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Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones

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ABSTRACT **Infectious vesicular stomatitis virus (VSV), the prototypic nonsegmented negative-strand RNA virus, was recovered from a full-length cDNA clone of the viral genome. Bacteriophage T7 RNA polymerase expressed from a recombinant vaccinia virus was used to drive the synthesis of a genome-length positive-sense transcript of VSV from a cDNA clone in baby hamster kidney cells that were simultaneously expressing the VSV nucleocapsid protein, phosphoprotein, and polymerase from separate plasmids. Up to 10^5 infectious virus particles were obtained from transfection of 10^6 cells, as determined by plaque assays. This virus was amplified on passage, neutralized by VSV-specific antiserum, and shown to possess specific nucleotide sequence markers characteristic of the cDNA. This achievement renders the biology of VSV fully accessible to genetic manipulation of the viral genome. In contrast to the success with positive-sense RNA, attempts to recover infectious virus from negative-sense T7 transcripts were uniformly unsuccessful, because T7 RNA polymerase terminated transcription at or near the VSV intergenic junctions.**

The ability to recover infectious virus from cDNA clones of RNA virus genomes facilitates molecular analyses of every aspect of the viral replicative cycle. For example, the demonstration that a plasmid encoding bacteriophage Qβ RNA gave rise to infectious phage when introduced into *Escherichia coli* allowed the experimental manipulation of the genomes of Qβ and other RNA phages (1). The subsequent finding that a cDNA clone of the entire genome of poliovirus gave rise to infectious virus particles when introduced into mammalian cells in culture extended these developments to animal viruses (2). Since then, infectious cDNA clones of a variety of positive-sense RNA virus genomes have been constructed, permitting the application of recombinant DNA technology to all aspects of the biology of these viruses (for a review, see ref. 3). In contrast to this success with positive-strand RNA viruses, attempts to generate infectious cDNA clones of negative-strand RNA viruses have until recently been unsuccessful. The genomes of negative-strand RNA viruses must be associated with their nucleocapsid protein to form the active ribonucleoprotein template for their RNA-dependent RNA polymerase (4). As a consequence of this and in contrast to positive-strand RNA viruses, naked genomic RNAs of negative-strand viruses are not infectious. Therefore the development of a system in which cDNA transcripts could be associated with the nucleocapsid protein and the viral polymerase was a prerequisite for the successful manipulation of the genomes of negative-strand RNA viruses. Several developments have permitted the manipulation of incomplete genomes of some of these viruses (5–15) and have led to the generation of infectious rabies virus from a positive-sense transcript of a complete genome-length cDNA clone (16).

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Working with the prototypic nonsegmented negative-strand RNA virus, vesicular stomatitis virus (VSV), our laboratory developed a system for the recovery of infectious defective interfering (DI) particles and VSV subgenomic replicons from cDNA clones (12, 17). In these experiments the production of infectious particles was supported entirely by viral proteins expressed in cells from plasmid vectors. These vectors were transcribed following transfection into cultured baby hamster kidney (BHK21) cells that were infected with the vaccinia virus (VV)–T7 RNA polymerase recombinant (vTF7-3; ref. 18) to provide a source of transcriptase. Negative-sense RNAs were generated from transcription plasmids that encoded subgenomic analogs or naturally occurring DI RNA, in cells simultaneously expressing the VSV nucleocapsid protein (N), phosphoprotein (P) and polymerase (L) from cloned versions of the genes. RNAs generated from these plasmids had two nonviral nucleotides at their 5′ termini and authentic 3′ termini that were created by the action of the self-cleaving ribozyme of hepatitis delta virus (HDV). Such RNAs were replication-competent and in the presence of the viral M and G proteins were assembled and budded from the cells to form infectious particles (12). This system has permitted an analysis of the cis-acting requirements for transcription and replication of VSV RNA and an examination of the balance between these two processes (ref. 17 and unpublished data); it also has allowed identification of the cis-acting requirements for the assembly and budding of VSV DI particles (ref. 19 and unpublished data).

The present paper describes the recovery of infectious VSV entirely from cDNA clones. We recovered up to 10^5 infectious virus particles from transfection of 10^6 cells. These particles were amplified upon passage, neutralized by VSV-specific antiserum, and shown to contain nucleotide sequence markers characteristic of the cDNA clone. In addition, experiments were carried out which addressed the question of why infectious virus was recovered from positive-sense transcripts of the VSV genomic cDNA, but not from negative-sense transcripts.

MATERIALS AND METHODS

Plasmid Construction and Transfections. A full-length cDNA clone of VSV was assembled from clones of each of the VSV genes and intergenic junctions by standard cloning techniques (20). These clones were assembled into a full-length cDNA and inserted in both orientations between the bacteriophage T7 promoter and a cDNA copy of the self-cleaving ribozyme from the antigenomic strand of HDV. The resulting plasmids were named pVSV1(+) and pVSV1(−) to reflect the polarity of the T7 transcript they generated: VSV antigenomic or genomic RNA, respectively. The T7 transcripts contained two non-VSV nucleotides (GG) at their 5′ ends but were cleaved by the HDV ribozyme to generate a 3′ terminus which

Abbreviations: VSV, vesicular stomatitis virus; DI, defective interfering; VV, vaccinia virus; HDV, hepatitis delta virus; araC, 1-β-D-arabinofuranosylcytosine; pfu, plaque-forming units.

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corresponded precisely to the 3′ end of the VSV antigenomic (Fig. 1) or genomic sequence, an essential requirement for VSV RNA replication (12). Transfection of plasmids into BHK21 cells infected with vTF7-3 was performed under the conditions and with quantities of support plasmids as described (12), and up to 5 μg of pVSV1(+) or pVSV1(−). Transfected cells were incubated at 31°C or 37°C. For some experiments, pVSV1(+) and pVSV1(−) were linearized by digestion at a unique *Nhe* I site located downstream of the T7 terminator in the pGEM-3-based plasmids.

To identify cDNA-derived virus unambiguously, several genetic markers were incorporated into the full-length cDNA clones. All five genes were of the Indiana serotype of VSV, but whereas the N, P, M, and L genes originated from the San Juan strain, the G gene (kindly provided by Elliot Lefkowitz, University of Alabama, Birmingham) was from the Orsay strain. In addition, the functional P clone has 28 nucleotide sequence differences from the published San Juan sequence (G.T.W.W. and A. W. LeGrone, unpublished data) and in the case of pVSV1(+) the 516 nt at the 5′ end of the VSV genome originated from pDI, the clone of DI-T RNA (12).

To examine the behavior of T7 RNA polymerase at a VSV intergenic junction, a *Bgl* II fragment that encompassed the N–P intergenic junction of VSV (nt 1236–1685) was inserted in both orientations into the unique *Bgl* II site of plasmid 8 (17). A positive-sense version of plasmid 8 was generated and used to accommodate the N–P intergenic junction sequence in an identical manner. These plasmids were named 8(−) and 8(+) to reflect the polarity of the T7 transcript they generated, with an additional suffix to indicate whether the N–P intergenic junction was in the natural (NP) or inverted (PN) orientation with respect to the surrounding VSV sequences (see Fig. 5 *Lower*).

Virus Production and Neutralization. The medium from transfected cells was harvested at 15–48 hr posttransfection and clarified by centrifugation at 14,000 × *g* for 5 min, and virus titers were monitored by plaque assay on BSC40 cells in the presence of 1-β-D-arabinofuranosylcytosine (araC, 25 μg/ml) to inhibit replication of VV. For neutralization assays, a mouse polyclonal antiserum raised against purified VSV was incubated with virus for 30 min at room temperature in Dulbecco's modified Eagle's medium. This approach also allowed titration of VV, by plaque assay in the absence of araC.

Virus Characterization. Virus amplification, radioactive labeling of RNA (with [^{3}H]uridine at 33 μCi/ml; 1 Ci = 37 GBq) and proteins (with [^{35}S]methionine at 10 μCi/ml or [^{3}H]leucine at 50 μCi/ml), and their electrophoretic analyses were performed as described (21). Viral RNA was purified from 10^8 plaque-forming units (pfu) of amplified cDNA-derived VSV and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) and a primer that annealed to negative-sense RNA at nt 11,026–11,043 of the VSV genome. Approximately 1/10th of this reaction mixture was used for DNA amplification by PCR. PCR mixtures contained the primer described above and a second primer that annealed to the extreme 3′ end of positive-sense VSV RNA (nt 11,161–11,144). PCR products were cloned and sequenced by standard techniques (20).

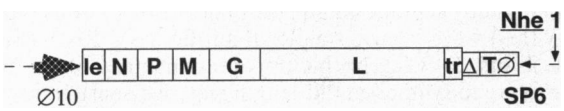

FIG. 1. The T7 transcription plasmid pVSV1(+) is illustrated, linearized at a unique *Nhe* I restriction site present within the vector. Ø10, T7 promoter; le, VSV leader gene; N, VSV nucleocapsid gene; P, VSV phosphoprotein gene; M, VSV matrix protein gene; G, VSV glycoprotein gene; L, VSV polymerase gene; tr, VSV trailer gene; Δ, HDV self-cleaving ribozyme; TØ, T7 terminator; SP6, SP6 promoter.

***In Vitro* Transcription.** RNA was generated *in vitro* by use of T7 RNA polymerase (GIBCO/BRL) according to the manufacturer's instructions, except that rNTP concentrations were increased to 2.5 mM and supplemented with [^{3}H]UTP (80 μCi/ml). The RNA products of *in vitro* transcription were resolved by electrophoresis in 1% agarose/6 M urea gels and visualized by fluorography. It should be noted that RNA mobility in the pH 3.0 agarose/urea gels is a function of base composition as well as size (22).

RESULTS

Construction of a Full-Length cDNA Clone of VSV and Recovery of Infectious Virus. A full-length cDNA of the RNA genome of VSV was assembled from clones of each of the five VSV genes and their intergenic regions and inserted into a pGEM-3-based transcription plasmid between the T7 promoter and the HDV ribozyme (Fig. 1). Plasmids containing the cDNA in both orientations were constructed and designated pVSV1(+) and pVSV1(−) to reflect the polarity of the T7 transcript they generated: VSV antigenomic or genomic RNA, respectively. pVSV1(+) was transfected into BHK21 cells that expressed T7 RNA polymerase from a VV recombinant, together with T7 transcription plasmids that separately encoded the VSV N, P, and L proteins. Control transfections included cells that received pVSV1(+) but no support plasmids, and cells that received the support plasmids but no pVSV1(+). After incubation at 31°C or 37°C, the culture media were harvested, diluted, and monitored by plaque assay for the presence of infectious VSV. Infectious virus was recovered reproducibly from cells that received both pVSV1(+) and the N, P, and L support plasmids (Fig. 2, plate 3; Table 1), but not in either of the two control transfections in which either pVSV1(+) or the VSV support plasmids were omitted (Fig. 2, plates 1 and 2, respectively; Table 1). Among the conditions tested, the highest level of recovery was 8×10^4

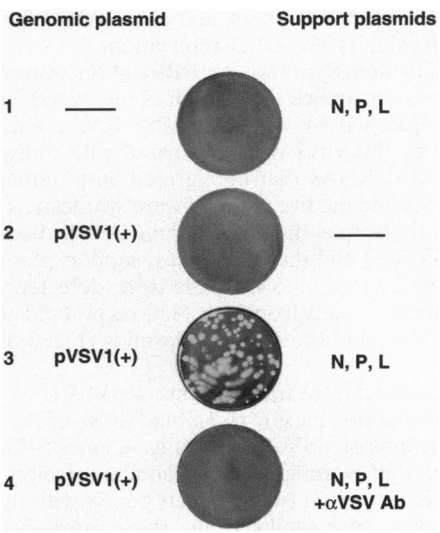

FIG. 2. Plaque assays of recovered virus. Monolayers of BHK21 cells were infected with vTF7-3 and transfected with pVSV1(+) and the N, P, and L support plasmids as indicated. After 45 hr of incubation at 37°C, the culture media were harvested and diluted 100-fold, and the infectious virus in 0.1-ml aliquots was determined by plaque assay using fresh monolayers of BSC40 cells. araC (25 μg/ml) was included in the agarose overlay to suppress the replication of VV. After 30 hr of incubation to allow VSV plaque formation, the monolayers were fixed, stained with crystal violet, and photographed. Shown are plaque assays of medium from cells that received N, P, and L support plasmids only (plate 1); pVSV1(+) only (plate 2); pVSV1(+) and N, P, and L support plasmids without (plate 3) or with (plate 4) subsequent incubation of the medium with anti-VSV antiserum (αVSV Ab).

Table 1. Recovery of infectious virus from pVSV1(+)

VSV plasmids transfected		Virus yield, pfu/ml	Virus recovered in *x/y* transfections
Genomic	Support		
pVSV1(+)	—	< 10	0/8
—	N, P, L	< 10	0/4
pVSV1(+)	N, P, L	< 10 to 8 × 10^4	9/12*
pVSV1(−)	N, P, L	< 10	0/27

Monolayers of BHK21 cells were infected with vTF7-3 and transfected with pVSV1(−) or pVSV1(+) and the N, P, and L support plasmids as indicated. After incubation at 31°C or 37°C for periods between 15 and 48 hr, the culture media were harvested and titers of infectious virus were determined by plaque assay in the presence of araC (25 μg/ml) to inhibit VV.

*The 3 transfection mixtures that failed to yield infectious virus were incubated at 31°C for 15, 21, and 37 hr. One of 9 transfections gave detectable virus only after an amplifying passage; the remaining 8 transfections yielded between 40 and 8 × 10^4 pfu/ml of supernatant.

pfu/ml in the 1.5 ml of medium from 10^6 cells that had received 5 μg of linearized pVSV1(+) and been incubated at 31°C for 45 hr. Plasmid pVSV1(−), which was designed to express a full-length negative-sense copy of the VSV genome, failed repeatedly to yield infectious virus (Table 1).

Neutralization of Recovered Virus by Anti-VSV Antiserum. The virus that yielded the plaques shown in Fig. 2, plate 3, was identified as VSV because plaque formation was completely inhibited by a mouse polyclonal antiserum raised against purified wild-type VSV (Fig. 2, plate 4). However, since the transfected cells had been infected with the VV recombinant vTF7-3 to provide T7 RNA polymerase, the harvested culture medium also contained infectious VV. Plaque assays performed in the presence of anti-VSV antiserum (and in the absence of araC) showed that under all conditions of transfection, VV titers of 1–2 × 10^6 pfu/ml were released from the infected transfected cells (data not shown). However, the VV plaques were less than 1/10th the size of the VSV plaques, easily distinguished from them, and completely suppressed by araC, which inhibits VV DNA replication.

RNA and Protein Synthesis Activities of Recovered VSV. To provide further evidence that the virus recovered from transfections of pVSV1(+) was VSV, the RNAs and proteins synthesized by this virus were compared with those made by authentic VSV. RNAs that comigrated with authentic VSV genomic RNA and the five mRNAs were synthesized following infection with samples harvested from transfections that received pVSV1(+) and the N, P, and L support plasmids (Fig. 3, lanes 2 and 5). No VSV RNAs were detected following passage of supernatants from transfections that did not receive both pVSV1(+) and the support plasmids (Fig. 3, lanes 1, 3, and 4).

Virus recovered from transfections of pVSV1(+) displayed a protein profile that closely resembled those of the San Juan and Orsay strains of VSV Indiana (Fig. 4, lanes 5–7). Further, the proteins that were specifically immunoprecipitated by a VSV-specific antiserum (which reacts poorly with the VSV M and P proteins) were similar in the three cases (Fig. 4, lanes 9–11), providing further evidence that the recovered virus was VSV. However, there were minor differences in the mobility of the proteins from the recovered virus, M protein providing the clearest example (Fig. 4, compare lane 5 with lanes 6 and 7). These different mobilities were characteristic of the proteins encoded by the support plasmids that had been used to construct pVSV1(+) (Fig. 4, lanes 2 and 4), and thus provided evidence that the genome of the recovered virus was derived from the cDNA clone.

VSV Recovered from the cDNA Clone Contained Characteristic Sequence Markers. During the construction of pVSV1(+), several nucleotide sequence markers were introduced within the 5′-terminal 516 nt, which originated from the cDNA clone of DI-T RNA (12). To examine the nucleotide sequence of the 5′ end of the genome of the recovered virus, reverse transcription–PCR was performed. RNA was purified from the recovered virus after 3 passages, and the region from nt 11,026 to the extreme 5′ end of the genome (nt 11,161) was amplified, cloned, and sequenced. In comparison to the published Indiana San Juan virus sequence the following nucleotide differences were noted; a G → A change at nt 11,038, an A → C change at 11,070, and an insertion of an A residue at 11,140 (data not shown). These results revealed that the nucleotide sequence of this region of the genome of the recovered virus was identical to the cDNA clone and, hence, that the recovered virus originated from pVSV1(+).

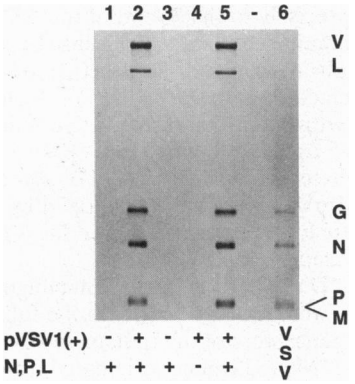

FIG. 3. RNA synthesis directed by the recovered virus. Supernatants from primary transfections were harvested and infectious virus was amplified in fresh, untransfected BHK21 cells in the presence of araC (25 μg/ml). Fresh BHK21 cells were then infected with the virus and viral RNA synthesis was analyzed by metabolic labeling with [^{3}H]uridine (33 μCi/ml) in the presence of actinomycin D (10 μg/ml) from 3–6 hr postinfection at 37°C. Labeled RNAs were analyzed by electrophoresis in 1.75% agarose/urea gels and visualized by fluorography. Plasmids present in original transfection mixtures were as follows. For experiment 1, transfection was performed at 37°C with support plasmids only (lane 1) or with circular pVSV1(+) and N, P, and L plasmids (lane 2). For experiment 2, transfection was performed at 31°C with pVSV1(+) linearized by digestion with *Nhe* I: lane 3, N, P, and L plasmids only; lane 4, pVSV1(+) only; lane 5, pVSV1(+) and N, P, and L plasmids (lane 2). Lane 6 shows VSV genomic RNA (V) and mRNAs (L, G, N, P, and M) labeled in cells infected with authentic wild-type VSV (Indiana serotype, San Juan strain). Note that the P and M mRNAs comigrate.

Genome-Length Negative-Sense RNA Transcripts of VSV Were Not Synthesized Efficiently by Bacteriophage T7 RNA Polymerase. In marked contrast to our success in recovering infectious VSV from pVSV1(+), attempts to generate infectious virus from negative-sense RNA transcripts were uniformly unsuccessful (Table 1). We therefore compared the ability of T7 RNA polymerase to synthesize genome-length positive- and negative-sense transcripts of VSV *in vitro*. pVSV1(+) and pVSV1(−) were linearized at the unique *Nhe* I site (see Fig. 1) and transcribed *in vitro* by T7 RNA polymerase in the presence of [^{3}H]UTP. The products were analyzed by electrophoresis 1% agarose/urea gels, followed by fluorography (Fig. 5). Whereas transcripts of pVSV1(+) were predominantly genome length, the majority of T7 transcripts of pVSV1(−) were clearly smaller than the VSV RNA (Fig. 5, lanes 2 and 3). Clearly this apparent inability of T7 RNA polymerase to synthesize full-length negative-sense transcripts of VSV RNA could explain the lack of infectivity of pVSV1(−).

The natural signal for transcriptional termination by T7 RNA polymerase is a strong hairpin structure followed by six U residues in the nascent RNA (23). A run of seven U residues exists at each of the intergenic junctions in the negative-strand

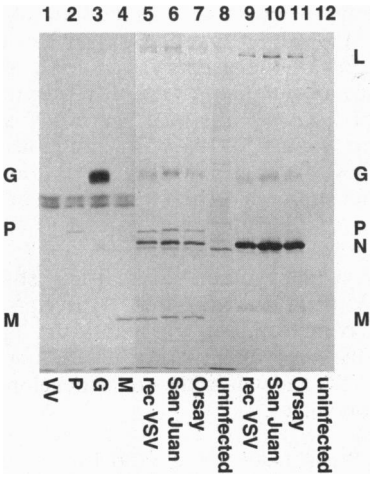

FIG. 4. Protein synthesis directed by the recovered virus. BHK21 cells were infected with recovered virus or authentic VSV at a multiplicity of infection of 5, and viral proteins were radiolabeled with [^{35}S]methionine (10 μCi/ml) at 1 hr postinfection for 5 hr in the presence of actinomycin D (10 μg/ml). Separate plates of BHK21 cells were infected with vTF7-3 and transfected with 5 μg of either the P, G, or M support plasmid, and proteins were labeled by incorporation of [^{3}H]leucine (50 μCi/ml) for 2 hr at 37°C. Cytoplasmic extracts were prepared and proteins were analyzed by electrophoresis in SDS/10% polyacrylamide gels either directly (lanes 5–8) or after immunoprecipitation with an antiserum raised against purified VSV (lanes 1–4, and 9–12). Lanes 1–4, vTF7-3-infected cells transfected with no DNA (lane 1), P support plasmid (lane 2), G support plasmid (lane 3), or M support plasmid (lane 4); lanes 5 and 9, cells infected with recovered (rec) VSV; lanes 6 and 10, cells infected with VSV Indiana (San Juan); lanes 7 and 11, cells infected with VSV Indiana (Orsay); lanes 8 and 12, uninfected cells. The VSV L, G, P, N, and M proteins are indicated for reference.

of VSV RNA, and among the transcription products from pVSV1(−) were four discrete RNAs of the appropriate size to represent the products of termination at the intergenic junctions (Fig. 5, lane 2). We therefore investigated the behavior of T7 RNA polymerase when transcribing a VSV intergenic junction in the negative sense, as compared with the positive sense. We focused on the N–P intergenic region, which was cloned in both orientations into transcription plasmids between the T7 promoter and the HDV ribozyme/T7 terminator cassettes (Fig. 5 *Lower*). Products of *in vitro* transcriptions from each of these circular plasmids in the presence of [^{3}H]UTP were analyzed by 1% agarose/urea gel electrophoresis and fluorography (Fig. 5). Plasmid 8(+)NP, which generated positive-sense transcripts of the NP intergenic junction (Fig. 5, *Lower*), gave the expected two RNAs that resulted from transcriptional termination at the T7 termination signal and the subsequent ribozyme-mediated self-cleavage to generate authentic VSV 3′ termini (Fig. 5, lane 5, solid and hatched arrowheads, respectively). The smaller, 200-nt product of self-cleavage had run off this gel. In contrast, plasmids 8(−)NP and 8(+)PN, which were designed to generate negative-sense transcripts of the N–P intergenic junction, each yielded a major smaller RNA product in addition to the expected products of T7 termination and self-cleavage (Fig. 5, lanes 4 and 6, open arrowhead). The sizes of these smaller RNAs were consistent with termination at or very close to the N–P intergenic junction, as shown by comparison with the size of the RNA made by runoff transcription from plasmid 8(+)PN linearized at the *Eco*RV site which is 7 nt from the N–P intergenic junction (Fig. 5, lane 7 and *Lower*). Further, the RNA products directed by pVSV1(−) indicate that similar termination occurred to a greater or lesser extent at the other intergenic junctions (Fig. 5, lane 2).

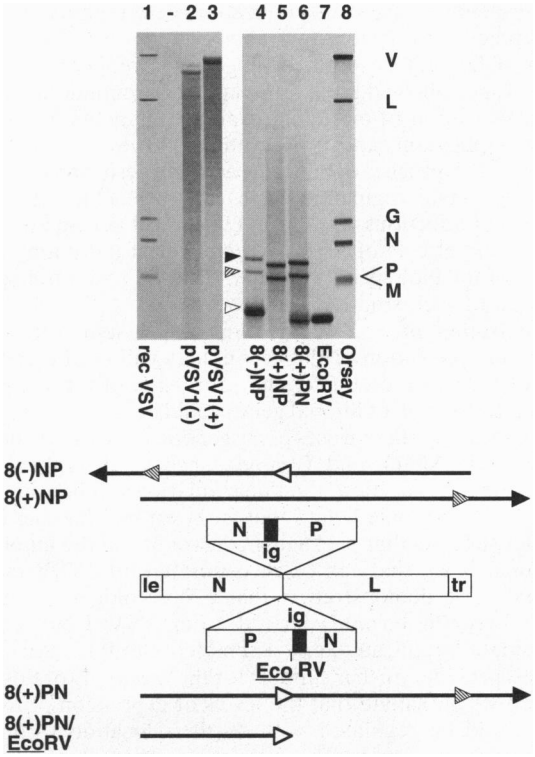

FIG. 5. T7 RNA polymerase terminates transcription at VSV intergenic junctions in the negative sense. (*Upper*) Labeled RNAs were synthesized from the indicated plasmids *in vitro* by T7 RNA polymerase in the presence of [^{3}H]UTP (80 μCi/ml) and were analyzed by electrophoresis in 1% agarose/urea gels. For comparison lanes 1 and 8 show the viral genomic RNA (V) and mRNAs (L, G, N, P, and M) labeled in cells infected with recovered (rec) VSV and authentic VSV Indiana (Orsay), respectively. Note that the P and M mRNAs comigrate. Products of *in vitro* transcription are shown for pVSV1(−) linearized by digestion with *Nhe* I (lane 2), pVSV1(+) linearized by digestion with *Nhe* I (lane 3), 8(−)NP circular DNA (lane 4), 8(+)NP circular DNA (lane 5), 8(+)PN circular DNA (lane 6), and 8(+)PN DNA linearized by digestion with *Eco*RV (lane 7). (*Lower*) Schematic representation of the VSV-specific regions transcribed by T7 RNA polymerase in the plasmids 8(−)NP, 8(+)NP, and 8(+)PN. The T7 transcripts generated from these plasmids are indicated by arrowheads in *Upper* and *Lower*: solid arrowheads, transcripts that terminated at the T7 terminator; hatched arrowheads, transcripts that terminated at the T7 terminator but were subsequently cleaved by the HDV ribozyme to yield authentic VSV 3′ genomic or antigenomic termini; open arrowheads, transcripts that terminated near the N–P intergenic junction. le and tr, VSV leader and trailer genes; ig, N–P intergenic region.

DISCUSSION

Implications of Recovery of Infectivity for Studies on Cis-Acting Elements and Trans-Acting Factors. This successful recovery of infectious VSV from cDNA clones is an achievement that will permit the genetic manipulation of the entire genome of VSV, the prototypic nonsegmented negative-strand RNA virus. VSV is one of the simplest nonsegmented negative-strand RNA viruses, having only five genes that encode five structural proteins and a nonstructural protein encoded in a second open reading frame of the P gene (24). The discovery that VSV packages its own polymerase (25) and the development of *in vitro* systems to monitor both transcription and replication allowed the initial investigations of transcription and replication of VSV (for reviews, see refs. 26 and 27). The expression and mutagenesis of individual viral proteins, and the ability to complement temperature-sensitive mutants of VSV with proteins generated from cDNAs, allowed evaluation of their roles in various steps of the virus life cycle. The studies

of trans-acting elements in replication were then extended by development of a system that allowed recovery of replicable RNAs of DI particles and of subgenomic replicons (12, 17). This advance allowed the manipulation of genomic sequences and investigation of cis-acting genomic elements in encapsidation, replication, and transcription. However, despite this progress, the precise details of each of these processes and their coordinate regulation have yet to be elucidated. The recovery of infectious virus from cDNA will permit an assessment of the effects of precisely engineered mutations on all aspects of the biology of VSV and it should revolutionize our approach to such studies.

Exploitation of VSV as a Viral Expression Vector. An important application of this technology will be the development of VSV as a vector for the expression of foreign genes. It seems likely that additional genes could be incorporated into VSV, as other rhabdoviruses carry genetic material in addition to the N, P, M, G, and L genes, such as the rabies virus pseudogene. In addition, the linear relationship between particle size and genome length that is shown by VSV and its DI particles suggests that packaging constraints on the amount of additional RNA that can be incorporated into VSV may be minimal. Our demonstration that a heterologous sequence placed between termini derived from VSV RNA can be encapsidated, replicated, and assembled into infectious particles (19) provides further support for the feasibility of this idea. Further, we anticipate that the levels of expression of foreign genes could be regulated both by their location within the genome and by altering the adjacent cis-acting sequences that function as their promoters. Also, when VSV infects a cell the ribonucleoprotein complex is delivered into the cytoplasm as a transcriptionally active unit, and primary transcription is therefore independent of genome replication. Thus it seems likely that vectors could be developed that would be transcribed but not replicated. Alternatively, by manipulation of the genes encoding the M and G proteins it should be possible to generate vectors that can replicate in the initially infected cells but are unable to spread from cell to cell.

The possibility of manipulating the cell tropism of viruses so that they deliver genes only to the target cells of interest would be a step forward in the development of viral vectors for gene delivery. With VSV, target cell specificity is mediated by the attachment glycoprotein, G, which permits the infection of virtually all animal cells that have been studied. This property has been exploited in generating pseudotypes of retrovirus vectors with an expanded host range (28). With VSV, pseudotypes are also formed, and it seems likely that further application of this technology could permit the development of VSV as a targetable gene-delivery vector.

Polarity of T7 Transcript and Effect on Recovery of Infectious Virus. In view of our past work where replicable VSV DI RNA and a variety of subgenomic replicons were recovered from negative-sense primary transcripts (12, 17), our failure to recover infectious virus from pVSV1(−) was unexpected. Analysis of the RNAs transcribed *in vitro* from pVSV1(−) demonstrated that T7 RNA polymerase did not generate significant amounts of genome-length negative-sense transcripts of VSV (Fig. 5, lane 2). An investigation of this problem revealed that T7 RNA polymerase partially terminated transcription at or near the VSV intergenic regions in the negative sense (Fig. 5, lanes 2, 4, and 6), but not in the positive sense (lanes 3 and 5). If such prematurely terminated transcripts were generated in transfected cells, they would have serious consequences for recovery of infectivity. These transcripts could compete with any genome-length RNAs for the N, P, and L proteins expressed from the support plasmids but would not be competent templates for replication because they lack the required 3′ terminus (17). Further, these transcripts could contribute to any antisense effect on the translation of mRNA produced from the support plasmids, as well as interfere with the encapsidation of the primary transcript. Therefore, we suggest that the cumulative effect of T7 termination at or around each of the VSV intergenic junctions is the primary reason for our failure to recover infectious virus from pVSV1(−). These findings may have implications for understanding the mechanism by which termination of transcription occurs at each of the intergenic junctions when the VSV polymerase is transcribing mRNA.

In summary, we have described the successful recovery of infectious VSV from positive-sense transcripts of a cDNA clone. This accomplishment will allow the application of recombinant DNA technology to investigation of all aspects of the biology of VSV and will facilitate the development of VSV as an expression vector.

Note Added in Proof. Lawson *et al.* (29) have recently presented findings similar to ours.

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