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# *cis*-Acting Signals Involved in Termination of Vesicular Stomatitis Virus mRNA Synthesis Include the Conserved AUAC and the U7 Signal for Polyadenylation

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**We investigated the *cis*-acting sequences involved in termination of vesicular stomatis virus mRNA synthesis by using bicistronic genomic analogs. All of the *cis*-acting signals necessary for termination reside within the first 13 nucleotides of the 23-nucleotide conserved gene junction. This 13-nucleotide termination sequence at the end of the upstream gene comprises the tetranucleotide AUAC, the tract containing seven uridines (U7 tract), and the intergenic dinucleotide (GA), but it does not include the downstream gene start sequence. Data presented here show that upstream mRNA termination is independent of downstream mRNA initiation. Alteration of any nucleotide in the 13-nucleotide sequence decreased the termination activity of the gene junction and resulted in increased synthesis of a bicistronic readthrough RNA. This finding indicated that the wild-type gene junction has evolved to achieve the maximum termination efficiency. The most critical position of the AUAC sequence was the C, which could not be altered without complete loss of mRNA termination. Reducing the length of the wild-type U7 tract to zero, five, or six U residues also totally abolished mRNA termination, resulting in exclusive synthesis of the bicistronic readthrough mRNA. Shortening the wild-type U7 tract to either five or six U residues abolished VSV polymerase slippage during readthrough RNA synthesis. Since neither the U5 nor U6 template was able to direct mRNA termination, these data imply that polymerase slippage is a prerequisite for termination. Evidence is also presented to show that in addition to causing polymerase slippage, the U7 tract itself or its poly(A) product constitutes an essential signal for mRNA termination.**

The genome of vesicular stomatitis virus (VSV), the prototypic nonsegmented negative-strand virus, consists of an 11,161-nucleotide RNA molecule with five genes flanked by the leader and trailer terminal promoter sequences in the order 3′ (leader)-N-P-M-G-L-(trailer) 5′. The functional template for VSV RNA synthesis is not the naked RNA genome but a structure known as the nucleocapsid complex. In this structure, the VSV genome is encapsidated with the viral nucleocapsid (N) protein and is associated with the viral phosphoprotein (P) and large polymerase (L) protein which together comprise the RNA-dependent RNA polymerase. The currently favored mechanism by which VSV mRNAs are transcribed proposes that the polymerase enters the genome at a single 3′ entry site and moves toward the 5′ terminus, synthesizing the leader RNA and mRNAs from each of the five genes in an obligatory sequential manner (1, 3). This sequential mode of transcription dictates that synthesis of downstream mRNAs can occur only following prior transcription and termination of the cistron immediately upstream.

The *cis*-acting signals that regulate the production of the five mRNAs are contained within a 23-nucleotide consensus sequence located at each of the four gene junctions (22, 24) (Fig. 1A). The gene junction sequence directs the transcribing VSV polymerase to polyadenylate and terminate synthesis of the upstream mRNA. Production of the VSV 3′ poly(A) tail is thought to occur by a process of reiterative transcription in which the polymerase repeatedly copies a run of U residues located on the genome template. However, the onset of reiterative transcription does not seem to guarantee that termination of mRNA synthesis will occur. The appearance of poly(A) sequences in bicistronic readthrough RNAs (12–14, 23) indicates that polymerase slippage and the consequent polyadenylation of upstream mRNAs is not the only factor involved in the termination process. It has been suggested that the mechanism of mRNA termination may be closely linked to, and depend on, the initial events of downstream mRNA synthesis (23, 27).

Transcription across the gene junction occurs by a mechanism which results in the absence of the intergenic dinucleotide in either the resultant up- or downstream mRNA products (22) and yields a downstream mRNA with a 5′ cap structure in which, unlike most other guanylyltransferase products, two of the three phosphates are derived from the capping GTP residue (2). Precisely how the gene junction directs the polymerase to accomplish these complex and possibly interconnected reactions during transcription is unknown. It is also unclear how the polymerase is influenced to ignore the gene junction signals during genomic RNA replication, although encapsidation of the nascent RNA strand by the VSV N protein may be partly responsible for changing the polymerase activity from transcriptase to replicase (4, 20).

To address these questions, our laboratory developed a system in which the VSV nucleocapsid complex could be assembled in cells entirely from the products of cDNA clones that allowed efficient generation of either replicable subgenomic analogs (19, 30) or full-length infectious VSV (18, 32). This approach allows manipulation of the VSV genomic RNA template to study the *cis*-acting signals that control RNA synthesis.

We previously described a VSV genome analog containing two transcriptional units separated by the authentic N/P gene junction. By site-directed mutation of this gene junction, we examined the role of the intergenic dinucleotide in transcription (6). We have extended that study to investigate the signals contained within the gene junction that are involved in signal-

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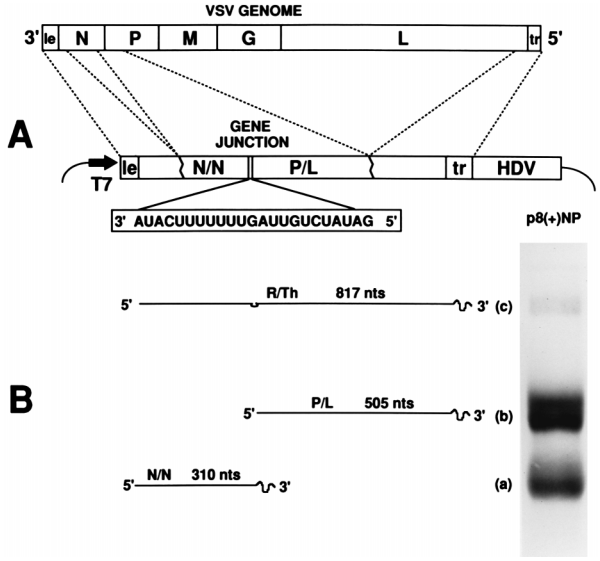


FIG. 1. Diagrammatic representation of the VSV genome, plasmid p8(+)NP, and the RNA species transcribed from the bicistronic genome analog that it encodes. (A) VSV sequences were inserted between the T7 RNA polymerase promoter (T7) and a copy of the self-cleaving hepatitis delta virus ribozyme (HDV) as described in Materials and Methods. Following transfection into vTF7-3-infected BHK cells, a positive-sense genome analog primary transcript having wild-type termini and the authentic N/P gene junction flanked by up- and downstream cistrons was generated. By associating with supporting N, P, and L proteins supplied from transfected plasmids, the positive-sense T7 RNA polymerase transcript was assembled into nucleocapsid structures competent for genome replication. Replication yielded the negative-sense RNAs that function as templates for both mRNA transcription and replication. The sequence of the negative-sense replication product at the gene junction is shown for reference. le, leader; tr, trailer. (B) The genome analog transcribed from p8(+)NP has the capacity to synthesize three mRNAs which are represented both diagrammatically and also as metabolically labeled, actinomycin D-resistant RNAs harvested from vTF7-3-infected cells containing plasmids expressing the genome analog and supporting N, P, and L proteins (described in Materials and Methods). The p8(+)NP template directs the synthesis of upstream N/N mRNA (a), downstream P/L mRNA (b), and a small quantity of a bicistronic readthrough mRNA (R/Th; c) which is synthesized when the transcription termination signals at the gene junction are ignored by the polymerase. This readthrough mRNA is synthesized from the p8(+)NP genome analog with a relative molar abundance of 1% and is seen only on long exposure autoradiographs or by analyzing the RNAs synthesized from templates having lower termination abilities than the wild-type template, which is 99%. nts, nucleotides.

ing the polymerase to terminate mRNA synthesis. The results show that these signals are located within the first 13 nucleotides of the conserved 23-nucleotide gene junction. These 13 nucleotides do not include the conserved gene start signal for the downstream transcription unit, indicating that termination of upstream mRNA occurs independently of initiation of downstream mRNA. In addition, we show that seven uninterrupted U residues, as found at the wild-type gene junction, are the minimum number that will promote mRNA termination, most likely because this is the minimum U-tract length required to direct reiterative copying by the VSV polymerase.

## MATERIALS AND METHODS

**Plasmid constructions.** Plasmid p8(+)NP directs bacteriophage T7 RNA polymerase to transcribe a positive-sense VSV genomic analog that has wild-type termini and contains two transcriptive units separated by the N/P gene junction. As described previously (6), this plasmid was constructed by juxtaposing nucleotides 1 to 215, 1236 to 1686, and 10897 to 11161 of the VSV genome between a promoter for T7 RNA polymerase and the cDNA encoding the self-cleaving ribozyme from the antigenomic strand of hepatitis delta virus (Fig. 1A). Following ribozyme self-cleavage, the RNA product synthesized by T7 RNA polymerase had a 3′ end that corresponded to the authentic 3′ end of the VSV antigenome but as a consequence of its construction contained two non-VSV nucleotides (GG) at its 5′ end. Mutations were introduced into the N/P gene junction of plasmid p8(+)NP by PCR amplification using an upstream oligonucleotide primer and a downstream mutagenic primer that spanned the gene junction. The amplified DNA fragments were digested with restriction endonucleases *Stu*I and *Eco*RV and inserted into the unique *Stu*I and *Eco*RV sites of p8(+)NP, corresponding to positions 1281 and 1393 of the VSV genome, replacing the wild-type sequence. All mutations were verified by sequence determination.

**Transfections.** Plasmids expressing VSV genomic analogs and the *trans*-acting N, P, and L proteins were transfected into BHK cells already infected with the recombinant vaccinia virus vTF7-3, which expresses the gene for T7 RNA polymerase (19). Fourteen hours posttransfection, VSV-specific RNAs were metabolically labeled with [$^3$H]uridine for 6 h in the presence of actinomycin D prior to harvest. Labeled RNAs were purified by phenol-chloroform extraction and ethanol precipitation, subjected to agarose-urea gel electrophoresis, and visualized directly by fluorography.

**Limited primer extension analysis.** The behavior of the VSV polymerase as it crossed the gene junction during readthrough RNA synthesis was analyzed by limited primer extension. RNAs were harvested from transfections in which the genome analog nucleocapsids were supplied as budded subviral particles that had been generated from prior transfections in which all five VSV proteins were supplied from cDNA support plasmids (19). This approach eliminated the need to supply the genome analog from a cDNA source, which could have complicated the interpretation of subsequent primer extensions. An oligonucleotide primer complementary to nucleotide positions 1418 to 1388 of the VSV sequence (2 to 34 nucleotides downstream of the N/P gene junction) was annealed to harvested RNAs and extended by using murine leukemia virus reverse transcriptase in the presence of the buffer system supplied by the manufacturer (Bethesda Research Laboratories), 450 μM dATP, dGTP, and ddCTP, and 5 μCi of [$^{35}$S]TTP (1,250 Ci/mmol) (Fig. 7). Reactions mixtures were incubated for 30 min at 37°C, subjected to a 5-min chase using 450 μM each of the four deoxynucleoside triphosphates, and then loaded on a 10% denaturing polyacrylamide gel. As a control for possible slippage of either the T7 polymerase or the murine leukemia virus reverse transcriptase on the gene junction sequences, control RNA templates were generated by transfection of genome analog cDNAs without supporting plasmids into BHK cells previously infected with vTF7-3. The resulting T7 transcripts were harvested and analyzed by limited primer extension.

**Quantitation.** The abundances of VSV-specific RNAs were determined by densitometric analysis of autoradiographs from at least three separate experiments, using a Howtek Scanmaster 3 scanner and Pdi Quantity One software. The termination ability of a template was defined as the molar ratio of upstream mRNA relative to the sum of the upstream mRNA and readthrough RNA and is expressed as a percentage. This quantity shows what percentage of transcripts that start at the beginning of the N gene terminate at the N/P gene junction, so that a template on which upstream mRNA was terminated without fail would have a termination ability of 100%.

Some of the mutants analyzed in this study showed slight variations in the ability to direct downstream mRNA initiation. The significance of these small variations will be assessed in future studies, and so this aspect of the results is not discussed in this report.

## RESULTS

**Behavior of the VSV polymerase at the wild-type N/P gene junction.** Plasmid p8(+)NP, shown diagrammatically in Fig. 1A, was designed to express a VSV genome analog with wild-type genomic termini flanking two cistrons separated by the wild-type N/P gene junction. Transfection of p8(+)NP into vTF7-3-infected cells, along with plasmids expressing *trans*-acting N, P, and L proteins, led to formation of nucleocapsid complexes functional for both genome replication and mRNA transcription (6). Two major RNA species of positive polarity were transcribed from the p8(+)NP genome analog, and these correspond to the mRNAs generated from the upstream (N/N) and downstream (P/L) cistrons (Fig. 1B) (6). The relative molar abundance of the downstream mRNA was approximately 79% of the abundance of the upstream mRNA, reflecting the polymerase attenuation that occurs at gene junctions (21% calculated from 20 separate experiments, with a standard deviation of 5.2 [6]). The level of polymerase attenuation calculated for the gene junctions during a VSV infection is between 29 and 32% (15), and comparison of these polymerase attenuation quantities indicates that the system that we have chosen for this study closely recreates the environment of a natural VSV infection.

An additional positive-stranded RNA of higher molecular

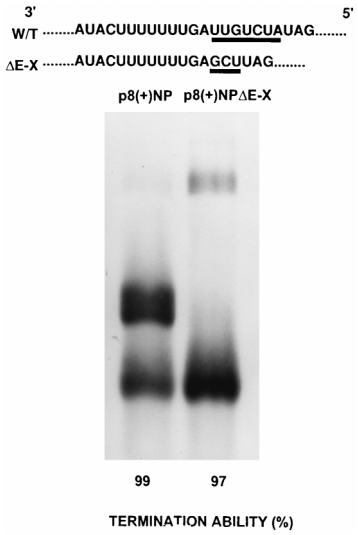


FIG. 2. Termination of upstream mRNA synthesis does not require initiation of the downstream mRNA species. A genome analog p8(+)NPΔE-X was constructed in which the first seven nucleotides of the P gene start sequence, 3′ UU GUCUA 5′, were replaced with the sequence 3′ GCU 5′, and this template was unable to transcribe downstream mRNA. The nucleotide sequences of the wild-type (W/T) and altered gene junctions are shown. The RNA products directed by this mutant genome analog were analyzed by direct metabolic labeling as described in Materials and Methods. A fluorogram of the dried gel is shown. The increased abundance of readthrough RNA synthesized is compensated by the increased abundance of the upstream mRNA made from this template.

weight, which corresponded to the bicistronic readthrough transcript generated when the VSV RNA polymerase failed to terminate synthesis of the upstream mRNA as it crossed the gene junction, was also transcribed from this template (Fig. 1B) (6). For the wild-type template p8(+)NP, the abundance of the readthrough RNA is very low (1% of total VSV-specific transcripts) and consequently is not discernible on the autoradiographs. The readthrough RNA is clearly visible as an RNA product from a template with reduced termination abilities, such as those analyzed in Fig. 3. Quantitation of the relative abundances of the upstream N/N mRNA and the readthrough product revealed that the wild-type gene junction had a termination ability of 99%.

The genome analogs described in this study have wild-type termini; thus, the major RNA synthetic activity performed by the VSV polymerase on these templates is transcription, with replication of the genomic analog being the minor event (30). For this reason, the replication product of these templates was not visible on the autoradiographs at the exposure length selected for best visualization of the products of transcription. That replication of these genome analogs occurred was evident by the positive polarity of the transcript RNAs (6) and was confirmed by the detection, using primer extension analysis, of a positive-sense RNA which represented the product of genome replication (5).

**Sequences downstream of the intergenic dinucleotide are not involved in mRNA termination.** To investigate whether downstream mRNA initiation was required for upstream mRNA termination, the conserved gene junction sequence was altered such that the first seven nucleotides of the downstream cistron (3′ UUGUCUA 5′), which includes the mRNA initiation sequence (3′ UUGUC 5′), were replaced with the sequence 3′ GCU 5′. Analysis of the RNAs synthesized by the resulting template [p8(+)NPΔE-X] revealed that the downstream P/L mRNA was not made (Fig. 2). In contrast, the upstream mRNA was synthesized and was unaltered in size and abundance compared to that of the wild-type template. Quantitation of the relative abundances of the upstream and

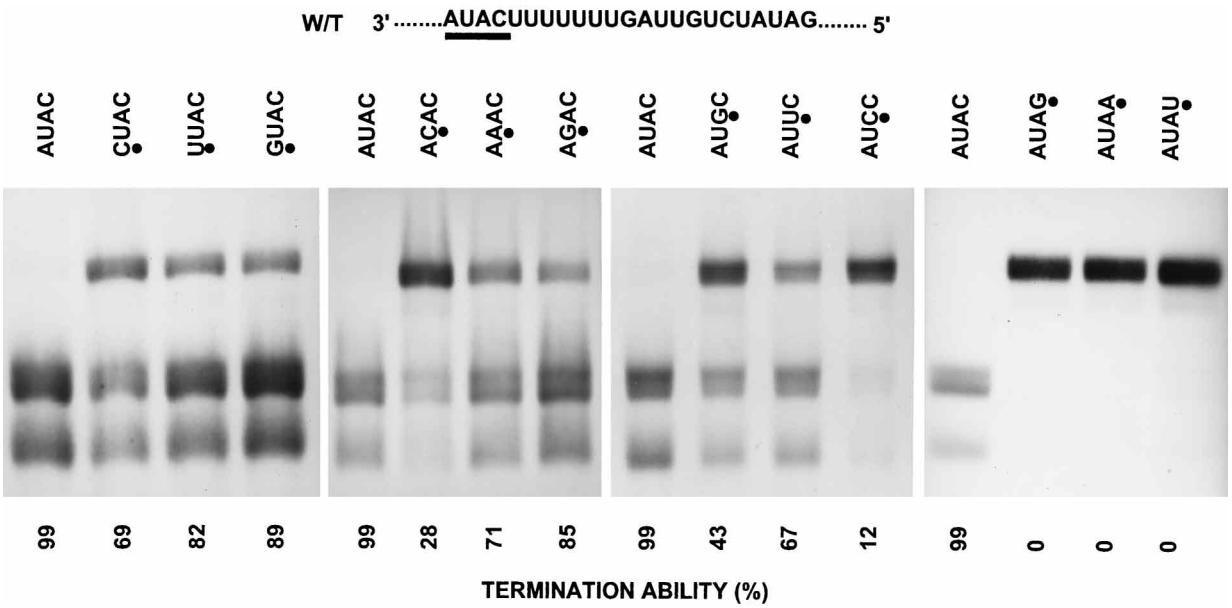


FIG. 3. Behavior of the VSV polymerase at a gene junction with alterations of the conserved tetranucleotide sequence AUAC. The sequence of the wild-type (W/T) N/P gene junction, with the AUAC tetranucleotide underlined, is shown above the gel. Metabolically labeled and actinomycin D-resistant RNAs transcribed from these altered templates were harvested from transfected cells (described in Materials and Methods), and the RNAs were analyzed by agarose-urea gel electrophoresis and fluorography. All alterations of the AUAC sequence resulted in increased synthesis of the readthrough RNA compared to wild type, indicating that the ability of each template to terminate synthesis of upstream mRNA had been reduced. The sequence of the tetranucleotide is shown above each lane, with a dot to identify the altered nucleotide. The termination ability expresses the molar ratio of upstream mRNA relative to the sum of the upstream mRNA and readthrough RNA and shows what percentage of transcripts that start at the beginning of the N gene terminate at the N/P gene junction, as described in Materials and Methods. For each template, this quantity represents the mean value determined from densitometric analysis of at least three separate experiments.

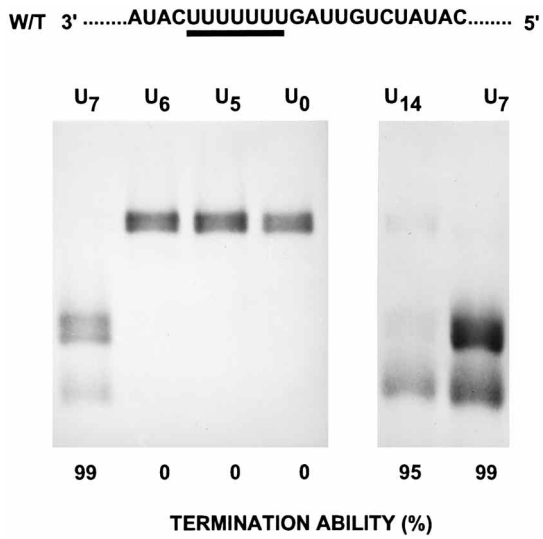


FIG. 4. Effects of altering the length of the conserved U7 tract on the ability of the gene junction to signal upstream mRNA termination. The gene junction of p8(+)NP was changed such that the resulting genome analogs had U tracts of either 0, 5, 6, or 14 U residues. The sequence of the N/P gene junction of wild-type (W/T) template p8(+)NP is shown above the gel, with the position of the U7 tract underlined. Metabolically labeled and actinomycin D-resistant RNAs synthesized by these altered templates in the transfection system were analyzed by agarose-urea gel electrophoresis as described in Materials and Methods. Each gel lane is marked with the number of U residues in place of the U7 tract at the gene junction of corresponding mutant templates. The termination ability calculated for each template represents the mean value determined from densitometric analysis of at least three separate experiments. Reducing the length of the U tract to any size less than the U7 found at the wild-type N/P gene junction abrogated termination. Doubling the length of the wild-type U tract from 7 to 14 gave a genome analog with only slightly reduced ability to terminate upstream mRNA synthesis but considerably reduced the ability to initiate downstream mRNA synthesis.

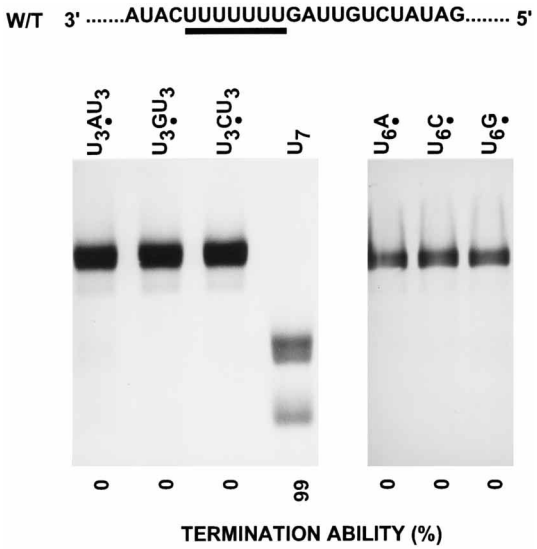


FIG. 5. Effects of interrupting the U7 tract on the ability of the gene junction to signal termination. The wild-type (W/T) gene junction was altered such that the U7 tracts were interrupted at either the fourth or the seventh U residue with each of the other three possible nucleotides. The gene junction sequence is shown above the gel, with the position of the U7 tract underlined. The metabolically labeled actinomycin D-resistant RNAs synthesized from the altered templates were harvested from transfected cells and analyzed by agarose-urea gel electrophoresis and fluorography as described in Materials and Methods. Each lane is marked with the sequence of the altered U7 tract present at the gene junction of the corresponding mutant template. The termination ability calculated for each template represents the mean value determined from densitometric analysis of at least three separate experiments. Interrupting the U7 tract at either the fourth or the seventh U position with any nucleotide resulted in complete loss of termination, indicating that the requirement for seven U residues in the U tract is not to maintain a crucial spacing of two spatially distinct elements within the gene junction but is to have a homopolymeric tract seven nucleotides in length.

readthrough RNAs by densitometric analysis revealed that the termination ability (see Materials and Methods) of the p8(+)NPΔE-X genome analog was 97%. These data show that termination of upstream mRNA synthesis is independent of downstream mRNA initiation. In addition this result demonstrates that sequences involved in termination of the upstream mRNA are located within the first 13 nucleotides of the conserved gene junction sequence. We previously demonstrated that the second position of the dinucleotide intergenic sequence had little effect on upstream mRNA termination (6); thus, the sequence required for transcriptional termination can be shortened further to just the first 12 nucleotides of the conserved gene junction (3′ AUACUUUUUUUG 5′).

**Effect of alteration of the AUAC tetranucleotide on mRNA synthesis.** Preceding the tract of seven uridines (U7 tract) within the gene junction is the conserved tetranucleotide sequence 3′ AUAC 5′ (Fig. 1A). To examine the role of this sequence in transcription termination, we constructed genome analogs in which each nucleotide of the AUAC sequence was altered in turn to each other possible residue. These genome analogs were incorporated into the transfection system, and their RNA transcription products were analyzed (Fig. 3). In each instance, the relative abundance of readthrough mRNA product was increased compared to that of the wild-type template, indicating that all of the altered templates had reduced abilities to terminate upstream mRNA synthesis (Fig. 3).

The effects of these changes on upstream mRNA termination varied according to both the position and the identity of the mutation. Several trends linking these variables emerged. Moving 3′ to 5′ along the sequence AUAC, the decrease in template termination ability with nucleotide substitution became more pronounced, such that a change at the A residue in the first position of the AUAC sequence resulted in better termination ability than when the identical change was introduced to the A residue at the third position of the AUAC sequence. Substituting the first A residue of the AUAC sequence with either U or G slightly decreased termination ability to give resulting termination ability of 89 or 82%, respectively. Replacement by a C reduced the termination ability to 69%. This trend, whereby out of the three possible substituting nucleotides the least termination was observed when the substituting nucleotide was a C, was also evident for the second and third positions of the AUAC sequence. This trend did not, however, extend to the fourth position of the AUAC sequence. When the C residue at this position was changed to any other possible nucleotide, termination was abrogated, leading to exclusive synthesis of the bicistronic readthrough.

To determine whether the AUAC tetranucleotide of the gene junction sequence was functional as a termination signal only when positioned directly in front of the U7 tract, we constructed a template in which the AUAC sequence and the U7 tract were separated by insertion of three nucleotides (GUA). In this altered template, the four nucleotides preceding the U7 tract were CGUA which had no identities with the wild-type AUAC. This mutant template directed exclusive synthesis of the readthrough RNA (termination ability of 0%), indicating that the AUAC sequence was not effective in sig-

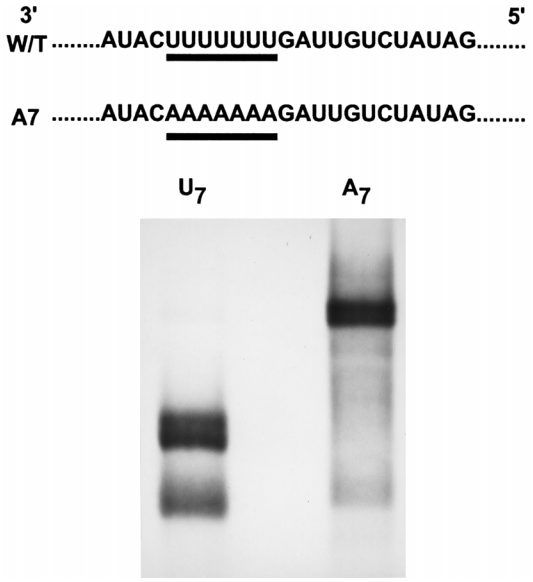


FIG. 6. The activity of VSV polymerase at a gene junction with an A7 tract in place of the U7 tract. The U7 tract at the gene junction of wild-type (W/T) template p8(+)NP was replaced with seven A residues, and the metabolically labeled and actinomycin D-resistant RNAs transcribed from this altered template were harvested from transfected cells as described in Materials and Methods. The major RNA species synthesized from this template was the readthrough RNA, indicating that the transcription termination ability of this template had been severely impaired.

naling termination unless directly juxtaposed against the U7 tract (not shown).

**Behavior of the polymerase at an intergenic junction with an altered U tract.** The U7 tract within the wild-type gene junction was either removed entirely or altered in length to contain 0, 5, 6, or 14 U residues, and the template activities of the resulting genome analogs were assessed (Fig. 4). Shortening the U tract to either five or six residues, or removing it altogether, led to exclusive synthesis of a readthrough transcript, showing that the gene junctions with shortened U7 tracts had lost the ability to direct termination of upstream mRNA synthesis. These findings indicate that the U tract is an essential termination signal and that it must contain a minimum of seven U residues.

The template with a tract of 14 uninterrupted U residues (U14 tract) directed termination of the upstream mRNA with an ability that was only slightly less than that of the wild-type genome analog, as evidenced by a slight increase in abundance of readthrough transcript and a correspondingly reduced termination ability of 95% (Fig. 4). Interestingly, the similar mobilities of the upstream mRNA transcribed from the genomic analogs having a U7 or U14 tract suggested that doubling the size of the U tract did not substantially change the length of the poly(A) tail. This result indicates that the extent of reiterative transcription during polyadenylation is not directly proportional to the length of the template region on which the stuttering occurs.

A quantitative assessment of the ratio of up- and downstream mRNAs synthesized from the U14 template indicated that despite having a wild-type initiation sequence, this template was impaired in its ability to direct initiation of downstream mRNA synthesis. A possible reason for this is that the U14 tract increases the likelihood of the polymerase dissociating from the template.

**Behavior of the VSV polymerase at a gene junction with an interrupted U7 tract.** The results described above showed that the U tract within the VSV gene junction had to be at least seven residues long in order to direct termination. To test whether this was due to a spacing requirement between two signals within the gene junction, we altered the U7 tract such that the U residue at the third or seventh position was separately replaced with either G, A, or C. Analysis of RNAs transcribed from these genome analogs revealed that all of the altered templates directed exclusive synthesis of the readthrough mRNA, with no detectable termination of upstream mRNA synthesis (Fig. 5). This result indicates that disruption of the U7 tract by substitutions at the third or seventh position abrogated the termination ability of the gene junction. These data show that the seven U residues of the U tract are required as a specific termination signal and are not required simply to separate two elements of the gene junction by seven nucleotides.

**Effects on polymerase activity of substituting the U7 tract with an A7 tract.** The results presented in the previous sections have identified the U7 tract within the conserved gene junction sequence as an essential signal for transcriptional termination. This sequence has been postulated to be copied in a reiterative manner by the VSV polymerase to give rise to the mRNA 3′ poly(A) tail (13). To test whether the VSV polymerase was capable of utilizing a seven-adenine (A7) tract to drive reiterative synthesis of a poly(U) tail during termination of upstream mRNA, we constructed a genomic analog [p8(+)NP·A7] in which the U7 tract was replaced by an A7 tract. Analysis of the RNA products synthesized from this altered template revealed that template p8(+)NP·A7 directed predominant synthesis of the bicistronic readthrough RNA, indicating that mRNA termination had been severely impaired (Fig. 6). We detected small amounts of an RNA whose mobility corresponded to that of the upstream mRNA, suggesting that a low level of upstream mRNA termination was directed by template p8(+)NP·A7. However, the abundance of this RNA species was at the detection limit of our current assay procedure, and we are now investigating alternative approaches to characterize the RNAs synthesized from this interesting template.

**Analysis of polymerase activity at the homopolymeric tract during synthesis of the readthrough RNA.** Previous studies have indicated that some polycistronic readthrough mRNAs contain untemplated polyadenylate tracts between the up- and downstream cistrons (12, 14). It is speculated that these sequences might arise when the polymerase slips on the U tract but for some reason then fails to terminate synthesis of the upstream transcript. Using a limited primer extension procedure, we investigated whether such intervening polyadenylate sequences were present in the readthrough RNAs generated from templates unable to direct termination of mRNA synthesis. The sequences present at the gene junction of readthrough RNAs might illuminate the requirements for directing polymerase slippage, which would allow us to probe the relationship between VSV polyadenylation and mRNA termination.

The number of intervening nucleotides between the cistrons of the readthrough RNA were analyzed by using RNAs harvested from transfections in which the genomic analogs were supplied as budded particles from a prior transfection. This avoided supplying a primary (cDNA-derived) transcript which would also be a template for the primer extension reaction. A primer whose 3′ end annealed three nucleotides from the 5′ end of the P/L cistron was extended by reverse transcriptase in the presence of dATP, dGTP, [$^{35}$S]dTTP, and ddCTP in place of dCTP so that termination would occur at the first G of the readthrough RNA template (Fig. 7A). The only primers that

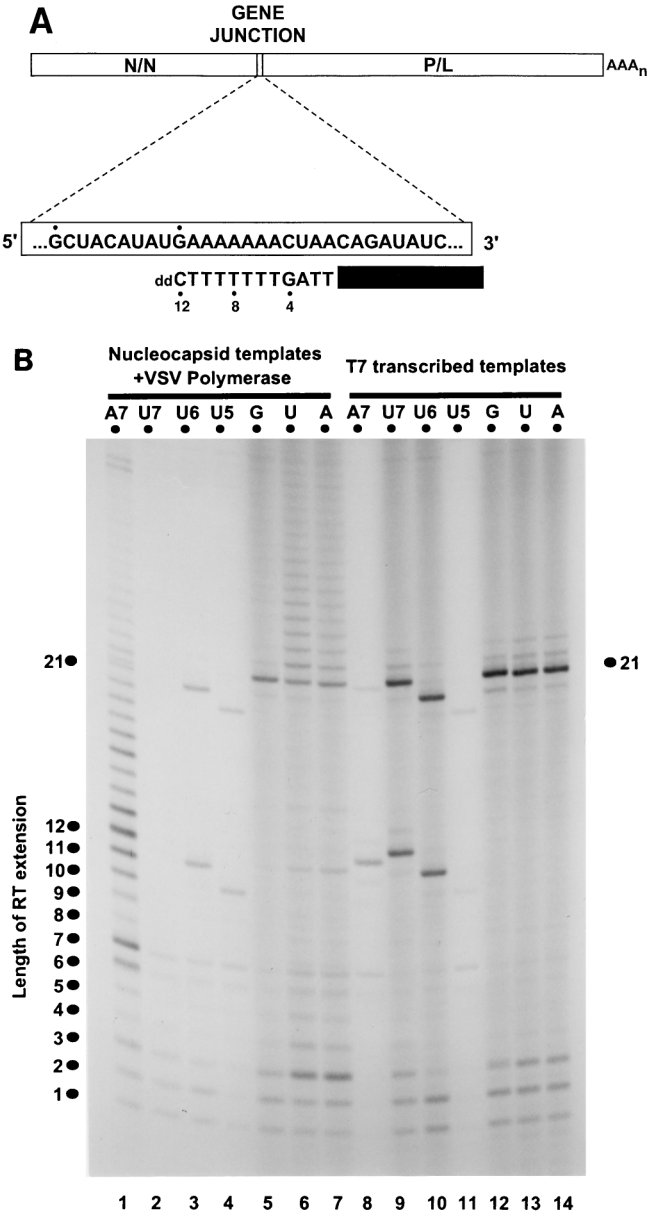


FIG. 7. (A) Diagrammatic representation of the limited primer extension procedure used to analyze the activity of the VSV polymerase at a gene junction during readthrough RNA synthesis. vTF7-3-infected cells were transfected with supporting N, P, and L plasmids and infected with VSV subviral particles containing various genome analogs known to direct exclusive synthesis of the readthrough RNA. Extension of an oligonucleotide primer (black rectangle) was limited by ddC to determine the number of nucleotides between the 3′ end of the primer and the G residue immediately downstream of the A7 tract on the RNA template (G residue marked ·). The only RNA species on which extension of more than three nucleotides could occur were the positive-sense genome and the readthrough RNA. On the wild-type VSV sequence shown, the first G residue is 12 nucleotides downstream from the 3′ end of the primer and the second G is 21 nucleotides downstream. (B) Detection of the products of VSV polymerase slippage by limited primer extension on readthrough RNAs synthesized from genomic analog templates that were unable to terminate upstream mRNA synthesis. RNAs synthesized from budded particle nucleocapsid templates were harvested from vTF7-3-infected cells and annealed to an oligonucleotide primer complementary to the 5′ end sequence of the downstream P/L mRNA (A). Extension of this primer by reverse transcriptase (RT) in the presence of ddC was terminated at the first G residue of the template RNA; for the wild-type sequence, this was 12 nucleotides downstream of the primer 3′ end. Termination products corresponding to the position of the second G of the readthrough template were also detected. To distinguish slippage by the VSV polymerase from slippage either by T7 RNA polymerase during synthesis of the primary transcript or by reverse transcriptase during primer extension, RNAs were also harvested from cells infected with vTF7-3 and transfected with cDNA templates that expressed only the altered genome analogs. The lanes are marked above with the nucleotide changes present at the gene junctions of the corresponding altered templates and below with numbers 1 to 14 to allow reference from the text. The lanes marked with G, U, or A identify the nucleotide to which the C of the AUAC tetranucleotide was changed. The number of nucleotides by which the primer was extended is indicated alongside. In all lanes, products that were the result of extensions which failed to terminate at the first template G residue, and which terminated at the second possible G, nine nucleotides downstream, were observed (A, marked ·).

could be extended into the N/N cistron were those that had annealed to either the readthrough RNA or the antigenome replication product. Since replication is only a minor activity of these templates by virtue of their wild-type terminal regions (30), the overwhelming majority of primer extensions would be on templates of the readthrough RNA species (Fig. 1 to 6). As a control to distinguish slippage by the VSV polymerase from slippage either by T7 RNA polymerase during synthesis of the primary transcript or by reverse transcriptase during primer extension, primary transcript RNAs were generated in vivo by transfecting cDNAs expressing the altered genome analogs into vTF7-3-infected cells, without supplying the N, P, and L plasmids necessary for replication. Any differences between the results of primer extension using these primary transcript RNAs and the RNAs generated from the budded particle nucleocapsid templates should be due to the behavior of the VSV polymerase.

With this assay procedure, the primary transcript generated by T7 RNA polymerase from wild-type template p8(+)NP gave rise to a primer extended by precisely 12 nucleotides, as expected (Fig. 7B, lane 9). Products resulting from primer extensions that terminated at the second G residue of the readthrough template nine nucleotides downstream were also observed (Fig. 7A [marked ·] and all lanes in Fig. 7B). If readthrough RNAs contained additional untemplated A residues as a result of polymerase slippage, the primer extension would result in a family of products that arose because the distance between the 5′ end of the primer and the first downstream G of the readthrough RNA template was longer than the 12 nucleotides found on the wild-type primary transcript or antigenome template RNA. The level of readthrough RNA directed by the wild-type intergenic sequence was, as explained above, too low to be characterized by this assay (Fig. 7B, lane 2). A population of primer extension products indicative of nontemplated A residues at the intergenic junction was detected when the C residue that precedes the U7 tract was changed to a G, U, or A (Fig. 7B; compare lanes 5 to 7 with lanes 12 to 14). For these three mutants, the population of products began after 21 nucleotides as the sequence alteration had removed the first G residue on the readthrough RNA template and termination now occurred on the next G residue located 9 nucleotides downstream (Fig. 7A, marked ·). This result shows that readthrough of these mutant gene junctions was accompanied by stuttering by the VSV polymerase on the U7 tract. Since these templates did not direct detectable termination of upstream mRNA synthesis (Fig. 3), this result agrees with previous findings that polymerase slippage is not in itself sufficient to cause transcriptional termination (12–14, 23). Our findings suggest that the C residue of the AUAC tetranucleotide is not important in causing polymerase slippage but that it may play an important role in allowing this slippage to result in productive termination. In contrast to the products of readthrough of 3′. . .AUA(G/U/A)U7. . .5′ gene junctions, the products from . . .AUACU5. . . or . . .AUACU6. . .

junctions did not contain untemplated residues (Fig. 7B, lanes 3 and 4), indicating that neither template with a shortened U tract could signal VSV polymerase slippage. Analysis of the readthrough RNA synthesized from template p8(+)NP·A7, which has the U7 tract replaced with an A7 tract, indicated that the polymerase was also able to slip on seven A residues presumably incorporating a long intervening poly(U) sequence (Fig. 7B; compare lanes 1 and 8). Since the termination ability of the A7 gene junction is severely impaired (Fig. 6), this result reinforces the conclusion that slippage is required but not sufficient for termination. It appears that an additional signal is present either within the U7 tract or alternatively within the reiteratively synthesized poly(A) tail on the nascent strand that is essential for transcriptional termination.

## DISCUSSION

In this study, we defined the *cis*-acting signals involved in termination of VSV mRNA synthesis. We first demonstrated that the mechanism by which upstream mRNA was synthesized and terminated was not dependent on the initiation of downstream mRNA. A genome analog [p8(+)NPΔE-X] with a gene junction altered such that it did not contain a consensus downstream mRNA initiation signal, and so could not make a downstream mRNA, directed the synthesis and termination of the upstream mRNA with essentially the same ability as the wild-type template. This observation explains how L mRNA synthesis is terminated when there is no initiation signal within the trailer sequence located downstream of the L-gene 5′ end (25, 26). A recently proposed model of VSV transcription has suggested that termination of upstream mRNA synthesis is mediated through a mechanism which depends on forward slippage of the nascent strand followed by a cleavage event mediated by GDP attack, resulting in the formation of a 5′ mRNA cap structure (27). Our findings would seem to be incompatible with this model since we have demonstrated that for template p8(+)NPΔE-X, termination can occur in a situation where synthesis of downstream mRNA and consequently cap addition is prevented. However, we cannot formally exclude the possibility of GDP-mediated cleavage, but our results show that the downstream product of such a putative cleavage event is not elongated.

Removal of the downstream mRNA initiation signal also indicates that the sequences involved in upstream mRNA termination must be located entirely within the first 13 nucleotides of the conserved gene junction sequence (3′ AUAC UUUUUUUGA 5′, negative sense). However we previously analyzed all 16 possible variations of the intergenic dinucleotide and showed that while the second position of the 3′ GA 5′ exhibited a major effect on initiation of the downstream mRNA, it had only a minor effect on termination of the upstream mRNA (6). Thus, we conclude that the signal for termination can be further shortened to the first 12 nucleotides of this sequence. Stillman and Whitt (29) analyzed the behavior of some of the 16 possible variations of the intergenic dinucleotide and also concluded that the intergenic junction played a role in transcript termination. In summary, the VSV transcription termination signal comprises 12 nucleotides which are the tetranucleotide sequence AUAC, the U7 tract, and the first nucleotide of the intergenic dinucleotide.

Any alteration of the wild-type AUAC tetranucleotide sequence that lies upstream of the U7 tract resulted in templates exhibiting increased readthrough RNA synthesis, and these templates were calculated to have a correspondingly lower termination ability than the wild type. Previously, we demonstrated that of 16 possible nucleotide combinations, the wild-type sequence of the intergenic dinucleotide promoted most effective mRNA termination (6). In this study we again show that the sequence of the wild-type provided the most effective termination signal, indicating that the highest possible transcription termination efficiency appears to have been selected for in VSV. RNA analysis of altered templates revealed a trend such that the termination ability of an altered template decreased as substitutions were made to the AUAC sequence in a 3′-to-5′ progression. Thus, changes to the 3′ A of the tetranucleotide sequence were less damaging to termination than identical substitutions made at the 5′ most A residue. The 5′ C residue of the AUAC was the most critical for termination. When the C of the tetranucleotide was changed to any other nucleotide, upstream mRNA termination was abolished, showing this residue to be of major importance during termination. It is interesting that for many other members of the family *Mononegavirales*, the sequences that have been implicated to act in transcriptional termination contain a C residue in the same position, immediately upstream of a U tract (9, 10, 28, 31).

Because of its sequence and position, the U7 tract located downstream of the AUAC sequence has been implicated in providing the template for addition of the 3′ poly(A) tail of VSV mRNAs (13, 25). It has been established that the poly(A) tail is synthesized by an activity of the viral RNA-dependent RNA polymerase and that the tail is added cotranscriptionally before initiation of the downstream mRNA (13). The process by which the 100- to 300-nucleotide-long poly(A) tail is templated by a 7-nucleotide tract is thought to involve a cycle consisting of backward slippage of the nascent strand in relation to the template, followed by nascent chain elongation and a further round of slippage (polymerase stuttering).

Templates which lacked the U tract, or those in which it was shortened to five or six U residues, were unable to terminate upstream mRNA synthesis, indicating that seven U residues is the minimum number that permits termination. Analysis of polymerase activity during synthesis of the readthrough RNA using limited primer extension indicated that the U5 or U6 tracts failed to direct VSV polymerase slippage. Since these templates differ from wild type only in the lengths of their U tracts, it is likely that their lack of termination ability originated from their inability to induce polymerase slippage, thus indicating that polymerase slippage is one essential feature of VSV transcriptional termination.

Substitution of the U7 tract by an A7 tract resulted in a template which directed almost exclusive synthesis of the readthrough RNA. Analysis of the readthrough RNAs made from this template indicated that the A7 tract signaled polymerase slippage. This observation coupled with the inability of the A7 tract to allow efficient termination supports several previous studies which indicated that reiterative transcription alone is not sufficient for termination of mRNA synthesis (12–14, 23). Since this template possessed the essential AUAC sequence immediately upstream of the homopolymeric tract and could still promote polymerase slippage, the inability to signal termination must be attributed to the U7 to A7 change itself. This observation suggests that an essential termination signal is located either in the U7 tract or in the poly(A) sequence incorporated into the nascent strand.

Doubling the length of the wild-type U7 tract to give a U14 tract decreased only slightly the ability of the template to signal termination of transcription (termination ability of 95%). Interestingly, analysis of the RNAs transcribed from this template showed that increasing the U-tract length severely affected the ability of the polymerase to initiate downstream mRNA synthesis. This finding indicates that for the U14 tem-

plate, once the polymerase has terminated the upstream mRNA, it is less likely to initiate downstream mRNA synthesis compared to the wild-type template. We have previously described many altered templates whose attenuation activities were increased over that of wild type, but these alterations were all made in the region of the gene junction which affected initiation of downstream mRNA (6). The U14 template is the only genome analog that we have analyzed for which polymerase attenuation has been dramatically affected by alteration of a sequence that is not associated with downstream mRNA initiation, suggesting that the increase in attenuation is occurring through a mechanism distinct from a reduced ability to initiate per se. It is possible that the U14 tract increases the likelihood of the polymerase dissociating from the nucleocapsid template.

Many paramyxoviruses edit their P genes to access alternative reading frames by insertion of nontemplated nucleotides into the nascent strand. This is achieved by backward slippage of the nascent strand relative to the template, followed by a continuation of RNA synthesis. The number of nucleotides inserted depends on how far the nascent strand slips backward, and this is specified by the template sequence which limits the base-pairing alternatives between the two strands after slippage (7, 8, 11, 16, 17, 21). VSV polyadenylation is thought to occur by a related mechanism, whereby repeated cycles of slippage and RNA synthesis in the vicinity of the gene junction U7 tract result in the synthesis of VSV poly(A) tails. The AUAC tetranucleotide of the template does not allow for any likely base-pairing alternatives between the A residues of the nascent strand following a backward slippage event, and so it is possible that during polyadenylation at the VSV gene junction, the sequence at which the two RNA strands are undergoing slippage cycles is entirely within the U7 tract. Consequently, interactions between template and nascent strands during polyadenylation would be weak rA:rU base pairs, and these pairings or equally weak dA:rU pairings have been previously implicated as slippery sequences (7, 17). The AUAC sequence may constitute an essential termination signal that exerts its effect by either interacting with a site on the VSV RNA polymerase or recruiting an accessory component involved in the termination process.

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