JOURNAL OF VIROLOGY, Mar. 1997, p. 1794–1801 Vol. 71, No. 3
0022-538X/97/$04.00+0

Role of the Intergenic Dinucleotide in Vesicular Stomatitis Virus RNA Transcription

JOHN N. BARR, SEAN P. J. WHELAN, AND GAIL W. WERTZ*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 14 October 1996/Accepted 19 November 1996

To investigate the role played by the intergenic dinucleotide sequence of the conserved vesicular stomatitis virus (VSV) gene junction in modulation of polymerase activity, we analyzed the RNA synthesis activities of bicistronic genomic analogs that contained either the authentic N/P gene junction or gene junctions that had been altered to contain either the 16 possible dinucleotide combinations, single nucleotide intergenic sequences, or no intergenic sequence at all. Quantitative measurements of the amounts of upstream, downstream, and readthrough mRNAs that were transcribed by these mutant templates showed that the behavior of the viral polymerase was profoundly affected by the nucleotide sequence that it encountered as it traversed the gene junction, although the polymerase was able to accommodate a remarkable degree of sequence variation without altogether losing the ability to terminate and reinitiate transcription. Alteration or removal of the intergenic sequence such that the U tract responsible for synthesis of the upstream mRNA poly(A) tail was effectively positioned adjacent to the consensus downstream gene start signal resulted in almost complete abrogation of downstream mRNA synthesis, thus defining the intergenic sequence as an essential sequence element of the gene junction. Many genome analogs with altered intergenic sequences directed abundant synthesis of a readthrough transcript without correspondingly high levels of downstream mRNA, an observation inconsistent with the shunting model of VSV transcription, which suggests that polymerase molecules are prepositioned at gene junctions, awaiting a push from upstream. Instead, the findings of this study support a model of sequential transcription in which initiation of downstream mRNA can occur only following termination of the preceding transcript.

The functional template for transcription and replication of vesicular stomatitis virus (VSV) is the nonsegmented negative-strand RNA genome encapsidated with the viral nucleocapsid protein (N). It is widely accepted that both of these RNA synthetic events are performed by the virus-encoded polymerase, which is a complex of the phosphoprotein (P) and the large protein (L) (3, 4, 10, 11). Available evidence indicates that during transcription, the polymerase moves in a sequential manner from the 3′ end of the genome toward the 5′ end and in so doing synthesizes six discrete RNA molecules via a mechanism whereby synthesis of a downstream mRNA depends on the polymerase having transcribed the upstream mRNA (1, 2). The six RNAs comprise (i) an uncapped and nonpolyadenylated RNA species transcribed from the leader gene and (ii) five mRNAs, all of which are capped and polyadenylated, corresponding to the five genes of VSV (3). Despite the sequential nature of VSV transcription, the five mRNAs are not made in equimolar quantities (27). Rather, their abundances decrease in the order N>P>M>G>L, which corresponds to the gene order from the 3′ end of the genome. It is believed that this gradient in mRNA abundance reflects differential levels of transcription that result from approximately one-third of the transcribing polymerase molecules failing to reinitiate mRNA synthesis following termination of an upstream mRNA (14).

The junctions between each of the five VSV genes are composed of conserved sequence elements (22) which are responsible for directing the VSV polymerase to terminate and polyadenylate the upstream mRNA and to initiate and cap the downstream mRNA. The 23-nucleotide consensus gene junction sequence has recently been shown to contain the signals that direct the polymerase to accomplish these functions (23). Two nucleotides of each gene junction are not incorporated into either mRNA (18, 22), but it is not known whether this is because they are not transcribed or because they are initially transcribed and then excised from the nascent transcript. A consequence of the strategy that VSV uses to synthesize the subgenomic RNAs is that during the first step of VSV genome replication, namely, synthesis of the antigenomic strand, the sequence elements at the gene junctions must be ignored by the advancing polymerase such that an intact complement of the genome is synthesized. The differential recognition of these signals is thought to be achieved, at least in part, by the ability of the nascent RNA to be encapsidated by the N protein, triggered when the abundance of the N protein reaches a critical level. The encapsidation event thus plays a crucial role in modulating the balance between transcription and replication (21).

Precisely how the *cis*-acting signals at the gene junction alter the behavior of the VSV polymerase during transcription is currently unknown, although this question is central to the most fundamental aspects of VSV molecular biology. To address this question, we have undertaken a comprehensive mutagenic analysis of the conserved sequence that represents the VSV gene junction by using a system developed in our laboratory which is able to reconstruct the entire replication cycle of VSV by using manipulatable cDNAs as the source of both genomic analog template and the essential *trans*-acting N, P, and L proteins (20, 28). By using this approach, the RNAs synthesized from the genomic analogs can be metabolically labeled and visualized directly without the use of either reporter gene methodology or blotting techniques. Although we could use this method to recover infectious VSV (29), for ease of manipulation we constructed a bicistronic minigenome

* Corresponding author. Mailing address: Department of Microbiology, BBRB 17/373, 845 19th St. South, University of Alabama at Birmingham, Birmingham, AL 35294-2170. Phone: (205) 934-0877. Fax: (205) 934-1636. E-mail: gail_wertz@micro.microbio.uab.edu.

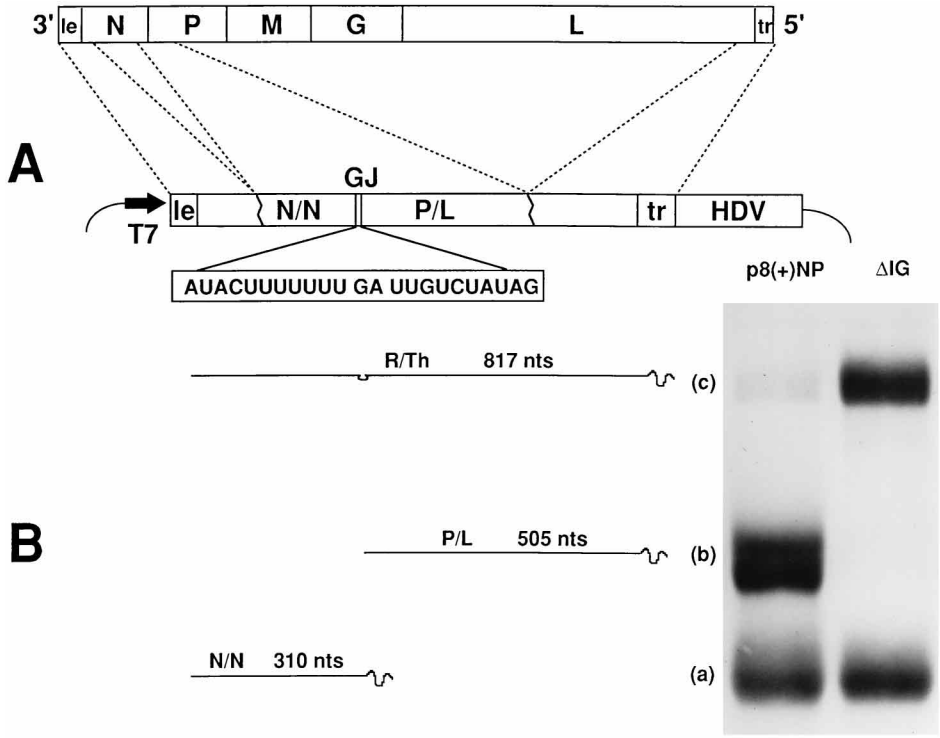

FIG. 1. Diagrammatic representation of plasmid p8(+)NP and the RNA synthesis activity of the bicistronic genome analog that it encodes. (A) The genomic analog transcribed from p8(+)NP includes the wild-type VSV 3′ and 5′ termini flanking an abbreviated N gene (N/N) upstream cistron and a fused partial P and L gene (P/L) downstream cistron separated by the N/P gene junction sequence (GJ; written in 3′ to 5′ orientation, negative sense). These VSV sequences were inserted between a T7 RNA polymerase promoter sequence (T7) and a copy of the self-cleaving hepatitis delta virus ribozyme (HDV). T7 RNA polymerase transcribes a positive-strand RNA from this plasmid which, after self-cleavage and encapsidation with VSV N protein, forms a functional template for the VSV polymerase. le, leader; tr, trailer. (B) The genome analog encoded by p8(+)NP has the capacity to transcribe three mRNA species, which are represented both diagrammatically and as metabolically labeled actinomycin D-resistant VSV RNAs synthesized in VTF7-3-infected BHK cells transfected with plasmids supplying the genome analog and *trans*-acting N, P, and L proteins (described in Materials and Methods). The major transcription products of the p8(+)NP template are an upstream (N/N) mRNA (band a), generated by the VSV polymerase initiating transcription at the leader/N gene junction and terminating transcription at the polyadenylation sequence of the N/P gene junction, and a downstream (P/L) mRNA (band b) which is initiated at the consensus start site of the N/P gene junction and terminated at the L gene/trailer junction. The minor RNA is a readthrough transcript (R/Th; band c), which arises when the termination signals of the gene junction are ignored. This transcript is synthesized at a relative molar abundance of 1.2% of total transcribed mRNA and is seen only on a long-exposure autoradiograph. To indicate the position of the readthrough transcript product more clearly, the RNAs transcribed from a template that has a reduced ability to terminate upstream mRNA synthesis [p8(+)NPΔIG, described in Results] is shown alongside (lane ΔIG). nts, nucleotides.

which contained only one gene junction. We mutated the consensus sequence elements at the gene junction of this genome analog and analyzed the RNA products directed by the altered templates. We found that the wild-type intergenic dinucleotide, GA, which occurs at three of the four VSV gene junctions, terminated synthesis of the upstream mRNA more efficiently that any other dinucleotide sequence although the wild-type sequence was not the most efficient at reinitiating transcription of the downstream mRNA. Taken together, these findings suggest that the degree of intergenic attenuation rather than the overall transcriptional activity has been optimized during the evolution of VSV.

MATERIALS AND METHODS

Plasmid constructions. As previously described (20, 28), plasmids used for generation of VSV genomic analogs were constructed by inserting VSV sequences between a promoter site for bacteriophage T7 and a cDNA encoding the self-cleaving ribozyme from the antigenomic strand of hepatitis delta virus (Fig. 1). Following T7 RNA polymerase transcription and subsequent ribozyme cleavage, the RNAs generated from these plasmids contained precise 3′ ends, but as a consequence of their construction, two additional non-VSV nucleotides (GG) were present at their 5′ ends. Plasmid p8(+)NP, designed to direct the transcription of a genomic analog containing the authentic 3′ and 5′ VSV termini and up- and downstream transcriptive units separated by the N/P intergenic junction, was constructed by inserting a PCR-amplified fragment comprising VSV nucleotides 1236 to 1685 into the unique *Bgl*II site of plasmid p8(+) (30). The upstream cistron of p8(+)NP comprised a partially deleted copy of the N gene from which nucleotides 211 to 1235 had been removed. The downstream cistron contained nucleotides 1383 to 1685 of the P gene fused to the last 205 nucleotides of the L gene. Previously, when T7 RNA polymerase generated a negative-sense transcript of the VSV genome, the sequence surrounding the N/P gene junction was recognized by T7 RNA polymerase as a termination signal (29); for this reason, all plasmids described here which contain the N/P gene junction were constructed such that the initial T7 RNA polymerase transcript was of positive polarity, indicated by the suffix (+). Sequence changes at the gene junction of p8(+)NP were introduced by PCR amplification using an upstream oligonucleotide primer and a downstream oligonucleotide primer spanning the gene junction, into which the desired alterations were incorporated. The resulting PCR-generated fragment was cleaved with *Stu*I and *Eco*RV and ligated into the unique *Stu*I and *Eco*RV sites of p8(+)NP, replacing the wild-type N/P gene junction sequence in each instance. The incorporated alterations were all checked by sequence determination.

Transfections. Plasmids expressing the genomic analogs and also support plasmids N, P, and L were transfected into vaccinia virus recombinant (VTF7-3)-infected BHK cells as previously described (20). VSV-specific RNAs were metabolically labeled by exposing the cells for 6 h to [^{3}H]uridine in the presence of actinomycin D (10 μg/ml) at 14 to 16 h posttransfection. Harvested RNAs were resolved by electrophoresis on 1.75% agarose-urea gels (20) and visualized by fluorography.

RNase H analysis. Harvested RNAs were incubated with an equal volume (20 μl) of 2× RNase H buffer (7) and 10 μl of 1× RNase H buffer with or without an appropriate oligonucleotide (0.5 μg) for 20 min at room temperature, as a predigestion annealing step. RNase H digestions were performed by the addition of 2 U of RNase H (Bethesda Research Laboratories) and incubation at 37°C for 20 min. Digested RNAs were precipitated by the addition of 145 μl of water, 5 μl of NaCl (4 M), 5 μl of yeast tRNA (10 mg/ml), and 500 μl of ethanol; analysis was done as described above.

Quantitation. Autoradiographs were subjected to densitometric analysis using a Howtek Scanmaster 3 scanner and Pdi Quantity One software. Molar quanti-

ties of the individual upstream, downstream, and readthrough transcripts were expressed as a percentage proportion of the total mRNA synthesis directed by each template. Densitometric analysis was performed on autoradiographs generated from at least three separate experiments, and the calculated quantities are shown in the figures along with standard deviation error bars. Percentage polymerase attenuation values relate the reduction in abundance of downstream mRNA compared to upstream mRNA, so that an attenuation value of 0% would be assigned to a junction at which upstream and downstream mRNAs were synthesized with equal molar abundance. The relative abundance of the readthrough mRNA species was used as a measure of the ability of a polymerase to terminate upstream mRNA synthesis, such that the higher the readthrough RNA abundance, the poorer the polymerase termination ability.

RESULTS

Behavior of the VSV polymerase at the wild-type N/P gene junction. Plasmid p8(+)NP, designed to express a genomic analog containing the wild-type VSV terminal sequences flanking two abbreviated VSV-specific transcriptional units separated by the authentic N/P gene junction, was constructed as described in Materials and Methods, and is shown diagrammatically in Fig. 1. RNA synthesis in the cell was initiated by transcription of the genomic analog from p8(+)NP by T7 RNA polymerase expressed from the vaccinia virus recombinant VTF7-3. Encapsidation of the resulting T7 RNA polymerase transcript with VSV N protein generated a functional template on which the VSV polymerase performed both genome replication and mRNA transcription. Two major VSV-specific RNA species corresponding in size to the individual transcription products of the upstream (N/N) and downstream (P/L) cistrons contained within the p8(+)NP genomic analog [Fig. 1, lane p8(+)NP, bands a and b] were synthesized in VFT7-3-infected cells transfected with plasmid p8(+)NP. An additional transcript of larger molecular weight is just visible [Fig. 1, lane p8(+)NP, band c]; this corresponds to the product that arises when the transcriptional termination signals at the gene junction are ignored, leading to a readthrough transcript containing the entire sequence of both cistrons. To indicate the position of the readthrough product more clearly, RNAs transcribed from an altered template [p8(+)NPΔIG, described below] from which the readthrough transcript is expressed in large quantities were compared with those RNAs expressed from the p8(+)NP template (Fig. 1, lane ΔIG, band c). For genomic analog templates with wild-type termini, as are present in p8(+)NP, the major RNA synthetic event is transcription, replication being only a minor activity (28); thus, at the exposure length of the autoradiographs shown, the replicated genomic RNA product is not visible. Replication of these template RNAs is evident by the positive polarity of the transcribed RNA species (see below) and was confirmed by detection using primer extension of a positive-sense RNA with the authentic 5′ end of the VSV antigenome, two nucleotides shorter than the primary T7 RNA polymerase transcript (5, 28). In some instances, the mRNA transcribed from the P/L cistron appeared as two closely migrating RNA species; we have previously suggested that this may be due to incomplete termination at the L/trailer junction, leading to a small population of downstream mRNAs being linked to the trailer (28).

The relative abundance of the readthrough RNA product transcribed from a genomic analog is a measure of that template's ability to terminate upstream mRNA synthesis, such that a template which directs a high abundance of the readthrough RNA product would have a poor termination ability. The relative abundance of the readthrough RNA transcribed from p8(+)NP amounted to 1.2% of total mRNA synthesized from this template, indicating that the wild-type N/P gene junction had a high termination ability. Once the polymerase had terminated upstream mRNA synthesis, the wild-type gene start signal was available to direct the polymerase to initiate downstream mRNA synthesis. In the case of the wild-type template p8(+)NP, the downstream mRNA was transcribed abundantly, but quantitation of the two mRNA species revealed that the upstream and downstream mRNAs were not transcribed in equimolar quantities. Studies on the abundance of VSV mRNAs synthesized during an infection have previously shown that the mRNA downstream of a gene junction is synthesized at an abundance which is 29 to 33% less than that of the corresponding upstream mRNA, the precise drop depending on which gene junction is in question (14). This decrease in abundance of downstream mRNA relative to upstream mRNA, termed polymerase attenuation, is believed to be responsible for the gradient in VSV mRNA transcription, presumably caused by a proportion of the polymerases dissociating from the template at each gene junction. During this study, we also observed a reduction in the molar quantities of downstream mRNA relative to upstream mRNA; after quantitation of mRNAs transcribed during 16 individual experiments using template RNA with a wild-type N/P gene junction, we calculated the mean polymerase attenuation to be approximately 21% (standard deviation of 5.2%). Given the differences in the two experimental systems, this value compares favorably to that previously calculated for polymerase attenuation in VSV-infected cells (32%), which indicates that the system used for this study generates an assay environment that closely resembles the situation of a natural VSV infection.

Identification of mRNA species transcribed from template p8(+)NP. To confirm that the three mRNA species transcribed by the VSV polymerase from the VSV genomic analogs were indeed the upstream, downstream, and readthrough transcripts, total cytoplasmic RNAs were incubated with strand- and sequence-specific oligodeoxynucleotides in the presence of RNase H (Fig. 2). The lowest-molecular-weight RNA transcribed from template p8(+)NP (Fig. 2A, band N/N) was cleaved only after the total RNA was incubated with a negative-sense oligonucleotide containing VSV N gene sequence {N/N(−), representing nucleotides 1235 to 1254 of VSV (Indiana [IND]) sequence)}, confirming that this RNA was of positive sense and represented the transcription product of the upstream N/N cistron. Likewise, the larger-molecular-weight RNA species (Fig. 2A, band P/L) was cleaved only when the total RNA was incubated with RNase H and a negative-sense oligonucleotide containing VSV P gene sequence [P/L(−), nucleotides 1665 to 1686], thus identifying this RNA species as the transcription product of the downstream P/L cistron. In each instance, incubation of total RNA with the positive-strand oligonucleotides N/N(+) and P/L(+), representing regions of the N (nucleotides 1235 to 1254) and P (nucleotides 1540 to 1565) genes, respectively, failed to mediate RNase H cleavage, as would be expected. To demonstrate that the large-molecular-weight RNA product transcribed from template p8(+)NP (Fig. 2B, band R/Th) contained sequences of both up- and downstream cistrons and thus was a readthrough transcript, total RNAs synthesized from template p8(+)NPΔIG, which expresses large quantities of this large-molecular-weight RNA, were incubated with RNase H and the same four oligonucleotides described above. As expected, only incubation of the total RNAs transcribed from template p8(+)NPΔIG with RNase H and either of the negative-sense oligonucleotides specific for the VSV N and P genes [Fig. 2B, lanes N/N(−) and P/L(−)] resulted in cleavage of this RNA.

Cleavage of the N/N mRNA after incubation with RNase H and the N/N(−) oligonucleotide was predicted to yield two RNA fragments with expected lengths of 165 and 125 nucleotides plus poly(A). Similarly, cleavage of the P/L mRNA with

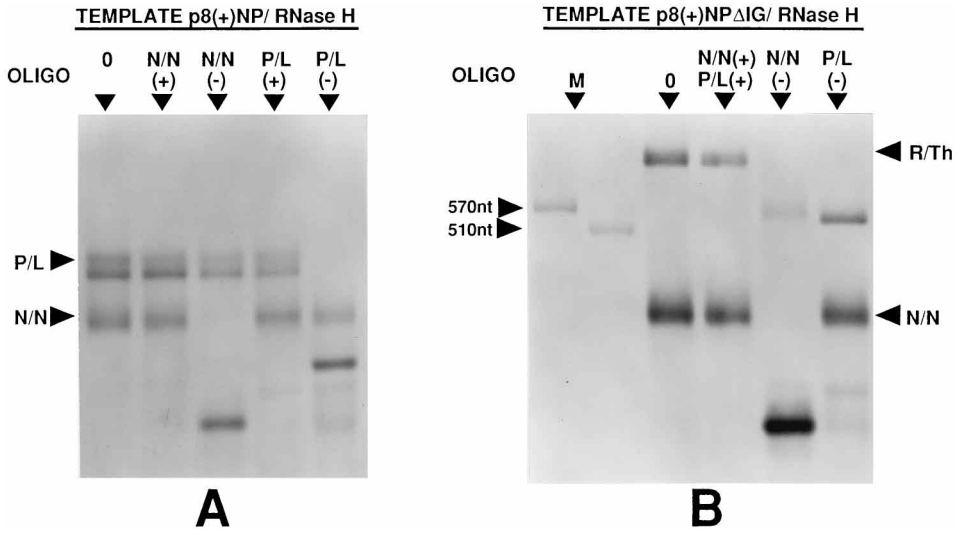

FIG. 2. Identification of the mRNAs transcribed by template p8(+)NP. Actinomycin D-resistant RNAs were metabolically labeled in VTF7-3-infected BHK cells transfected with plasmids encoding essential *trans*-acting N, P, and L proteins and either p8(+)NP, expressing a bicistronic genome analog with the wild-type N/P gene junction, or p8(+)NPΔIG, designed to encode a genome analog lacking an intergenic sequence. (A) Labeled RNAs were incubated with sequence- and strand-specific oligonucleotides in the presence of RNase H and then subjected to agarose-urea gel electrophoresis to confirm both that the VSV-specific mRNAs were of positive polarity and also that they contained sequences transcribed from the up- and downstream cistrons of the template. Lane 0 contains total RNAs transcribed from template p8(+)NP incubated with RNase H without any oligonucleotide. All other lanes are identified with the positive or negative polarity of the oligonucleotide included in the incubation and also the identity of the genome analog cistron that the oligonucleotide represented (N/N upstream mRNA or P/L downstream mRNA). (B) To demonstrate that the RNA of slowest mobility (readthrough [R/Th]) contained sequences of both the up- and downstream cistrons of p8(+)NP and was of positive polarity, RNAs transcribed from altered template p8(+)NPΔIG (described in Results) that include an increased quantity of the slow-mobility product were incubated with the same set of oligonucleotides as used for panel A, and the corresponding lanes are labeled by the same convention. Lane M contains RNAs of known size, and these are labeled accordingly (nt, nucleotides).

RNase H and the P/L(−) oligonucleotide should yield RNA species with predicted lengths of 277 and 208 nucleotides plus poly(A). RNase H- and oligonucleotide N/N(−)-mediated cleavage of the readthrough RNA was predicted to yield RNAs with lengths of 165 and 632 nucleotides plus poly(A), whereas oligonucleotide P/L(−)-mediated cleavage was expected to yield RNA fragments 589 and 208 nucleotides plus poly(A). The gel system used in this study resolves RNA species on the basis of both size and sequence composition; consequently, the mobilities of the RNA species generated by RNase H cleavage correspond well to their expected sizes.

All three mRNA species were found to be polyadenylated, as evidenced by an increase in mobility on agarose-urea gels after the RNAs had been incubated with RNase H and oligo(dT) (5). The ability of an mRNA to be polyadenylated plays a crucial role in determining the activity of the polymerase at a gene junction, and this relationship is under investigation in our laboratory.

Behavior of the VSV polymerase at a gene junction without an intergenic sequence. Plasmid p8(+)NPΔIG was designed to transcribe an altered template in which the GA dinucleotide intergenic sequence was entirely removed such that the U7 tract directly abutted the consensus downstream mRNA initiation signal (UUGUC [Fig. 1]). This alteration almost completely inhibited the ability of the resulting template to direct downstream mRNA synthesis (Fig. 3, lane ΔIG). Following densitometric analysis of the mRNA species expressed from this template, we calculated the polymerase attenuation across the gene junction of this altered template to be 98%, compared to 21% for the wild-type N/P junction. The level of attenuation that occurs across a gene junction is a quantity that relates just to the ability of a polymerase molecule to reinitiate downstream mRNA synthesis once it has terminated synthesis of the upstream mRNA. A template for which polymerase attenuation is increased over that calculated for the wild-type template p8(+)NP (21%) thus demonstrates a lessened ability to initiate downstream mRNA synthesis. The altered gene junction of this template was still able to direct upstream mRNA termination, although the increased abundance of the readthrough transcript compared with the abundance of that expressed from the wild-type p8(+)NP template indicated that the upstream mRNA termination ability had been reduced.

Behavior of the VSV polymerase at a gene junction with a single-nucleotide intergenic sequence. The GA dinucleotide intergenic sequence within the consensus gene junction of p8(+)NP was substituted with each of the four individual nucleotides, and the mRNAs transcribed from the altered templates were analyzed. In all cases, the quantity of readthrough transcript was increased over that transcribed from the template p8(+)NP, indicating that a single nucleotide as the intergenic sequence results in a gene junction with a lessened ability to direct upstream mRNA termination compared to the wild type (Fig. 3, lanes G, A, C, and U). Reduction of termination ability was less pronounced for the templates having a G or A residue as the intergenic sequence (readthrough transcript relative abundance of 6.7 or 6.4%, respectively) than for the templates having C or U (16.8 or and 24.9% readthrough, respectively). All four altered templates showed increased polymerase attenuation compared to template p8(+)NP, indicating that having a single nucleotide as the intergenic sequence resulted in a gene junction less able to initiate downstream mRNA synthesis. Incorporation of just a G and just an A residue as the intergenic sequence resulted in polymerase attenuation values that were very similar (30 and 32%, respectively) but higher than that for the template p8(+)NP (21%). The most dramatic effect on downstream mRNA initiation was seen when either C or U was substituted in place of the GA intergenic sequence, and this observation is supported by the polymerase attenuation quantities calculated for these templates (86 and 95%, respectively).

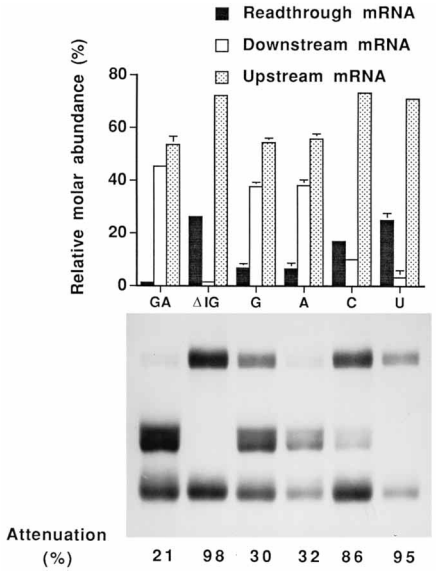

FIG. 3. Effects of alteration of the conserved GA dinucleotide intergenic sequence on the polymerase signaling ability of the gene junction. The wild-type N/P gene junction of plasmid p8(+)NP was subjected to site-specific mutagenesis such that the GA intergenic sequence was either changed to incorporate each individual nucleotide alone or removed entirely. Plasmids expressing the wild-type and altered templates were transfected into cells as described in Materials and Methods. RNAs transcribed from these templates were metabolically labeled with [^{3}H]uridine in the presence of actinomycin D and analyzed by agarose-urea gel electrophoresis and fluorography. Lane ΔIG contains the RNAs transcribed from template p8(+)NPΔIG, which has no intergenic sequence; the remaining lanes are marked with the intergenic sequence present at the gene junction of each corresponding template (in negative sense). Relative molar abundances of upstream, downstream, and readthrough mRNAs transcribed from these templates were calculated by densitometric analysis of autoradiographs generated from three separate experiments and are represented in a bar chart, with each group of three bars positioned above the corresponding intergenic sequence. The standard deviation calculated for each plotted quantity is represented with an error bar. Where error bars are not visible, deviation was negligible. The polymerase attenuation quantity that was calculated to occur across each of the gene junctions indicates the percentage decrease in downstream mRNA abundance relative to upstream mRNA abundance and is displayed underneath each corresponding lane.

Behavior of the VSV polymerase at gene junctions with altered dinucleotide intergenic sequences. The GA dinucleotide intergenic sequence within the gene junction of p8(+)NP was changed to all 15 other possible combinations, and the RNA synthesis characteristics of the altered templates were analyzed (Fig. 4). Compared to template p8(+)NP, which has the wild-type N/P gene junction, a common feature of the altered templates was that they all exhibited a reduced ability to terminate upstream mRNA synthesis, as evidenced by an increased relative abundance of the readthrough transcript. Interestingly, the dinucleotide intergenic sequence of the template which demonstrated the second-most-effective upstream mRNA termination ability was CA, which is present at the P/M gene junction of VSV (IND). From the analysis of the relative abundances of the three mRNAs transcribed from the altered templates, many general trends relating polymerase signaling abilities to the presence of particular nucleotides within the intergenic sequence emerged. By keeping the second position of the dinucleotide sequence constant and altering only the first position, it is evident that for each nucleotide in turn, the templates exhibiting the best termination abilities were demonstrated predominantly by templates having G in the first position (GG, GA, GU, and AC). Conversely, the poorest upstream mRNA termination abilities were always those with a U residue as the first position (UG, UA, UU, and UC). Using the same approach but looking at the second position, we found that the templates exhibiting best termination abilities were those having an A in this position (GA, AA, UA, and CA), and those templates with the least termination abilities were those with a U in the second position (GU, AU, UU, and CU). In agreement with these trends, the template showing the worst upstream mRNA termination ability overall had the intergenic sequence UU, whereas, as stated previously, the template with the best termination ability overall had the intergenic sequence GA.

While alteration of the intergenic sequence did in all cases affect the ability of the template to terminate upstream mRNA synthesis, in many instances the major effect of the alteration was on initiation of the downstream mRNA. The polymerase attenuation calculated for the wild-type N/P gene junction in this study was 21% (standard deviation of 5.2%); interestingly, the polymerase attenuation quantities calculated for five templates having dinucleotide sequences of GG, AG, AA, UA, and CA were lower than this value. For the templates having GG and CA dinucleotide sequences, the polymerase attenuation values (20 and 18%, respectively) are not significantly different from that of the wild type. However, for the AG, AA, and UA templates, the attenuation quantities of 12, 14, and 10%, respectively, indicate that these altered templates are able to direct more polymerase molecules to reinitiate downstream mRNA synthesis than the wild-type N/P gene junction template. As described above for the abilities of altered templates to direct upstream mRNA termination, trends relating dinucleotide alteration to polymerase attenuation also became evident. The presence of either a G or an A residue at the second position of the dinucleotide sequence resulted in a level of polymerase attenuation acting across the gene junction which was similar to that of the template p8(+)NP. However, when the second position of the dinucleotide was changed to either a C or particularly a U residue, attenuation was increased compared to that for p8(+)NP. Interestingly, these trends were independent of the nucleotide identity of the first position of the dinucleotide intergenic sequence. Comparison of the activities of templates having dinucleotide intergenic sequences with those of templates having just a single nucleotide indicated that while the identity of the first nucleotide per se has little effect of polymerase attenuation, the presence of a second nucleotide at the intergenic sequence considerably affects polymerase signaling (compare templates GA, AA, UA, and CA with a template having just A as the intergenic sequence). The trend that nucleotides G or A are more effective than the nucleotide C, and particularly U, in promoting effective downstream mRNA initiation when in the second position of the dinucleotide matches the trend found for activities of templates with single-nucleotide intergenic sequences, described above.

Behavior of the VSV polymerase at gene junctions with additional nucleotides at the intergenic sequence. The intergenic sequence (GA) of the wild-type template p8(+)NP was altered to GAA, GAAA, and GAAAAA, and the relative abundances of the three mRNAs transcribed from these templates were determined (Fig. 5). These altered gene junctions all demonstrated an ability to terminate upstream mRNA synthesis that was almost identical to that seen for template p8(+)NP. This finding was perhaps not surprising, as in each instance the first dinucleotide sequence that the advancing polymerase will encounter as it crosses the gene junction will be the GA dinucleotide. The polymerase attenuation exhibited by the gene junctions with GAA and GAAA intergenic sequences was also very similar to that calculated for the p8(+)NP template (18 and 19%, respectively, compared to

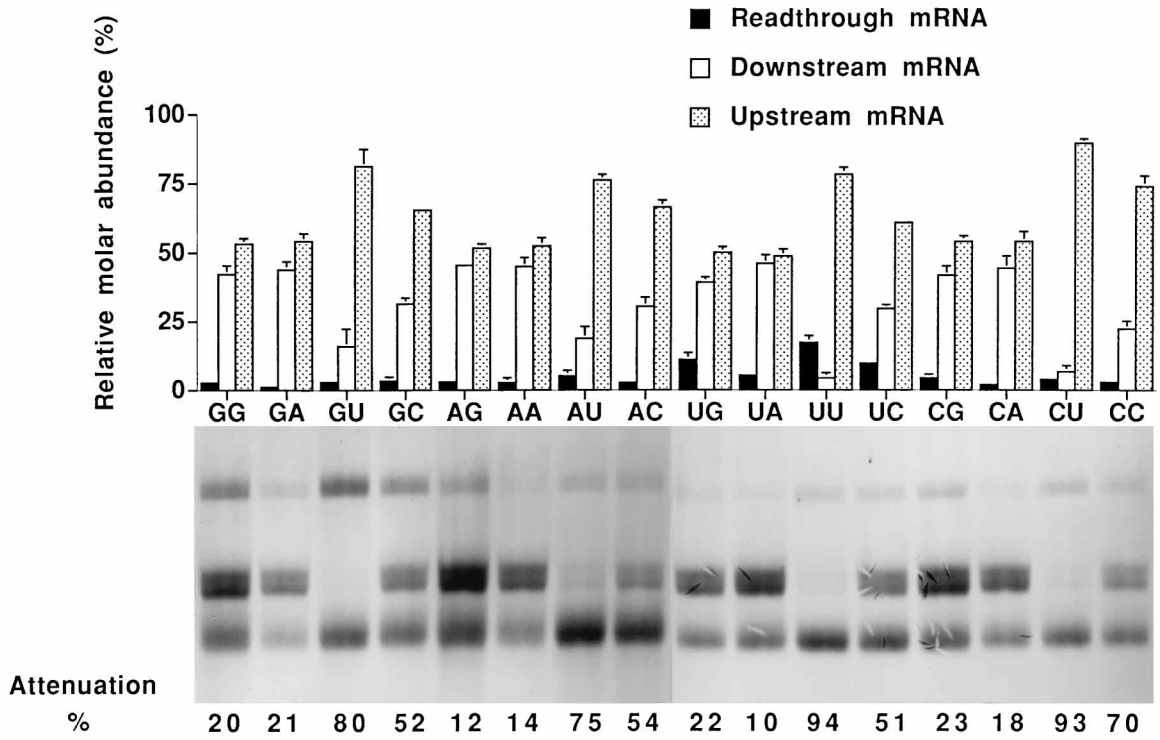

FIG. 4. Effects of sequence alterations at the intergenic dinucleotide on the polymerase signaling ability of the gene junction. The wild-type N/P gene junction of plasmid p8(+)NP was altered such that the GA dinucleotide was changed to all 15 other possible dinucleotide sequences. Plasmids expressing the wild-type and altered templates were transfected into cells as described in Materials and Methods. RNAs transcribed from these templates were metabolically labeled with [^{3}H]uridine in the presence of actinomycin D and analyzed by agarose-urea gel electrophoresis and fluorography. The lanes are labeled with the corresponding intergenic sequence (in negative sense) present at the gene junctions of the wild-type and altered templates. Relative molar abundances of upstream, downstream, and readthrough mRNAs transcribed from these templates were calculated by densitometric analysis of autoradiographs generated from three separate experiments and are represented in a bar chart, with each group of three bars positioned above the corresponding intergenic sequence. The standard deviation calculated for each plotted quantity is represented with an error bar. Where error bars are not visible, deviation was negligible. The polymerase attenuation quantity that was calculated to occur across each of the gene junctions indicates the percentage decrease in downstream mRNA abundance relative to upstream mRNA abundance and is displayed underneath each corresponding lane.

21% for GA), thus indicating that downstream mRNA initiation was not significantly affected by these extensions. In contrast, extension of the intergenic sequence by the addition of four A residues considerably increased polymerase attenuation (35%).

Interestingly, insertion of the tetranucleotide sequence GCUC immediately downstream of the intergenic sequence had an effect on the RNA synthesis activity of the resulting template more dramatic than to that of the GAAAAA mutant, which also has an insertion of four nucleotides. Alteration of the template in this way almost entirely prevented initiation of the downstream mRNA, as shown by the polymerase attenuation value calculated for this template (92%). The inability of this template to initiate downstream mRNA synthesis is not due to the altered spacing of the sequence elements, since the same spacing in the GAAAAA mutant is still permissible to mRNA initiation. It is more likely that the inability of this template to direct mRNA initiation at the gene junction is due to the inserted sequence itself.

DISCUSSION

In this study, we analyzed how elimination, alteration, or extension of the dinucleotide intergenic sequence affects the ability of the gene junction to modulate polymerase activity. The essential requirement for an intergenic sequence within the gene junction for maintenance of correct polymerase signaling was demonstrated by the almost complete inability of template p8(+)NPΔIG, in which the intergenic sequence has been removed, to direct initiation of downstream mRNA synthesis. Exchange of the dinucleotide intergenic sequence for either a G, A, U, or C single nucleotide diminished the ability of the altered template to both terminate upstream mRNA transcription and initiate downstream mRNA synthesis. A gene junction with a single-nucleotide intergenic sequence is thus unable to signal the polymerase to perform either termination or initiation of mRNA synthesis with the ability of the dinucleotide gene junction of template p8(+)NP. For the templates with either G or A as the single-nucleotide intergenic sequence, the relative abundances of all three mRNAs were virtually identical. It would seem that in the context of a single-nucleotide intergenic sequence, the G or A nucleotide offers the same polymerase signaling activity for both upstream mRNA termination and downstream mRNA initiation.

When in conjunction with the rest of the consensus gene junction sequence, the presence of the GA dinucleotide, which is found at three of the four VSV (IND) gene junctions, resulted in synthesis of the least quantity of readthrough transcript. This result indicated that of all 16 possible intergenic sequences, the dinucleotide of the N/P gene junction promoted the most effective termination of upstream mRNA synthesis. The intergenic sequence CA had a termination ability which was the second most effective; interestingly, this is the intergenic sequence which is found at the P/M gene junction of VSV (IND) (22). Analysis of RNAs expressed in VSV (IND)-infected cells has revealed that of all the four gene junctions, the P/M junction gives rise to the largest quantity of a bicis-

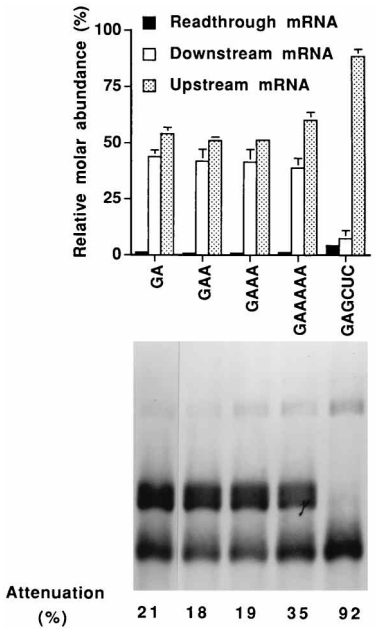

FIG. 5. Effects of increasing the size of the dinucleotide intergenic sequence on the polymerase signaling ability of the gene junction. The GA dinucleotide found at the N/P gene junction of plasmid p8(+)NP was extended by site-specific mutagenesis to either GAA, GAAA, GAAAAA, or GAGCUC. Plasmids expressing the wild-type and altered templates were transfected into cells as described in Materials and Methods. RNAs transcribed from these templates were metabolically labeled with [^{3}H]uridine in the presence of actinomycin D and analyzed by agarose-urea gel electrophoresis and fluorography. The lanes are marked according to the intergenic sequence found at the gene junction of the corresponding templates. Relative molar abundances of upstream, downstream, and readthrough mRNAs transcribed from these templates were calculated by densitometric analysis of autoradiographs generated from three separate experiments and are represented in a bar chart, with each group of three bars positioned above the corresponding intergenic sequence. The standard deviation calculated for each plotted quantity is represented with an error bar. Where error bars are not visible, deviation was negligible. The polymerase attenuation quantity that was calculated to occur across each of the gene junctions indicates the percentage decrease in downstream mRNA abundance relative to upstream mRNA abundance and is displayed underneath each corresponding lane.

tronic readthrough transcript (17). This previous study estimated that the P/M readthrough transcript was between three and five times more abundant than the N/P readthrough transcript, whereas in our system, this difference is approximately two-fold.

Those templates that are altered such that their ability to signal upstream mRNA termination is reduced direct increased synthesis of readthrough transcripts. Such readthrough species are believed to be capable of directing protein synthesis only from the upstream cistron (30), and thus it would seem that maximal gene expression of up- and downstream cistrons could occur only when both the termination of upstream mRNA synthesis was occurring with the highest efficiency and polymerase attenuation was as close to zero as possible. While three of the four gene junctions of VSV contain the intergenic sequence GA, which we have shown signals most effective upstream mRNA termination, we identified three alternative dinucleotide sequences (AG, AA, and UA) which gave levels of polymerase attenuation significantly lower than that of the wild type; thus, it appears that absolute maximal gene expression is a characteristic which has not been selected for in VSV.

The intergenic sequence, GA, of VSV (IND) is either identical to or closely related to the intergenic sequences of many other nonsegmented negative-strand viruses (12, 13, 16, 24). It will be interesting to see if in these and other viruses the consensus intergenic sequences, and more broadly speaking the entire gene junction sequences, have been selected to direct similarly attenuated mRNA synthesis.

Perhaps the most dramatic effect on the polymerase signaling ability of an altered gene junction was observed when the intergenic sequence was altered such that the seven-nucleotide-long U tract responsible for directing synthesis of the mRNA poly(A) tail became positioned adjacent to the consensus gene start signal (UUGUC), in which case downstream mRNA initiation was almost entirely abrogated. This arrangement of sequence signals presented itself when the intergenic sequence was removed entirely, was changed to the single nucleotide U, or was changed to the dinucleotide UU. In these instances, the marked effect on the polymerase signaling ability of the template was unlikely to be due to the effective extension of the U tract alone, since wild-type intergenic sequences preceded by extended U tracts still allow high levels of downstream mRNA initiation (5). We speculate that juxtaposing the seven U residues with the mRNA initiation signal provides an effectively extended U tract on which the polymerase synthesizes the poly(A) tail of the upstream mRNA. The polymerase then recognizes the first non-U residue that it encounters after the U tract as an effective intergenic sequence, which is an essential requirement for termination to occur. The polymerase will now have moved past the UUGUC initiating signal, and this effectively prevents initiation of downstream mRNA synthesis. The primary function of the intergenic sequence would then seem to be in providing a separating sequence between the U tract and the UUGUC initiation signal, thus permitting upstream mRNA termination to occur, which is a prerequisite for initiation of the downstream mRNA to occur. A less dramatic level of attenuation was observed for those templates that had been altered such that the second nucleotide position of the intergenic sequence which precedes the UUGUC initiation sequence was changed to a U residue. In these instances, we believe that termination occurs by virtue of the U tract and the first nucleotide of the intergenic sequence, but then mRNA initiation is reduced since the UUGUC signal is partially obscured by the presence of the U residue directly upstream.

Compared with template p8(+)NP, the extremely similar levels of polymerase attenuation that were calculated to occur for the templates with extended GAA and GAAA intergenic sequences indicate that the polymerase possesses a limited flexibility in the position at which it can effectively initiate downstream mRNA synthesis, and since further enlargement of the intergenic sequence to GAAAAA increases attenuation, these results suggest that this flexibility extends by at least two but fewer than four nucleotides. This finding suggests that when a consensus mRNA initiation signal does not appear in the usual position directly after the dinucleotide intergenic sequence, the polymerase may be able to advance a short distance until a permissible start sequence is found. However, the increased polymerase attenuation calculated for the template with the intergenic sequence GAAAAA would suggest that moving four additional nucleotides along the genome results in a proportion of polymerase molecules failing to reinitiate downstream mRNA synthesis. The more marked attenuation that was observed for the template having a nonhomopolymeric extension (intergenic sequence GAGCUC) indicates that the tolerance exhibited by the polymerase to extension of the intergenic dinucleotide is sequence specific.

The VSV (New Jersey) polymerase must possess a tolerance to spacing between gene end and gene start sequences, since the sequence between the G and L genes of this serotype is not the conserved dinucleotide, GA, that is found at the other

three gene junctions but is a sequence that extends for 21 nucleotides (16). It is not known whether this extended intergenic sequence reduces the efficiency at which downstream L mRNA initiation occurs any more than the attenuation that occurs at the other three gene junctions, but from the results presented here, we speculate that this extended intergenic would considerably down-regulate initiation of the L gene mRNA. A scenario leading to increased attenuation at the G/L gene junction would be reminiscent of many other nonsegmented negative-strand RNA viruses which use various strategies to apparently down-regulate polymerase expression. Human respiratory syncytial virus (8) and the rhabdovirus sigma (25) both contain an overlap between the polymerase gene and the preceding cistron, and this arrangement has been speculated to attenuate transcription of the polymerase mRNA. Rabies virus (strains ERA and PV) (9, 19, 26), infectious hematopoietic necrosis virus (15), and viral hemorrhagic septicemia virus (6) all have an additional gene junction between the G and L genes, and because of gene junction attenuation that is observed during rhabdovirus transcription (14), this arrangement is predicted to reduce downstream mRNA production.

Throughout this investigation, we consistently observed that for a template altered to signal decreased termination of upstream mRNA, there was without exception a coincident decrease in downstream mRNA initiation. In addition, none of the altered templates described here directed the synthesis of more downstream mRNA than upstream mRNA. Both of these observations are in agreement with a basic tenet of the sequential transcription model, which does not allow for initiation of a downstream mRNA by direct entry of a polymerase molecule to the mRNA start site. A variation of the currently favored model of sequential transcription proposes that rather than have a single polymerase molecule traverse, and transcribe mRNAs from, the entire template in a continual manner, polymerase molecules are lined up at the intergenic junction and can initiate only when pushed by an advancing polymerase. Our findings, however, eliminate such a possibility since those altered templates which synthesize high levels of readthrough transcripts would also be expected to transcribe high levels of the downstream mRNA, and they do not.

While our findings have shown that in many instances alterations of the intergenic sequence have little effect on the function of the gene junction, the extreme conservation of the gene junction sequence would seem to indicate that viruses with the sequence GA have a selective advantage. The function of the gene junction is to modulate polymerase activity such that during transcription, the flanking genes are expressed as mRNA in quantities that render the virus most fit in the population. It may be that even slight alteration in the signaling ability of a gene junction and the subsequent change in the abundance at which the upstream and downstream mRNAs are synthesized are debilitating to the fitness of a virus and thus may render such a virus less competitive during the infectious cycle. We are now in a position to relate the pattern of mRNA synthesis that we observe by using a particular altered template in our genome analog system to the effects that such a mutation would have on the overall fitness of a virus by incorporation of the alteration into infectious virus via our full-length VSV cDNA.

ACKNOWLEDGMENTS

We thank members of the Wertz and Ball laboratories for helpful suggestions throughout the course of this study.

This work was funded by NIH grant R37 AI12464 to G.W.W.

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. **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.
3. **Banerjee, A. K.** 1987. Transcription and replication of rhabdoviruses. Microbiol. Rev. **52:**66–87.
4. **Banerjee, A. K., G. Abraham, and R. J. Colonno.** 1977. Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. **34:**1–8.
5. **Barr, J. N., S. P. Whelan, and G. W. Wertz.** Unpublished results.
6. **Basurco, B., and A. Benmansour.** 1995. Distant strains of the fish rhabdovirus VHSV maintain a sixth functional cistron which codes for a nonstructural protein of unknown function. Virology **212:**741–745.
7. **Cavanagh, D., and T. Barrett.** 1988. Pneumovirus like characteristics of the mRNA and proteins of turkey rhinotracheitis virus. Virus Res. **11:**241–256.
8. **Collins, P. L., R. A. Olmsted, M. K. Spriggs, P. R. Johnson, and A. J. Buckler-White.** 1987. Gene overlap and site specific attenuation of transcription of the viral polymerase L gene of human respiratory syncytial virus. Proc. Natl. Acad. Sci. USA **84:**5134–5138.
9. **Conzelmann, K. K., J. H. Cox, L. G. Schneider, and H. J. Thiel.** 1990. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology **175:**485–499.
10. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis virus B and T virions. J. Virol. **10:**297–309.
11. **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.
12. **Galinski, M. S.** 1991. Annotated nucleotide sequence and protein sequences for selected *Paramyxoviridae*, p. 537–568. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York, N.Y.
13. **Gupta, K. C., and D. W. Kingsbury.** 1984. Complete sequence of the intergenic and mRNA start signals in the Sendai virus genome: homologies with the genome of vesicular stomatitis virus. Nucleic Acids Res. **12:**3829–3841.
14. **Iverson, L. E., and J. K. Rose.** 1981. Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. Cell **23:**477–484.
15. **Kurath, G., K. G. Ahern, G. D. Peerson, and J. C. Leong.** 1985. Molecular cloning of the six mRNAs of species of infectious haematopoietic necrosis virus, a fish rhabdovirus, and gene order mapping by R-loop mapping. J. Virol. **53:**469–476.
16. **Luk, D., P. S. Masters, D. S. Gill, and A. K. Banerjee.** 1987. Intergenic sequences of the vesicular stomatitis genome (New Jersey serotype): evidence for two transcription start sites within the L gene. Virology **160:**88–94.
17. **Masters, P., and C. E. Samuel.** 1984. Detection of in vitro synthesis of polycistronic mRNAs of vesicular stomatitis virus. Virology **134:**277–286.
18. **McGeoch, D. J.** 1977. Structure of the gene N: gene NS intercistronic junction in the genome of the vesicular stomatitis virus. Cell **17:**673–681.
19. **Morimoto, K., A. Ohkubo, and A. Kawai.** 1989. Structure and transcription of the glycoprotein gene of attenuated HEP-flurry strain of rabies virus. Virology **173:**465–477.
20. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
21. **Patton, J. T., N. L. Davis, and G. W. Wertz.** 1984. N protein alone satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. J. Virol. **49:**303–309.
22. **Rose, J. K.** 1980. Complete intergenic and flanking gene sequences from the genome of vesicular stomatitis virus. Cell **19:**415–421.
23. **Schnell, M. J., L. Buonocore, M. A. Whitt, and J. K. Rose.** 1996. The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. J. Virol. **70:**2318–2323.
24. **Spriggs, M. K., and P. L. Collins.** 1986. Human parainfluenza virus type 3: messenger RNAs, polypeptide coding assignments, intergenic sequences, and genetic map. J. Virol. **59:**646–654.
25. **Teninges, D., F. Bras, and S. Dezélée.** 1993. Genomic organisation of the sigma rhabdovirus: six genes and a gene overlap. Virology **193:**1018–1023.
26. **Tordo, N., O. Poch, A. Ermine, G. Keith, and F. Rougeon.** 1986. Walking along the rabies virus genome: is the large G-L intergenic region a remnant gene? Proc. Natl. Acad. Sci. USA **83:**3914–3918.
27. **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.
28. **Wertz, G. W., S. Whelan, A. W. LeGrone, and L. A. Ball.** 1995. Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. Proc. Natl. Acad. Sci. USA **91:**8587–8591.
29. **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.
30. **Wong, T. C., and A. Hirano.** 1987. Structure and function of bicistronic RNA encoding the phosphoprotein and matrix protein of measles virus. J. Virol. **61:**584–589.