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Mutational Analyses of the Intergenic Dinucleotide and the Transcriptional Start Sequence of Vesicular Stomatitis Virus (VSV) Define Sequences Required for Efficient Termination and Initiation of VSV Transcripts

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We have used dicistronic vesicular stomatitis virus (VSV) minigenomes to dissect the functional importance of the nontranscribed intergenic dinucleotide and the conserved transcription start sequence found at the beginning of all VSV genes. The minigenomes were generated entirely from cDNA and contained the G and M protein genes, flanked by the leader and trailer regions from the Indiana serotype of VSV. All mutations were made either within the nontranscribed M-G intergenic dinucleotide or within the transcription start sequence of the downstream G gene. Immunofluorescence microscopy and immunoprecipitation analysis of the mutated minigenomes indicated that the first three nucleotides of the transcriptional start sequence are the most critical for efficient VSV gene expression, whereas the nontranscribed, intergenic dinucleotide and the other conserved nucleotides found at the 5′ mRNA start sequence can tolerate significant sequence variability without affecting G protein production. RNA analysis indicated that nucleotide changes in the transcriptional start sequence which resulted in reduced G protein expression correlated with the amount of transcript present. Therefore, this conserved sequence appears to be required for efficient transcript initiation following polyadenylation of the upstream mRNA. While the minimum sequence for efficient transcription (3′-UYGnn-5′) is similar to that of other rhabdoviruses, it is not homologous to the start sites for viruses from the *Paramyxoviridae* or *Filoviridae* families. Using Northern blot analysis, we also found that some nucleotide changes in the nontranscribed intergenic region resulted in higher levels of read-through transcription. Therefore, the nontranscribed intergenic dinucleotide plays a role in transcript termination.

Vesicular stomatitis virus (VSV) is an enveloped, nonsegmented negative-strand RNA virus which is considered the prototype for the *Rhabdoviridae* family. The genome of VSV is approximately 11 kb in size and contains five genes which are sequentially and discontinuously transcribed by the virally encoded RNA-dependent RNA polymerase (1, 3, 21). Because the genome is in the negative, or noncoding, sense the polymerase must be packaged in the virion during virus assembly and remain associated with the ribonucleocapsid (RNP) core during virus entry and uncoating. Once RNPs enter the cytoplasm of the cell, the viral polymerase, which is composed of two subunits, the phosphoprotein (P) and the large catalytic subunit (L), transcribes five mRNAs which encode a total of seven proteins. Two of these proteins (C and C′) are translated from the phosphoprotein (P) mRNA by using alternative initiation codons (33) and are minor proteins which are not found in virus particles (22). The other proteins include the nucleocapsid (N), matrix (M), and glycoprotein (G). In addition to the initial transcription of viral mRNAs, the polymerase is responsible also for replication of the VSV genomic RNA via synthesis of a full-length positive-sense replicative intermediate (RI), which is then used as a template for synthesis of full-length negative-sense genomes.

One model of VSV transcription has proposed that the viral polymerase initiates transcription from the extreme 3′ terminus of the genomic RNA (14) and then genes are sequentially transcribed by a start-stop mechanism (14, 21); however, there is some evidence that the viral polymerase can initiate at internal sites on the genome (36). In accordance with the 3′ entry model, the viral polymerase first transcribes a small 47-nucleotide (nt) RNA called the leader which is complementary to the extreme 3′ end of the genome but which does not encode a protein product. Then each of the five mRNAs encoding the VSV proteins is synthesized. Because VSV gene expression is both sequential and polar (20), the five major proteins are expressed in amounts equivalent to their mRNA abundance, which follows the order of genes on the genome (e.g., N > P > M > G > L). The discontinuous nature of VSV transcription results from the polymerase transcribing each successive downstream gene at a level approximately 30% less than that of the adjacent upstream gene (20). Although the mechanism responsible for this attenuation phenomenon is not known, it may result from dissociation of the polymerase from the RNP template after pausing as it polyadenylates the 3′ end of each mRNA.

The *cis*-acting signals involved in both termination/polyadenylation of an upstream mRNA and reinitiation at the next downstream gene have been tentatively identified based on sequences which are conserved at each gene junction. For both the Indiana and New Jersey serotypes of VSV (VSV_I and VSV_{NJ}), the mRNA coding region terminates with the sequence 3′-$AUACU_7$-5′. The polymerase presumably stutters at this sequence to transcribe the poly(A) tail (32). The next mRNA is initiated downstream of two intergenic nucleotides, which are usually 3′-GA-5′ (30). Because the 5′ ends of all

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VSV mRNAs contain the sequence 5′-AACAGnnAUC-3′ (with n being any nucleotide), it is thought that this sequence may be important for reinitiation following polyadenylation of the upstream mRNA. Alternatively, it may play some role in capping such as signaling the polymerase to pause and facilitate the addition of the 5′ cap. Until recently, it has not been possible to examine the role of *cis*-acting signals in VSV replication and transcription due to the inability to introduce site-specific mutations into the VSV genome. Now several different systems are available that make the study of *cis*-acting sequences involved in the transcription, replication, and assembly of VSV feasible (24, 28, 35, 39).

In this study, we have used a transcriptionally active, dicistronic VSV minigenome to examine the importance of the nontranscribed intergenic dinucleotide as well as the conserved sequence found at the beginning of all VSV genes for the synthesis of viral mRNAs. With one exception where the second nucleotide of the nontranscribed intergenic region is a U, we show that the specific sequence of the intergenic dinucleotide has little to no influence on the expression of genes on either side of the intergenic junction. However, the intergenic sequence is important for efficient transcript termination of upstream genes. In contrast, we found that a U at the second position of the intergenic dinucleotide severely reduced G gene expression because the polymerase attempted to initiate transcription at that position. This mutation effectively shifts the G mRNA start sequence by one nucleotide, resulting in a mRNA start sequence which is not tolerated by the VSV polymerase. In addition, we found that positions 4 through 10 of the conserved start sequence can tolerate a significant number of nucleotide substitutions, whereas all but one nucleotide change at positions 1 to 3 essentially eliminated gene expression. Our data also indicated that there is an absolute requirement for the VSV polymerase to initiate efficient transcription at a U, which could also explain why a uridinylate is found at the 3′ ends of both the genomic RNA and RI.

MATERIALS AND METHODS

Expression plasmids and minigenome constructs. Plasmids encoding the wild-type G and M minigenome (GMMG) as well as the VSV_I N, P, G, and L proteins have been described elsewhere (16, 35). To generate minigenome mutants with nucleotide changes in the G-M intergenic region (IG mutants), we performed region-specific mutagenesis using a PCR-based strategy. Three different genome-sense, degenerate oligonucleotides (wild-type sequence is 5′-CATAGTG acgcgtAAACAGATC**GAT**CTCTGTT*AG*TTTTTTTCATAGGG-3′) having both single and multiple substitutions in either the intergenic dinucleotide (italics), the first five nucleotides of the G mRNA (underlined), or nt 8 to 10 of the G mRNA (boldface) were used with an antigenome-sense oligonucleotide (MW-28, 5′-TATAGGGCCCTCG**CGAAGACAAACAAACCATTATTATC**-3′) complementary to the leader region (boldface) to generate three sets of PCR products from a wild-type pBS-GMMG template. The primer used to generate the IG mutants was synthesized by delivering equimolar amounts of all four phosphoramidites at each target position, giving a theoretical misincorporation rate of 75%. The primer used to generate the nt 1–5 mutants had a calculated misincorporation rate of 32.5% and was synthesized by simultaneous delivery of a 76.5 mM mixture of all four phosphoramidites together with an equal volume of a 100 mM solution of the wild-type base at each of the target nucleotides. The primer used for the nt 8–10 mutants was synthesized by delivering a mixture of 55% wild-type and 15% the other three bases at each target nucleotide, resulting in a theoretical misincorporation rate of 45%. Following PCR amplification using standard conditions (16), the PCR products were digested with *Mlu*I and *Stu*I and the resulting 210-bp fragments were used with a *Stu*I-to-*Bgl*II fragment from the M gene to replace the corresponding regions in the wild-type pBS-GMMG plasmid. Plasmids containing mutations in the target sequences were identified by direct sequence analysis using a Thermal Cycle dideoxy sequencing method (New England Biolabs). Any single nucleotide changes that were not found in the pool of clones were subsequently generated with primers that had specific changes at the desired positions.

Minigenome expression and immunofluorescence analysis. Baby hamster kidney (BHK-21) cells in 35-mm-diameter dishes were transfected with 10 μg of plasmid DNA encoding either wild-type or mutant VSV minigenomes, together with 5, 4, and 1 μg of plasmids encoding the N, P, and L proteins, respectively, using TransfectACE (31). Indirect immunofluorescence was performed between 18 and 24 h posttransfection essentially as described previously (35), with the following modification. To detect both G protein on the cell surface and cytoplasmic M protein, fixed cells were first incubated with the G protein-specific monoclonal antibody I1 (25) and then a lissamine rhodamine sulfonyl chloride (LRSC)-labeled, affinity-purified donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). After washing, the cells were permeabilized with 1% Triton X-100 in phosphate-buffered saline containing 10 mM glycine and then incubated with an M protein-specific monoclonal antibody (23H12 [25]) and a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse secondary antibody. Because the FITC-linked secondary antibody can react with both G- and M-specific monoclonal antibodies, we determined the level of G protein actually detected by the FITC-linked secondary antibody. Cells expressing the N, P, and L proteins were infected with P4 supernatants containing wild-type minigenome particles. At 24 h after minigenome infection, fixed cells were first incubated with the G protein-specific monoclonal antibody I1 and then the LRSC-labeled, affinity-purified donkey anti-mouse secondary antibody. After washing, the cells were then incubated with a FITC-conjugated donkey anti-mouse secondary antibody. Very low levels of G protein were detected with the FITC-conjugated secondary antibody. Therefore, any G protein detected by the FITC-conjugated secondary antibody did not contribute significantly to the resulting signal for detection of M protein.

Minigenome passaging. Minigenomes were passaged essentially as described previously (35) except that cells were transfected with 5, 4, and 1 μg of plasmids encoding the N, P, and L proteins, respectively, prior to addition of the P1 supernatant. To obtain high-titer supernatants of minigenome mutants that could not be passaged efficiently due to insufficient G protein expression, cells were transfected with plasmids encoding the minigenomes and the N, P, and L proteins as described above, as well as 3 μg of a plasmid encoding G protein (pBS-G). Minigenome expression and passaging were monitored by detecting M protein expression, using immunofluorescence microscopy.

Immunoprecipitation of G and M protein. Cells expressing the N, P, and L proteins were infected with P3 or P4 supernatant containing either wild-type or mutant minigenome particles. At 4 h after minigenome infection, the cells were rinsed once with methionine-free, serum-free Dulbecco modified Eagle medium and then pulsed for 15 min with 50 μCi of [^{35}S]methionine in 1 ml of the same medium, using Express labeling mix (DuPont/NEN) at 37°C. Following the pulse, the radioactive medium was removed and the cells were lysed immediately with 1 ml of detergent lysis buffer (10 mM Tris [pH 7.4], 66 mM EDTA, 1% Triton X-100, 0.4% deoxycholic acid, 0.02% sodium azide, 0.1 trypsin inhibitor unit of aprotinin). One-tenth to one-third of the cell lysate from a 35-mm-diameter plate was immunoprecipitated essentially as described previously (16) except that the antigen-antibody complexes were formed for 1.5 h at 37°C, using 3 μl a VSV polyclonal antibody (TN-1), and the antibody-protein A complexes were formed for 1 h at 37°C. The immunoprecipitated proteins were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions and visualized by fluorography (6). Densitometry was performed by digitizing fluorograms with a Hewlett-Packard ScanJet IIcx flatbed scanner and then determining band intensities by using NIH Image.

RNA analysis (RNase protection, primer extension, and Northern blot assays). Total RNA was isolated at 4 h after P3 or P4 minigenome infection or VSV infection (recombinant VSV_I [24]) by the method of Chomczynski and Sacchi (9). Following extraction with phenol-chloroform and precipitation with isopropanol, the resuspended pellets were treated with RNase-free DNase as instructed by the manufacturer (Promega), extracted again with phenol-chloroform, and ethanol precipitated. The RNA pellets were resuspended in diethyl pyrocarbonate-treated deionized H_2O, and their concentrations were determined spectrophotometrically.

RNA probes used in the RNase protection assay were generated by in vitro transcription with T7 RNA polymerase and [α-^{32}P]CTP. The template contained sequences from the *Dra*I site at nt 1565 in the G gene to the *Sac*I site at nt 2016 in the M gene of pBS-GMMG, which were cloned into the *Eco*RV and *Sac*I sites of pBS-SK+ (Stratagene). Runoff transcripts were produced after the template had been linearized with *Stu*I located at position 1917 in the M gene, resulting in a 407-nt probe which contained 55 nt from the pBS-SK+ polylinker region. Individual templates were made for each of the mutant minigenomes. Approximately 3×10^6 cpm of labeled RNA probe was ethanol precipitated with 0.03 to 3.0 μg of total RNA and 10 μg of tRNA. The pellet was resuspended in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 1 mM EDTA, 0.4 M NaCl, 80% formamide], incubated at 85°C for 5 min, and immediately transferred to 50°C for an overnight incubation. To digest unhybridized RNA, the tubes were cooled to room temperature and then incubated at 30°C for 1 h in 300 μl of digestion buffer (0.3 M NaCl, 10 mM Tris [pH 7.5], 5 mM EDTA, 0.04 U of RNase T_1 per ml, 1 μg of RNase A per ml). The digestion was stopped by the addition of 10 μl of 10% SDS and 100 μg of proteinase K. After a 30-min incubation at 37°C, 10 μg of tRNA was added and the sample was phenol-chloroform extracted and ethanol precipitated. The pellets were resuspended in 2.5 μl of loading dye and electrophoresed on a 6% sequencing gel. The protected species were visualized following autoradiography and quantitated by densitometry as described above.

For primer extensions, we used oligonucleotide MW-38 (5′-CTATGGTG AACTTGCAATTCACCCC-3′), complementary to a sequence approximately

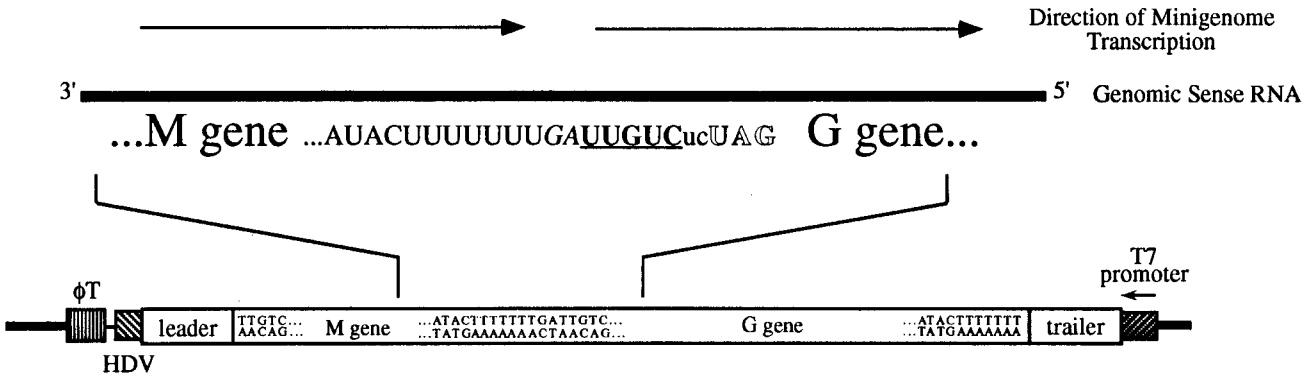

FIG. 1. Conserved sequence elements at the M-G gene junction. A diagram of pBS-GMMG is shown. The polyadenylation stop signal of the G gene is fused to the trailer region, and the conserved transcription start site of the M gene is fused to the leader region. The locations of the hepatitis delta virus ribozyme sequence (HDV) and the T7 terminator (φT) are also shown. Expression from the T7 promoter results in the synthesis of a genome-sense RNA which becomes encapsidated by the N protein and serves as a template for the synthesis of the M and G mRNAs by the VSV polymerase. The sequence of the M-G gene junction is enlarged, and the regions that were mutated are highlighted. Mutations were introduced into the M-G intergenic dinucleotide (italics), nt 1 to 5 (underlined) of the conserved transcription start for the G protein gene, or nt 8 to 10 (outline font), which are also conserved.

90 nt from the 5′ end of the G mRNA. The primer was end labeled in a standard kinase reaction (5 mM oligonucleotide, 1× kinase buffer, 100 μCi of [γ-^{32}P]ATP, 10 U of T4 polynucleotide kinase [New England Biolabs]) for 45 min at 37°C, and the kinase was inactivated by incubation at 70°C for 5 min; after ethanol precipitation and washing, the labeled primer was resuspended in 100 μl of distilled H_2O. Between 5 and 10 μg of the total RNA was ethanol precipitated with 5×10^4 cpm of the primer, and the pellet was resuspended in 30 μl of hybridization buffer (40 mM PIPES [pH 6.8], 1 mM EDTA, 0.4 M NaCl, 80% formamide), incubated at 85°C for 5 min, and immediately transferred to 30°C for overnight incubation. The total RNA used for the primer extension and RNase protection assay was isolated from the same minigenome infection. Hybridized RNAs were ethanol precipitated, and the pellet was resuspended in 25 μl of primer extension reaction buffer (0.55 mM each of the four deoxynucleoside triphosphates, 33 U of RNasin, 50 mM Tris-Cl [pH 8.2], 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM KCl, 50 μg of bovine serum albumin per ml). The reaction was initiated by addition of 50 U of avian myeloblastosis virus reverse transcriptase (Promega), the reaction mixture was incubated for 1.5 h at 42°C, and then the reaction was stopped by the addition of 1 μl of 0.5 M EDTA and 2 μl of RNase A (0.5 μg/ml). After a 15-min incubation at 37°C, the cDNA reaction was ethanol precipitated, and the pellet was washed with 70% ethanol, resuspended in 3 μl of loading dye, and then electrophoresed on a 6% sequencing gel next to a sequencing ladder. The sequencing ladder was generated by using the same end-labeled primer and wild-type pBS-GMMG plasmid as a template, using the Thermal Cycle protocol (New England Biolabs).

For Northern blot analysis, total or poly(A) RNA (Qiagen) was isolated at 6 h after P3 or P4 minigenome infection. The sample RNA was denatured by heating to 65°C in denaturing sample buffer [1× 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, 6.5% formaldehyde, 50% formamide] and then fractionated on 1% agarose-formaldehyde gels in 1× MOPS buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA) as described previously (2). The RNA was transferred to Nytran (Schleicher & Schuell), cross-linked by UV irradiation, and then examined by Northern blot analysis using standard procedures (2).

The membranes were prehybridized in a solution of 50% deionized formamide, 0.1 M PIPES (pH 6.8), 0.5 M NaCl, 10× Denhardt's solution, 0.2% SDS, 50 μg of denatured salmon sperm DNA per ml, and 50 μg of tRNA per ml for 2 to 3 h at 68°C. Hybridization occurred in 5 ml of fresh solution containing 5×10^6 cpm of the probe for 15 to 18 h at 68°C. After hybridization at 68°C, the temperature was raised to 75°C for 2 h. The membranes were then washed four times for 30 min each in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium acetate)–0.01% SDS at 68°C. RNA probes were generated as described above. The templates used to generate the G mRNA-specific probe and the M mRNA-specific probe were described previously (35).

RESULTS

To examine the role of the conserved sequences found at the beginning of each gene in VSV gene expression, we mutated three different regions of the G-M gene junction in the G and M minigenome, GMMG (Fig. 1). These regions consisted of (i) the nontranscribed dinucleotide found between the M and G genes, (ii) the first 5 nt of the transcription start sequence for the G protein gene, and (iii) nt 8 to 10 of the G protein mRNA, which are found also at the 5′ ends of all VSV mRNAs. A total of 40 mutants which had either single or multiple nucleotide substitutions were generated (Table 1).

G and M protein expression from mutated minigenomes. Initially we used immunofluorescence microscopy to determine if any of the nucleotide changes affected M and/or G protein expression (Fig. 2). We predicted that M protein expression should not be affected even if the mutations prevented termination of the M protein message since polyadenylation of an M-G dicistronic mRNA should occur normally at the G gene termination/polyadenylation signal. As expected, we found that all of the mutants expressed M protein at or near wild-type levels. Interestingly, mutations in nt 8 to 10 of the mRNA start site and most of the mutations in the nontranscribed dinucleotide did not appear to significantly decrease G protein expression from minigenomes. However, when the second nucleotide of the intergenic dinucleotide (3′-GA-5′) was changed from an A to a U, the expression of G protein, as determined by immunofluorescence microscopy, was almost completely eliminated (data not shown). For positions 1 to 5, several nucleotide changes reduced the amount of G protein expressed. For example, all substitutions at the first position reduced G protein expression to undetectable levels (Fig. 2E and F), while substitution of U with C at the second position diminished G protein expression by approximately 50% (Fig. 2C and D). Similar results were found for mutations at the third nucleotide of the start sequence, while changes at nt 4 and 5 had less effect on G protein expression (not shown). Taken together, these data suggested that only the first three positions of the transcription start sequence are critical for VSV gene expression and that there can be significant flexibility in the sequence of the nontranscribed, intergenic dinucleotide and the other conserved nucleotides found at the 5′ mRNA start sequence for normal levels of VSV gene expression.

Replication and passage of mutant minigenomes. To determine the effect of the mutations on virus assembly, we attempted to passage each of the mutant minigenomes. We have shown previously that cells transfected with plasmids encoding wild-type GMMG and the N, P, and L proteins produce infectious particles that can be sequentially passaged on cells expressing the N, P, and L proteins only (35). Supernatants from cells expressing either wild-type or mutant minigenomes, together with the N, P, and L proteins, were used to infect a second set of cells that had been transfected with the N, P, and

TABLE 1. Passaging of mutant GMMGs

Mutated region	Sequence[a]	Passage efficiency[b]
Intergenic dinucleotides	GA	++
	C-	++
	U-	++
	A-	++
	-C	++
	-U	−
	-G	++
	UC	++
	UG	++
	AC	++
	CU	−
	UU	−
	AU	−
nt 1–5 of mRNA start	UUGUC	++
	C----	−
	A----	−
	G----	−
	-C---	++
	-A---	+
	-G---	+
	--C--	+
	--U--	+
	--A--	+
	---C-	++
	---A-	++
	---G-	++
	----U	++
	----A	++
	----G	+
	GGA--	−
	--UA-	+
	---GA	+
nt 8–10 of mRNA start	UAG	++
	C--	++
	A--	++
	-C-	++
	-U-	++
	--U	++
	GC-	++
	AU-	++
	CC-	++
	A-A	++
	GUA	++

[a] The wild-type genomic sequence (3′ to 5′) is indicated on the first line for each group.

[b] Transfer of supernatants containing either wild-type GMMG or mutant GMMG particles to cells expressing the N, P, and L proteins only resulted in approximately a 10-fold increase in cells expressing M protein (++), a 2-fold increase (+), or no cells expressing M protein (−) compared to the number of M-positive cells determined from the previous passage. The data are compiled from three rounds of passaging.

L plasmids only. At 22 to 24 h postinfection, the cells were examined for M protein expression by immunofluorescence microscopy.

For wild-type GMMG, there was at least a 10-fold increase in the number of positive cells with each passage, and after two passages, about 30% of the cells expressed M protein. Similar results were obtained for minigenomes having mutations at nt 8 to 10 of the 5′ mRNA start site (Table 1). In addition, all intergenic dinucleotide mutants, except those with a U at the second position, passaged as efficiently as wild-type GMMG. In contrast, IG mutants with a U at the second position and several of the mutants with substitutions in the nt 1–5 region produced either no or significantly fewer infectious GMMG particles. For example, when the first U of the start sequence was changed to any other nucleotide, no infectious particles were produced. Typically minigenomes with mutations at the second and third nucleotides were able to passage, but there was never more than a twofold increase in the number of cells expressing M protein after each passage. The only exception was when the second U was changed to a C, which resulted in near-wild-type passaging efficiency. Minigenomes with single-nucleotide changes at positions 4 and 5 passaged at or near wild-type efficiency, but when both nucleotides were changed (e.g., when nt 4 and 5 were changed from UC to GA), there appeared to be a significant reduction in the amount of virus produced. For all mutant GMMGs, the ability to passage and amplify particles correlated with the levels of G protein expressed as determined by immunofluorescence microscopy. Therefore, these results indicated that mutations which did not significantly reduce G protein expression had no detectable effect on the replication or assembly of minigenome particles.

Quantitation of G and M protein expression from mutated minigenomes. The data obtained by both immunofluorescence microscopy and passaging indicated that the first 3 nt of the start sequence are the most critical for G protein expression. In addition, the majority of these mutations resulted in varied levels of G protein expression, as opposed to all-or-none expression. Therefore, to more rigorously determine the amount of G and M protein expressed from each of the GMMG mutants, we radioactively labeled cells that had been infected with third- or fourth-passage supernatants and quantitated the amounts of G and M protein present following immunoprecipitation. To obtain high-titer supernatants for mutants that

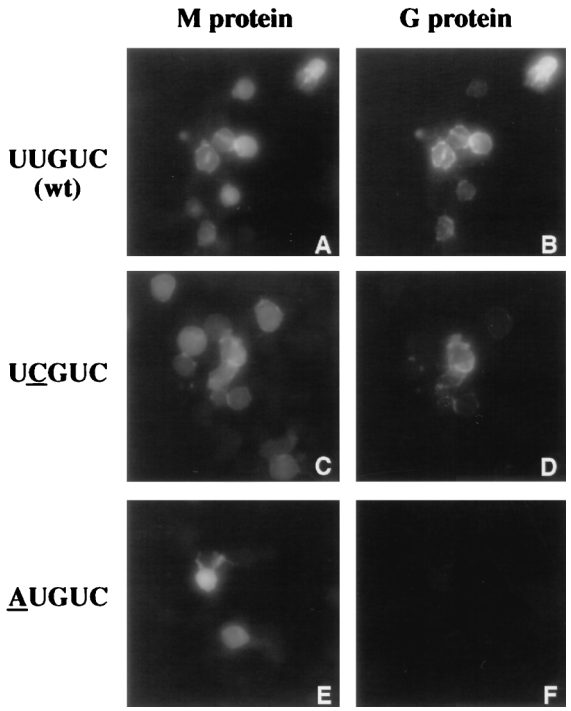

FIG. 2. Immunofluorescence of cells expressing G and M proteins from wild-type and mutant minigenomes. Two nt 1–5 mutants are shown. The altered nucleotide is underlined. BHK-21 cells were infected with vTF7-3 and then transfected with plasmids encoding the N, P, and L proteins together with either wild-type (wt) or mutant GMMG. The cells were fixed at 24 h posttransfection and examined for both G protein on the cell surface and cytoplasmic M protein as described in Materials and Methods. (A, C, and E) Cells expressing M protein (FITC-labeled cells); (B, D, and F) the same cells immunoprobed for G protein on the cell surface (LRSC-labeled cells).

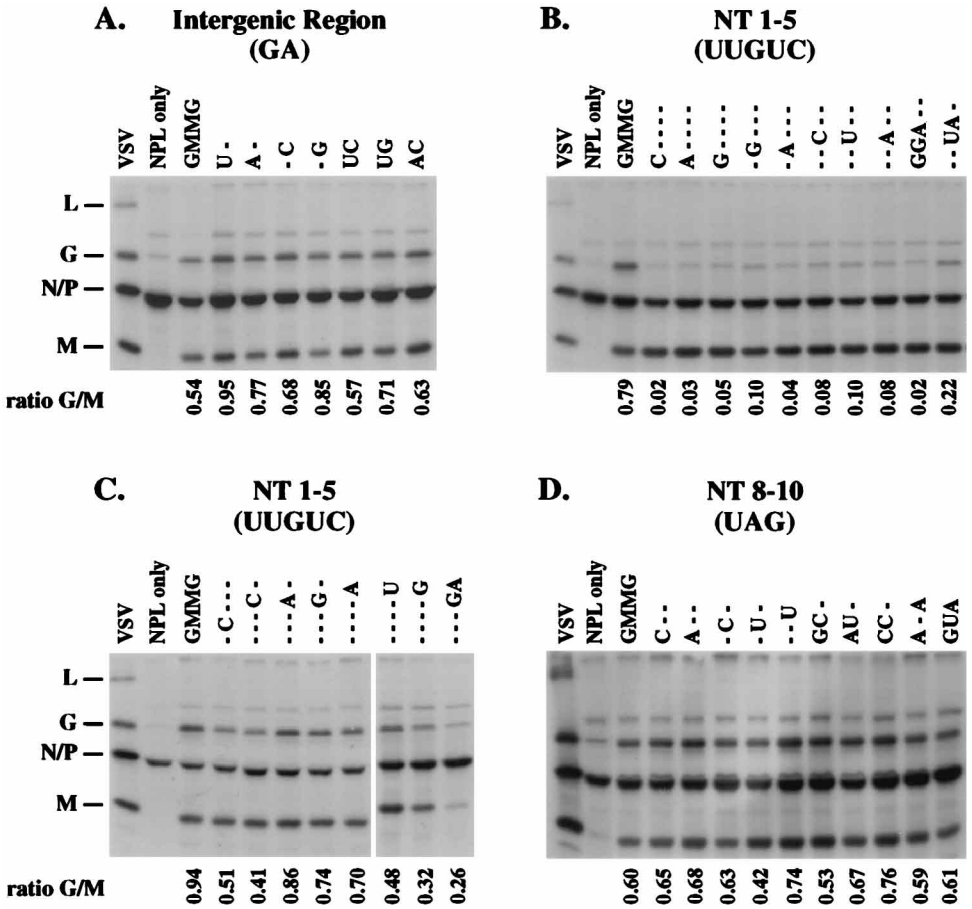

FIG. 3. Immunoprecipitation of VSV proteins expressed in wild-type and mutant GMMG-infected cells. Cells expressing the N, P, and L proteins were infected with P3 or P4 supernatant containing GMMG particles, and viral proteins were labeled for 15 min with [^{35}S]methionine at 4 h postinfection. The cells were lysed immediately; viral proteins were immunoprecipitated and then analyzed by reducing SDS-polyacrylamide gel electrophoresis. Each panel includes immunoprecipitates from cells infected in parallel with wild-type VSV or cells expressing the N, P, and L proteins (NPL) only. The positions and nucleotide substitutions of the mutants are indicated above the lanes. The ratios of G to M protein for the mutants are shown below the lanes and were determined by densitometry of digitized fluorograms. Background levels, due to cross-reactivity with vaccinia virus-encoded proteins, are shown in the NPL-only lanes and were subtracted from the G and M levels determined for wild-type and mutant GMMGs.

did not passage (e.g., nt 1 mutants), G protein was expressed in *trans* together with the N, P, and L proteins in the primary transfection and during each subsequent passage. When G protein was provided in *trans*, there was at least a 10-fold increase in the number of M-positive cells with each passage, and after three to four passages, the supernatants contained approximately 10^5 infectious particles per ml. For the final minigenome infection in which the cells were radioactively labeled, the plasmid encoding G protein was omitted. To determine if there was any G protein expression due to carryover of the plasmid encoding the G protein, cells were transfected with 10 μg of pBS-SK+ vector plasmid to mimic transfection with the plasmids encoding the N, P, and L proteins and then infected with a P4 supernatant of wild-type GMMG that had been previously passaged with the N, P, L, and G plasmids. To prevent G expression from the input wild-type GMMG particles, the P4 supernatant was first neutralized with a VSV polyclonal antibody. No G protein expression was detected in these samples by immunofluorescence microscopy.

Figure 3 shows the relative levels of G and M protein expressed from some of the mutants. To ensure that the immunoprecipitation was quantitative, each of the supernatants was subjected to a second round of immunoprecipitation. Although significant amounts of labeled N protein were precipitated from the supernatants, virtually no G or M protein was detected (data not shown). To compare the mutants and to normalize for differences in the amount of infectious virus present in each of the supernatants, we calculated the ratio of G protein to M protein as described in the figure legend. The results are also shown graphically in Fig. 4. Since there was a 1.7-fold variation in the G-to-M ratio for wild-type GMMG in each set of immunoprecipitations (compare the GMMG lanes in Fig. 3), we considered differences greater than 3-fold to be important. As indicated by the immunofluorescence and passaging data, most intergenic mutations and mutations in nt 8 to 10 had no effect on G protein expression (Fig. 3A and D, respectively, and Fig. 4). However, for the nt 1–5 mutants that required G protein in *trans* to passage and the IG mutants with a U at the second position, G protein expression was reduced 4- to 40-fold (Fig. 3B; results for IG mutants not shown). As noted above, all substitutions in the first 3 nt of the transcription start signal, except for a pyrimidine substitution (U to C) at nt 2, reduced G protein expression significantly. For nt 2–5 mutants that could passage without G complementation, the amount of G protein expressed ranged from wild-type levels to a threefold reduction (Fig. 3C). The observation that single-nucle-

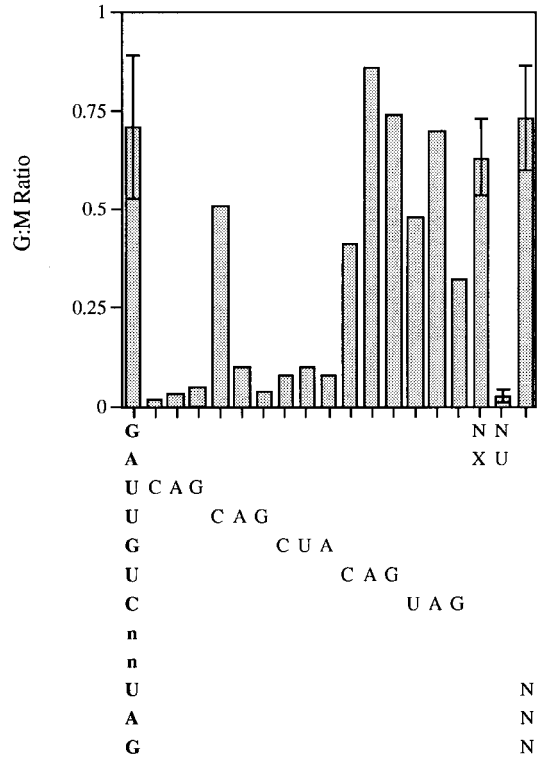

FIG. 4. Graph of G-to-M protein ratios obtained from immunoprecipitation assays. The wild-type sequence is in boldface. The ratios for wild-type GMMG, the IG mutants, and the nt 8–10 mutants are a combined average from all mutants with nucleotide changes in those regions. Wild-type GMMG, $n = 4$; NX IG mutants, $n = 7$; NU IG mutants, $n = 4$; nt 8–10 mutants, $n = 10$. N indicates any nucleotide, and X corresponds to G, C, or A.

otide changes of U to G and C to A at positions 4 and 5, respectively, had little effect on G protein expression but the double substitution at positions 4 and 5 of UC to GA reduced G expression approximately fourfold suggested that nt 4 and 5 are important but less so than nt 1 to 3. These results also indicated that infectious GMMG particles could still be produced even when G protein expression was reduced as much as 70%.

Levels of G and M mRNA expression. To determine the basis for the reduced levels of G protein expression, we examined the RNA species present in both wild-type and mutant minigenome-infected cells by using an RNase protection assay. The probe that was used could detect both G and M mRNAs as well as the RI. The protected positive-sense RNA species expected after digestion are diagrammed in Fig. 5A. Since the probe overlaps the area that was mutated, individual probes with the corresponding nucleotide changes were generated for each mutant minigenome.

We examined the relative level of G mRNA expressed from each of the mutants, using the M mRNA to normalize for differences in the amount of GMMG particles used to infect cells. We then compared the levels of G mRNA expressed from the mutants to that expressed from wild-type GMMG. The mutant minigenomes which had changes within nt 8 to 10 and the intergenic dinucleotide that expressed G protein all expressed wild-type levels of G mRNA (Fig. 5B, lanes 11 to 13). However, mutations in nt 1 to 5 either eliminated transcription from the G gene (lanes 4 and 5) or reduced the amount of G mRNA between 50 and 90% (lanes 6 to 10). These results indicated that the mutations in nt 1 to 5 possibly affected transcript initiation, although we cannot rule out the possibility that this difference was due to reduced message stability. It should be noted that even though the G-specific signals in lanes 6 through 10 were similar, there was less total VSV RNA in lanes 9 and 10 and the relative amounts of G and M mRNA for these mutants were approximately 50% of that found for wild-type GMMG.

In addition to the 158-nt G-specific fragment, there were additional RNA species that migrated slightly faster when total RNAs from wild-type VSV, GMMG, and several of the mutants were examined (Fig. 5B, arrowhead). The heterogeneity of these RNAs suggested that they were nonspecific. However, for the mutant in which nt 5 was changed to an A (NT-5A; lane 10), there was an additional protected fragment which appeared to be specific and had a length approximately 5 nt

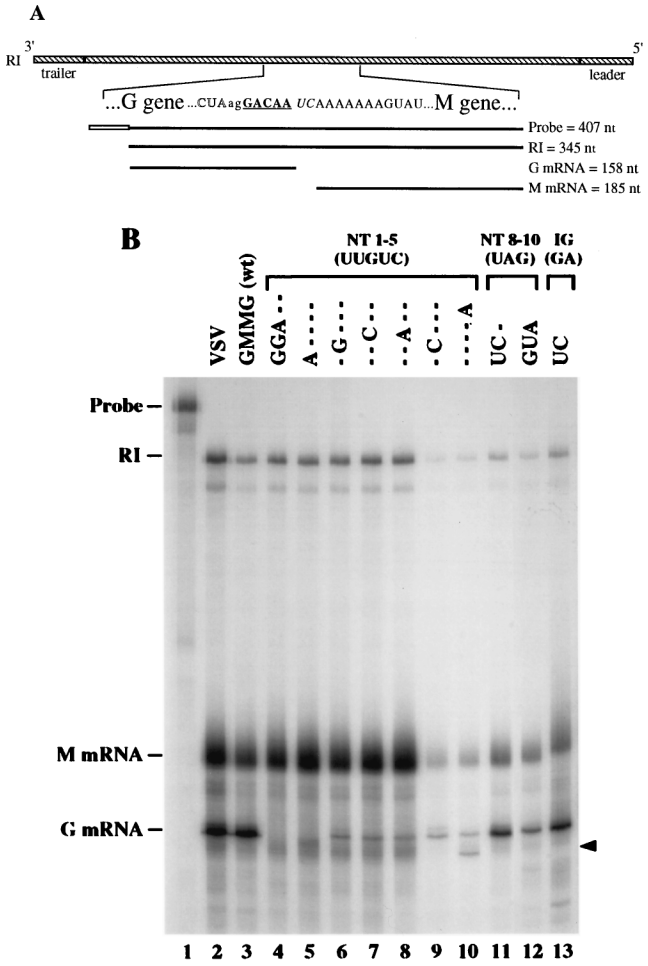

FIG. 5. RNase protection assay of total RNAs purified from wild-type and mutant GMMG-infected cells. (A) The antisense RNA probe spans the G-M intergenic regions, enabling it to detect RI, G mRNA, and M mRNA with different sizes of the protected RNA species. The open box represents 55 nonhomologous nucleotides from the pBS-SK+ polylinker region. Other notations are as in Fig. 1. (B) Total RNA was extracted from cells that were transfected with plasmids encoding the N, P, and L proteins and then infected with GMMG particles from a P4 supernatant or from cells that were infected with wild-type recombinant VSV. The RNA was hybridized with the antisense RNA probe, and after digestion, the protected species were fractionated on a denaturing 6% polyacrylamide gel. Lanes 2 and 3, VSV and wild-type GMMG [GMMG (wt)], respectively; lanes 4 to 10, nt 1–5 GMMG mutants; lanes 11 and 12, nt 8–10 GMMG mutants; lane 13, IG GMMG mutant.

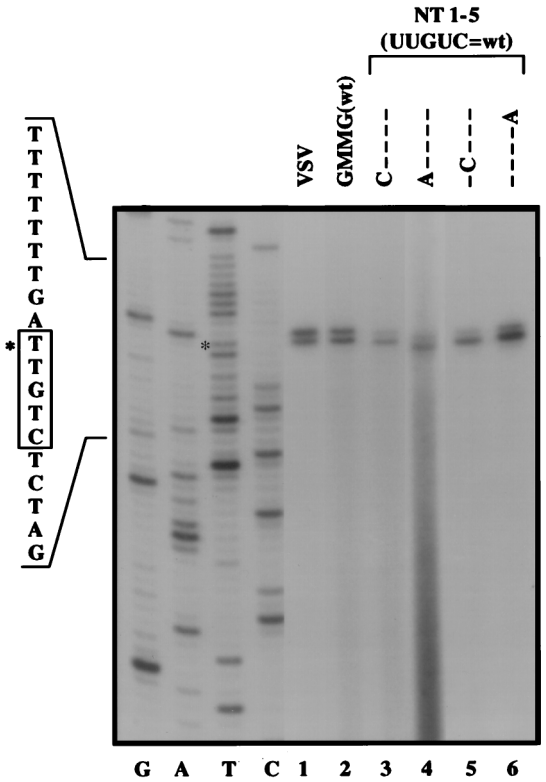

FIG. 6. Primer extension analysis of RNAs purified from GMMG-infected cells. The total RNAs isolated were hybridized to an end-labeled oligonucleotide complementary to sequences in the G mRNA. Following the extension reaction, the products were run on a denaturing 6% polyacrylamide gel. A sequencing ladder generated from pBS-GMMG and the end-labeled oligonucleotide is shown also. The conserved nt 1–5 region is boxed, and the position of the initiating nucleotide is marked by an asterisk. The RNA generated for the NT-5A mutant was isolated from cells that were infected with the same supernatant stock as used for the RNase protection assay. To obtain equivalent band intensities, 2 to 100 times more total RNA was used for mutant GMMG extension reactions, and digitized autoradiographs from different exposures were used to assemble the figure. wt, wild type.

shorter than that predicted for the G mRNA species. One possibility is that there were two populations of G mRNAs present, one which initiated at nt 1 and another which initiated at nt 5. Alternatively, a revertant could have arisen during passaging of the mutant minigenome, resulting in a mismatch at nt 5 between the probe and the G mRNA. To determine if the smaller RNA resulted from initiation at nt 5, we used primer extension to identify the G mRNA start site utilized by this and several of the other mutant minigenomes (Fig. 6). The results show that the 5′ end of the G transcript expressed from NT-5A (lane 6) was identical to that found in VSV- and wild-type GMMG-infected control cells (lanes 1 and 2). One major product in all of these cases mapped to the first nucleotide of the 5′ mRNA start sequence. Another product which was 1 nt longer and detected with both VSV and GMMG RNAs presumably resulted from partial extension through the cap at the 5′ end of the G mRNA (13, 38). Therefore, these results indicated that the near-normal levels of G protein expression observed for NT-5A did not result from two RNA transcripts having alternative start sites.

Although no G mRNA was detected by RNase protection assays for several of the nt 1–3 mutants, specific products were detected when the equivalent of 100 times more total RNA than that used for wild-type GMMG was analyzed by primer extension. Interestingly, for NT-1A (Fig. 6, lane 4) and NT-1G (data not shown), the primer extension products were 1 nt shorter than the primer extension products from VSV and wild-type GMMG RNAs, indicating that transcripts initiated at the second U of the start sequence in these mutants. However, for NT-1C (lane 3), the primer extension product was the same size as that produced from VSV and wild-type GMMG RNAs, indicating that the transcript initiated at the first position of the start sequence. For this mutant, the first nucleotide is now a C. These data suggested that in addition to the sequence requirements defined by the first 3 nt, there appears to be an absolute requirement by the VSV polymerase to initiate transcription at a pyrimidine.

Levels of M-G dicistronic mRNA from IG mutant GMMGs. Previously, it had been hypothesized that the intergenic dinucleotide plays a role in the termination of upstream transcripts. This hypothesis arose from observations of increased read-through transcription at the P-M intergenic junction of VSV_I (19, 26). The P-M intergenic dinucleotide is 3′-CA-5′, while 3′-GA-5′ is the sequence found at the other gene junctions in both VSV_{NJ} and VSV_I.

To determine if an intergenic dinucleotide of 3′-CA-5′ specifically results in read-through transcription, total RNA from N, P, and L plasmid-transfected and minigenome (wild type wt or mutant)-infected BHK-21 cells was examined by Northern blot analysis. Figure 7A shows the RNA species produced by the IG mutants had near-wild-type levels of G protein expression. For comparison, total RNA from cells infected with wild-type VSV_I was also examined (lane 1). The G and M antisense RNA probes that were used detected G mRNA, M mRNA, and two types of M- and G-specific species, which were the RI and M-G dicistronic messages. The RI and M-G mRNAs from GMMG-infected cells were predicted to be approximately the same size because the later is polyadenylated (Fig. 7B, lane 3). The ratio of RI and M-G mRNAs to M mRNA was determined for each mutant. Single-nucleotide changes at either the first position (lanes 3 to 5) or the second position (lanes 6 and 7) resulted in near-wild-type to slightly higher levels of M-G positive-sense RNA. Interestingly, the level of M-G dicistronic message for the 3′-CA-5′ mutant was similar to that found in wild-type VSV-infected cells (lane 3). In contrast, nucleotide changes at both positions (lanes 8 to 10) resulted in significantly higher levels of M-G positive-sense RNA compared to wild-type GMMG-infected cells. These data indicated that nucleotide changes in the intergenic region do indeed lead to an increase in the frequency of read-through transcription.

To confirm that the increase in read-through occurred during transcription, as opposed to an increase in the synthesis of RI, poly(A) RNA was isolated from wild-type or mutant GMMG-infected cells and examined by Northern blot analysis (Fig. 7B). Significantly higher levels of dicistronic message remained for the double mutant, 3′-AC-5′ (lane 3), compared to wild-type GMMG, the 3′-CA-5′ mutant, and mutant GMMGs with nucleotide changes within the 5′ mRNA start sequence (lanes 1, 2, 4, and 5). These results indicated the composition of the intergenic dinucleotides is important for efficient transcript termination and may play a role in regulating the amount of translatable message available for certain VSV genes.

To determine the basis for the reduced levels of G protein expression from intergenic mutants with a U at the second position, total RNA was examined by Northern blot analysis as described above (Fig. 8A). These mutations also resulted in an increase in the frequency of read-through transcription, as indicated by the increase in the RI and M-G dicistronic mRNA-to-M mRNA ratio (lanes 3 to 5). In addition, the levels of G mRNA, but not M mRNA, were dramatically reduced.

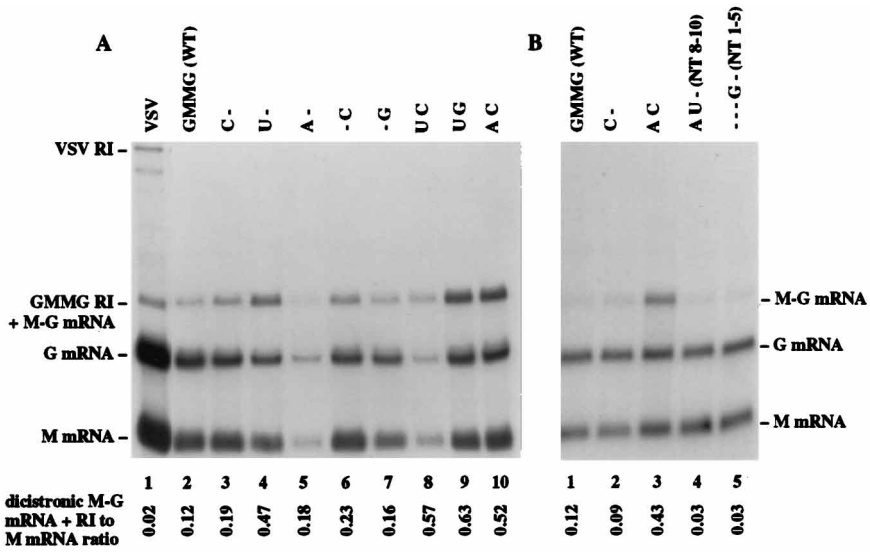

FIG. 7. Northern blot analysis of total RNAs and poly(A)$^+$ RNAs purified from wild-type (WT) and IG mutant minigenomes. Approximately 3 μg of total RNA (A) or poly(A) RNA (B) was fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with two antisense RNA probes specific for either G or M mRNA sequence. The RNAs used for panels A and B were isolated from two separate minigenome infections. The ratios of RI and M-G mRNAs to M mRNA are shown at the bottom.

These data indicate that the mutations also affected initiation of G mRNA. One explanation is that the polymerase attempted to initiate transcription at the second nucleotide of the intergenic dinucleotide (which has been changed to a U). The start sequence would then be shifted by one nucleotide such that a U instead of a G would be present in the third position (e.g. 3′-*UUUGUC-5′ instead of 3′-*UUGUC). Initiation at this nucleotide would not be optimal for the VSV polymerase since the mutational analysis of the start sequence indicated that the third nucleotide of the start sequence must be a G for efficient mRNA expression.

To determine the initiating nucleotide for the low levels of G mRNA expressed from these mutants, we used a primer extension assay (Fig. 8B). The results show that the major product for the 3′-CU-5′, 3′-AU-5′, and 3′-GU-5′ mutants (lanes 4, 6, and 7) is 1 nt larger than the product for VSV and wild-type GMMG (lanes 1 and 2, respectively) and the double IG mutant, 3′-UG-5′ (lane 3), which does not result in a drastic

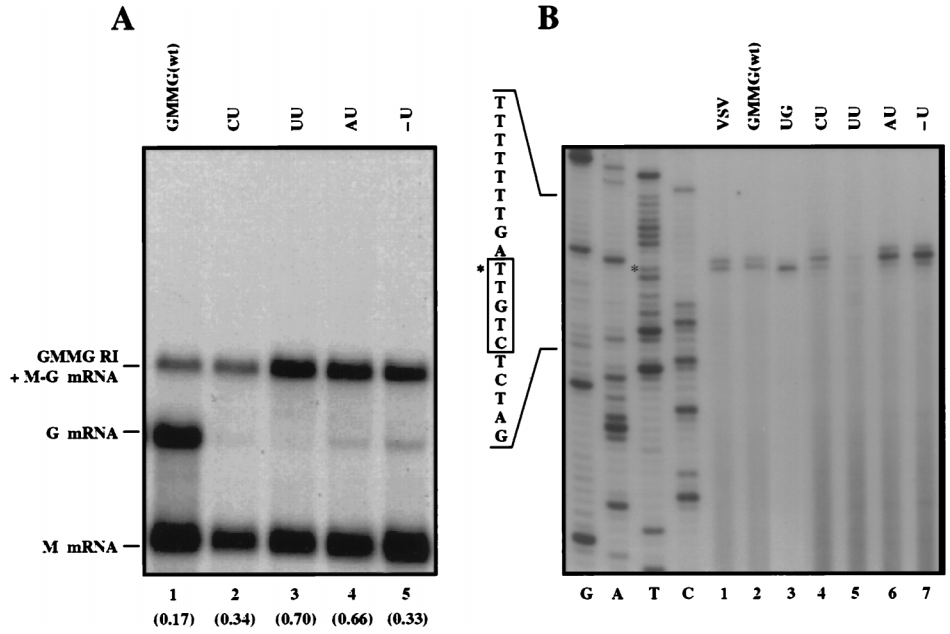

FIG. 8. Northern blot and primer extension analysis of RNA isolated from IG mutants that reduce G protein expression. (A) Northern blot analysis. Approximately 3 μg of total RNA was probed essentially as described for Fig. 7. Lane 1, wild-type GMMG [GMMG(wt)]; lanes 2 to 4, IG mutants with a U at the second position. (B) Primer extension analysis. The initiating nucleotide for the G mRNA was determined essentially as described for Fig. 6. To obtain equivalent band intensities, 2 to 100 times more total RNA was used for mutant GMMG primer extension reactions, and digitized autoradiographs from different exposures were used to assemble the figure.

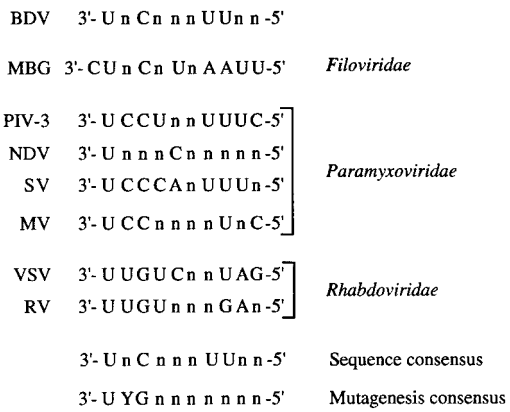

FIG. 9. Comparison of consensus sequences found at the beginning of each gene for viruses from the *Rhabdoviridae*, *Filoviridae*, and *Paramyxoviridae* families. Consensus sequences were obtained from references 4, 11, 12 and 17. n = G, A, U, or C; Y = C or U. BDV, Borna disease virus (unclassified); MBV, Marburg virus; NDV, Newcastle disease virus.

decrease in G protein expression. In contrast to the other IG mutants, it was difficult to determine at which nucleotide the 3′-UU-5′ mutant initiated since there were RNA species which were both longer and shorter than the wild-type G mRNA. Changing both intergenic nucleotides to U's resulted in a G-M gene junction that contained 11 consecutive U's (7 in the polyadenylation signal of M, 2 in the intergenic junction, and 2 at the beginning of the G 5′ start sequence). It appears that the polymerase may initiate transcription at any of these U's as well as the U at position 4 of the 5′ start sequence. These results and the data for the nt 1 mutants indicate that the VSV polymerase can initiate transcription either at the first U encountered after transcription of the upstream gene, regardless of the 5′ start sequence of the downstream gene, or at a pyrimidine in the context of an optimal start sequence.

DISCUSSION

All nonsegmented negative-strand RNA viruses, including members of the *Rhabdoviridae*, *Paramyxoviridae*, and *Filoviridae* families, contain conserved sequences at the beginning and ends of each gene which are thought to serve as transcriptional start and polyadenylation stop signals, respectively. Previously a consensus 5′ start sequence based on the sequences of individual viruses from all three families was suggested to be 3′-UxCxxxUUxx-5′ (15), although the greatest homologies exist within each of the individual families (Fig. 9). The results from our mutational analysis indicate that the transcription start sequence of VSV can tolerate significant sequence variability. With these data we have defined the minimal sequence necessary for wild-type levels of gene expression by the VSV polymerase as 3′-UYGnnnnn-5′ (where Y is either U or C). This minimal sequence is not homologous to the previously suggested consensus sequence, and comparisons with other negative-strand viruses show that only rabies virus (RV), another rhabdovirus, contain this minimal sequence (Fig. 9). Therefore, it appears that viruses from different families have maintained a unique set of conserved nucleotides which no doubt reflect fundamental differences in the requirements of the polymerase for transcript initiation and possibly other modifications such as mRNA capping.

What role does the conserved sequence at the mRNA start site play in VSV transcription? For the mutations that had an effect, the levels of G protein correlated with the levels of transcript present. In addition, we did not observe an increase in read-through at the M-G gene junction during transcription with these mutants; therefore, it appears that the conserved sequence does not function as a transcriptional stop signal; rather, it may be part of a template recognition element required for the polymerase to reinitiate transcription following polyadenylation of the upstream mRNA. Similar results were recently obtained with deletion mutants of respiratory syncytial virus minigenomes (23).

Another possibility for the role for the conserved sequences at the mRNA (5′) start site is that they are important for mRNA modifications such as capping and methylation by the VSV polymerase (5). Capping by the VSV polymerase is a relatively complex process which occurs on nascent transcripts only (5). Presumably, the polymerase initiates transcription and then pauses while the cap is added. In addition to methylation at the N^7 position of the guanine cap, the polymerase also doubly methylates the first nucleotide (at the 2′-*O*-ribose and the N^6 position of adenosine) and singly methylates the second (A) nucleotide of nascent transcripts in vivo (27, 29). Each of these modifications may require additional pausing and a recognition event involving the conserved sequences before processive elongation occurs. Two RNA species which differed by one nucleotide were observed when the 5′ start sites of G transcripts were identified by primer extension. Presumably the longer species resulted from partial extension through the cap at the 5′ end of the G mRNA. It appears that different levels of this RNA species are produced from wild-type GMMG and some of the mutants. Further experiments are needed to determine if the amount of the larger primer extension product is indeed an accurate representation of the levels of capped message.

Although single mutations at positions 4 and 5 and all mutations at positions 8 through 10 did not appear to affect G protein expression significantly, these experiments were performed in vaccinia virus-infected cells. Vaccinia virus encodes cytoplasmic capping enzymes (7), which may mask the effects of mutations that affected one of these posttranscriptional modifications made by the VSV polymerase. By introducing several of the mutations at positions 4 through 10 into full-length cDNA clones of VSV encoding an additional reporter gene, we should be able to determine if any of these mutations affect the capping activity of the VSV polymerase.

Changing the first nucleotide of the start sequence to any other nucleotide drastically reduced the levels of G transcript expressed. Interestingly, changing it from a pyrimidine to a purine also altered the site at which the transcripts initiated to the second U of the start sequence. These data suggest that there is an absolute requirement by the polymerase to initiate transcription at a pyrimidine.

For mutant GMMGs with a U at the second position of the intergenic dinucleotide, the VSV polymerase initiated low levels of transcription at this U. Presumably the reduction in G transcript levels from these mutants is due to shifting the context of the start sequence by one nucleotide, resulting in a suboptimal initiation signal for the VSV polymerase. Transcript initiation at alternative sites, which is defined by the first U encountered following the intergenic region, has several implications relevant to the mechanism of VSV transcription. First, it supports the idea that the polymerase scans through the intergenic region. Unless the polymerase first comes to a pyrimidine in the context of a start sequence, it will initiate transcription at the first U that it encounters. Second, it suggests that the start sequence may not be an initiation signal but instead may be important for mRNA modifications such as capping or methylation. This theory is supported by the fact

that the polymerase will initiate transcription at a U found in the intergenic region even though there is a U in the context of a wild-type start sequence one nucleotide downstream. Since the 5′ capping and methylation modifications occur concomitantly with transcription, one model to account for the reduction in G transcript levels is that for genes with altered start sequences, transcription elongation may not occur efficiently if the polymerase is unable to cap or methylate the nascent transcript.

Another interesting aspect of VSV transcription is the site-specific attenuation that occurs at each gene junction. There is a 29 to 33% reduction in transcription across each gene junction, resulting in a cumulative effect on the transcription of downstream genes. The exact mechanism of this attenuation is unknown. It is thought that it may be sequence specific or a result of polymerase pausing due to polyadenylation, reinitiation, or capping. It appears that transcription from minigenome templates exhibits a similar degree of attenuation, since the ratios between the G and M mRNAs of VSV and of the wild-type GMMG are similar. The specific activities of the portion of probe protected by the G and M mRNAs in the RNase protection assays are equivalent; therefore, values of 0.65 and 0.58 for VSV and GMMG, respectively, represent decreases of approximately 35 to 40% across the M-G gene junction (data and calculations not shown).

The conservation of the intergenic region for both VSV_I and VSV_{NJ} suggested that this sequence also plays a role in transcription. Previously, it has been speculated that the dinucleotide is involved in termination of the polyadenylation reaction and/or the reinitiation of the downstream mRNA. This hypothesis was supported by reports of increased read-through transcription across the P-M gene junction of VSV_I (19, 26). The P-M intergenic dinucleotide in VSV_I is 3′-CA-5′, compared to the 3′-GA-5′ found at all VSV_{NJ} and the other VSV_I gene junctions. Northern blot analysis indicated that nucleotide changes in the intergenic dinucleotide lead to an increase in read-through transcription at that junction. Therefore, the intergenic dinucleotide does play a role in transcript termination and appears to be a separate *cis*-acting sequence that is not part of the 5′ mRNA start signal. Although there is a loss of downstream gene transcription from intergenic mutants with a U at the second position, this defect appears to be due to a shift and therefore an alteration in the start sequence.

By comparing the nontranscribed intergenic regions of other nonsegmented negative-strand RNA viruses, some notable trends are apparent. First, the intergenic regions of VSV, Sendai virus (SV), parainfluenza virus type 3 (PIV3), and measles virus (MV) are conserved within the individual viruses and show homology between each other (3′-GAA-5′ for SV, PIV-3, and MV; 3′-GA-5′ for VSV [8, 18, 30, 34]); however, the intergenic regions of RV and respiratory syncytial virus contain both length and sequence variability (10, 37). Second, the intergenic regions of all of these RNA viruses typically begin with a guanine and/or end with an adenine. Third, although VSV and RV are related members of the *Rhabdoviridae* family and have sequence homology at the polyadenylation stop signal and 5′ mRNA start sequences, the intergenic regions appear to differ significantly, supporting the idea that the intergenic region may be a separate *cis*-acting sequence that is not part of the polyadenylation or 5′ mRNA start signals. At what point does the intergenic dinucleotide exert its function? The polymerase may recognize this sequence before stuttering at the polyadenylation site, or perhaps this sequence is important for efficient termination and release of the polyadenylated transcript. The intergenic dinucleotide is not the only signal needed for termination, since GMMG mutants with changes at both intergenic nucleotides continue to express high levels of G protein message. Possibly the intergenic dinucleotide acts in concert with the conserved polyadenylation stop signal found at the end of each gene. Also, passaging data indicated that wild-type GMMG appears to have no replicative advantage over GMMGs with mutated intergenic regions. Further investigation with full-length recombinant VSV will be necessary to determine if this region provides some advantage to the virus that is not evident with the minigenome system.

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