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## Vesicular Stomatitis Virus mRNA Capping Machinery Requires Specific *cis*-Acting Signals in the RNA<sup>∇</sup>

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**Many viruses of eukaryotes that use mRNA cap-dependent translation strategies have evolved alternate mechanisms to generate the mRNA cap compared to their hosts. The most divergent of these mechanisms are those used by nonsegmented negative-sense (NNS) RNA viruses, which evolved a capping enzyme that transfers RNA onto GDP, rather than GMP onto the 5' end of the RNA. Working with vesicular stomatitis virus (VSV), a prototype of the NNS RNA viruses, we show that mRNA cap formation is further distinct, requiring a specific *cis*-acting signal in the RNA. Using recombinant VSV, we determined the function of the eight conserved positions of the gene-start sequence in mRNA initiation and cap formation. Alterations to this sequence compromised mRNA initiation and separately formation of the GpppA cap structure. These studies provide genetic and biochemical evidence that the mRNA capping apparatus of VSV evolved an RNA capping machinery that functions in a sequence-specific manner.**

The 7<sup>m</sup>GpppN cap structure found at the 5' terminus of eukaryotic mRNAs is required for efficient translation (27) and mRNA stability (13). The cap structure is formed through a series of enzymatic reactions. An RNA triphosphatase removes a single phosphate from the 5' end of the RNA to yield 5' ppN. The diphosphate is then capped by an RNA guanylyl-transferase which transfers Gp from a Gppp donor to form the cap structure, GpppN. The cap is further modified by a guanine-N-7 (G-N-7) methylase which transfers a methyl group from *S*-adenosyl-L-methionine (SAM) to yield 7<sup>m</sup>GpppN and *S*-adenosyl homocysteine (14). This methylated cap can then be subsequently methylated by a ribose-2'-O (2'-O) methylase to yield 7<sup>m</sup>GpppN<sup>m</sup>.

Many viruses of eukaryotes have evolved unusual strategies to generate capped mRNA, including the nonsegmented negative-sense (NNS) RNA viruses. Studies with vesicular stomatitis virus (VSV), a prototype of the NNS RNA viruses, demonstrated that the capping enzyme transfers RNA onto Gpp, rather than Gp onto the 5' end of the RNA (1, 28). The mRNA cap methylase activities of VSV are also unusual in that the G-N-7 and 2'-O methylase activities use a single SAM binding site (24). Thus, for these viruses, each step of mRNA cap formation is distinct from that of the host.

Much understanding of gene expression strategies of NNS RNA viruses comes from studies of VSV. The VSV genome comprises 11,161 nucleotides (nt) of RNA. This is delivered into the cytoplasm of the host cell as a transcription-competent core in which the RNA is completely encapsidated by the nucleocapsid (N) protein and associated with the RNA-dependent RNA polymerase (RdRP). The viral components of the

RdRP are a 29-kDa phosphoprotein (P) and a 241-kDa large (L) polymerase subunit (10, 11). In response to a promoter, the VSV RdRP initiates synthesis at the first gene-start (GS) sequence (3' UUGUCNNUAC 5') (25, 40, 41). The nascent mRNA chain is capped and methylated (1, 26), and in response to a specific gene-end (GE) sequence (3'-AUACUUUUUU GA-5'), the RdRP polyadenylates and terminates (4, 5, 18). The RdRP then encounters a downstream GS sequence and initiates mRNA synthesis. Productive reinitiation is not 100% efficient, such that 30% less mRNA is produced from each subsequent gene (19). This start-stop sequential transcription of the genomic RNA yields progressively less of each of five mRNAs encoding in order the viral N, P, matrix (M), attachment glycoprotein (G), and L proteins (2).

The GS and GE elements govern the process of mRNA synthesis, and these sequence elements have been extensively characterized (39). Mutation of the first three positions of a VSV GS sequence reduces mRNA initiation (34) and result in accumulation of aborted transcripts (35). These transcripts were not reactive with an antibody raised against trimethyl-guanosine (35), suggesting that either polymerase processivity or a step in formation of the methylated cap required a specific RNA sequence. Capping enzymes typically modify RNA in a sequence-independent manner. However, recent work indicates that some viral capping machines may have evolved to specifically recognize sequences found in the viral RNA. For Sendai virus (SeV), an NNS RNA virus in the *Paramyxovirus* family, a fragment of L protein was shown to G-N-7 methylate capped 5-nt-long RNAs provided that they corresponded in sequence to viral mRNA (29). Similarly, purified VSV L protein was shown to cap 5-nt transcripts that corresponded in sequence to the beginning of a viral mRNA but not heterologous transcripts (28). Together these data indicate that the NNS RNA virus capping and methylating enzymes function in a sequence-specific manner. However, the viral capping machinery does not normally respond to exogenous transcripts (33), and it is uncertain how these observations of the require-

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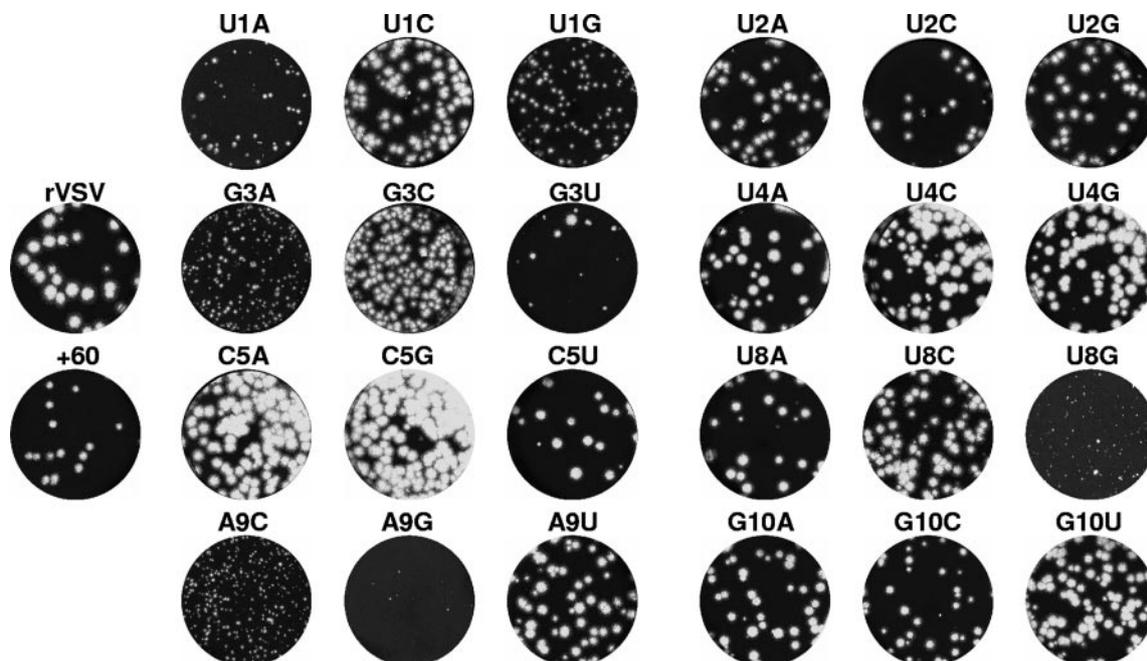


FIG. 1. Plaque morphologies of viruses containing mutations to the conserved GS. The plaque morphology is shown at 40 h postinoculation compared to rVSV. Differing dilutions of the viruses were plated to emphasize the uniform plaque size of the small-plaque viruses.

ment of *trans* capping of 5-nt RNAs relate to cap formation of viral mRNAs which occurs during their synthesis.

In this study, we used infectious recombinant VSV (rVSV) to evaluate whether cap formation required specific RNA sequence elements. We employed a strategy to evaluate the role of each position of the first GS sequence in mRNA initiation and cap formation without compromising virus viability. Biochemical analysis of a panel of 24 recombinant viruses in which each of the conserved GS positions was altered identified specific requirements for mRNA initiation and showed that formation of the GpppA cap requires a separate RNA sequence element. These studies show that the RNA capping machinery of VSV and by extrapolation other NNS RNA viruses is unusual not only with regard to the mechanism of action of the enzyme but also in the requirement for a specific sequence element in the RNA.

#### MATERIALS AND METHODS

**Generation of recombinant viruses.** Plasmid pVSV1(+)-60, containing an infectious cDNA clone of the VSV genome with a 60-nt transcriptional unit inserted at the leader-N gene junction, was constructed as described previously (32). The eight conserved residues of the GS of the 60-nt gene were modified to the three alternate residues by site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA). The presence of the mutation was confirmed by sequence analysis. A total of 24 mutations were generated, named to reflect the nucleotide substitutions (Fig. 1). rVSV was recovered from cDNA by transfection of BHK-21 cells infected with a recombinant vaccinia virus (vTF7-3) that expressed T7 RNA polymerase as described previously (12, 37). Cell culture fluids were collected at 48 to 96 h posttransfection, and virus was amplified once in BHK-21 cells. Individual plaques were isolated on Vero cells, and large stocks were generated in BHK-21 cells and purified as described above. Viral titer was determined by plaque assay on Vero cells, and protein content was measured with the Bradford reagent (Sigma Chemical Co., St Louis, MO). The 60-nt gene of the purified viruses was sequenced again, and these stocks were used for *in vitro* transcription reactions.

**Transcription and analysis of viral RNA.** Viral RNA was synthesized *in vitro* as described previously (3, 41). Purified rVSV (20  $\mu$ g) was activated by incubation with detergent for 5 min at room temperature. RNA synthesis reactions were performed in the presence of nucleotide triphosphates (1 mM ATP and 0.5 mM each of CTP, GTP, and UTP). Total RNA was extracted, purified, and subjected to secondary manipulations as follows. The RNA cap structure was removed by tobacco acid pyrophosphatase (TAP; Epicenter) as previously described (23). For exonuclease digestion, total RNA was dephosphorylated using Antarctic phosphatase (New England Biolabs [NEB]), and a single phosphate was added using T4 polynucleotide kinase (NEB) according to the manufacturer's instructions. Where indicated, the resulting RNAs were subsequently treated with Terminator exonuclease (Epicenter) according to the manufacturer's instructions. The products of RNA synthesis were detected by a primer extension assay using VSV Le+, 60, and N primers as described previously (41). Quantitative analysis was performed by densitometric scanning of autoradiographs or using a phosphorimager (GE Healthcare; Typhoon) and ImageQuant TL software (GE Healthcare, Piscataway, NJ).

**Analysis of the terminal sequences by 5' rapid amplification of cDNA ends (RACE).** Purified RNAs synthesized by detergent-activated virus *in vitro* were treated with TAP prior to ligation to a sequence-specific oligonucleotide using T4 RNA ligase (NEB). The products of this reaction were subjected to reverse transcription using Superscript III (Invitrogen) reverse transcriptase (RT) and a primer designed to anneal at positions 33 to 16 within the 60-nt RNA. The resulting cDNA RNA hybrids were amplified by PCR. The products of this reaction were purified and ligated into pGEMTEZ (Promega), and the sequence was determined.

#### RESULTS

**Viral mutants with altered GS sequences.** A consequence of the start-stop mechanism of sequential mRNA synthesis in NNS RNA viruses is that perturbing initiation at a GS reduces expression of all genes downstream. To dissect the role of the VSV GS sequence in mRNA initiation and cap formation, we developed a strategy that allowed mutation of the first GS sequence without compromising virus viability. Prior work showed that polymerase initiates mRNA synthesis directly at

TABLE 1. Summary of the phenotypic properties of the GS mutants

Virus	Mean plaque size (mm)	mRNA (% rVSV +60) <sup>a</sup>		Presence of cap <sup>b</sup>
		60	N	
rVSV	4.8	NA <sup>c</sup>	307	
+60	2.1	100	100	+
U1A	1.1	15	100	-
U1C	4.1	63	167	-
U1G	1.3	26	66	-
U2A	2.5	58	149	-
U2C	2.4	155	134	+
U2G	2.8	81	85	-
G3A	0.7	32	89	-
G3C	1.8	57	154	-
G3U	1.3	116	145	+/-
U4A	2.2	148	76	+
U4C	2	127	115	+
U4G	2.5	197	120	+
C5A	2.8	104	143	+
C5G	2.6	130	174	+
C5U	3.5	138	123	+/-
U8A	3.3	63	65	+
U8C	2.6	179	171	+
U8G	0.3	8	89	ND <sup>d</sup>
A9C	0.6	107	83	+
A9G	0.2	211	95	+
A9U	2.9	111	99	+
G10A	3	113	105	+
G10C	2.2	89	51	+
G10U	2.5	143	76	+

<sup>a</sup> Data normalized by comparison with Le+ levels.

<sup>b</sup> The presence (+) or absence (-) of cap on the +60 RNA was assessed by the sensitivity of the +60 RNA to exonuclease (Fig. 3) and also its sensitivity to TAP (Fig. 2). +/-, partially capped.

<sup>c</sup> NA, not applicable.

<sup>d</sup> ND, not determined.

the first GS sequence (41) and that genes of 60 nt are sufficient for mRNA initiation and termination (38). We hypothesized that compromising initiation by modifying the GS of a 60-nt gene inserted at the first position of the genome would permit access of polymerase to the N GS sequence, enabling recovery of infectious virus and analysis of viral mRNA synthesis *in vitro*. Each of the eight conserved positions of the 60-nt GS was mutated to the three alternate nucleotides, and 24 viable viruses were recovered (Fig. 1). Several mutants were compromised in growth, with U1A/G, G3A/C/U, and A9C showing a reduction in plaque diameter ranging from 0.6 to 1.8 mm compared to 2.1 mm for rVSV +60. The most striking defect in growth was observed for mutants U8G and A9G, which possessed 0.2- to 0.3-mm or pinpoint plaques (Fig. 1; Table 1). A region spanning nt 18 to 462 of each virus was amplified by RT PCR, and sequence analysis confirmed the presence of the desired mutation; no additional mutations were observed in the sequenced region (data not shown). The objective of this study was to define the effect of these mutations on initiation of mRNA synthesis and mRNA cap formation. Consequently the molecular basis of this intriguing plaque morphology was not examined further.

**GS mutations affect mRNA synthesis.** To examine the effect of mutations in the first GS sequence on RNA synthesis, transcription reactions were performed *in vitro* and the products were detected by primer extension. A schematic of the 3' end

of the VSV genome and the products detected by this primer extension assay is shown in Fig. 2A. Three primers were used, one designed to anneal to positions 47 to 34 of Le+, a second designed to anneal to positions 33 to 16 within the 60-nt mRNA, and a third designed to anneal to positions 80 to 55 within the N mRNA. Extension of the leader primer by RT on RNA synthesized by rVSV +60 yielded a 47-nt product corresponding to the positive-sense leader RNA, Le+ (Fig. 2A). For rVSV +60, the major product detected by the 60 primer was 34 nt long, which presumably corresponded to RT extending onto the cap structure (Fig. 2A). To confirm this, RNAs were treated with TAP to cleave the GpppA cap structure prior to primer extension. This resulted in detection of a major 33-nt RT product that mapped to position U1 of the GS (Fig. 2A). As a control, the effect of TAP cleavage on the leader and N mRNA was also examined. For rVSV +60, the N primer gave a major 81-nt RT product corresponding to the capped 5' end of the N mRNA and a 141-nt product corresponding to detection of the capped 60-N readthrough transcript (Fig. 2A). TAP treatment resulted in a single nucleotide shift for each of these RT products, consistent with the presence of a cap structure (Fig. 2A, 80- and 140-nt products). By contrast, TAP treatment had no effect on the leader RNA synthesized by rVSV +60 (Fig. 2A).

Identical analysis was performed for each of the viral mutants, and the results of typical experiments are shown in Fig. 2B and C. Although equivalent amounts of virus were used to synthesize RNA *in vitro*, the yield of RNA varied between mutants. To quantify the effect of GS mutations on RNA synthesis, the relative amounts of Le+, 60, and N mRNA were determined by phosphorimager analysis. Each virus makes Le+, and its synthesis does not depend upon initiation at the +60 GS. Consequently, we normalized the levels of +60 and N mRNA relative to the amount of leader RNA synthesized by each mutant (Table 1). As predicted, the major effect of the GS mutations was on accumulation of the +60 mRNA. This was most evident for some U1, U2, G3, and U8 mutations which reduced the abundance of the 60 mRNA with U8G, decreasing productive initiation to the limit of detection (Table 1; Fig. 2B). Despite these reduced levels of 60 mRNA, levels of N mRNA were only modestly different from those of rVSV +60 (Table 1; Fig. 2C), suggesting that synthesis of the 60 mRNA was not essential for synthesis of N mRNA.

**GS mutations affect mRNA cap formation.** To determine whether any of the GS mutations specifically affected the formation of the GpppN cap structure, we examined the sensitivity of the 60 mRNA to TAP cleavage prior to primer extension (Fig. 2B). For rVSV +60, TAP treatment resulted in the detection of a 33-nt-long major species that mapped to the authentic initiation site (Fig. 2B, lane 27), although a small fraction of the product was 34 nt. This is consistent with the majority of the 60-nt mRNAs containing the 5' cap structure GpppA. The +60 mRNA synthesized by U2C, U4A/C, C5A/G/U, A9U, and G10A/C/U was similarly sensitive to TAP cleavage, resulting in a major RT product at 33 nt which mapped to position +1 of the GS (Fig. 2B). Mutant U4G was also sensitive to TAP cleavage, yielding a 33-nt product (Fig. 2B, lane 25), although in contrast to U4A and U4C a significant 34-nt product still remained. Mutants U8C and A9C/G showed some sensitivity of the 60 mRNA to TAP cleavage

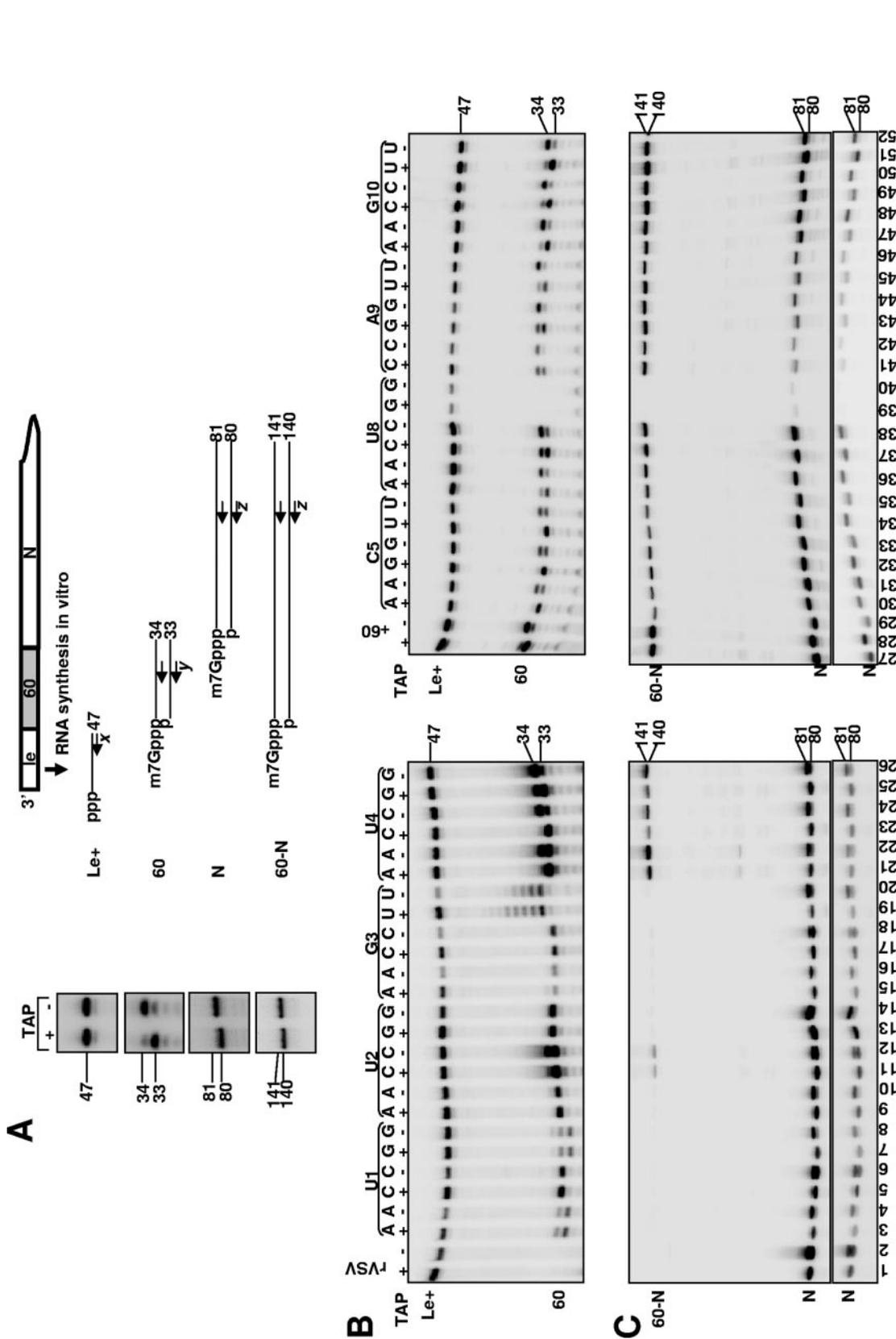


FIG. 2. GS mutations affect synthesis of the 3' end of the rVSV +60 genome is shown together with the products of in vitro RNA synthesis. These include the leader RNA (Le+), the 60-nt mRNA (60), the N mRNA (N), and a 60-N readthrough transcript. Reverse transcriptions were performed using primers that were end labeled with [ $\gamma$ - $^{32}$ P]ATP and designed to anneal to the leader RNA (Le+) or 60-nt (60) or N mRNA, shown by x, y, and z, respectively. Products were analyzed on denaturing 6% polyacrylamide gels and detected using a phosphorimager. The sizes of the RT products are indicated to the left of the gels and alongside the specific transcripts detected. Where indicated, samples were treated with TAP to hydrolyze the cap structure prior to detection by primer extension. Under these conditions RT cannot extend onto the cap, thus yielding a product that is a single nucleotide shorter. (B) Detection of the leader RNA and the +60 mRNA for each recombinant virus. The identities of the viruses are indicated at the top of the lanes, and the sizes of the products are marked to the right of the gels. (C) Detection of the N mRNA and the 60-N readthrough transcript. The identities of the viruses are indicated above the lanes in panel B; the sizes of the products are indicated to the right of the gel. A light exposure of the gel is shown below to allow easier visualization of the differences between the samples treated with TAP for some of the mutants.

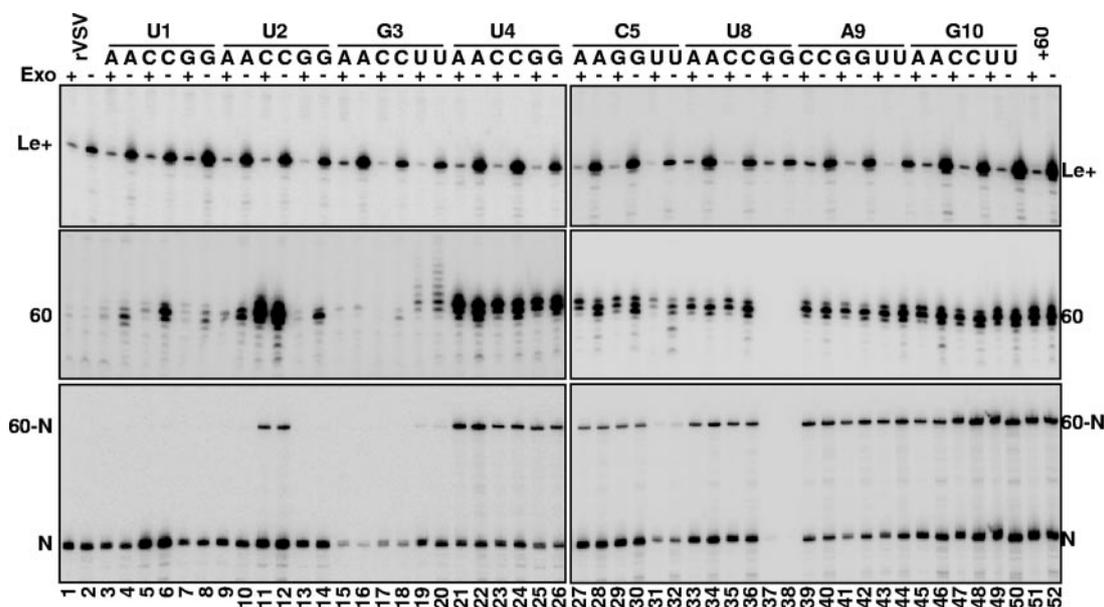


FIG. 3. Exonuclease sensitivities of the products of RNA synthesis. A primer extension analysis of the products of in vitro RNA synthesis is shown. Where indicated (+), samples were treated with alkaline phosphatase followed by polynucleotide kinase and exonuclease to cleave RNAs that lacked a cap structure prior to primer extension. Reverse transcriptions were performed using a primer designed to anneal to the Le+, 60, or N mRNA. Products were analyzed on denaturing 6% polyacrylamide gels and detected using a phosphorimager.

(Fig. 2B, lanes 37, 41, and 43), but the majority of the RT products were 34 nt long, indicating either that the cap structures were resistant to TAP cleavage or that the position of transcription initiation was altered (Fig. 2B, lanes 37, 38, and 41 to 44). In contrast, mutants U1A/C/G, U2A/G, and G3A/C yielded RT products that were unaltered by TAP treatment, suggesting that these mutants did not generate capped RNAs (Fig. 2B, lanes 3 to 10 and 13 to 18). Owing to the ladder of products observed for mutant G3U and the lack of 60 mRNA synthesized by U8G, this assay was unable to determine an effect of these mutations on cap formation (Fig. 2B, lanes 19, 20, 39, and 40).

As controls for these manipulations, the products synthesized from the N gene were also detected. Treatment of the RNAs with TAP prior to primer extension resulted in a major 80-nt RT product that corresponds to position +1 of the N GS sequence (Fig. 2C, +TAP, compare + and - TAP). This demonstrates that failure of TAP to cleave the 60 mRNA for many viral mutants was not due to the presence of an inhibitor in the purified RNA. The major difference observed between the viruses was the accumulation of the 60-N readthrough transcript. For mutants U1, U2, G3, and U8G dramatically diminished quantities of the readthrough transcript were seen (Fig. 2C, lanes 3 to 18, 39, and 40). This is consistent with the amount of 60 mRNA seen for these mutants.

To confirm these findings, we examined the sensitivity of RNA to exonuclease cleavage (Fig. 3). Exonuclease degrades RNAs with 5'-monophosphate termini, whereas those containing a 5'-triphosphate or blocking cap structure are resistant. Consequently, the triphosphate leader RNA and the capped N mRNA should be resistant to exonuclease digestion. To render all uncapped RNA sensitive to exonuclease digestion, we treated RNA with alkaline phosphatase to remove the terminal phosphates, followed by T4 polynucleotide kinase to generate

a 5'-monophosphate terminus. This treatment will not affect capped RNAs but should render Le+ and uncapped mRNA sensitive to exonuclease digestion. The products of this reaction were then analyzed by primer extension using the Le+, 60, and N primers as described above. This treatment resulted in the specific loss of Le+ for each mutant while having no significant effect on the abundance of the N mRNA (Fig. 3, odd-numbered lanes, bands Le+ and N, respectively). In contrast, the effect of this treatment on the +60 mRNA varied. Specifically, exonuclease treatment had no significant effect on the detection of the +60 mRNA synthesized by rVSV +60 and mutants U2C, U4A/C/G, C5A/G, U8A/C, A9C/G/U, and G10A/C/U (Fig. 3, lanes 11, 21 to 29, 33, 35, 39 to 49, and 51), confirming that these transcripts were capped. In contrast, the +60 transcript generated by mutants U1A/C/G, U2A/G, and G3A/C was sensitive to exonuclease degradation (Fig. 3, lanes 3 to 9 and 13 to 17), consistent with its being uncapped. Mutants G3U and C5U showed an intermediate phenotype with some resistance of the +60 RNA to exonuclease cleavage, suggesting that some of the transcripts were capped (Fig. 3, lanes 19 and 31). The gel shown of the +60 transcripts was overexposed to permit the visualization of products that are produced in low abundance, such as those from mutant U1A. As a consequence, additional products are seen mapping to the +60 GS. These products likely reflect an intrinsic property of RT, which can add a single nontemplated nucleotide at the 3' end of the RNA or can stall in response to methyl groups in the RNA. Irrespective of this, these data show that VSV mRNAs contain a specific sequence for cap formation and that the first three positions of the GS sequence are most critical for this activity, with position 5 making a more modest contribution.

**Mutations to the GS that alter the position of initiation.** The position to which the 5' end of the +60 transcript mapped was altered for mutants U1A/G and G3U as judged by the primer

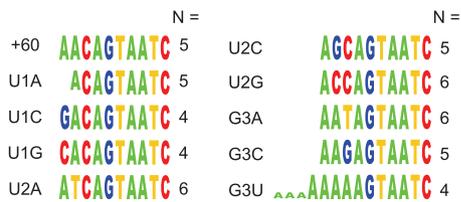


FIG. 4. Sequence analysis of the 5' ends of transcripts from the 60-nt gene. The sequence of the 5' ends was determined by 5' RACE. A pictogram is shown to indicate the sequences and their relative frequencies. The heights of the letters represent the frequencies with which a base was identified out of  $n$  ( $N$ ) independent experiments.

extension analysis (Fig. 2 and 3). The most striking of these was G3U, which generated a ladder of products that extended several nucleotides prior to the GS site (Fig. 2B, lane 20). This was reminiscent of the stuttering exhibited by paramyxoviral polymerases in response to RNA editing sites (31). To examine the sequence at the 5' end of the mRNAs produced, we performed 5'-RACE analysis on the U1, U2, and G3 mutants. The sequences obtained are represented as pictograms in Fig. 4. With the exception of mutants U1A and G3U, the mutants produced transcripts that were direct copies of the 60-nt gene and mapped to the authentic position of initiation. For U1A, initiation at +2 was observed in four of five independent samples, with the remaining sequence corresponding to initiation at +3. Transcripts synthesized by G3U contained nontemplated A residues at the 5' terminus, with the majority containing a single additional A residue, although up to four were detected. These data show that mutation of the first GS sequence of the VSV genome can alter the abundance and position of initiation of mRNA synthesis, as well as mRNA cap formation. They also demonstrate that the initiation of mRNA synthesis is remarkably tolerant of mutations in the first GS but that a specific signal is required for mRNA cap formation.

## DISCUSSION

We performed a genetic and biochemical analysis of the role of the conserved positions in the VSV GS sequence in RNA synthesis and mRNA cap formation. We generated 24 mutations in the first GS sequence and determined the effect of these changes on transcription *in vitro*. These experiments showed that the GS provides specific *cis*-acting signals for initiation of mRNA synthesis and separate signals for formation of the GpppA cap structure. Thus, the capping apparatus of VSV functions in a sequence-specific manner. Combined with earlier work that demonstrated that cap formation involves transfer of RNA onto GDP (1, 28) and that the mRNA cap methylase activities use a single SAM binding site (24), these studies reveal that each of the steps of mRNA cap formation of VSV is distinct from those of the host.

**A specific sequence element required for mRNA cap formation.** To date, capping machines of several eukaryotes and their viruses and parasites have been characterized, and these typically function in a sequence-independent manner. Among the best studied of all viral capping machines is that encoded by vaccinia virus, which will promiscuously add a cap structure to RNA. In this study, we provide evidence that one function of the GS sequence of VSV is to dictate the efficient capping of

the viral mRNAs. This is consistent with a recent study which showed that 5-nt transcripts were capped by the VSV L protein *in vitro* provided that they corresponded to the first 5 nt of a viral mRNA (28). Similarly, capped 5-nt transcripts corresponding to SeV mRNA were shown to be methylated by the SeV L protein (29). While these studies were consistent with the capping and methylating enzymes of NNS RNA viruses functioning in a sequence-specific manner, how these *trans* modifications of short RNAs *in vitro* relate to cotranscriptional mRNA cap formation during viral RNA synthesis was not certain. In general, our findings are consistent with and extend these studies. However, some differences are apparent, such as the very modest defect that we found in cap formation on modification of position C5 compared to no detectable cap formation when this position was altered in the 5-nt transcripts (24). We suggest that this reflects a property of using such short RNAs for *trans* cap formation that can be overcome in the context of virus.

Earlier work demonstrated that the first three positions of an internal VSV GS sequence were critical for mRNA synthesis (34, 35) and provided a consensus sequence of UYG as essential to drive expression of a reporter gene from a VSV subgenomic replicon. This system depended upon the presence of a recombinant vaccinia virus to supply T7 RNA polymerase (12) as a source of transcriptase necessary to drive the expression of the subgenomic replicon and the VSV N, P, and L proteins from transfected plasmids (30). Vaccinia virus contains its own promiscuous capping machinery; thus, these studies could not address a specific sequence requirement for cap formation. Subsequent *in vitro* work showed a relaxed sequence requirement of YNG for initiation (35) and provided evidence for premature termination of mRNA synthesis if a transcript was not capped and methylated. Our findings are, however, consistent with and extend these observations. Specifically, we analyzed each of the conserved positions of the first GS for RNA initiation and mRNA cap formation. We found that, with the exception of mutation U8G, transcription of the first gene was detected for each mutant, thus demonstrating that the first GS is remarkably tolerant of mutation for initiation of mRNA synthesis. We further found that changes to positions 1 to 3 that deviated from the consensus 3'-UYG yielded transcripts that did not acquire the GpppN cap structure. Individual substitutions at positions 4, 5, 8, 9, and 10 yielded RNAs that were capped, although some reduction in efficiency was seen for the C<sub>5</sub> substitutions as judged by their sensitivity to exonuclease.

Our study also supports a model for mRNA synthesis in which cap formation impacts polymerase processivity. Specifically, failure to form a cap structure on the +60 mRNA was accompanied by a failure to detect the +60-N readthrough transcript (Fig. 2, lanes 3 to 20). How might cap formation affect polymerase processivity? Previously, we speculated that the conserved sequence elements found at the 5' end of VSV mRNAs or the addition of the cap structure could act as a regulatory target during transcription (38). Perhaps a region of L or a cofactor recognizes the cap, which makes polymerase processive and also allows recognition of GE sequences. However, mutant U2C synthesized abundant +60 mRNA, some of which was capped, and yet only trivial quantities of the +60-N readthrough transcript (Fig. 2, lanes 11 and 12). This suggests

that the presence of the cap and the sequence itself might impact the processivity of the polymerase.

Although a specific sequence requirement has not been demonstrated for other viral capping reactions, flavivirus cap methylation was recently shown to depend upon recognition of a specific viral RNA sequence element (9). This sequence element differs for G-N-7 and ribose-2'-O methylation, which remarkably are carried out by the same enzyme (42). While we did not analyze the specific sequence requirements for cap methylation in this study, studies with SeV support the idea that G-N-7 methylation occurs in a sequence-specific manner (29). Remarkably, the two methylase activities of VSV share a single SAM binding site (24) and may share the same active-site residues (23). This suggests that the evolutionarily distinct flaviviruses and rhabdoviruses may have evolved a similar mechanism for mRNA cap methylation.

**The role of the NNS RNA virus GS sequence in mRNA synthesis.** To date, the most extensive analysis of any NNS RNA virus GS sequence was performed with human respiratory syncytial (RS) virus (20, 21). For human RS virus, 9 of the 10 genes contain the sequence 3'-CCCCGUUUA(U/C)-5' and the L gene contains the sequence 3'-CCCUGUUUUA-5'. Mutagenesis of the consensus human RS virus GS showed that positions 1, 3, 6, 7, and 9 were the most sensitive to substitutions, whereas position 5 was relatively insensitive (20, 21). Although the effect of these mutations on mRNA cap formation was not examined, analysis of the transcripts synthesized by templates in which position 1 of the GS was altered showed that the nucleotide incorporated into the mRNA was not always templated but instead was frequently the wild-type G (20). This "quasitemplated" initiation was speculated to occur through stuttering of polymerase on positions 2 to 4 of the GS sequence or by the preloading of the polymerase with GTP for initiation.

In the present study, we also found evidence for polymerase slippage at a VSV GS sequence, specifically for mutant G3U. This substitution creates an unusual sequence at the leader first gene junction for VSV, GAAAUUUUC, which shares some similarity to the stuttering sequences (3'-UnCn-5') encountered within the P genes of certain *Paramyxoviridae*. This sequence governs the insertion of G residues into the edited mRNA to allow access to each of the open reading frames (15, 16, 36). Manipulation of this sequence can lead to the insertion of A residues, presumably through stuttering of polymerase on the U tract. Precisely how the stuttering undertaken by the VSV polymerase at this altered first GS sequence is controlled is uncertain, but we obtained evidence for insertion of up to eight A residues. These findings again underscore the idea that polymerase slippage and reiterative transcription are fundamental properties of NNS RNA virus polymerase molecules.

**Recombinant viruses with mutations in the first GS sequence.** A tenet of the start-stop model of sequential transcription is that compromising initiation at a GS sequence should prevent polymerase access to the subsequent GS (39). Consequently mutations in the first GS sequence that decrease mRNA initiation would be predicted to reduce initiation at all subsequent GS sequences. In this study we found that compromising initiation at the +60 GS was not accompanied by an equally dramatic reduction in initiation at the N GS. This was most apparent for mutant U8G, in which transcription of the

60-nt gene either aborts prior to the length required for detection in the primer extension assay (33 nt) or fails to initiate at all. In either event, the polymerase gains access to the N GS sequence possibly by internal loading or by scanning along the template. We anticipated that recombinant viruses bearing each of the desired mutations would be viable, although we were surprised by the diversity of their plaque size. Some mutations increased plaque size, whereas others diminished plaque size or generated pinpoint plaques. The explanation for this diminished plaque morphology is not apparent, as there was no obvious defect in the abundance of RNAs synthesized in vitro by mutants such as G3A, A9C, and A9G with dramatically diminished plaque size. We have not yet done a comprehensive analysis of the replication kinetics of these 25 new recombinant viruses, nor have we examined RNA synthesis in cells or the ability of these viruses to replicate in mice, which may shed light on this observation. However, in this paper we show that the viral capping machinery requires specific *cis*-acting signals in the RNA and describe a panel of viruses, some of which presumably now overproduce an uncapped triphosphate RNA. We are mindful of the fact that triphosphate RNAs have been reported to serve as potent triggers of innate immune responses when transfected into cells (17, 31). However, as most clearly illustrated by mutants U2A and U2G, not all viruses defective in +60 cap formation exhibited a small-plaque phenotype (Fig. 2B; Table 1). Further experiments will thus be required to determine the molecular basis of the intriguing plaque morphologies exhibited by these mutants.

In summary, we examined the role of the conserved VSV GS in viral RNA synthesis and defined specific sequences that are required for mRNA initiation and mRNA cap formation. These sequences overlap with one another and yet are clearly distinct and thus demonstrate that the mRNA capping apparatus functions in a sequence-specific manner. These findings further underscore the unusual nature of the mRNA capping machinery of the NNS RNA viruses, which renders them attractive targets for antiviral intervention. Specifically, this work shows that in addition to the viral capping enzyme itself, the *cis*-acting signals that are essential for cap formation are also targets. In addition, this study raises the interesting question of why NNS RNA viruses have evolved a sequence-specific capping apparatus. Each NNS RNA virus has a GS sequence that is conserved for its 5 to 10 genes, and this sequence differs from that employed by the RdRP to initiate synthesis at the 3' end of the viral genomic and antigenomic RNAs. These latter initiations produce full-length antigenomes and genomes, respectively, as well as two short leader RNAs of poorly understood function. For VSV these are a 47-nt Le<sup>+</sup> and a 45-nt Le<sup>-</sup>. These RNAs share the common 5' sequence pppApCpGpApG and are not capped (8). It is tempting to speculate that a function of these RNAs, such as their role in RNA encapsidation (6, 7) and/or their localization in the nuclei of cells (22), requires them to remain uncapped and that this might explain the origin of a sequence-specific capping apparatus.

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