JOURNAL OF VIROLOGY, Jan. 1999, p. 444–452 Vol. 73, No. 1
0022-538X/99/$04.00+0

Overlapping Signals for Transcription and Replication at the 3′ Terminus of the Vesicular Stomatitis Virus Genome

TONG LI AND ASIT K. PATTNAIK*

Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Florida 33136

Received 7 July 1998/Accepted 8 October 1998

Transcription and replication signals within the negative-sense genomic RNA of vesicular stomatitis virus (VSV) are located at the 3′ terminus. To identify these signals, we have used a transcription- and replication-competent minigenome of VSV to generate a series of deletions spanning the first 47 nucleotides at the 3′ terminus of the VSV genome corresponding to the leader gene. Analysis of these mutants for their ability to replicate showed that deletion of sequences within the first 24 nucleotides abrogated or greatly reduced the level of replication. Deletion of downstream sequences from nucleotides 25 to 47 reduced the level of replication only to 55 to 70% of that of the parental template. When transcription activity of these templates was measured, the first 24 nucleotides were also found to be required for transcription, since deletion of these sequences blocked or significantly reduced transcription. Downstream sequences from nucleotides 25 to 47 were necessary for optimal levels of transcription. Furthermore, replacement of sequences within the 25 to 47 nucleotides with random heterologous nonviral sequences generated mutant templates that replicated well (65 to 70% of the wild-type levels) but were transcribed poorly (10 to 15% of the wild-type levels). These results suggest that the minimal promoter for transcription and replication could be as small as the first 19 nucleotides and is contained within the 3′-terminal 24 nucleotides of the VSV genome. The sequences from nucleotides 25 to 47 may play a more important role in optimal transcription than in replication. Our results also show that deletion of sequences within the leader gene does not influence the site of transcription reinitiation of the downstream gene.

The prototypic rhabdovirus, vesicular stomatitis virus (VSV), is an enveloped, nonsegmented, negative-strand RNA virus with a genomic RNA that is 11,161 nucleotides long (38). Within the virion core, the genomic RNA is tightly wrapped around by the RNA-binding nucleocapsid protein (N) forming ribonuclease-resistant nucleocapsid structure (N-RNA) that serves as the template for transcription and replication of the genome by the viral RNA-dependent RNA polymerase. The viral RNA polymerase is a complex of the phosphoprotein (P) and the large protein (L) (11, 13, 32). Genetic and biochemical studies have suggested that the L protein carries all the enzymatic activities necessary for generation of mature viral mRNAs, i.e., ribonucleotide polymerization activity, methyl- and guanylyltransferase activity, and poly(A) polymerase activity (19, 20, 42). P serves as an accessory protein required for the functions of the L protein, and differential phosphorylation of the P protein at different domains appears to influence the transcriptase and replicase activities of the L protein (8, 10, 35, 37).

Following entry of the virus into susceptible cells and uncoating of the viral nucleocapsid in the cytoplasm, the negative-sense nucleocapsid template is first transcribed by the template-associated viral RNA polymerase complex. The polymerase is thought to initiate transcription at the extreme 3′ end of the genome (12) and generates a small 47-nucleotide-long uncapped and nonpolyadenylated leader RNA and five capped and polyadenylated mRNAs for the five structural proteins of VSV, namely, N, P, M, G, and L. However, recent studies have also suggested that the viral RNA polymerase may initiate transcription by entering at the internal sites in the genome (9). Transcription from the viral genome is sequential, which reflects the physical order of the genes from the 3′ end of the genome (1, 3). Furthermore, transcription is attenuated at each of the gene junctions, resulting in the generation of a gradient in the molar amounts of the mRNAs which also follows the gene order (22, 49). Following translation of the mRNAs, the negative-sense nucleocapsid template is used by the viral polymerase for replication, resulting in the synthesis of a full-length positive-sense antigenomic RNA in the form of nucleocapsid. The antigenomic RNA is subsequently used for further rounds of replication to generate the genomic-sense nucleocapsids.

During the replicative cycle of VSV, the RNA synthetic events, such as transcription, replication, and encapsidation, are controlled by various interactions between the RNA template, the nucleocapsid protein (N), and the RNA polymerase complex. It has been proposed that the interaction between the N protein and the nascent RNA strand is critical for the switch from transcription to replication (6). The *cis*-acting signals that mediate the RNA synthetic events are located at the termini as well as at the intergenic junctions in the viral genome. With the recent development of methods that allow genetic manipulation of the genomes of VSV and its defective interfering (DI) particles (26, 30, 33, 47, 51), it has been possible to address many of the long-standing questions relating to the role(s) and requirements of various sequence elements at the termini and intergenic junctions in transcription and replication of the viral genome. With cDNAs encoding transcription- and replication-competent minigenomes or minireplicons of VSV, it has been shown by mutational analysis that the first three nucleotides of the conserved sequence 3′ UUGUC 5′, found at each of intergenic junctions, are required for efficient transcription (48);

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Miami School of Medicine, P. O. Box 016960 (R-138), Miami, FL 33136. Phone: (305) 243-6711. Fax: (305) 243-4623. E-mail: apattnaik@mednet.med.miami.edu.

the 3′ AUAC$(U)_7$ 5′ sequence element is required for polyadenylation and subsequent transcription termination (4, 21); the dinucleotide 3′ (G/C)A 5′ may be required for transcription termination (48), whereas other studies suggest that the dinucleotide may function as a spacer element between the transcription termination and reinitiation signals (5, 21). It has also been shown that the termini of VSV and its DI particle genome contain all the necessary signals for replication (34, 50) and that the termini of DI particle genome and the 3′ terminus of VSV antigenome contain a replication enhancer sequence (RES) that upregulates replication (30). The presence of RES element in the DI genome has been proposed to be a key factor for efficient replication of DI RNA as well as for asymmetric levels of replication of genomic and antigenomic RNAs of VSV (30).

Unlike the 3′ terminus of the viral antigenomic RNA, which contains only the signals for replication, the 3′ terminus of the viral genome must contain the signals for both transcription and replication. By using reconstituted in vitro transcription system and synthetic VSV nucleocapsids (31), it was shown that only the 3′-terminal 22 nucleotides were sufficient to serve as excellent transcription templates (44). To examine the transcription and replication signals at the 3′ terminus in the context of VSV genome, we have undertaken a deletion mutational analysis of the first 47 nucleotides corresponding to the leader gene. We have analyzed the mutant templates for their ability to replicate to generate the genomic- and antigenomic-sense RNA products as well as to transcribe. Our results show that the first 24 nucleotides contain overlapping signals for both transcription and replication. Downstream sequences from nucleotides 25 to 47 do not influence replication appreciably;however, they appear to be required for optimal levels of transcription. These results suggest that sequences from nucleotides 25 to 47 within the leader gene may play a more important role in optimal transcription than in replication. Furthermore, deletions within the 3′ terminus of VSV genome do not influence the site at which transcription of mRNA is reinitiated.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK-21) cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) containing 7.5% heat-inactivated fetal bovine serum (FBS) and the antibiotics penicillin G (100 U/ml), streptomycin (20 μg/ml), and kanamycin (20 μg/ml). Thymidine kinase-negative human 143B cells were also maintained as monolayers in Eagle's MEM supplemented with 5% FBS. Stocks of VSV (Indiana serotype, San Juan strain) and the recombinant vaccinia virus (vTF7-3) carrying the bacteriophage T7 RNA polymerase gene (15) were prepared in BHK-21 cells, and infectivity titers of these viruses were determined by plaque assay with BHK-21 and 143B cells, respectively.

Minigenome and protein expression plasmids. Plasmids pN, pP, and pL, encoding the VSV nucleocapsid protein (N), the phosphoprotein (P), and the large protein (L), respectively, under the control of the T7 RNA polymerase promoter have been described previously (36).

The plasmid p9BN (Fig. 1A), encoding a VSV antigenomic minireplicon, has also been described previously (30). Transcription from p9BN by T7 RNA polymerase and subsequent cleavage by the hepatitis delta virus ribozyme generate a positive-sense VSV antigenomic RNA (9BN) of 1,618 nucleotides.

Site-directed mutagenesis. A series of deletion mutants (Fig. 1B) spanning the first 47-nucleotide region of the 5′ terminus of the VSV antigenomic RNA (which corresponds to the complementary sequences of the 3′ terminus of VSV genome) was generated, with p9BN as the template. These plasmids were designated p9BNΔ3′1-6, p9BNΔ3′7-12, p9BNΔ3′13-18, p9BNΔ3′19-24, p9BNΔ3′25-30, p9BNΔ3′31-36, p9BNΔ3′37-42, p9BNΔ3′43-47, p9BNΔ3′25-36, and p9BNΔ3′37-47, which contained deletion of nucleotides 1 to 6, 7 to 12, 13 to 18, 19 to 24, 25 to 30, 31 to 36, 37 to 42, 43 to 47, 25 to 36, and 37 to 47 at the 3′ terminus of the VSV genome, respectively. Two more substitution mutants, p9BN-m1 and p9BN-m2, encoding minireplicons (9BN-m1 and 9BN-m2) in which sequences from 25 to 36 and 37 to 47 were replaced by random heterologous sequences GUCAAGCUACGU and GUCAAGCUAGC, respectively, were also generated. All these mutants were generated by the PCR megaprimer method (40). A negative-sense primer containing the desired sequence deletions or substitutions and a primer containing the unique *Fsp*I site that annealed to sequences within the β-lactamase gene of the vector were used to amplify a fragment of approximately 1,200 bp by PCR, with p9BN as the template. The fragment was then used as megaprimer in a second round of PCR amplification, with p9BN as the template, along with another negative-sense primer containing a unique *Bgl*II site (at position 210 of the VSV genome) that annealed to nucleotides 226 to 209 of the N gene. The second PCR product (~1.4 kbp) was digested with *Bgl*II and *Fsp*I and subcloned into p9BN plasmid digested with the same enzymes. After transformation of competent DH5α cells, bacterial colonies carrying the recombinant plasmids were screened and the mutant plasmids were identified by nucleotide sequencing. Standard methods of plasmid subcloning and preparation (2, 39) were used.

Virus infections and DNA transfections. BHK-21 cells were grown in 60-mm-diameter plates or 35-mm-diameter 6-well plates to about 90% confluency. These cells were infected with the recombinant vaccinia virus (vTF7-3) at a multiplicity of infection of 10 PFU per cell. Forty-five minutes after virus adsorption at 37°C, inoculum was removed, the cells were washed twice with Dulbecco's modified MEM (DMEM) without serum and transfected with various plasmid DNAs with lipofectin (Gibco/BRL, Gaithersburg, Md.) according to the manufacturer's specifications or with a transfection reagent prepared in the laboratory as described previously (7). At 4 to 5 h posttransfection, medium from transfected cells was removed, cells were washed twice with DMEM containing 2% FBS, and incubated with the appropriate volume of the same medium. For RNA replication and transcription assays in 60-mm-diameter plates, 3 μg of pN, 2 μg of pP, 1 μg of pL, and 5 μg of p9BN or mutant p9BN plasmids were used. These plasmid amounts were reduced by half when 6-well plates were used in the experiments.

Metabolic labeling and analyses of RNA. At 15 to 16 h posttransfection, cells were pretreated with 15 μg of actinomycin D per ml of DMEM at 37°C for 45 min and subsequently exposed to 15 μCi each of [^{3}H]uridine and/or [^{3}H]adenosine per ml of DMEM containing 2% FBS and the same concentrations of actinomycin D for 6 to 8 h. After labeling, cytoplasmic extracts were prepared in lysis buffer as described previously (30, 36). Replicated RNAs in nucleocapsids were immunoprecipitated by polyclonal anti-VSV antibodies. RNAs were recovered from immunoprecipitated nucleocapsids by extraction with phenol and chloroform and precipitation with ethanol. The RNAs were subsequently resolved by electrophoresis in agarose-urea gels (28) and detected by fluorography (25). For transcription studies, cytoplasmic extracts were collected as described above. Labeled RNAs present in extracts were purified by phenol-chloroform extraction, analyzed by agarose-urea gel electrophoresis, and detected by fluorography as described above.

Primer extension analysis. At 24 h posttransfection, cytoplasmic extracts from transfected cells were harvested. Total unlabeled RNA from the extracts was recovered by extraction with phenol and chloroform and subjected to RNase-free DNase (Promega Biotech, Madison, Wis.) digestion to remove residual transfected plasmid DNAs that may have been present in the RNA preparations. After digestion, RNA was extracted with phenol-chloroform and recovered by ethanol precipitation. Dried RNA pellet was resuspended in 5 μl of H_2O, boiled for 1 min, and quick-chilled ice-water. Annealing was performed by mixing the template RNA with 1 μl (20 ng/μl) of minus-sense N gene primer 5′ CCTCAT TTGCAGG 3′ (which anneals to N mRNA at a site that is 80 nucleotides from its 5′ terminus) and 2 μl of 5× first-strand synthesis buffer (Gibco/BRL). The mixture was heated at 65°C for 5 min and slowly cooled to room temperature. The following reaction components were added at the final concentrations of 2 mM (each) dGTP, dCTP, and dTTP; 0.1 mM dATP; 10 mM dithiothreitol; 0.1 mM Tris (pH 8.0); 0.01 mM EDTA; 0.75 mCi of [^{35}S]dATP (1,250 Ci/mmol); and 20,000 U of Moloney murine leukemia virus reverse transcriptase per ml of reaction volume. The primer extension reaction was performed at 37°C for 90 min. The reaction was terminated by adding 6 μl of sequencing reaction stop solution (United States Biochemicals, Cleveland, Ohio). The samples were denatured at 85°C for 3 min, and 4 μl of the reaction products was electrophoresed in a 6% polyacrylamide sequencing gel alongside a sequence ladder generated with the same primer and p9BN template. The primer extension products were detected by autoradiography.

RESULTS

In order to identify the replication and transcription signals at the 3′ terminus of the VSV genome, we used the plasmid p9BN (30) encoding an antigenomic minireplicon RNA (9BN) containing the VSV N gene and part of the L gene flanked by the 3′- and 5′-terminal sequences from the VSV antigenome. The processes of encapsidation, replication, and transcription of 9BN RNA in transfected cells in the presence of the viral proteins are schematically depicted in Fig. 1A. Briefly, in transfected cells, the antigenomic RNA template generated from p9BN plasmid by T7 RNA polymerase is encapsidated by the N protein and replicated by the VSV RNA polymerase (P and L proteins) to produce the full-length minus-sense RNA,

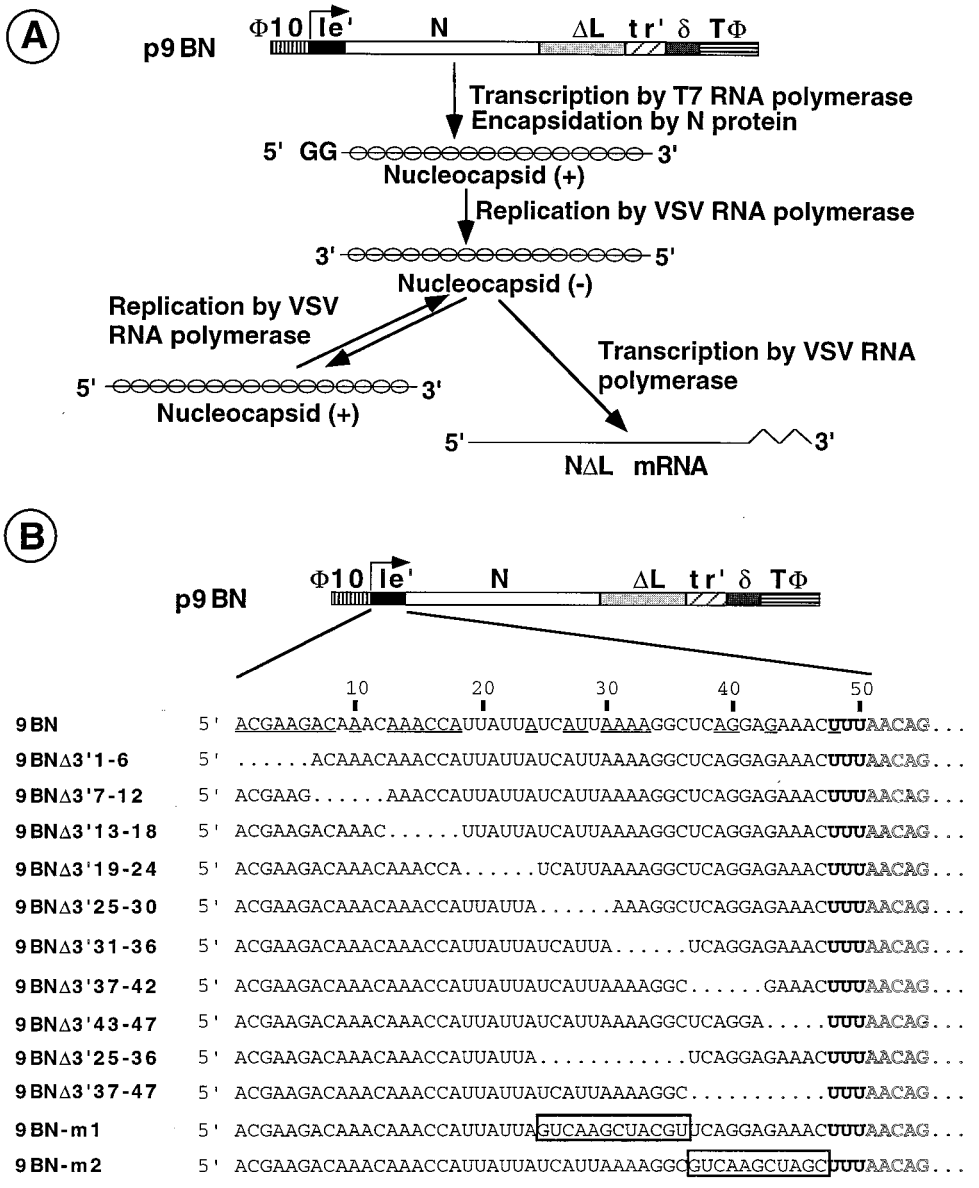

FIG. 1. (A) The plasmid p9BN encoding the antigenomic plus-sense minireplicon. Only the relevant regions of the plasmid are shown. ϕ10, Tϕ, and δ represent the T7 RNA polymerase promoter, the terminator, and the hepatitis delta virus ribozyme sequences, respectively; le′ and tr′ represent complementary sequences of leader and trailer regions of the VSV genome; N and ΔL represent the coding sequences of the N and part of the L gene of VSV. In plasmid-transfected cells, the antigenomic minireplicon, synthesized from p9BN by T7 RNA polymerase, is encapsidated by the viral N protein to generate plus-sense nucleocapsid with two additional guanosine residues at the 5′ terminus. Replication of this nucleocapsid by VSV RNA polymerase generates the genomic minus-sense nucleocapsid, which serves as the template for transcription by VSV RNA polymerase to synthesize NΔL mRNA and also for replication to generate the antigenomic plus-sense nucleocapsid. The plus-sense RNA synthesized by VSV RNA polymerase differs from the plus-sense RNA synthesized by T7 RNA polymerase by the absence of the two 5′-terminal guanosine residues. (B) Various mutant minireplicons with deletion or substitution of nucleotides (as shown) at the 5′ terminus of the antigenomic minireplicon (9BN), which corresponds to the leader gene sequences at the 3′ terminus of genomic-sense RNA. Sequences of the first 55 nucleotides at the 5′ terminus of the antigenomic RNA are shown. They correspond to 47 nucleotides of leader RNA sequence, three nontranscribed intergenic nucleotides (UUU, bold-faced), and the first five nucleotides (AACAG, outlined) of N mRNA. Underlined sequences represent nucleotides that are complementary to the sequences at the 3′ terminus of the antigenome. Deleted nucleotides within the leader region are represented by dots. Random heterologous sequences that replace leader sequences are shown in boxes.

which in turn serves as the template for further rounds of replication as well as for transcription to generate NΔL mRNA (30).

Replication signal(s) at the 3′ terminus of VSV genome. A series of six- and five-nucleotide deletions spanning the 5′-terminal 47-nucleotide region of the antigenomic-sense minireplicon RNA was generated (Fig. 1B). These templates, when replicated by VSV polymerase, generate genomic-sense templates with deletion of sequences at the 3′ terminus, and therefore the effect of these deletions on transcription and replication could be examined. These mutants were designated 9BNΔ3′1-6, 9BNΔ3′7-12, 9BNΔ3′13-18, 9BNΔ3′19-24, 9BNΔ3′25-30, 9BNΔ3′31-36, 9BNΔ3′37-42, and 9BNΔ3′43-47 (Fig. 1B). Since the wild-type and mutant minireplicon RNAs must be first encapsidated by the N protein to serve as templates for replication, we initially examined the ability of mutant RNAs to be encapsidated by the N protein. When cells expressing the N protein and the wild-type or mutant minireplicon templates were labeled with [^{3}H]uridine, almost similar amounts of labeled wild-type or mutant minireplicons were immunoprecipitated from the cell extracts (data not shown),

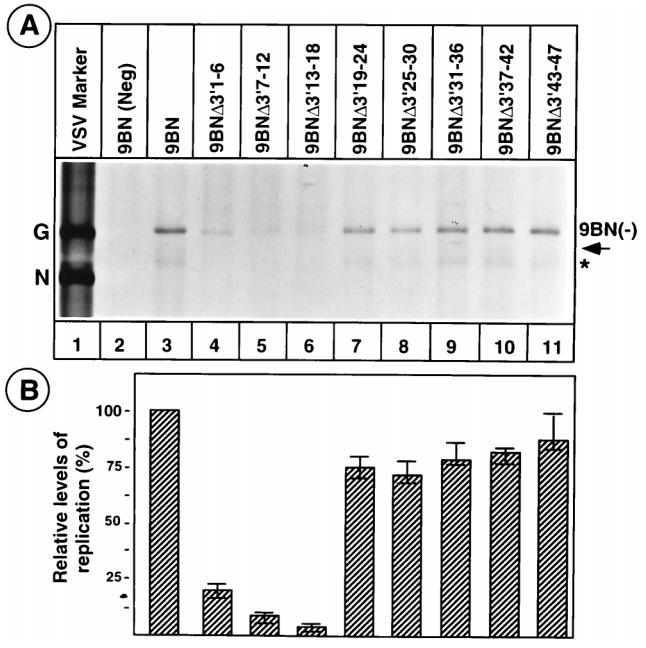

FIG. 2. (A) Replication of various mutant antigenomic minireplicons to generate genomic-sense 9BN(−) RNA. Cells infected with vTF7-3 were transfected with plasmids encoding the N, P, and L (or without L in the negative control, lane 2) proteins and either p9BN or deletion mutant plasmids. At 16 h posttransfection, cells were labeled for 6 h with [^{3}H]uridine in the presence of actinomycin D. Replicated RNAs in the nucleocapsids were immunoprecipitated, purified, and analyzed by agarose-urea gel electrophoresis as described in Materials and Methods. G and N represent the G mRNA and N mRNA of VSV isolated from infected cells. The migration position of plus-sense RNA is indicated by an arrow. 9BN(−) is the negative-sense replication product. An asterisk indicates the band of NΔL mRNA, which is sometimes immunoprecipitated (30, 50) by anti-VSV antibodies. (B) Relative levels of replication of deletion mutant minireplicons to produce genomic-sense products. Histograms show averages and range of levels of replication of various deletion mutants (described at the top of panel A) from three independent experiments.

suggesting that encapsidation of mutant minireplicons was unaffected by deletion of sequences at the 5′ terminus. This was surprising, since the encapsidation signal(s) is presumed to reside at the 5′ terminus of the RNA. It is possible that the six-nucleotide deletions are not large enough to disrupt the encapsidation signal or that deletion of small regions could be functionally replaced by adjacent sequences.

The ability of the minireplicon RNAs to be replicated in cells expressing the viral proteins N, P, and L was then analyzed. Results (Fig. 2) show that the first three deletion mutants, Δ3′1-6, Δ3′7-12, and Δ3′3-18, replicated to produce very low levels (approximately 5 to 19%) of genomic-sense 9BN(−) RNA (Fig. 2A, lanes 4 to 6 and Fig. 2B) compared to the wild-type template (lane 3). The other mutants with deletion of sequences spanning nucleotides 19 to 47 replicated relatively well (Fig. 2A, lanes 7 to 11 and Fig. 2B), and the levels of replication of these mutants were at least 75% of that of the parental template. It should be noted that in these replication assays, only the genomic-sense 9BN(−) replication products could be detected. The antigenomic-sense 9BN(+) replication products (whose relative migration position in this gel is indicated by the arrow in Fig. 2A) were barely detectable even upon longer exposure of the fluorogram because the genomic-sense replication products accumulate at much greater levels (>90%) than the antigenomic-sense replication products (14, 30). From the data shown in Fig. 2A, it is also possible that the mutant templates may have lost the ability to replicate to generate the antigenomic-sense replication products. Nevertheless, it appears that deletion of sequences within the first 18 nucleotides significantly affects replication, whereas deletion of downstream sequences seems to have a less dramatic effect on replication.

As shown in Fig. 1, the mutant plasmids encode antigenomic RNAs containing deletions at their 5′ termini, so replication from the 3′ termini of these mutant templates to generate the genomic-sense RNAs was considered to remain unaffected. However, synthesis of plus-sense RNAs from the newly synthesized minus-sense RNAs might be affected because the templates now contain the deletions at its 3′ terminus, from where replication is initiated. Thus, the minus-sense RNAs that we detected in Fig. 2A (lanes 4 to 6) may have been generated from the first round of replication only (if the deletions on the negative-sense RNAs were lethal for further rounds of replication) or may be the accumulated products of many rounds of replication at low levels (if the deletions reduced but did not abrogate replication). To distinguish between these possibilities, we wanted to examine the synthesis of plus-sense replication products from these mutant templates. The replication assay in the presence of actinomycin D (as shown in Fig. 2A) is not sensitive enough to allow clear detection and quantitation of the plus-sense replication products. More sensitive methods, such as Northern blotting or RT-PCR, cannot distinguish the plus-sense RNA replication products of the VSV RNA polymerase from the plus-sense RNA synthesized from the transfected plasmid by the T7 RNA polymerase because of their similar size. During replication, the 5′ terminus of T7 RNA polymerase-derived transcripts containing extra nonviral nucleotides (two guanosine residues) is corrected by VSV RNA polymerase (Fig. 1A). The replication products of VSV polymerase are two nucleotides shorter than the templates produced by the T7 RNA polymerase (33). Therefore, we examined the plus-sense replication products by analyzing their 5′ termini by primer extension analysis.

In a control experiment, total RNA from BHK-21 cells infected with vTF7-3 and transfected with p9BN and the plasmids encoding the VSV proteins, N, P, and L (L plasmid was omitted in the negative control) were isolated and subjected to primer extension analysis with a negative-sense primer complementary to the N gene coding sequences 80 nucleotides downstream of the N mRNA start site. The primer can hybridize to all plus-sense RNA species, including the plus-sense RNA replication products, and generate extension products. Under conditions of replication, the 5′ termini of four different RNA species, as follows, could be mapped (Fig. 3A, lane 6): (i) the RNA transcripts with two extra guanosine residues (the top thick band of the doublet marked a), which were generated from the p9BN plasmid by T7 RNA polymerase; (ii) the VSV polymerase-derived replication products (the bottom band marked by a dot in the doublet a), which are two nucleotides shorter; (iii) the N mRNA synthesized by T7 RNA polymerase (the doublet marked b) from transfected pN plasmid; and (iv) the NΔL mRNA synthesized from 9BN(−) template by VSV polymerase (the doublet marked c). Each of the primer extension products migrated as doublets, since their templates contained a mixture of uncapped and capped RNAs as a result of capping at the 5′ terminus by vaccinia virus guanylytransferase or by VSV RNA polymerase. Under conditions (in the absence of L protein) in which replication and transcription did not occur (Fig. 3A, lane 5), the bottom band of the doublet a, representing the VSV RNA polymerase-derived plus-sense RNA replication products, as well as the doublet c, representing the VSV RNA polymerase-derived transcription products, was not detected. Furthermore, the intensity of the primer

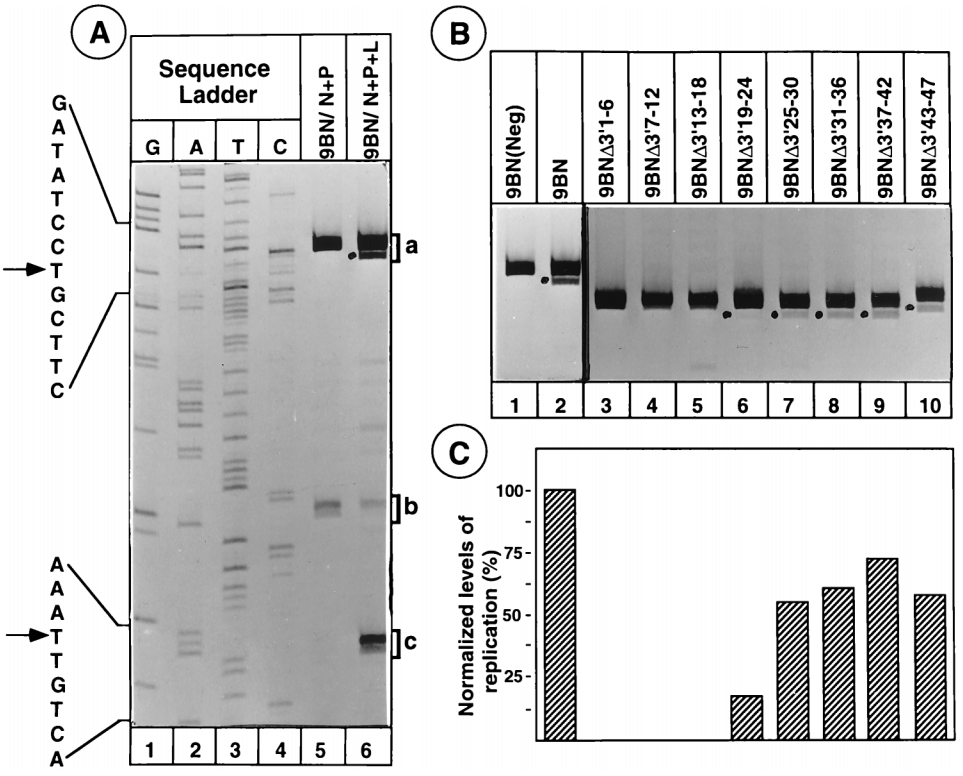

FIG. 3. (A) Primer extension analysis to detect 5′ termini of various plus-sense RNA products in transfected cells. Lane 6, extension products of RNAs from cells after transcription and replication; lane 5, extension products of RNAs without transcription or replication. The extension products labeled a, b, and c are described in the text. The 5′ terminus of the replication product (indicated by a dot in lane 6) maps to the T residue (identified by the top arrow on the left) corresponding to the first nucleotide (U) in the VSV genome. The 5′ terminus of the NΔL mRNA transcription product maps to the first T residue (identified by the bottom arrow on the left) of the transcription initiation signal UUGUC in the VSV genome. The more intense top bands in doublets c and b most likely represent the extension product of capped mRNA. (B) Analysis of plus-sense RNA replication products from mutant templates by primer extension. Replication products are identified by dots in the lanes. Only the top portion of the gel is shown. (C) Average normalized replication of various mutants (shown at the top of panel B) from two separate experiments as determined with the following formula: Normalized levels of plus-sense replication products (%) = relative levels of plus-sense replication products/relative levels of minus-sense template × 100.

extension products was proportional to the amount of total RNA used in the reaction (data not shown), indicating that primer extension analysis can be used to quantitatively detect the plus-sense replication products.

By using primer extension analysis, we next examined replicability of the deletion mutant minireplicons to generate the plus-sense RNA products. Results (Fig. 3B) show that plus-sense RNA replication products could be detected for mutants Δ3′19-24, Δ3′25-30, Δ3′31-36, Δ3′37-42, and Δ3′43-47 (lanes 6 to 10, respectively), as evidenced by the presence of the faster-migrating primer extension products (shown by dots), although levels of replication of these mutants were lower than that of the parental template (lane 2). No plus-sense RNA replication products could be detected for the mutants Δ3′1-6, Δ3′7-12, and Δ3′13-18, even upon longer exposure of the gel, indicating that these mutant templates had lost the ability to replicate. It is noteworthy that all the mutant templates, with or without the ability to be replicated, still contained the introduced deletions (as indicated by the primer extension products, which are six or five nucleotides shorter than their parental counterpart), suggesting that VSV RNA polymerase did not correct back these deletions in order to generate the competent templates for replication.

Since the amount of plus-sense RNA replication products depends on the amount of minus-sense RNA templates, we normalized the levels of plus-sense RNA replication products based on the amount of the corresponding templates as shown in Fig. 2B. Results (Fig. 3C) suggest that the first 24 nucleotides at the 3′ terminus of VSV genome contain the signal(s) for replication, whereas the sequences from nucleotides 25 to 47 may not be as critical for replication. These results suggest that the low levels of minus-sense replication products that were detected in lanes 4 to 6, Fig. 2A, represent only the products from the initial round of replication by the VSV RNA polymerase of plus-sense RNAs synthesized from the transfected plasmid by the T7 RNA polymerase.

Transcription signal(s) at the 3′ terminus of VSV genome. We next examined the 3′ terminal sequences of VSV genome that are required for mRNA transcription. Total RNA from transfected cells radiolabeled in the presence of actinomycin D was analyzed in agarose-urea gel. Results (Fig. 4A) show that no NΔL mRNA was detected from the first three deletion mutants, Δ3′1-6, Δ3′7-12, or Δ3′13-18 (lanes 4 to 6, respectively), even after much longer exposure of the fluorogram. The levels of NΔL mRNA from Δ3′25-30, Δ3′31-36, Δ3′37-42, and Δ3′43-47 (lanes 8 to 11, respectively) were 25% to 35% of that of the wild-type template (lane 3), whereas Δ3′19-24 template supported transcription at a level of only about 10% (lane 7). When the levels of transcription were normalized by considering the levels of minus-sense template (as shown in Fig. 2), the results (Fig. 4B) suggested that transcription activity of the mutants Δ3′25-30, Δ3′31-36, Δ3′37-42, and Δ3′43-47 were 35 to 45% of the level of wild-type template. The mutant Δ3′19-24 was 12 to 15% as active as the wild-type template, whereas the

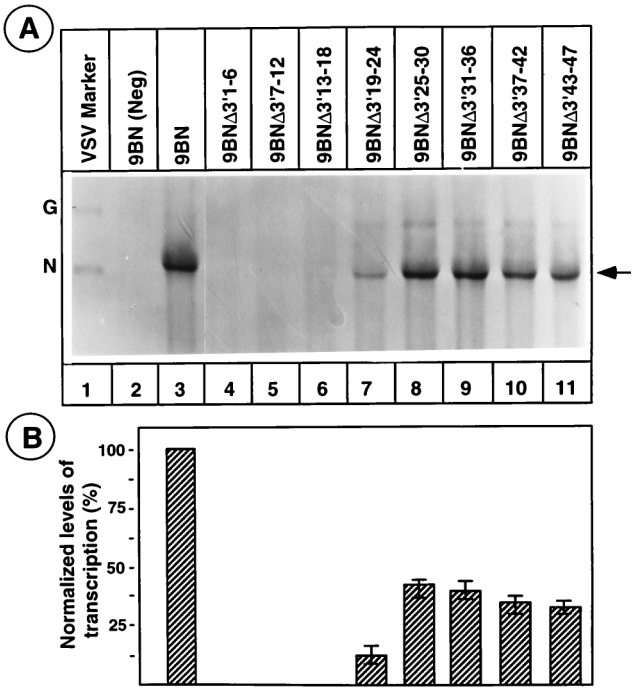

FIG. 4. (A) Analysis of transcription from the mutant templates. Cells were infected with vTF7-3 and transfected with plasmids encoding N, P, and L and wild-type or mutant minireplicons. At 16 h posttransfection, cells were treated with actinomycin D and labeled with [^{3}H]uridine for 6 h. Total labeled RNA from these cells was analyzed by electrophoresis in agarose-urea gel. Lane 2 shows RNA from the negative control (no L plasmid in transfection) sample. An arrow indicates the NΔL mRNA transcription product. (B) Normalized levels of transcription from the mutant templates. Values were obtained by using the formula: Nomarlized transcription (%) = relative levels of transcription/relative levels of minus-sense template × 100. Histograms represent the averages and ranges of values from three separate experiments.

three deletion mutants Δ3′1-6, Δ3′7-12, and Δ3′13-18 were completely inactive in transcription. Furthermore, it must be pointed out that the transcription signal(s) at the 3′ terminus of minus-sense template is much stronger than the replication signal(s), since the transcription products represented greater than 95% of the total RNA synthesized from the minus-sense template.

In the experiment described in Fig. 4, the plasmid (p9BN) encoding an antigenomic-sense minireplicon was used. This minireplicon must first be replicated to produce the genomic-sense templates for transcription, so transcription activity was dependent upon prior replication of the input template. In order to directly assess the effect of deletions on transcription, a genomic-sense minireplicon template containing the luciferase reporter gene (10) was used. A series of minireplicon templates containing the deletions (as described in Fig. 1B) at the 3′ terminus was generated. These mutant templates as well as the wild-type template contained deletions of sequences 6 to 12 at the 5′ terminus, rendering the templates inactive in further rounds of replication and amplification (30). This was necessary to directly compare the transcription activities of the wild-type and mutant templates in the absence of replication and subsequent amplification. When transcription activities of these mutant templates were determined as a function of luciferase enzyme activity in transfected cells, the results (data not shown) confirmed the data shown in Fig. 4B.

Do downstream sequences play role in replication and/or transcription? Results from previous experiments (Fig. 2 to 4) suggested that the first 24 nucleotides at the 3′ terminus of VSV genome contained the essential signal(s) for replication and transcription. Furthermore, the six-nucleotide deletions spanning nucleotides 25 to 47 did not appear to have any major negative effects on transcription or replication. It is possible that this region contains functionally redundant signals and that the six-nucleotide deletions may have only a partial effect on replication and/or transcription. To investigate if larger deletions within this region would have a more dramatic effect on replication and/or transcription, we generated two mutant plasmids, p9BN Δ3′25-36 and p9BN Δ3′37-47 (Fig. 1B), that encode plus-sense minireplicon templates with deletions of nucleotides 25 to 36 and 37 to 47 at their 5′ termini. The initial round of replication of these RNAs would generate minus-sense genomic RNA templates with the corresponding deletions at the 3′ end.

When the plus-sense mutant minireplicon templates were analyzed for their ability to replicate to generate minus-sense genomic RNA [9BN(−)], both the mutant templates replicated at levels similar to that of the parental template (Fig. 5A). When replication of minus-sense genomic RNAs to produce plus-sense RNA was examined by primer extension analysis, it was found that the larger deletion mutants replicated to generate plus-sense RNAs at levels 40 to 45% of that of the parental template (Fig. 5B and 5D). However, transcription activities of these deletion mutants were significantly lower than that of the parental template (Fig. 5C and D). These two

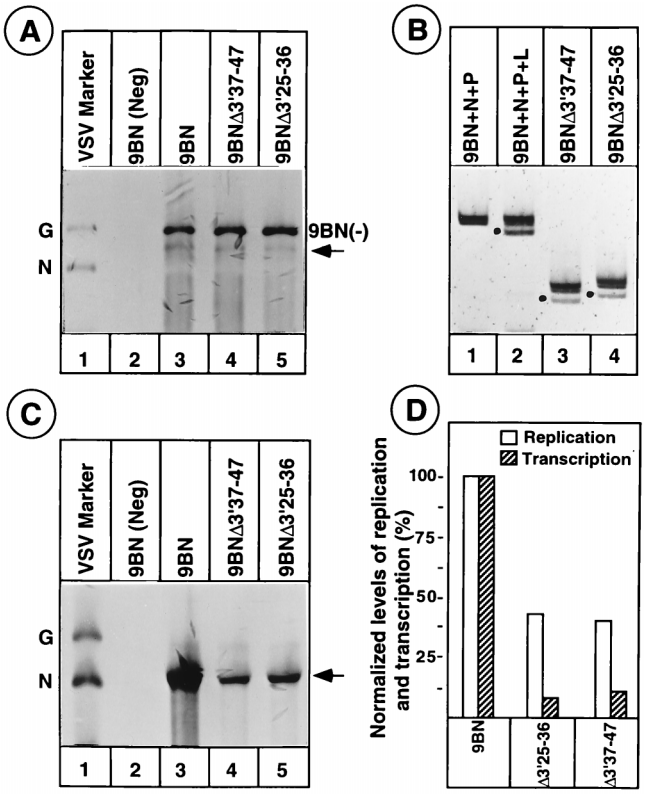

FIG. 5. Transcription and replication activities of larger deletion mutant templates. (A) Analysis of negative-sense replication products [p9BN(−)] as described in Fig. 2A. Lane 2 shows replication products from the negative control (no L plasmid in transfection) sample. (B) Analysis of plus-sense replication products (identified by dots in lanes 2 to 4) by primer extension analysis as described in the legend for Fig. 3B. (C) Analysis of NΔL mRNA transcription products (indicated by arrow) as described in the legend for Fig. 4A. (D) Normalized levels of transcription and replication from minus-sense templates, as described in the legends for Fig. 3C and 4B.

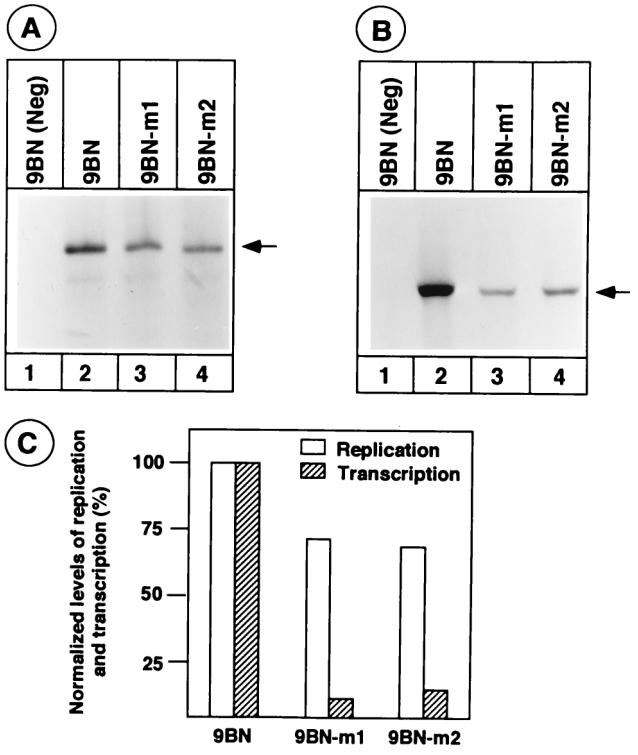

FIG. 6. Transcription and replication activities of substitution mutant minireplicons. (A) Analysis of minus-sense replication products (indicated by arrow) as described in the legend for Fig. 2A. Lane 1 contains RNA from the negative control (no L) sample. (B) Analysis of NΔL mRNA transcription product (indicated by arrow) as described in the legend for Fig. 4A. (C) Normalized levels of transcription and replication from negative-sense templates as described in the legends for Fig. 3C and 4B.

mutant templates were transcribed at levels 10 to 12% of the wild-type template. It is possible that nucleotides 25 to 47 are not important for transcription but might be required simply to correctly space the transcription start signal relative to the 3′ terminus. To address this possibility, we generated two substitution mutant minireplicons, 9BN-m1 and 9BN-m2 (Fig. 1B), in which the sequences 25 to 36 and 37 to 47 were replaced with random heterologous sequences. Analyses of these mutants showed that both mutants replicated to generate minus-sense RNA at levels about 75% of that of the wild-type template (Fig. 6A), but primer extension analysis and subsequent normalization (as performed in the experiment shown in Fig. 3B and C), showed that plus-sense RNA synthesis was 65 to 70% of the wild-type levels (Fig. 6C). However, transcription activity of these templates was significantly reduced and represented 10 to 15% of the wild-type levels (Fig. 6B and C). These results suggest that sequences from nucleotides 25 to 47 at the 3′ terminus of VSV genome may be necessary only for optimal levels of transcription.

Primer extension analysis to examine the 5′ terminus of mRNAs from the mutant minireplicons. We next examined the site of reinitiation of transcription from these mutant templates by examining the 5′ termini of the NΔL mRNAs produced from the mutant templates. Total RNA from transfected cells was isolated and subjected to primer extension analysis with the primer as described in the experiment shown in Fig. 3A. This primer hybridizes to the VSV RNA polymerase-derived NΔL mRNA transcription product and generates the extension products (the doublet marked c in Fig. 3A). Results (Fig. 7) show that the 5′ termini of NΔL mRNAs from the mutant templates mapped to the first T residue at the authentic site of transcription initiation (UUGUC in the viral genome) (lanes 10 to 14) as seen for the wild-type template (lane 6). The mutant templates with deletions of sequences 1 to 6, 7 to 12, and 13 to 18 did not produce the primer extension products (lanes 7 to 9), since these templates were transcriptionally inactive (Fig. 4). Low levels of primer extension products from RNA generated from Δ3′19-24 mutant template (lane 10) reflect the relatively low level of transcription from this template. Furthermore, the NΔL mRNAs synthesized from the two larger deletion mutants Δ3′25-36 and Δ3′37-47 were also initiated from the same initiation site (data not shown). These results indicate that deletions within the leader gene do not affect the site of reinitiation of transcription from the gene immediately downstream.

DISCUSSION

The 3′ terminus of the negative-sense genomic RNA of VSV is presumed to contain the *cis*-acting signals for transcription and replication of the genome. In this study, using a minige-

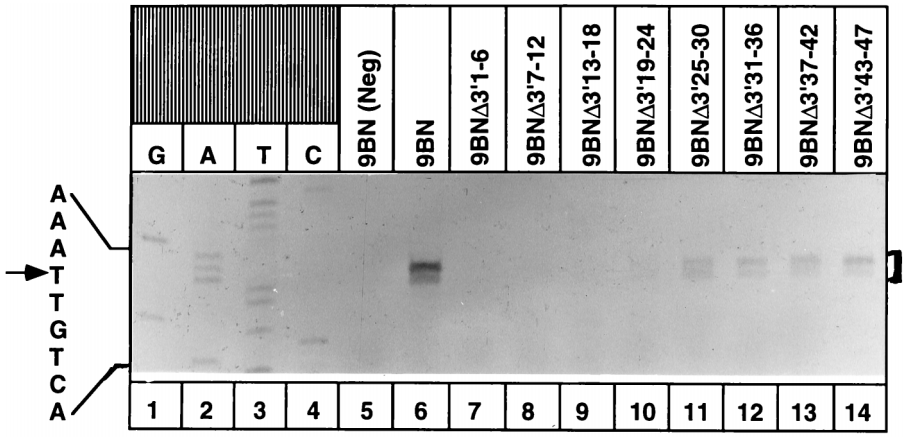

FIG. 7. Primer extension analysis to examine the 5′ terminus of transcription products from the mutant minireplicons. Analysis was performed as described in the legend for Fig. 3A, and the data shown in the figure represent the extension products of NΔL mRNA. Lane 5 shows the extension products of RNA from a negative control experiment in which L plasmid was omitted from the transfection mixture. The 5′ terminus of uncapped NΔL mRNA from wild-type and mutant minireplicon templates maps to the T residue (shown by the arrow in the sequence ladder) of the transcription initiation site, UUGUC, in the viral genome.

nomic RNA of VSV, we have examined the role of sequences within the first 47 nucleotides (corresponding to the leader gene) at the 3′ terminus in mediating transcription and replication by the viral RNA polymerase under in vivo conditions. From the data presented, the first 24 nucleotides appear to contain the signals necessary for transcription and replication. The downstream sequences from nucleotides 25 to 47 appear to be necessary for optimal levels of transcription.

The observation that mutant templates with a deletion of sequences within the first 18 nucleotides were completely defective in transcription as well as in replication, whereas the template with deletions of nucleotides 19 to 24 was partially active, suggests that the signals for transcription and replication are contained within the first 24 nucleotides and that they overlap. It is possible that both transcriptase and replicase recognize the same sequence to initiate transcription and replication. In fact, the 3′-terminal 18 nucleotides have been implicated as having a major role(s) in RNA synthesis. Our results are consistent with the proposal from sequence analysis showing that the first 18 nucleotides at the 3′ termini of various serotypes and strains of VSV have strong homology (16, 23) and therefore have been thought to be involved in the initiation of RNA synthesis (transcription and replication). Furthermore, with the reconstituted synthetic VSV nucleocapsids under in vitro transcription conditions, it has been shown that the first 15 to 17 nucleotides at the 3′ end of the negative-strand RNA are required for optimal transcription (44). In addition, only the first three nucleotides, 3′ UGC, which are invariant in all rhabdoviruses, are absolutely essential for transcription, and nucleotides at positions 4 to 17 are not as critical as the overall length of the sequence for optimal transcription (44). However, it should be noted that in these in vitro transcription reactions, the reconstituted synthetic templates were very small (22 nucleotides), and therefore the processivity of the polymerase and the contribution of downstream nucleotides to transcription elongation by the polymerase could not be assessed. In the data present here, synthesis of mature mRNA (NΔL mRNA) form various mutant templates in vivo was measured. Although analysis of leader RNA synthesis is the most direct approach to assess the effects of these deletions, considering the sequential mode of transcription of VSV genes (1, 3), synthesis of mRNA could also be used for such studies. While our data are generally consistent with those obtained previously (44), we suggest that the first 19 to 24 nucleotides at the 3′ terminus of negative-sense VSV genome are essential for transcription. Our data, however, cannot rule out the possibility that the mutant templates with deletions of sequences within the first 24 nucleotides were active in transcription initiation to generate short initiated transcripts.

Data shown in Fig. 5 and 6 suggest that deletion of larger regions spanning nucleotides 25 to 47 within the leader gene or replacement of these sequences with random heterologous sequences affected transcription more dramatically than replication. It is possible that these regions contain functionally redundant signals for replication and that deletion or substitution of one or the other region has a less adverse effect on replication. But the importance of these sequences in optimal levels of transcription could be realized from these mutants which rendered the templates transcriptionally less active (about 10 to 15% of the wild type). It seems, therefore, that the sequence requirements for optimal transcription are different from those for replication. The observation that optimal transcription requires the sequences downstream is consistent with the data from dimethyl sulfate methylation protection studies (24), which suggest a sequence-specific high-affinity binding site for the P protein at the 3′-terminal nucleotides 16 to 30 (3′ . . . GGUAAUAAUAGUAAU . . . 5′) corresponding to the middle part of leader gene. It was proposed (17) that this AU-rich sequence element may be analogous to the Goldberg-Hogness box located near the transcription initiation sites of eukaryotic genes (18). Another line of evidence that indicates the importance of this sequence in transcription is derived from studies with chimeric synthetic nucleocapsids of Indiana and New Jersey serotypes of VSV. The divergent RNA sequence of the leader gene (nucleotides 22 to 50/51) appeared to be a major determinant of serotype specificity for transcription and an indication that this sequence may be important for transcription elongation rather than initiation (45). These observations together with the data presented here support the conclusion that downstream sequences (nucleotides 25 to 47) are important for optimal levels of transcription, which may be mediated by interaction with the P protein of the polymerase complex. The findings that certain nucleotide insertions within this region of the leader gene downregulate transcription without significantly affecting replication (29, 50) further support this conclusion.

It is not known how the downstream sequences mediate optimal levels of transcription. In addition to the primary sequence, secondary structures within this AU-rich region might be involved. A potential stem-loop structure has been predicted to exist in the central region (nucleotides 16 to 33) of the leader RNA (17), suggesting that complementary sequences at the corresponding positions within the 3′ terminus of minus-sense VSV genome may contain similar structures and may play an important role in regulating transcription. This is also the region at which the P protein interacts with the template (24), so it is possible that transcription activity of the RNA template is modulated by the presence of specific sequences as well as secondary structures. However, the existence of RNA secondary structures in the N protein-associated nucleocapsid templates and their involvement in signaling RNA synthetic events must await further investigation.

In a previous study (30), we showed that the 3′ terminus of the VSV antigenome as well as the DI particle genome and antigenome contain a minimal promoter (within nucleotides 1 to 24) for replication and another element (RES, nucleotides 25 to 45) that enhances replication. The differential presence of this enhancing element was proposed to account for asymmetric levels of synthesis and accumulation of genomic and antigenomic RNAs in VSV-infected cells (30, 41, 43, 46). A similar situation might exist at the 3′ terminus of the VSV genome for transcription in that the first 24 nucleotides contain the minimal promoter element for transcription (and replication) and that downstream sequences from nucleotides 25 to 47 are necessary for optimal transcription. Further studies on this region may provide important clues as to how these sequences mediate optimal transcription from the 3′ end of the VSV genome.

Results shown in Fig. 7 suggest that the transcription reinitiation site at the leader-N gene junction remains unaffected by deletion of nucleotides within the leader gene. It is obviously important to examine synthesis and termination of leader RNA from these mutant templates. It must be pointed out that analysis of leader RNA from transfected cells has been difficult because of its small size and relative instability (27). However, preliminary data (not shown) on leader RNA analysis indicate that the minigenome templates that are transcriptionally active also generate the leader RNA. A more detailed investigation is currently under way to examine this possibility.

In conclusion, our results show that the first 24 nucleotides at the 3′ terminus of the negative-sense genome of VSV contain overlapping signals for transcription and replication. The

sequences downstream within the leader gene are necessary for optimal levels of transcription.

ACKNOWLEDGMENTS

We thank Nathan Englund for technical assistance, Michelle Perez for preparation of the manuscript, and Leroy Hwang for comments and suggestions on the manuscript. We also thank Merck and Co., Inc., Rahway, N.J., for a gift of actinomycin D.

This investigation was supported by Public Health Service grant AI 34956 from the National Institutes of Health.

REFERENCES

1. **Abraham, G., and A. K. Banerjee.** 1976. Sequential transcription of the genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA **73:**1504–1508.
2. **Ausubel, F., R. Brent, E. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl.** 1988. Current protocols in molecular biology, John Wiley and Sons, Inc., New York, N.Y.
3. **Ball, L. A., and C. N. White.** 1976. Order of transcription of genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA **73:**442–446.
4. **Barr, J. N., S. P. J. Whelan, and G. W. Wertz.** 1997. *cis*-Acting signals involved in termination of vesicular stomatitis virus mRNA synthesis include the conserved AUAC and the U7 signal for polyadenylation. J. Virol. **71:** 8718–8725.
5. **Barr, J. N., S. P. J. Whelan, and G. W. Wertz.** 1997. Role of the intergenic dinucleotide in vesicular stomatitis virus RNA transcription. J. Virol. **71:** 1794–1801.
6. **Blumberg, B. M., M. Leppert, and D. Kolakofsky.** 1981. Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. Cell **23:**837–845.
7. **Campbell, M. J.** 1995. Lipofection reagents prepared by a simple ethanol injection technique. BioTechniques **18:**1027–1032.
8. **Chang, T. L., C. S. Reiss, and A. S. Huang.** 1994. Inhibition of vesicular stomatitis virus RNA synthesis by protein hyperphosphorylation. J. Virol. **68:** 4980–4987.
9. **Chuang. J. L., and J. Perrault.** 1997. Initiation of vesicular stomatitis virus mutant polRI transcription internally at the N gene in vitro. J. Virol. **71:** 1395–1400.
10. **Das, T., A. K. Pattnaik, A. M. Takacs, T. Li, L. N. Hwang, and A. K. Banerjee.** 1997. Basic amino acid residues at the carboxy-terminal eleven amino acid region of the phosphoprotein (P) are required for transcription but not for replication of vesicular stomatitis virus genome RNA. Virology **238:**103–114.
11. **De, B. P., and A. K. Banerjee.** 1985. Requirements and functions of vesicular stomatitis virus L and NS proteins in the transcription process in vitro. Biochem. Biophys. Res. Commun. **26:**40–49.
12. **Emerson, S. U.** 1982. Reconstitution studies detect a single RNA polymerase entry site on the vesicular stomatitis virus genome. Cell **31:**635–642.
13. **Emerson, S. U., and Y.-H. Yu.** 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **15:**1348–1356.
14. **Finke, S., and K.-K. Conzelman.** 1997. Ambisense gene expression from recombinant rabies virus: random packaging of positive- and negative-strand ribonucleoprotein complexes into rabies virions. J. Virol. **71:**7281–7288.
15. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:** 8122–8126.
16. **Giorgi, C., B. Blumberg, and D. Kolakofsky.** 1983. Sequence determination of the (+) leader RNA regions of the vesicular stomatitis virus Chandipura, Cocal, and Piry serotype genomes. J. Virol. **46:**125–130.
17. **Grinnell, B. W., and R. R. Wagner.** 1984. Nucleotide sequence and secondary structure of VSV leader RNA and homologous DNA involved in inhibition of DNA-dependent transcription. Cell **36:**533–543.
18. **Gruss, P., R. Dhar, and G. Khoury.** 1981. Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. USA **78:**943–947.
19. **Hercyk, N., S. M. Horikami, and S. A. Moyer.** 1988. The vesicular stomatitis virus L protein possesses the mRNA methyltransferase activities. Virology **163:**222–225.
20. **Hunt, D. M., E. G. Smoth, and D. W. Buckley.** 1984. Aberrant polyadenylation by a vesicular stomatitis virus mutant is due to an altered L protein. J. Virol. **52:**515–521.
21. **Hwang, L. N., N. Englund, and A. K. Pattnaik.** 1998. Polyadenylation of vesicular stomatitis virus mRNA dictates efficient transcription termination at the intercistronic gene junctions. J. Virol. **72:**1805–1813.
22. **Iverson, L. E., and J. K. Rose.** 1981. Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. Cell **23:**477–484.
23. **Keene, J., M. Schubert, R. Lazzarini, and M. Rosenberg.** 1978. Nucleotide sequence homology at the 3′-termini of RNA from vesicular stomatitis virus and its defective interfering particles. Proc. Natl. Acad. Sci. USA **75:**3225–3229.
24. **Keene, J. D., B. J. Thornton, and S. U. Emerson.** 1981. Sequence-specific contacts between the RNA polymerase of vesicular stomatitis virus and the leader RNA gene. Proc. Natl. Acad. Sci. USA **78:**6191–6195.
25. **Laskey, R.** 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. Methods Enzymol. **65:**363–371.
26. **Lawson, N. D., E. A. Stillman, M. A. Whitt, and J. K. Rose.** 1995. Recombinant vesicular stomatitis viruses from DNA. Proc. Natl. Acad. Sci. USA **92:** 4477–4481.
27. **Leppert, M., L. Rittenhouse, J. Perrault, D. Summers, and D. Kolakofsky.** 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell **18:**735–747.
28. **Lerach, H., D. Diamond, J. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical examination. Biochemistry **16:**4743–4751.
29. **Li, T.** 1998. Identification and characterization of cis-acting signals for RNA replication and transcription of VSV and its defective interfering particle. Ph.D. thesis. University of Miami, Coral Cables, Fla.
30. **Li, T., and A. K. Pattnaik.** 1997. Replication signals in the genome of vesicular stomatitis virus and its defecting interfering particles: identification of a sequence element that enhances DI RNA replication. Virology **232:**248–259.
31. **Moyer, S. A., S. Smallwood-Kentro, A. Haddad, and L. Prevec.** 1991. Assembly and transcription of synthetic vesicular stomatitis virus nucleocapsids. J. Virol. **65:**2170–2178.
32. **Naito, S., and A. Ishihama.** 1976. Function and structure of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. **251:**4307–4314.
33. **Pattnaik, A. K., L. A. Ball, A. LeGrone, and G. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
34. **Pattnaik, A. K., L. A. Ball, A. LeGrone, and G. W. Wertz.** 1995. The termini of VSV DI particle RNAs are sufficient to signal RNA encapsidation, replication, and budding to generate infectious particles. Virology **206:**760–764.
35. **Pattnaik, A. K., L. Hwang, T. Li, N. Englund, M. Mathur, T. Das, and A. K. Banerjee.** 1997. Phosphorylation within domain I of the phosphoprotein of vesicular stomatitis virus is required for transcription, but not replication. J. Virol. **71:**8167–8175.
36. **Pattnaik, A. K., and G. W. Wertz.** 1990. Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. J. Virol. **64:**2948–2957.
37. **Richardson, J. C., and R. W. Peluso.** 1996. Inhibition of VSV genome RNA replication by monoclonal antibodies specific for the viral P protein. Virology **216:**26–34.
38. **Rose, J., and M. Schubert.** 1987. Rhabdovirus genomes and their products, p. 129–166. *In* R. R. Wagner (ed.), The rhabdoviruses. Plenum Press, New York, N.Y.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sarkar, G., and S. S. Sommer.** 1990. The "megaprimer" method of site-directed mutagenesis. BioTechniques **8:**404–407.
41. **Schincariol, A. L., and A. Howatson.** 1972. Replication of vesicular stomatitis virus. Virology **49:**766–783.
42. **Schubert, M., G. G. Harmison, C. D. Richardson, and E. Meier.** 1980. Site on the vesicular stomatitis virus genome specifying polyadenylation at the end of the L gene. J. Virol. **34:**550–559.
43. **Simonsen, C. C., S. Batt-Humphries, and D. F. Summers.** 1979. RNA synthesis of vesicular stomatitis virus-infected cells. In vivo regulation of replication. J. Virol. **31:**124–132.
44. **Smallwood, S., and S. A. Moyer.** 1993. Promoter analysis of the vesicular stomatitis virus RNA polymerase. Virology **192:**254–263.
45. **Smallwood, S., E. Richards-Sumners, and S. A. Moyer.** 1994. Determinants of serotype specificity in transcription of vesicular stomatitis virus synthetic nucleocapsids. Virology **199:**11–19.
46. **Soria, M., S. P. Little, and A. S. Huang.** 1974. Characterization of vesicular stomatitis virus nucleocapsids. I. Complementary 40S RNA molecules in nucleocapsids. Virology **61:**270–280.
47. **Stillman, E. A., J. K. Rose, and M. A. Whitt.** 1995. Replication and amplification of novel vesicular stomatitis virus minigenomes encoding viral structural proteins. J. Virol. **69:**2946–2953.
48. **Stillman, E. A., and M. A. Whitt.** 1997. Mutational analyses of the intergenic dinucleotide and transcriptional start sequences of vesicular stomatitis virus (VSV) define sequences required for efficient termination and initiation of VSV transcripts. J. Virol. **71:**2127–2137.
49. **Villarreal, L. P., M. Breindl, and J. J. Holland.** 1976. Determination of molar ratios of vesicular stomatitis virus induced RNA species in BHK-21 cells. Biochemistry **15:**1663–1667.
50. **Wertz, G. W., S. Whelan, A. LeGrone, and L. A. Ball.** 1994. Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. Proc. Natl. Acad. Sci. USA **91:** 8587–8591.
51. **Whelan, S. P. J., L. A. Ball, J. N. Barr, and G. W. Wertz.** 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc. Natl. Acad. Sci. USA **92:**8388–8392.