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Recombinant vesicular stomatitis viruses from DNA

(rhabdovirus/viral replication/viral assembly)

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ABSTRACT **We assembled a DNA clone containing the 11,161-nt sequence of the prototype rhabdovirus, vesicular stomatitis virus (VSV), such that it could be transcribed by the bacteriophage T7 RNA polymerase to yield a full-length positive-strand RNA complementary to the VSV genome. Expression of this RNA in cells also expressing the VSV nucleocapsid protein and the two VSV polymerase subunits resulted in production of VSV with the growth characteristics of wild-type VSV. Recovery of virus from DNA was verified by (*i*) the presence of two genetic tags generating restriction sites in DNA derived from the genome, (*ii*) direct sequencing of the genomic RNA of the recovered virus, and (*iii*) production of a VSV recombinant in which the glycoprotein was derived from a second serotype. The ability to generate VSV from DNA opens numerous possibilities for the genetic analysis of VSV replication. In addition, because VSV can be grown to very high titers and in large quantities with relative ease, it may be possible to genetically engineer recombinant VSVs displaying foreign antigens. Such modified viruses could be useful as vaccines conferring protection against other viruses.**

Rhabdoviruses are membrane-enveloped viruses that are widely distributed in nature where they infect vertebrates, invertebrates, and plants. These viruses have single, negative-strand RNA genomes of 11,000–12,000 nt (1). Because the genome is the negative sense, rhabdoviruses must encode and package an RNA-dependent RNA polymerase in the virion (2). This enzyme transcribes genomic RNA to make subgenomic mRNAs encoding the 5–6 viral proteins and also replicates full-length positive-sense and negative-sense RNAs. The same basic genetic system is also used by the paramyxoviruses and filoviruses.

The prototype rhabdovirus, vesicular stomatitis virus (VSV), grows to very high titers in most animal cells and can be prepared in large quantities. As a result, VSV has been widely used as a model system for studying the replication and assembly of enveloped RNA viruses. The study of VSV and related negative-strand viruses has been limited by the inability to perform direct genetic manipulation of the virus by using recombinant DNA technology. The difficulty in generating VSV from DNA is that neither the full-length genomic nor antigenomic RNAs are infectious. The minimal infectious unit is the genomic RNA tightly bound to 1250 subunits of the nucleocapsid (N) protein (3) and smaller amounts of the two virally encoded polymerase subunits, L and P. To reconstitute infectious virus from the viral RNA, it is necessary first to assemble the N protein–RNA complex that serves as the template for transcription and replication by the VSV polymerase. Although smaller negative-strand RNA segments of the influenza virus genome can be packaged into nucleocapsids *in vitro* and then rescued in influenza-infected cells (4, 5), systems for packaging the much larger rhabdoviral genomic RNAs *in vitro* are not yet available.

In the past 4 years, systems for replication and transcription of DNA-derived minigenomes or small defective RNAs from rhabdoviruses (6, 7) and paramyxoviruses (8–13) have been described. In these systems, RNAs are assembled into nucleocapsids within cells that express the viral N protein and polymerase proteins. Although these systems have been very useful, they do not allow genetic manipulation of the full-length genome of infectious viruses.

A pioneering study showing the recovery of rabies virus (a rhabdovirus) from a complete cDNA clone was published recently (14). An important feature of the approach used for rabies was initiation of the infectious cycle by expressing the antigenomic RNA rather than the genomic RNA in cells expressing the viral N, P, and L proteins. The rationale for starting with the antigenomic RNA was that, unlike the genomic RNA, it would not be able to hybridize to the N, P, and L mRNAs supplied to generate the nucleocapsid and initiate replication. This strategy avoids a potentially deleterious antisense problem in which the mRNAs encoding the N, P, and L proteins would hybridize to the negative-strand genomic RNA. We report here that the strategy of expressing the full-length positive strand (14) is also successful for VSV, allowing us to generate recombinant VSVs from DNA.

MATERIALS AND METHODS

Plasmid Construction. The plasmid pVSVFL(+) expressing the 11,161-nt positive-strand (antigenomic) VSV RNA sequence was constructed from four DNA fragments cloned into pBluescript SK(+) (Stratagene). The starting plasmid for the construction, pVSVFL(−), expressed the complete negative-sense VSV genomic RNA (Indiana serotype) from a T7 promoter. This plasmid was generated in a nine-step cloning procedure that involved joining the five original cDNA clones of the VSV mRNAs (15–17) with gene junction fragments and terminal fragments. These fragments were generated by reverse transcription and PCR (RT–PCR) (18) from VSV genomic RNA (M.A.W., R. Burdine, E.A.S., and J.K.R., unpublished work). To facilitate engineering of the VSV genome and to provide genetic tags, unique *Mlu* I and *Nhe* I restriction enzyme sites were introduced by oligonucleotide-directed mutagenesis into the 5′ and 3′ noncoding regions flanking the VSV glycoprotein gene before construction of the full-length genome.

In the initial step of constructing pVSVFL(+) we used primers 5′-CCGGCTCGAGTTGTAATACGACTCACTATAGGGACGAAGACAAACAAACCATTATTATC-3′ and 5′-GAACTCTCCTCTAGATGAGAAC-3′ to amplify (18) a 2124-nt fragment from pVSVFL(−) (no. 1, Fig. 1*A*). This fragment corresponds to the 3′ end of the VSV genome. The first primer introduced an *Xho* I site and a T7 promoter (underlined)

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Abbreviations: VSV, vesicular stomatitis virus; VSV_I and VSV_{NJ}, VSV Indiana and New Jersey serotype, respectively; RT–PCR, reverse transcription–PCR; rVSV, recombinant VSV; G_I and G_{NJ}, Indiana-serotype and New Jersey-serotype glycoprotein, respectively.

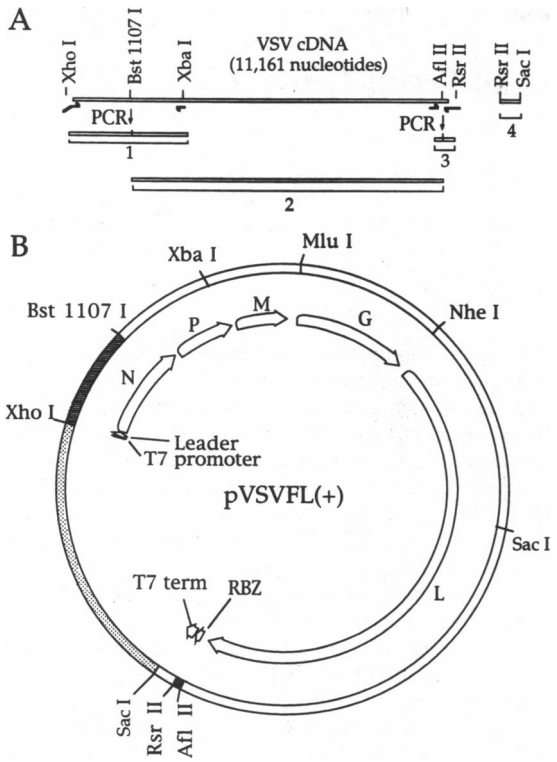

FIG. 1. Plasmid DNA construction. (*A*) Diagram illustrates the cloned VSV genomic sequence and the four DNA fragments (numbered 1–4) used to generate plasmid pVSVFL(+). Horizontal arrows represent PCR primers used to generate fragments 1 and 3. (*B*) Diagram of plasmid pVSVFL(+) that gives rise to infectious VSV. Locations of the VSV genes encoding the five proteins N, P, M, G, and L are shown. The stippled region from *Sac* I to *Xho* I represents the pBSSK$^+$ vector sequence, and the hatched segments represent the regions of the VSV genome generated by PCR. Transcription from the T7 promoter generates the complete positive-strand VSV RNA. RBZ, ribozyme.

immediately preceding the sequence complementary to the 3′ end of the VSV genome. The second primer covered a unique *Xba* I site present in the VSV *P* gene. The PCR product was digested with *Xho* I and *Xba* I and cloned into pBluescript SK(+) (Stratagene) that had been digested with *Xho* I and *Xba* I. The resulting plasmid carrying the sequence corresponding to the 3′ end of the VSV genome preceded by a T7 promoter was designated pBSXX. Note that an additional T7 promoter is also present upstream of the *Xho* I site in the vector. Next we generated the sequence corresponding to the 5′ end of the VSV genome and part of the hepatitis delta virus ribozyme (7, 19). A 147-nt PCR product (no. 3, Fig. 1*A*) was amplified from pVSVFL(−) with primers 5′-AGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCACGAAGACCACAAAACCAG-3′ and 5′-ATGTTGAAGAGTGACCTACACG-3′. The first primer contained 39 nt of the sequence encoding the hepatitis delta virus ribozyme (underlined) followed by 19 nt complementary to the 3′ end of the VSV antigenomic RNA. The second primer hybridized within the *L* gene (Fig. 1*A*). The PCR product was digested with *Afl* II and *Rsr* II, and the 80-nt *Afl* II–*Rsr* II fragment was ligated to a 225-nt *Rsr* II–*Sac* I fragment (no. 4, Fig. 1*A*) derived from a plasmid designated pBS-GMG (30). Fragment 4 contained the T7 terminator sequence and the remainder of the sequence encoding the hepatitis delta virus ribozyme. Ligated products were digested with *Afl* II and *Sac* I, and the 305-nt *Afl* II–*Sac* I product was cloned into the *Afl* II and *Sac* I sites of a modified pBSXX vector that contained an *Afl* II site inserted at the unique *Not* I site within the polylinker. This plasmid containing the *Afl* II–*Sac* I fragment was designated pBXXAS. To complete the construction, a 10,077-nt *Bst* 1107 I–*Afl* II fragment (no. 2, Fig. 1*A*) containing 90% of the VSV sequences from pVSVFL(−) was inserted into the unique *Bst* 1107 I and *Afl* II sites of pBXXAS. The final plasmid was designated pVSVFL(+). The sequences in this plasmid generated by PCR (hatched sequences, Fig. 1*B*) were determined and contained no errors. We also prepared a plasmid in which the sequence of the VSV Indiana serotype (VSV_I) *G* gene (*Mlu* I–*Nhe* I) was replaced with the *G* gene from the New Jersey serotype of VSV (VSV_{NJ}) (20). This plasmid is called pVSVFL(+)$_{I/NJG}$ and has only a single T7 promoter.

Transfection and Recovery of Recombinant VSV (rVSV). Baby hamster kidney cells (BHK-21; American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM)/5% fetal bovine serum. Cells on 10-cm dishes (≈70% confluent) were infected at a multiplicity of infection of 10 with vTF7-3 (21). After 30 min, plasmids encoding the VSV antigenomic RNA and the N, P, and L proteins were transfected into the cells by using a calcium phosphate transfection kit according to directions supplied (Stratagene). The coding regions for N, P, and L proteins were each expressed in pBluescript SK(+) from the T7 promoter. Plasmid amounts were 10 μg of pVSVFL(+), 5 μg of pBS-N, 4 μg of pBS-P, and 2 μg of pBS-L. After 24- to 48-hr incubation at 37°C in 3% CO_2/air cells were scraped from the dish and subjected to three rounds of freeze–thawing (−70°C, 37°C) to release cell-associated virus. Debris was pelleted from the cell lysates by centrifugation at 1250 × *g* for 5 min. Five milliliters of this lysate was added to ≈10^6 BHK cells on a 10-cm plate in 10 ml of DMEM/5% fetal bovine serum. After 48 hr the medium was clarified by centrifugation at 1250 × *g* for 10 min and passed through a filter to remove most of the vaccinia virus (0.2-μm pore size; Gelman). One milliliter was then added directly to BHK cells that had been plated on a coverslip in a 35-mm dish. After 4 hr, the cells were fixed in 3% paraformaldehyde and stained with monoclonal antibody I1 to the VSV Indiana-serotype glycoprotein (G_I) (22) or with monoclonal antibody 9B5 (23) to the VSV New Jersey-serotype glycoprotein (G_{NJ}) protein followed by goat anti-mouse rhodamine-conjugated antibody (Jackson ImmunoResearch). Cells were then examined by indirect immunofluorescence using a Nikon Microphot-FX microscope equipped with a ×40 planapochromat objective. When VSV recovery was successful, 100% of the cells showed the typical bright stain for G protein characteristic of a VSV infection.

Preparation and Analysis of VSV RNA and Protein. VSV and wild-type VSV isolated from single plaques (≈10^5 plaque-forming units) were used to infect a monolayer of BHK cells (≈80% confluent) on a 10-cm dish in 10 ml of DMEM/5% fetal bovine serum. After 24 hr cell debris and nuclei were removed by centrifugation at 1250 × *g* for 5 min, and virus was then pelleted from the medium at 35,000 rpm in a Beckman SW41 rotor for 1 hr. Virus pellets were resuspended in 0.5 ml of 10 mM Tris·HCl, pH 7.4, for protein analysis. For RNA isolation, virus was resuspended in 0.2 ml of 0.5% SDS/0.2 M sodium acetate, pH 8.0, followed by extraction with phenol/$CHCl_3$. RNA was precipitated with 95% (vol/vol) ethanol and 5 μg of carrier tRNA. RNA was pelleted by centrifugation at 12,000 × *g* for 15 min and resuspended in water with 1 unit of RNasin (Promega). For analysis of RNA by RT–PCR, primer pairs flanking either the distinctive *Nhe* I or *Mlu* I sites were used. The first-strand DNA synthesis reaction was done in 50 μl of PCR buffer (Promega) containing 5 mM $MgCl_2$, 1 mM dNTPs, 1 unit of RNasin (Promega), 1 unit of avian myeloblastosis virus reverse transcriptase (Promega), 0.75 μM primer, and ≈0.25 μg of VSV genomic RNA. Incubation was at 42°C for 15 min followed by 5 min at 99°C and 5 min at 5°C. PCR was done by addition of 0.5 unit of *Taq* polymerase, adjustment of $MgCl_2$ concentration to 1.25 mM, and addition of the second primer (0.75 μM). The reaction was subjected to

20 thermal cycles: 95°C, 1 min; 60°C, 1.5 min. The reaction was then incubated at 60°C for 7 min.

Direct sequencing of VSV genomic RNA was done according to a described protocol based on the dideoxynucleotide chain-termination method (24), except that [α-^{33}P]dATP (Amersham) was used. Each reaction included ≈0.25 μg of VSV genomic RNA.

RESULTS

To construct a cDNA clone encoding the entire 11,161-nt VSV genome, we initially joined the individual cDNA clones of the VSV mRNAs by using small DNA fragments generated by RT–PCR that covered the four gene junctions. Correct genomic terminal sequences were also generated by RT–PCR of the VSV genome, and these were joined to the other DNAs by using restriction sites. This initial clone was constructed with a T7 promoter directing synthesis of the full-length negative-strand VSV RNA. Despite numerous attempts, we were unable to recover VSV from cells expressing the VSV genomic RNA and the VSV N, P, and L proteins. The recent report that infectious rabies virus could be recovered from a clone expressing the complete rabies positive-strand (antigenomic) RNA (14) prompted us to redesign the VSV construct to express the VSV antigenomic RNA. The construction strategy is described in *Materials and Methods* and in Fig. 1. The entire VSV sequence as well as a T7 promoter, terminator, and hepatitis delta virus ribozyme sequence were cloned in pBluescript SK(+) between the *Xho* I and *Sac* I sites (Fig. 1*B*). An additional T7 promoter is also present upstream of the *Xho* I site in the plasmid. A slightly different cloning strategy was used to generate plasmids lacking the upstream T7 promoter, and VSV has also been recovered from these constructs.

Recovery of VSV from DNA. To determine whether we could recover VSV from plasmid DNA, we infected cells with vaccinia vTF7-3 (21) to provide cytoplasmic T7 RNA polymerase. These cells were then transfected with pVSVFL(+), which expresses the antigenomic VSV RNA from a T7 promoter, and three other plasmids that express the VSV N, P, and L proteins. Expression of the N protein was required to assemble nascent VSV antigenomic RNA into nucleocapsids. Once formed, these nucleocapsids should serve as templates for synthesis of minus-strand RNA by the L/P polymerase complex. Encapsidated minus-strand RNA should then be a template for transcription, initiating the VSV infectious cycle.

The initial recovery experiment used two 10-cm plates of BHK cells (≈5 × 10^6 cells each). At 24 hr after the infection with vaccinia vTF7-3 and transfection with the four plasmids, cells and medium were frozen and thawed to release any cell-associated VSV, and the clarified lysates were added to fresh BHK cells. After 48 hr, both plates showed severe cytopathic effect that could have been due either to vaccinia virus or to recovered VSV. One milliliter of each supernatant was then added to small dishes of BHK cells on coverslips. After 2 hr, one of these coverslips showed rounded cells characteristic of a VSV infection, whereas the other did not. After 4 hr, cells on both coverslips were fixed, stained with appropriate antibodies, and examined by indirect immunofluorescence microscopy to detect the VSV G protein. All cells on the coverslip showing rounded cells revealed intense fluorescence characteristic of G protein expression during VSV infection (data not shown). Subsequent passaging and analysis described below showed that VSV had been recovered from the transfection. The other coverslip showed no G expression, and no VSV could be recovered after passaging.

On the basis of the frequency with which rabies virus (14) and VSV minigenomes (30) were recovered, we anticipated that recovery of complete VSV would be a rare event. The initial recovery of VSV from only one of two transfections suggested the possibility that the initial titer in the positive lysate was very low. To examine this titer, we infected BHK cells on coverslips with one-tenth of the lysate (1 ml) derived from each initial transfection. After 8 hr, the cells were examined for expression of G protein by indirect immunofluorescence. A scan of the entire coverslip revealed no VSV infection from the negative lysate and only five small areas of infection (two to six cells each) from the lysate that gave rise to VSV G expression on subsequent passaging. The initial titer was therefore very low, as we suspected, and likely represented a total of ≈50 infectious particles, probably derived from a VSV infection initiated in only one cell out of 2 × 10^7 transfected. This low rate of recovery of infectious VSV is typical of that observed in several experiments.

Analysis of Viral Proteins. Subsequent passages and plaque assays of VSV recovered in three independent experiments revealed plaques that were detectable in <16 hr and titers up to 2 × 10^9 plaque-forming units/ml characteristic of VSV. For further verification that VSV had been recovered, the proteins in virus pelleted from the medium were examined by SDS/PAGE. Fig. 2 shows the Coomassie-stained gel of proteins of VSV recovered from recombinant DNA (rVSV) and wild-type VSV. The mobilities and relative amounts of the five viral proteins are indistinguishable in the wild-type and recombinant virus.

Identification of Sequence Tags. In pVSVFL(+), the VSV nucleotide sequence was altered by oligonucleotide-directed mutagenesis to generate unique *Mlu* I and *Nhe* I restriction enzyme sites in the 5′ and 3′ noncoding regions of the glycoprotein gene. To verify that these sites were present in recovered virus, we carried out reverse transcription of genomic RNA purified from wild-type or recombinant virions using primers upstream of each restriction site. The reverse transcription products were then amplified by PCR using an additional primer downstream of each restriction site. The presence of the genetic tag in the recombinant virus was verified by digestion of the PCR products with the appropriate restriction enzymes. With this method, the presence of both the *Mlu* I and *Nhe* I sequences in the recovered virus RNA was verified, and the results for the *Nhe* I site are shown in Fig. 3. Sequences from wild-type VSV and rVSV were amplified in parallel, and a 620-nt fragment was obtained in both cases (lanes 3 and 5). No product was obtained when reverse transcriptase was omitted from the reactions before PCR (lanes 1 and 2), indicating that the PCR product was derived from RNA, not from contaminating DNA. After digestion with *Nhe* I, expected fragments of 273 and 347 bp were obtained from rVSV RNA, whereas the DNA derived from the wild-type RNA remained undigested (lanes 4 and 6).

Direct Sequencing of Tagged Genomic RNA. The presence of additional restriction sites in the DNA generated by PCR provided strong evidence that VSV had been recovered from

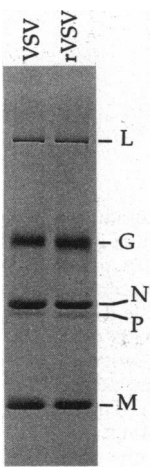

FIG. 2. Proteins present in wild-type VSV and rVSV. Proteins from 1% of the virus recovered from ≈5 × 10^6 infected BHK cells were separated by SDS/10% PAGE and visualized by staining with Coomassie brilliant blue. Positions of the five VSV proteins are indicated.

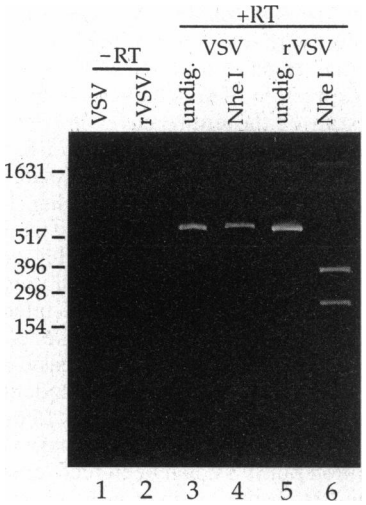

FIG. 3. Identification of a restriction enzyme recognition sequence in the rVSV. A 620-nt segment of genomic RNA isolated from wild-type and rVSV was amplified by RT–PCR using the primers 5′-CATTCAAGACGCTGCTTCGCAACTTCC-3′ and 5′-CATGAATGTTAACATCTCAAGA-3′. Controls in which reverse transcriptase (RT) was omitted from the reaction are indicated. DNA samples were either digested with *Nhe* I or left undigested (undig.) before electrophoresis on a 6% polyacrylamide gel, as indicated. DNA was detected by staining with ethidium bromide. Sizes of DNA markers are indicated at left.

DNA. To ensure that identification of the genetic tags by PCR had not resulted from inadvertent contamination by plasmid DNA, we carried out direct sequence analysis of the genomic RNA with reverse transcriptase and a primer hybridizing upstream of the *Nhe* I site. The sequence from the autoradiogram shown in Fig. 4 is in exact agreement with the published sequence of the VSV G mRNA (16), except that the 4-nt changes used to generate the *Nhe* I site (GCACAA to GCTAGC) are present. These results show unequivocally that the sequence tag is present in the genomic RNA.

VSV_I Virus Carrying G_{NJ}. There are two serotypes of VSV, designated VSV_I and VSV_{NJ}. The glycoproteins of the two serotypes share ≈50% sequence identity (20). In earlier studies we found that G_{NJ} could complement a mutant of VSV_I that makes a defective glycoprotein (25). It therefore seemed likely that a rVSV in which the G_I gene was replaced by the G_{NJ} gene would be viable, despite the extensive sequence divergence. To generate such a recombinant, the G_{NJ} cDNA was amplified by PCR using primers that introduced *Mlu* I and *Nhe* I sites within the 5′ and 3′ noncoding regions at each end of the gene. The amplified DNA was cloned into pBluescript, and the G_{NJ} protein was expressed in BHK cells using the vaccinia–T7 system. The protein expressed was shown to have membrane fusion activity below pH 6.0, indicating that it was functional (data not shown). This G_{NJ} cDNA was then cloned into the unique *Mlu* I and *Nhe* I sites of the full-length construct after removal of sequences encoding G_I. rVSV was recovered, essentially as described above except that the initial transfection was allowed to proceed for 48 hr before the freeze–thaw step. After the first passage, expression of the G_{NJ} protein was verified by indirect immunofluorescence using a monoclonal antibody specific to G_{NJ} (23). The virus was then plaque-purified and grown. To examine the proteins present in the recombinant virus, virus recovered from cells infected with VSV_I, VSV_{NJ}, and the recombinant $VSV_{I/NJG}$ was analyzed by SDS/PAGE followed by Coomassie staining. The VSV_I G, N, P, and M proteins each have mobilities distinct from their VSV_{NJ} counterparts (Fig. 5, lanes 1 and 3). The recombinant $VSV_{I/NJG}$ shows the mobility difference in only the G protein as expected (lane 2). The presence of the *Nhe* I and *Mlu* I sites in the recombinant was also verified (data not shown).

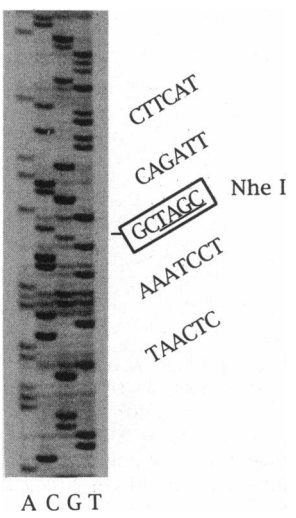

FIG. 4. Autoradiogram showing the sequence of genomic RNA from rVSV. RNA prepared from rVSV was sequenced by the dideoxynucleotide chain-termination method with reverse transcriptase. The written sequence corresponds to nt 1553–1602 in the G mRNA (16). Underlined sequence represents the 4 nt that were changed to generate the *Nhe* I site.

DISCUSSION

The results presented here establish that infectious VSV can be recovered from recombinant DNA. This prototype rhabdovirus is now amenable to the type of detailed genetic analysis that has been available in other viral systems for many years. Two earlier studies were important to our success in this venture. The first established a system in which the RNA from VSV-defective interfering particle could be derived from DNA and replicated by providing the N, P, and L proteins in trans (7). We adapted that system including the use of the hepatitis delta virus ribozyme (19) to generate the presumably critical 3′ end of the initial transcript (7). The second study showed that rabies virus could be recovered from DNA when the positive-strand, antigenomic RNA was expressed in the presence of rabies N, P, and L proteins (14). Although it was only speculated for rabies virus, we believe that using the positive strand was critical to our success with VSV because we have not recovered virus starting with an equivalent construct encoding the genomic RNA.

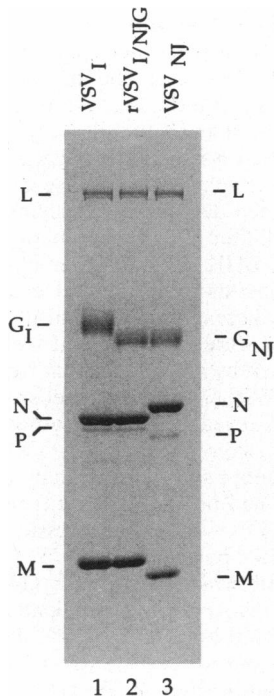

FIG. 5. Protein analysis of rVSV expressing G_{NJ}. Proteins from 1% of the virus pelleted from the medium of ≈5×10^6 BHK cells infected for 24 hr with wild-type VSV_I (lane 1), $rVSV_{I/NJG}$ (lane 2), or wild-type VSV_{NJ} (lane 3) were separated by SDS/10% PAGE. Proteins were visualized by staining with Coomassie brilliant blue; positions of viral proteins are indicated.

Why is the initial event of generating VSV so rare, apparently occurring in only 1 in 10^7–10^8 transfected cells? One possibility is that our clone contains a sequence error that is only corrected by a rare mutational event. We believe this is not the case because the clone was completely sequenced before assembly, and differences from published sequences were corrected, or the proteins were shown to be functional in complementation assays. Also, the frequency of recovery is actually higher than expected based on our observations with minigenomes encoding one or two VSV proteins (30). In these cases we found that a transcribing and replicating minigenome (≈2-kb RNA) was recovered in ≈1 in 10^2 transfected cells expressing the RNA with the N, P, and L proteins. Addition of a second cistron (an additional 0.85-kb RNA) encoding the M protein dropped the recovery rate to ≈1 in 10^3 transfected cells. If there is a 10-fold drop in recovery rate for each additional kilobase of RNA added, one can easily rationalize an even lower frequency of recovery for the 11,161-kb genome than we observed. Although these minigenomes encode negative-sense RNAs, the comparison of the frequency of recovery to that of the full-length-plus construct is probably valid because expression of the N, P, and L mRNAs would not generate mRNAs complementary to the minigenome.

Although the rate-limiting step in generation of infectious VSV is unknown, it is probably at the level of synthesis and encapsidation of the large antigenomic RNA, which must occur before replication and transcription. The complete encapsidation with N protein probably has to occur on the nascent RNA to protect it from degradation, and the cells in which this occurs must also produce appropriate amounts of L and P proteins to initiate replication. Once this has occurred, however, the transcription and translation of the genome should generate additional N, P, and L proteins, as well as the G and M proteins required for budding of infectious virus.

The recovery of VSV from DNA opens numerous aspects of the viral life cycle to genetic analysis. The studies of the genetic signals involved in transcription and replication have so far been confined to analysis of defective RNAs that do not encode viral proteins (7, 26). These and other signals can now be examined in the context of a VSV infection occurring in the absence of a vaccinia virus infection. The system we have described also provides an opportunity to study the roles of individual viral protein domains and modifications in viral assembly and replication. Previously these analyses have been confined to *in vitro* systems or to analysis using the complementation of naturally occurring mutants, where synthesis of the mutant protein can complicate the analysis.

Perhaps even more exciting is the potential to use VSV as a vector to express other proteins. The experiment in which we recovered VSV_I carrying G_{NJ} (Fig. 5) illustrates that viable recombinants can be made. For reasons that are unclear the titers of recombinant virus were at least 10-fold lower than those obtained with either parent. The lower titer apparently did not result from a defect in viral assembly because the amounts of proteins in wild-type and recombinant virions at the end of infection were comparable (Fig. 5). Our previous experiments showed that a foreign glycoprotein carrying the appropriate cytoplasmic tail signal could be incorporated into the VSV envelope (27). This result suggests the possibility of generating rVSVs carrying novel proteins in their envelopes. If these viruses were appropriately attenuated, they could be useful as vaccines against other viral diseases.

We do not know yet whether there will be strict limits on the maximum size of the genome that can be packaged. The truncated genomes of defective interfering particles are replicated and packaged very well; thus we suspect that there will be flexibility in the maximum length as well. In addition, other rhabdoviruses encode additional proteins from extra genes (28). Presumably a longer nucleocapsid can be packaged as a longer bullet-shaped particle. Because of the modular nature of the VSV genome, with conserved gene end and start sequences at the gene junctions (1), it should be relatively easy to engineer additional genes into VSV. It is not known whether additional unselected sequences will be maintained, given the high mutation rate of RNA viruses, but the preservation of a nonessential pseudogene (14, 29) in rabies virus during growth in tissue culture suggests that additional cistrons may be tolerated.

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