proteins and transfected with pSDI-CAT was transferred to cells transfected with plasmids encoding N, P, and L proteins (+L) or N and P proteins (−L). Cells were harvested 48 h after infection and analyzed by Northern hybridization with a CAT-specific probe (A) and CAT assay (B). Sizes are indicated in kilobases.

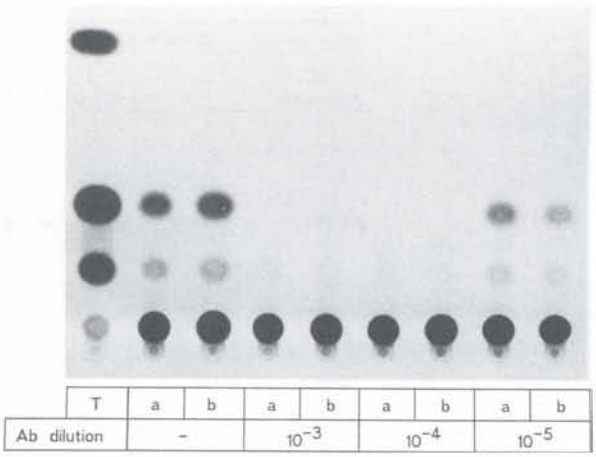


FIG. 6. Neutralization of SDI-CAT infectivity. Culture supernatant from cells transfected with pSDI-CAT and expressing N, P, M, G, and L proteins was incubated with the indicated dilutions of the RV neutralizing MAb E543 and used to infect cells expressing N, P, and L proteins. CAT activity was determined 48 h after infection. T, CAT activity in cells transfected with pSDI-CAT. a and b represent independent neutralization experiments.

demonstrated directly by Northern hybridization with a CAT-specific DNA probe. Whereas in cells transfected with pSDI-CAT, because of incomplete ribozyme autolysis, CAT-specific RNAs exceeding the SDI-CAT genome size of 1 kb were observed (not shown; see Fig. 2), only one broad hybridization signal was obtained in cells infected with SDI-CAT (Fig. 5A). The recognized RNAs should consist of the SDI-CAT genome and polyadenylated mRNAs which, according to the location of transcriptional signals within the SDI-CAT genome, are of approximately the same size. Oligo(dT)-cellulose chromatography confirmed that polyadenylated RNAs were indeed present (not shown).

To determine whether infection was mediated by the glycoprotein of SDI-CAT particles, supernatants were incubated with serial dilutions of a neutralizing monoclonal antibody (MAb) directed against the RV glycoprotein (MAb E543 [21]). Transmission of CAT activity was efficiently inhibited, dependent on dilution of the antibody (Fig. 6). This result indicated that SDI-CAT particles possess an envelope which is similar to that of standard rabies virions.

As for SDI-CAT, infectious particles were also assembled and released from cells transfected with pSDI-blue and the whole set of RV protein-encoding plasmids. Expression of β-Gal in the fresh cells after transfer of supernatants was strictly dependent on the presence of RV proteins N, P, and L. Northern hybridization of total RNA from cells infected with SDI-blue-containing supernatants with a *lacZ* DNA probe revealed correctly sized SDI-blue RNA of about 4 kb (Fig. 7).

Subsequent multiple passages of SDI-CAT and SDI-blue could be performed in cells expressing all five RV proteins from plasmids. Passage and expression of CAT and β-Gal, respectively, were also supported by standard RV. Cells infected with SDI-CAT or SDI-blue generated in transfection experiments and superinfected with RV SAD B19 showed CAT and β-Gal activities, respectively. For subsequent multiple passages of CAT and β-Gal activities and the respective recombinant RNAs, transfer of the resulting supernatants to fresh cultures was sufficient (Fig. 7). Thus, the recombinant genomes containing foreign sequences can be propagated by standard virus like natural defective RNAs.

DISCUSSION

In this report, we describe a system which allows direct experimental investigation of RV transcription and replication mechanisms and the signals and factors involved. It is shown that intracellularly produced RNAs may be encapsidated, replicated, transcribed, and incorporated into virions by RV proteins expressed also from cDNA clones.

The approaches taken so far to rescue engineered RNAs of unsegmented negative-stranded viruses are similar but differ in the source providing the necessary viral functions as well as in the design of the rescued RNAs. On the one hand, constructs have been made by flanking the CAT reporter gene with 3′- and 5′-terminal viral RNA sequences. Detection of encapsidation and transcription of the transfected RNAs by standard helper paramyxoviruses relies on the enzymatic activity of the reporter gene product (5, 8, 9, 16). On the other hand, the recombinant RNAs mimicked DI RNAs naturally selected for their ability to replicate efficiently (3, 4, 17). Only for the latter type of RNAs, replication and virion assembly by proteins expressed from plasmids were successful and replication products could be demonstrated by direct biochemical means.

In the experiments described here, we generated RNAs which possess standard RV 3′ and 5′ noncoding regions, in the case of SDI-1 these being the only constituents. Insertion of the CAT gene between the viral sequences led to a construct comparable to the RNAs reported to be rescued by Sendai virus, respiratory syncytial virus, or parainfluenza virus type 3. Insertion of the *lacZ* reporter sequence instead of CAT not only increased the size of the genome analog but also provided a means to analyze single cells.

The cDNA constructs were designed in a way to allow intracellular transcription of RNAs with termini very similar to authentic genome ends. However, detectable amounts of RNA starting with the correct RV 5′-end sequence were not transcribed by T7 RNA polymerase. For VSV, it has been shown

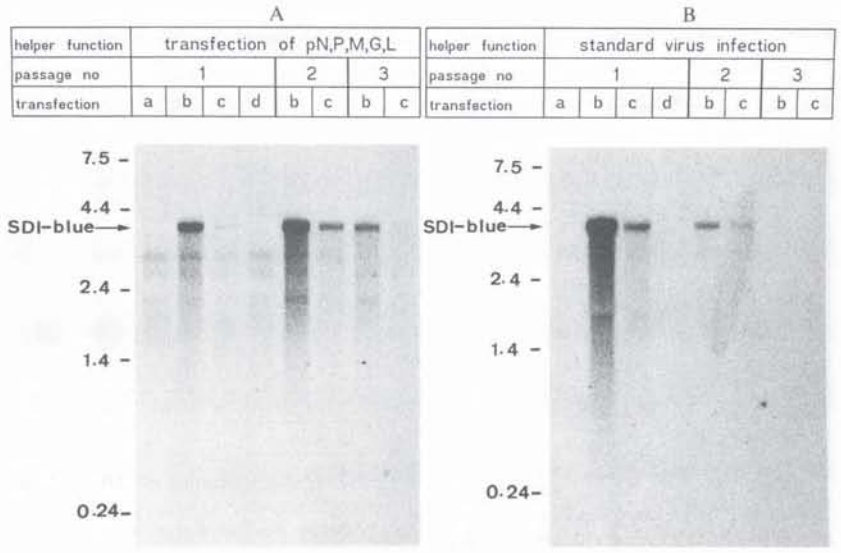


FIG. 7. Successive passages of SDI-blue in cells expressing all RV proteins from plasmids (A) and in cells infected with standard RV SAD B19 (B). Supernatants from cells transfected with pSDI-blue and plasmids encoding N, P, and L (a), N, P, M, G, and L (b and c, independent experiments), or N and P (d) proteins were used for infection of cells which had been transfected with protein-encoding plasmids 2 h previously. Subsequent passages 2 and 3 in transfected cells were performed accordingly. For passages with standard RV, cells were first infected with the supernatant from cells transfected with pSDI-blue and 1 h later with RV SAD B19. For passages 2 and 3, no additional infection with standard virus was carried out. Total RNA from cells was analyzed by Northern hybridization with a *lacZ*-specific probe. Sizes are indicated in kilobases.

that some extra nucleotides at the 5′ end of the synthetic RNA genomes are trimmed by the viral polymerase (17); therefore, three G residues were inserted between the T7 promoter and the RV sequence. In vTF7-3-infected cells, the resulting transcripts were capped, as indicated by primer extension and RNA sequencing experiments (not shown). RNAs ending with the viral 3′-terminal residue, shown to be critical for replication of the VSV DI RNA (17), were generated in the transfected cell by the autolytic activity of the HDV ribozyme (1). Despite capping, the extra three nucleotides at the 5′ end, and the expected presence of a 2′,3′-cyclic phosphate at the 3′-terminal ribose residue by the action of the HDV ribozyme (18), the T7 RNA polymerase transcripts were recognized by RV proteins expressed from transfected plasmids.

In the case of pSDI-1 transcripts, successful and efficient encapsidation and subsequent replication by RV polymerase could readily be demonstrated by Northern hybridization using total RNA of transfected cells. The presence of positive-stranded RNAs, which served as an indicator for encapsidation and replication, was observed only in cells cotransfected with N-, P-, and L-encoding plasmids. Further confirmation of RV polymerase-based transcription was provided by the finding of specific subgenomic RNAs. In contrast to the described synthetic VSV DI RNA, which is of the copyback type and contains terminal sequences derived only from the viral 5′ end (17), the recombinant RV RNAs described in this report contain the original viral 3′-terminal sequence and, accordingly, the start signal for transcription of subgenomic RNAs. The size composition and the hybridization pattern with specific oligonucleotides of the observed SDI-1-specific RNAs is consistent with the assumption that correct transcription of a leader RNA of 58 bases and mRNA consisting of 146 bases and heterogeneous poly(A) tails had occurred. Accordingly, all *cis*-acting signals for encapsidation, replication, and transcription of leader and mRNA are located within the RV 3′ and 5′ noncoding sequences present in SDI-1 RNA.

Once started within the terminal regions, encapsidation of the RNA transcripts apparently proceeds independently of primary RNA sequence. Substitution of RV coding regions with the bacterial CAT and *lacZ* genes resulted in replication and expression of the foreign sequences by RV proteins. Although low CAT activity was observed in cells infected with vaccinia virus and transfected with CAT DNA, which is most likely due to vaccinia virus RNA polymerase transcription (19), successful encapsidation and transcription of SDI-CAT RNA could be monitored by standard CAT assays. As indicated by the level of CAT expression as well as by the finding that plus-stranded SDI-CAT RNAs could be demonstrated directly by Northern analysis of total RNA from cells transfected with pSDI-CAT (not shown), encapsidation occurs efficiently. In contrast, correct encapsidation of pSDI-blue transcripts seems to be a rarer event. According to fluorescence analyses with anti-N MAbs, transfection of pT7T-N was successful in 50 to 80% of cells. It was estimated that packaging of pSDI-blue transcripts and subsequent expression of β-Gal occurred in only 1 of 1,000 cells correctly transfected with all plasmids and infected with vTF7-3. Accordingly, and in contrast to SDI-1 or SDI-CAT, positive-stranded SDI-blue RNAs could not be detected in transfected cells by Northern hybridization using oligonucleotide L1M as a probe. It appears most likely that packaging efficiency decreases with increasing length of the recombinant RNAs. The size of SDI-blue RNA, 3,980 bases, is approximately 17 times the size of SDI-1 and one-third of the standard RV genome size. Encapsidation efficiencies of at most 5% of negative-stranded transcripts which were achieved for the 2.2-kb VSV DI RNA in the same expression system (17) fit well into this hypothesis.

The transient vaccinia virus/T7 polymerase system was used to express individual RV proteins from plasmids. In contrast to helper virus-based rescue systems, this approach allowed not only efficient rescue of synthetic RNAs containing the necessary *cis*-acting signals but also dissection of RV protein func-

tions. It was shown that the synthetic minigenomes are biologically active only in the presence of functional RV polymerase proteins. Positive-stranded RNAs were observed only in the presence of the complete set of N, P, and L proteins in transfected cells. Omission of any one of these proteins abolished replication, thus providing evidence that for RV polymerase function, both L and P proteins and a template RNA associated with N protein are necessary and sufficient. Investigation of protein interactions concerning RV replicase and transcriptase functions may now be envisaged.

Moreover, it was shown that RNPs containing the recombinant SDI-CAT and SDI-blue RNAs may be rescued into infectious virus particles upon simultaneous expression of N, P, M, G, and L proteins, implicating a potential use of RV-based vectors. Both M and G proteins were indispensable for generation of complete virions. Particles whose infectivity could be blocked by anti-G MAbs neutralizing standard RV were generated. Thus, the whole viral replicative cycle, including assembly and budding of infectious virions which in structure should correspond to standard rabies virions, was reconstituted entirely from DNA-encoded proteins and RNAs. Detailed structure-function analyses of RV gene products in each step of the cycle are now possible.

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