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A Conserved Motif in Region V of the Large Polymerase Proteins of Nonsegmented Negative-Sense RNA Viruses That Is Essential for mRNA Capping[▽]

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Nonsegmented negative-sense (NNS) RNA viruses cap their mRNA by an unconventional mechanism. Specifically, 5′ monophosphate mRNA is transferred to GDP derived from GTP through a reaction that involves a covalent intermediate between the large polymerase protein L and mRNA. This polyribonucleotidyltransferase activity contrasts with all other capping reactions, which are catalyzed by an RNA triphosphatase and guanylyltransferase. In these reactions, a 5′ diphosphate mRNA is capped by transfer of GMP via a covalent enzyme-GMP intermediate. RNA guanylyltransferases typically have a KxDG motif in which the lysine forms this covalent intermediate. Consistent with the distinct mechanism of capping employed by NNS RNA viruses, such a motif is absent from L. To determine the residues of L protein required for capping, we reconstituted the capping reaction of the prototype NNS RNA virus, vesicular stomatitis virus, from highly purified components. Using a panel of L proteins with single-amino-acid substitutions to residues universally conserved among NNS RNA virus L proteins, we define a new motif, GxxT[n]HR, present within conserved region V of L protein that is essential for this unconventional mechanism of mRNA cap formation.

The 5′ terminus of eukaryotic mRNA is modified by the addition of a 7^mGpppN cap structure. The cap structure is essential for mRNA stability, mRNA transportation, splicing of pre-mRNAs, and translation (13, 28). Formation of this structure requires a series of enzymatic reactions. An RNA triphosphatase (RTPase) hydrolyzes the 5′ triphosphate (pppN) end of mRNA to yield a 5′ diphosphate (ppN). This is capped by an RNA guanylyltransferase (GTase) which transfers Gp derived from GTP to form the cap structure. The cap is subsequently methylated by guanine-N-7 (G-N-7) methyltransferase (MTase) to yield 7^mGpppN, which can be further methylated by ribose-2′-O (2′-O) MTase to yield 7^mGpppN^m (14, 36).

Cap formation in nonsegmented negative-strand (NNS) RNA viruses involves a different reaction mechanism. For vesicular stomatitis virus (VSV) (2), spring viremia of carp virus (16), and respiratory syncytial virus (RSV) (5), the underlined phosphates of the 5′ GpppN triphosphate bridge were shown to be derived from GDP rather than GMP. Recent studies with VSV demonstrated that this reaction does not involve transfer of guanylate onto the mRNA, but rather involves a polyribonucleotidyltransferase activity (29). Here, the mRNA capping reaction proceeds via a covalent intermediate between the 241-kDa viral polymerase protein L and the 5′ monophosphate mRNA. This monophosphate mRNA is transferred onto GDP derived from GTP to yield the GpppA mRNA cap. Consequently, this mechanism of cap formation is in marked contrast with those catalyzed by conventional GTases. The crystal struc-

tures of representative GTases have been solved and their reaction mechanisms studied in biochemical detail (17). GTases typically contain a KxDG motif, in which the lysine forms the covalent intermediate with GMP, and consistent with the distinct mechanism such a motif is absent from the VSV L protein. In contrast to our knowledge of how RNA GTases catalyze formation of the mRNA cap, cap formation by polyribonucleotidyltransferases is not well understood and the active-site residues have not been mapped. Consequently, the location of the capping activity within the 2,109-amino-acid VSV L protein has not been determined.

The NNS RNA viruses share a common strategy for gene expression. The template for RNA synthesis is a protein-RNA complex in which the genomic RNA is encapsidated by the viral nucleocapsid (N) protein and associated with the RNA-dependent RNA polymerase (RdRP). The minimal viral components of the RdRP were shown for VSV to comprise the L protein and an accessory phosphoprotein (P) (11). The RdRP sequentially copies the genomic RNA into five capped and polyadenylated mRNAs (1, 4). The mRNAs acquire their 5′ mRNA cap structure and 3′ poly(A) tail during their synthesis, and the enzymatic activities that catalyze these reactions are provided by L protein.

Sequence alignments between representative NNS RNA viruses identified six regions of conservation in the L protein (CRI to -VI) separated by regions of lower sequence homology (33). For Sendai virus (SeV), CRI is implicated in recruitment of N protein during replication and interaction with P protein (8) and CRII appears to play a role in template binding (27, 38). Whether these regions of L mediate the same function in VSV has not been determined. However, it is clear for all NNS RNA virus L proteins that CRIII contains the polymerase active-site motif, and consistent with this, modification of VSV L protein residue D714, which is predicted to coordinate a

catalytically essential Mg^{2+} ion, inhibits all RdRP activity (37). In addition, CRVI of SeV and VSV L protein has been shown to function as the mRNA cap methylase (15, 21, 22, 30). Based on these assignments, we suspected that either CRIV or -V might serve as the mRNA capping enzyme. This idea is consistent with a prior study that described a small-molecule inhibitor of RSV RNA synthesis in vitro (23). This inhibitor resulted in the synthesis of short uncapped viral RNAs. Viral mutants resistant to this inhibitor were selected, and the resistance mutations were mapped to CRV, suggesting that CRV of L plays a role in either mRNA cap formation or polymerase processivity.

In this study, we established a system to study cap formation in vitro using purified recombinant L protein. Guided by phylogenetic comparisons among NNS RNA virus L proteins, we generated 19 L protein variants with single-amino-acid changes to residues in CRV of L that are highly conserved among the NNS RNA viruses. We analyzed the ability of these L proteins to support RNA synthesis and cap formation in vitro. These studies define a new motif that is conserved among the NNS RNA virus L proteins that are essential for mRNA cap formation.

MATERIALS AND METHODS

Purification of N RNA template and RNP complex. Nucleocapsid RNA (N RNA) template was purified from recombinant VSV essentially as described previously (31). Briefly, 4 mg purified virus was disrupted on ice for 1 h in 20 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 5% glycerol, 5 mM EDTA, 3.5 mM dithioerythritol, 20% dimethyl sulfoxide, and 1.0 M LiCl. Template was recovered by centrifugation (190,000 × g, 3.5 h) through a glycerol step gradient of 0.25 ml each of 40, 45, and 50% glycerol in TED (20 mM Tris-Cl [pH 8.0], 1 mM EDTA, 2 mM dithioerythritol) supplemented with 0.1 M NaCl. The pellet was resuspended in 0.3 ml of TED plus 10% glycerol and disrupted on ice again, except Triton and EDTA concentrations were reduced to 0.05% and 1 mM, respectively. The N RNA template was isolated by banding in a 3.6-ml 20 to 40% (wt/wt) CsCl gradient (150,000 × g, 2.5 h), recovered by side puncture, and diluted fourfold with 10 mM Tris-Cl (pH 8.0) plus 0.1 mM EDTA. Template was recovered following centrifugation (150,000 × g, 1.5 h) through a 0.5-ml cushion (50% glycerol, TED buffer, 0.1 M NaCl).

The RNP complex was purified as described previously (6). Briefly, 1 mg of purified VSV was disrupted (0.4 M NaCl, 0.2% Triton X-100) for 1 h on ice. The RNP complex was recovered as pellet following centrifugation (160,000 × g, 1 h) through a 4-ml 10% sucrose NTE (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris-Cl [pH 7.4]) cushion. Both N RNA and RNP pellets were resuspended in 0.3 ml NTE and stored in aliquots at −80°C.

Expression and purification of recombinant VSV polymerase. Plasmids carrying functional VSV L and P genes were described previously (32). To facilitate protein purification, hexahistidine tags were introduced at the N terminus of VSV L and P and were separately inserted into pFASTBAC DUAL (Invitrogen) under control of the polyhedrin promoter. A third plasmid, designed to coexpress L and P, was generated by insertion of N His-L under control of the polyhedrin promoter and an untagged version of P under control of the P10 promoter. Recombinant baculoviruses (BV) were recovered following transfection of bacmid DNA into *Spodoptera frugiperda* Sf21 cells using Cellfectin (Invitrogen). Mutagenesis of the L gene was performed using QuikChange (Stratagene, La Jolla, CA). A 2-kb fragment containing the desired mutations was sequenced prior to subcloning into pFASTBAC DUAL. This confirmed that no other mutations were introduced. For expression of polymerase components, Sf21 cells were infected at a multiplicity of infection of 10 with recombinant BV. At 72 h postinfection, cells were scraped into media and washed twice with ice-cold phosphate-buffered saline, and the cells were recovered by centrifugation. Cells were suspended in lysis buffer (50 mM NaH_2PO_4, 10% glycerol, 0.2% NP-40, 300 mM NaCl, 10 mM imidazole [pH 8.0]) supplemented with EDTA-free protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication. The cell lysates were clarified by centrifugation (30,000 × g, 40 min) and incubated with Ni-nitrilotriacetic acid (NTA)-agarose beads (Qiagen) for 2 h. The resin was then washed with 20 bed volumes of wash buffer (50 mM NaH_2PO_4, 10% glycerol, 300 mM NaCl, 20 mM imidazole [pH 8.0]), and the His-tagged proteins were eluted in elution buffer (50 mM NaH_2PO_4, 10% glycerol, 300 mM NaCl, 250 mM imidazole [pH 7.0]).

Where indicated, the His-L and His-P proteins were purified further by ion-exchange chromatography through either a Mono S HR 5/5 or a Mono Q HR 5/5 column (GE Healthcare), respectively. Prior to loading, the fractions eluted from Ni-NTA-agarose were diluted twofold in buffer A (50 mM Tris-HCl [pH 7], 0.1 M NaCl, 10% glycerol, 1 mM dithiothreitol [DTT]), which had also been used to equilibrate the columns. Columns were washed with 5 column volumes of buffer A, and the proteins were eluted with a 20-column-volume gradient of 0.1 to 1 M NaCl in buffer A. Peak fractions were pooled and dialyzed against 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 15% glycerol, and 1 mM DTT. The purified proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue stain. Protein amounts were determined by Bradford assay.

Reconstitution of viral RNA synthesis in vitro. Viral RNA was synthesized in vitro either using purified RNP complex from virus particles or using purified polymerase from insect cells. Reconstituted reactions were typically performed using 1 μg of N RNA template, 1 μg of purified L, and 0.5 μg of purified P, nucleoside triphosphates (1 mM ATP and 0.5 mM each CTP, GTP, and UTP), and 30% (vol/vol) rabbit reticulocyte lysate (Promega). Where indicated, reaction mixtures were supplemented with 15 μCi of [α-^{32}P]GTP (3,000 Ci mmol^{-1}) (Perkin-Elmer, Wellesley, MA). After 5 h of incubation at 30°C, RNA was purified, resuspended in 60 μl of water, and analyzed by electrophoresis in 6% acid-agarose-urea gels (20).

Primer extension assay. Minus-sense oligonucleotides, corresponding to nucleotides (nt) 27 to 47 and 130 to 115 of the complete VSV genome sequence, were used in primer extensions as described previously (43). Products were analyzed by electrophoresis on 6% polyacrylamide gels and detected by PhosphorImager. Where indicated, products of in vitro transcription reactions were treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure, prior to reverse transcription.

Analysis of the mRNA cap structure. To directly examine the cap structure, RNA was digested with TAP (Epicenter, Madison, WI), as previously described (21, 22). Products were analyzed by thin-layer chromatography (TLC) on polyethyleneimine-F cellulose sheets (EM Biosciences). Plates were developed using 1.2 M LiCl and the spots were visualized using a PhosphorImager. Markers 7^{m}Gp and Gp were visualized by UV shadowing at 254 nm.

Characterization of the 5′ termini by terminator exonuclease. Purified RNAs were treated with TAP and alkaline phosphatase (AP), followed by T4 polynucleotide kinase (PNK) and exonuclease (Exo). Reaction mixtures were then treated with proteinase K in presence of 0.5% SDS, followed by phenol-chloroform extraction, and precipitated with ethanol and glycogen. RNA samples were analyzed by electrophoresis on acid-agarose gels, and products were detected by using a PhosphorImager.

Synthesis of 5′-triphosphorylated oligo-RNAs. RNA (pppApApCpApG or pppApCpGpApA) was synthesized using partially double-stranded synthetic DNA templates containing a T7 polymerase promoter as described previously (29). Transcription reaction mixtures contained 100 nM of annealed template; 0.2 U μl^{-1} of T7 polymerase (NEB); and 1 mM of ATP, CTP, and GTP in T7 reaction buffer (40 mM Tris-HCl [pH 7.9], 6 mM $MgCl_2$, 10 mM DTT, and 2 mM spermidine). Where indicated, reaction mixtures contained 20 cpm pmol^{-1} or 3 × 10^4 cpm pmol^{-1} of [α-^{32}P]GTP. Following 4 h of incubation, DNA was digested with 3 U of RQ1 DNase (Promega) and the RNA was recovered by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation. RNA was washed with 70% ethanol, dried, and resuspended in H_2O. Following addition of formamide stop solution, samples were boiled for 3 min and then resolved by electrophoresis on 20% polyacrylamide–7 M urea gels. Transcripts were visualized by autoradiography, and the 5-nt products were excised, crushed, and soaked in 0.3 M ammonium acetate (pH 5.2). Eluted RNA was recovered by ethanol precipitation, and the sequence was confirmed by digestion with RNase T$_1$.

RNA *trans* capping assay. The RNA *trans* capping assay was performed essentially as described previously (29). Briefly, 5 μM of 5′-triphosphorylated 5-nt oligo-RNAs was incubated with 0.5 μM of [α-^{32}P]GTP or [α-^{32}P]dGTP (400 cpm fmol^{-1}) and 300 ng of His-L in capping buffer (50 mM MOPS [morpholinepropanesulfonic acid; pH 5.8], 1 mM $MnCl_2$, 2 mM DTT, 5% glycerol, and 0.1 mg/ml bovine serum albumin), at 30°C for 2 h. Reactions were stopped by addition of Tris-HCl (pH 8) to 100 mM, followed by treatment with AP and subsequent digestion with proteinase K. Reaction products were extracted by phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, washed, dried, and resuspended in H_2O.
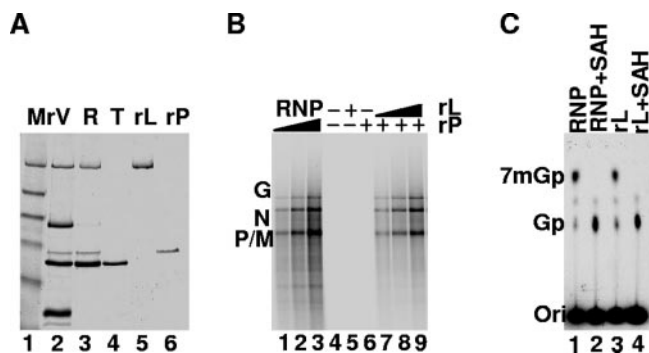
FIG. 1. Reconstitution of VSV mRNA synthesis in vitro. (A) Virus (V), ribonucleoprotein cores (R), N RNA template (T), and BV-expressed recombinant L (rL) and P (rP) proteins were purified and analyzed by SDS-PAGE. An image of the Coomassie blue-stained gel is shown. Lane 1, molecular weight standards (Mr); lane 2, 5 μg of purified VSV; lane 3, 3 μg of RNP; lane 4, 1.5 μg of N RNA template; lane 5, 1 μg of rL; lane 6, 0.5 μg of rP. (B) In vitro transcription reactions using 5, 10, and 15 μg of purified RNP (lanes 1 to 3) or reconstituted from 1 μg of N RNA, 0.5 μg of P, and 0.5, 1, or 2 μg L (lanes 4 to 9) were performed in the presence of [α-^{32}P]GTP. Purified RNA was analyzed on acid-agarose gels and detected using a PhosphorImager. (C) TLC analysis of the cap. Transcription reactions were performed in the presence of SAM or SAH and [α-^{32}P]GTP. Purified RNAs were digested with TAP, and the products were analyzed by TLC. Plates were dried, and spots were visualized with a PhosphorImager. The migration of markers 7^{m}Gp and Gp is shown.

Quantitative analysis. Quantitative analysis was performed by using a PhosphorImager (GE Healthcare, Typhoon) and ImageQuant TL software (GE Healthcare, Piscataway, NJ). Statistical analysis was performed on three to five independent experiments. The significance of the values was determined by a paired Student's t test.

RESULTS

Expression and purification of functional VSV polymerase. We developed a system to analyze RNA synthesis using purified recombinant polymerase. In this system, N RNA template was purified from infectious VSV (Fig. 1A, lane 4) and the viral P and L proteins were expressed in Sf21 cells from recombinant BV. The P and L proteins were purified by Ni-NTA agarose and subsequent anion-exchange chromatography to yield highly purified L (Fig. 1A, lane 5) and P (Fig. 1A, lane 6). As a control, the RNP complex was purified from infectious VSV (Fig. 1A, lanes 2 and 3). To examine whether recombinant RdRP was functional for RNA synthesis, template was mixed with the indicated quantity of purified P and L proteins and in vitro RNA synthesis was performed in the presence of [α-^{32}P]GTP. The products were analyzed by acid-agarose gel electrophoresis. In reactions programmed with N RNA template as well as the P and L proteins, full-length VSV N, P, M, and G mRNAs were detected and their abundance increased with increasing amounts of L (Fig. 1B, lanes 7 to 9). These mRNA products were indistinguishable from those synthesized by RNP complexes isolated from purified VSV (Fig. 1B, lanes 1 to 3). RNA synthesis was not detected when reactions were programmed with N RNA template alone (Fig. 1B, lane 4) or when incubated with L alone (Fig. 1B, lane 5) or P alone (Fig. 1B, lane 6). These data show that recombinant RdRP was functional for mRNA synthesis in vitro.

To demonstrate the mRNAs contained a cap structure, RNA was synthesized in vitro in the presence of [α-^{32}P]GTP and the methyl donor S-adenosyl-L-methionine (SAM) or inhibitor S-adenosyl-L-homocysteine (SAH). Purified RNA was digested with TAP, and the products were resolved by TLC. Cleavage of VSV mRNAs by TAP should yield Gp or, if the cap structure is methylated, 7^{m}Gp. Consistent with this, TAP cleavage of RNA synthesized by the RNP or recombinant RdRP yielded a product that comigrated with the Gp or 7^{m}Gp markers (Fig. 1C). Quantitative analysis showed that 7^{m}Gp accounted for 91% of the released cap structure for RNA synthesized by the recombinant RdRP, and this was indistinguishable from the 94% observed for the RNP complex (Fig. 1C). These results show that the purified recombinant RdRP supports the synthesis of full-length, capped, and methylated mRNA in vitro.

Alterations to CRV of L protein. Available evidence suggested that CRV of L may play a role in capping. Sequence alignments of this region of L identified residues that are conserved among the NNS RNA viruses (Fig. 2). We introduced substitutions to each of the residues highlighted by an asterisk on Fig. 2 to generate a total of 17 single-amino-acid substitutions in this region. In general, these residues were selected owing to their potential to coordinate a metal ion, which is a predicted function of this region, or were selected based on their universal conservation or proximity to such residues. Although the alignment shown (Fig. 2) reveals that W1188 is universally conserved, it was not modified as our initial alignment of the entire L protein sequence did not identify this position as conserved. In addition to these 17 single-amino-acid substitutions, we engineered substitutions at two key positions in the active site for ribonucleotide polymerization in region III. With the exception of H1294A, H1296A, and C1302A, which were expressed without P, the other L proteins were coexpressed with P in Sf21 cells (Fig. 3). During the course of this study, we discovered that coexpression with P protein was not essential for efficient L protein expression in insect cells or the subsequent purification of functional L protein. In addition, no significant differences were seen between reactions reconstituted with P and L that were separately purified versus those in which P and L were copurified (data not shown).

Effect of substitutions in CRV of L protein on mRNA synthesis in vitro. To examine the effect of substitutions to CRV of L protein on RNA synthesis, we reconstituted RNA synthesis in vitro using purified recombinant L. Standard reaction mixtures contained 1 μg of template, 1 μg of purified L, and 0.5 μg of purified P, and synthesis was carried out in the presence of [α-^{32}P]GTP. RNA was purified and analyzed by acid-agarose gel electrophoresis (Fig. 4). Based on this analysis, the L proteins fell into three groups. The first comprised the two substitutions in CRIII of L, G713A and D714A, and the CRV substitutions P1104A, Y1152A, H1294A, H1296A, and C1302S, which failed to synthesize RNA (Fig. 4A, lanes 2, 3, 8, 12, and 19 to 21). The second group comprised C1081A, R1090A, R1096A, G1100A, E1108A, C1120A, L1153A, and S1155A, which synthesized full-length VSV mRNA, at 10 to 60% of wt levels (Fig. 4A, lanes 4 to 7, 9, 10, 13, and 15). The third group comprised G1154A, T1157A, H1227A, and R1228A, which exhibited a remarkable phenotype. Under

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                1    250    500    750    1000   1250   1500   1750   2000  2109
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              │        │  I  │ II  │ III  │ IV   │     V     │  VI  │      │
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VSIV      1069 HLRRGSCKMWTCSATHADTLRYKSW-----GRTVIGTTVPHPLEML-GPQHRKETPCAPCNTSGFN----YVSVHCPDGT------HDVFS
VSNJV     1069 HYRLGDNQIWSCSASRADILRYKSW-----TRKVVGTTVPHPLEMH-GPPSKKERPCQLCNSSGLT----YISVHCPKGT------IDVFN
RABV      1081 QTPQRVGGVWPCSSERADLLREISW-----GRKVVGTTVPHPSEML-GLLPKSSISCTCGATGGGN-P-RVSVSVLPSF-----DQSFF
SYNV      1130 VNTQDGHAIGSCPTRDSKMLRNWTW-----GKNIIGVTTPHPLGYL-KRERHS----ESSSCDN----N-YIRVLTKRIG-----NSWEL
MEASV     1120 RKRNVLIDKESCSVQLARALRSHMWARLARCRPIYCLEVPDVLESMRCHLIRRHETCVCECGS----VNYGWFFVPSGC-----QLDDI
SENDV     1120 PVKDNIEYEYMCSVELAVGLRQKMWIHLTYGRPIHGLETPDPLELLRCTFIEGSEVCKLCRSEGAD--PIYTWFYLPDNT------DLDTL
PIV2      1128 PGYLNYITDQTCSIDISRSLRKLSWSSLLNGRTLEGCLETPDPIEVVNGFLIVGTGDCDFCMQGD----DKFTWFFLPMGI------IIDGN
PIV3      1120 IVSDKIRYEDMCSVDLAIALRQKMWIHLSGGRMISCLETPDPLELLSCVIITGSEHCKICYSSDGT--NPYTWMYLPGNI-----KIGSA
PIV5      1124 PTYLKAMTLETCSIDIARNLRKLSWAPLLGGRNLEGCLETPDPIEITAGALIVGSGYCEQCAAGD----NRFTWFFLPSGI------EIGGD
NDV       1100 RSNHPLVSSNMCSLTLADYARNRSWSPLTGGRKILCVSNPDTIELVEGEILSVSGGCTRCDSGD----EQFTWFHLPSNI------ELTDD
EBOZ      1094 -ILAEALTQITCTVDLAQILREYSWAHILEGRPLIGATLPCMIEQFKVFWLKPYEQCPQCSNAKQPGGKPFVSVAVKKHI-VSAWPNASR
HRSV      1186 DKREILSMENLSITELSKYVRERSWSL----SNIVGVTSPSIMYTMD------------------IKYTTSTISSGTIIEKYNVNSL
consensus               cs  la  lR sW     gr v Gv P pleml g        c  c      y   vp  i

                             ****  *                                                          **
VSIV      1145 SRG--PL-PAYLGSKTSESTSILQPWERESKVPLIKRATRLRDAISWFVEPDSKLAMTILSNIHSLTGEEWTKRQHG--FKRTGSALHRF
VSNJV     1145 RRG--PL-PAYLGSNTSESTSILQPWEKESKIPIIKRATRLRDAISWFIPPESPLSTCILNNIQALTGEDWSSKQHG--FKRTGSALHRF
RABV      1158 SRG--PL-KGYLGSSTSMSTQLFHAWEKVTNVHVVKRALSLESINWFITRDSNLAQALIRNIMSLTGPDFPLEEAP-VFKRTGSALHRF
SYNV      1201 RRG--QF-RPYFGSYTEEKFKMTTLASAYGDESILKRAIKIQKLLGWRYHQGSSLYNLIQKILTCVTDADPNKFLPL-PDEITGDVEHRY
MEASV     1201 DKETSSLRVPYIGSTTDERTDMKLAFVRAPS-RSLRSAVRIATVYSWAYGDDDSSWNEAWLLARQRANVSLEELRVITPISTSTNLAHRL
SENDV     1203 TNGSPAIRIPYFGSATDERSEAQLGYVRNLS-KPAKAAIIAMVYTWAYGTDEISWMEAALIAQTRANLSLENLKLLTPVSTSTNLSHRL
PIV2      1209 PETNPPIRVPYIGSRTEERRVASMAYIKGAT-HSLKAALRGAGVYIWAFGDTVVNWNDALDIANTRVKISLEQLQTLTPLPTSANITHRL
PIV3      1203 ETGISSLRVPYFGSVTDERSEAQLGYIKNLS-KPAKAAIIRIAMIYTWAFGNDEISWMEASQIAQTRANFTLDSLKILTPVATSTNLSHRL
PIV5      1207 PRDNPPIRVPYIGSRTEERRVASMAYIRGAS-SSLKAVLRLAGVYIWAFGDTLENWIDALDLSHTRVNITLEQLQSLTPLPTSANLTHRL
NDV       1181 TSKNPPMRVPYLGSKTQERRAASLAKIAHM-SPHVKAALRASSVLIWAYGDNEVNWTAALTIAKSRCNINLEYLRLLSPLPTAGNLQHRL
EBOZ      1183 ISWTIGDGIPYIGSRTEDKIGQPAIKPKCP-SAALREAIELASRLTWVTQGSSNSDLLIKPFLEARVNLSVQEILQMTPSHYSGNIVHRY
HRSV      1251 TRGERGPTKPWVGSSTQEKKTMPVYNRQVL-TKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLTYEKAKKLFPQYLSVNYLHRL
consensus          l pylGS T ek      w r     ik alrl   W y              i        l p  tgnv HR

                                                                                          *  *      *
VSIV      1230 STSRMSHGGFASQSTAALTRLMATTDTMR---DL--GDQNFDFLFQATLLYAQITT-T---VARDGWITSCTD----HYHIAC-KSCLRP
VSNJV     1230 STSRMSNGGFASQSPATLTRMIATTDTMR---DF--GTKNYDFMFQASLLYGQMTT-S---ISRYGTPGSCTD----HYHIRC-KGCIRE
RABV      1244 KSARYSEGGYSSVCPNLLSHISVSTDTMS---DLTQDGKNYDFMFQPLMLYAQTWT-SEL-VQRDTRLRDSTF----HWHLRC-NRCVRP
SYNV      1287 HDMATKHGGIPSNLIHLYTHASCNTSTFI---NHSKGAANESLHFQAAIIWTCM-Q-SICRTSASSSVSDIS-----HYHEAC-NQCIVK
MEASV     1290 RDRSTQVKYSGTSLVRVARYTTISNDNLS--FVISDKKVDTNFIYQQGMLLGLGVLETLFRLEKDTGSSNTVL----HLHVET-DCCVIP
SENDV     1292 KDTATQMKFSSATLVRASRFITISNDNMA--LKEAGESKDTNLVYQQIMLTGLSILGLKGKPLIL----HLHLNN-GCCIME
PIV2      1298 DDGATTLKFTPASSYAFSSYTHISNDQQY---LEIDQRVVDSNIIYQQLMITGLGIIETYHNPPIRTSTQEITL----HLHTSS-SCCVRS
PIV3      1292 KDTATQMKFSSTSLIRVSRFITMSNDNMS--IKEANETKDTNLIYQQIMLTGLSVFEYLFRLEETTGHNPIVM----HLHIED-ECCIKE
PIV5      1296 DDGTTTLKFTPASSYTFSSTFHISNDEQY--TLTNDKTADSNIYQQLMITGLGILETWNNPPVNRTFEESTL----HLHTGA-SCCVRP
NDV       1270 DDGITQMTFTPASLYRCHLTFFTYPMILKG--YSLKKESKRGMWFINRVMLLGLSLIESIFPMTTTRTYDEITL----HLHSKF-SCCIRE
EBOZ      1272 NDQYSPHSFMANRMSNSATRLIVSTNTLGEFSGGGQSARDSNIIFQNVINYAVALFDIKFRNTEATDIQYNRA----HLHLT-KCCIRE
HRSV      1340 TVSSRPCEFPASIPAYRTTNYHFDTSPINRILTEKYGDEDIDIVFQNCISFGLSLMSVVEQFTNVCPNRIILIPKLNEIHLMKPPIFTGD
consensus         d  t  y       t  t d m        lfq  ll gl v  t            h Hi     clr
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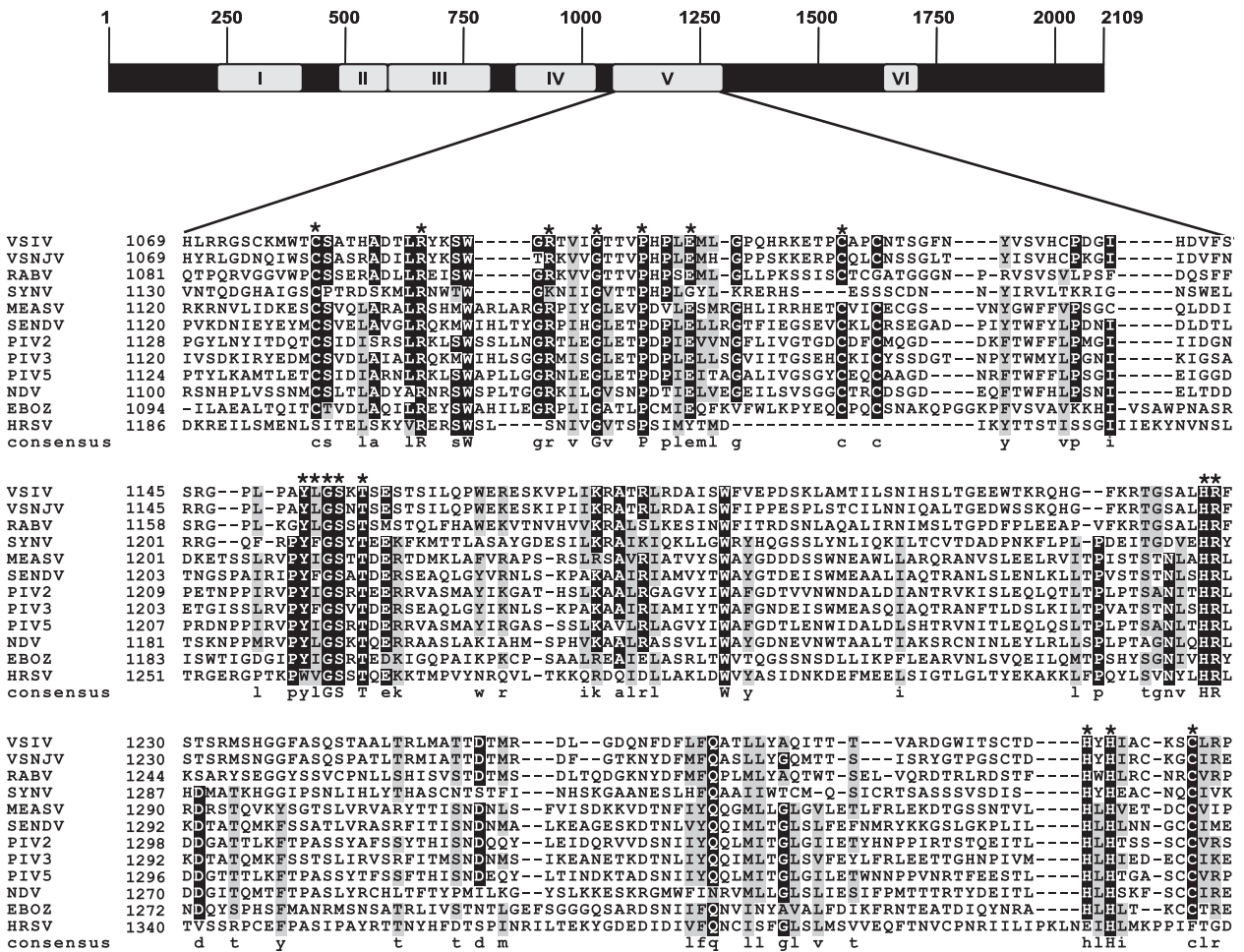
FIG. 2. Alignment of CRV of the L protein of NNS RNA viruses. The alignment was generated using CLUSTALW, and conserved residues were emphasized by BOXSHADE. Residues modified in the present study are highlighted by asterisks. The consensus sequence is shown. L proteins and their accession numbers are as follows: VSIV, VSV Indiana (P03523); VSNJV, VSV New Jersey (P16379); RABV, rabies virus (P16289); SYNV, Sonchus yellow net virus (P31332); MEASV, measles virus (P12576); SENDV, SeV (Q06996); PIV2, human parainfluenza virus 2 (P26676); PIV3, human parainfluenza virus 3 (P12577); PIV5, parainfluenza virus 5 (Q03396); NDV, Newcastle disease virus (P11205); HRSV, human RSV (P28887); EBOZ, Ebola virus Zaïre (Q05318).
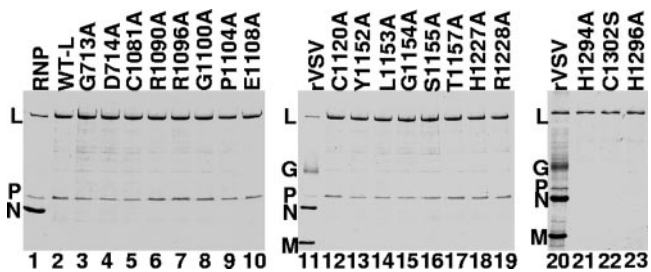

FIG. 3. Purification of L protein with substitutions in CRV. Recombinant BV coexpressing the indicated L and P were used to infect Sf21 cells. Purified protein was analyzed by SDS-PAGE. Markers of 3 μg of RNP or recombinant VSV (VSV) are shown.

standard conditions, full-length mRNA was not detected for G1154A and T1157A; rather short transcripts were observed (Fig. 4A, lanes 14 and 16). Similarly, H1227A and R1228A yielded short transcripts, although some RNA comigrated with full-length VSV N mRNA (Fig. 4A, lanes 17 and 18). In this system, the quantity of mRNA synthesized is dependent upon the amount of L protein (Fig. 1B). Therefore, for L proteins in groups 1 and 3, we increased the amount of polymerase up to 3 μg and reexamined RNA synthesis in vitro. Under these conditions, RNA synthesis was still undetectable for D714A, P1104A, Y1152A, H1294A and C1302S (data not shown and Fig. 4B, lane 2). In contrast, some full-length N, P, M, and G mRNA was observed for H1296A (Fig. 4B, lane 7). Full-length N mRNA was also observed for G1154A, T1157A, H1227A, and R1228A, although the abundance was less than 1% of that of the wt. Consistent with the reconstitutions using 1 μg of L protein, the major products observed were short (Fig. 4B, lanes 3 to 6). These ranged from 100 nt to the full-length mRNA, although the majority were <400 nt (Fig. 4B, lane 1).

Changes to CRV of L protein inhibit mRNA cap formation. To determine the effect of mutations to CRV of L on the position at which polymerase initiated RNA synthesis and to examine whether mRNAs were capped, we used a primer extension assay. A schematic of the 3′ end of the VSV genome
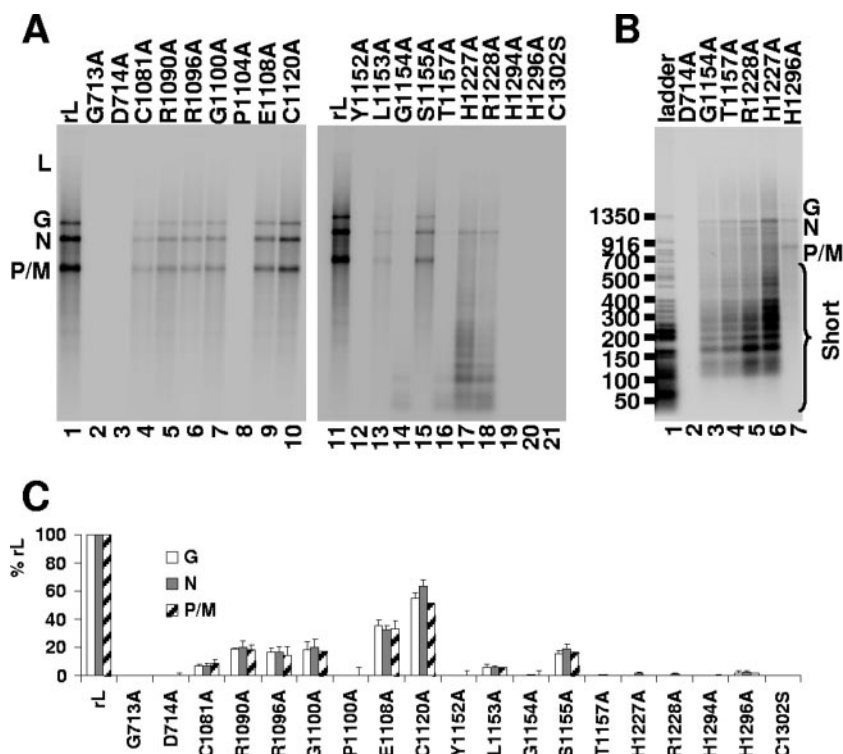
FIG. 4. Substitutions in CRV of L protein are accompanied by defects in mRNA synthesis in vitro. (A) Transcription reactions performed in vitro in the presence of 1 μg of N RNA template, 0.5 μg of recombinant P (rP), and 1 μg of recombinant L (rL) and [α-^{32}P]GTP were analyzed by electrophoresis on acid-agarose gels. The identity of the mRNAs is shown on the left. Note that 1/4 the total reaction was analyzed for wt L, whereas 1/2 the reaction was analyzed for each mutant. (B) As in panel A, except with 3 μg of L. Lane 1, 50-bp DNA size ladder, labeled by T4 PNK in the presence of [γ-^{32}P]ATP. (C) Quantitative analysis. For each mRNA, the mean ± standard deviation was expressed as a percentage of that observed for recombinant VSV (rVSV) for three independent experiments.

and the products detected by this assay are shown (Fig. 5A). Two primers were used: one designed to anneal to positions 47 to 34 of the positive-sense leader RNA, Le+, and a second designed to anneal to positions 80 to 55 within the N mRNA. Extension of the leader primer by reverse transcriptase (RT) on RNA synthesized by wt L protein yielded a 47-nt product, which corresponds to Le+ (Fig. 5B, lanes 3 and 23). This product was absent when L was omitted from the reaction (Fig. 5B, lane 1). Extension of the N primer yielded an 81-nt product which corresponds to RT extending onto the cap structure (Fig. 5B, lanes 3 and 23). To confirm this, RNAs were treated with TAP to cleave the cap prior to reverse transcription. This resulted in detection of an 80-nt product that mapped to position U1 of the N gene start (Fig. 5B, lanes 4 and 24). As control, TAP cleavage had no effect on the size of the RT products from the uncapped leader RNA (Fig. 5B, lanes 3, 4, 23, and 24).

The RNA products synthesized by each polymerase were examined in an identical manner. Consistent with the failure to observe RNA synthesis (Fig. 4), no products were detected for G713A, D714A, P1104A, Y1152A, H1294A, and C1302S. Reactions reconstituted with C1081A, R1090A, R1096A, G1100A, E1108A, C1120A, L1153A, S1155A, and H1296A yielded N mRNA products whose migration and sensitivity to TAP were indistinguishable from those to wt L (Fig. 5B, lanes 9 to 16, 19 to 22, 27, 28, 31, 32, 43, and 44), indicating that they were capped. In contrast, extension of the N primer by RT on

RNA synthesized by G1154A, T1157A, H1227A, and R1228A yielded only the 80-nt RT product, even in the absence of TAP. This suggested that the RNA lacked a cap structure (Fig. 5B, lanes 29, 30, and 33 to 38). Each of these L proteins synthesized the 47-nt uncapped leader RNA (Fig. 5B). Taken together, these data show that the N mRNA synthesized by G1154A, T1157A, H1227A, and R1228A lacked a cap structure.

To directly show that the products were uncapped, transcription reactions were carried out in the presence of [α-^{32}P]GTP and 1 mM SAM or SAH. RNA was purified and digested with TAP, and the products were resolved by TLC. For wt L, transcription reactions performed in the presence of SAM yielded a single product that comigrated with a 7^{m}Gp marker, which confirmed that the RNA was capped and methylated (Fig. 5C, lane 1). Cleavage of RNA synthesized in the presence of SAH yielded Gp, which represents the unmethylated cap structure (Fig. 5C, lane 2). In contrast, when reactions were reconstituted with G1154A, T1157A, H1227A, and R1228A, 7^{m}Gp was below the limit of detection (Fig. 5C, lanes 3 to 6). A low level (<5%) of Gp was observed (Fig. 5C, lanes 3 to 6), indicative of a defective capping activity. Consistent with the data of the primer extension assay, TAP cleavage of RNA synthesized by G1100A, E1108A, and C1120A yielded 7^{m}Gp, which confirmed that their RNAs were capped and methylated (Fig. 5C, lanes 7 to 9). These data directly show that RNA synthesized
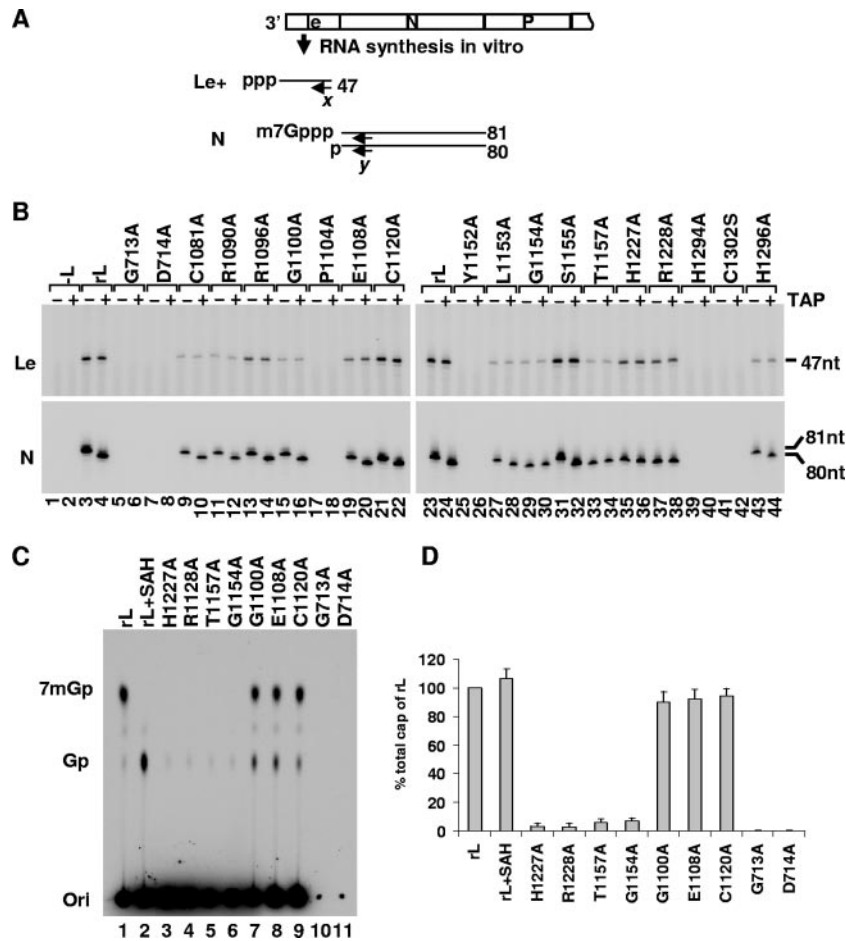
FIG. 5. Substitutions in CRV of L protein result in defects in mRNA cap formation. (A) Schematic of the 3′ end of the VSV genome and the products of in vitro RNA synthesis. Leader RNA (Le+) and N mRNAs were detected by primer extension using primers denoted *x* and *y*, respectively. (B) Products were analyzed on denaturing 6% polyacrylamide gels and detected using a PhosphorImager. The size of the RT product is shown. Where indicated, samples were treated with TAP to hydrolyze the cap structure. Under these conditions, RT cannot extend onto the cap, thus yielding a 1-nt-shorter product. rL, recombinant L. (C) Viral mRNA was synthesized as for panel A, except 15 μCi of [α-^{32}P]GTP was incorporated into the reaction. Purified RNAs were digested with TAP and analyzed by TLC. Plates were dried, and spots were visualized using a PhosphorImager. Migration of markers 7^{m}Gp and Gp and the chromatographic origin are shown. (D) Quantitative analysis performed on three independent experiments. Released Gp (mean ± standard deviation) was expressed as a percentage of the total released Gp from wt L.

by G1154A, T1157A, H1227A, and R1228A lacked a 5′ cap structure.

Cap-defective L proteins generate transcripts with 5′ triphosphate termini. The direct cap analysis demonstrated that G1154A, T1157A, H1227A, and R1228A were defective in mRNA cap formation. To determine whether the RNA contained a 5′ triphosphate (5′ pppRNA) or had been processed to a monophosphate (5′ pRNA), we used an exonuclease sensitivity assay. Terminator Exo is a 5′-to-3′ processive enzyme that degrades 5′ pRNA. In contrast, 5′ pppRNA, or RNA that contains a 5′ cap, is resistant. To render all uncapped RNA sensitive to Exo, RNA was treated with AP to remove the terminal phosphates, followed by T4 PNK to generate a 5′ pRNA. This treatment will not affect capped mRNAs (Fig. 6A). To render capped RNA sensitive to Exo digestion, RNA was first treated with TAP to remove the cap and generate 5′ pRNA (Fig. 6A).

RNA was synthesized in vitro in the presence of [α-^{32}P]GTP, purified, subjected to the indicated treatment, and analyzed on acid-agarose gels (Fig. 6B). Treatment with TAP and Exo led to complete degradation of all mRNA synthesized by wt L protein, whereas either enzyme alone had no effect (Fig. 6B, lanes 1 to 4). This indicated that the mRNA synthesized by wt L was fully capped. Consistent with this, treatment of RNA with AP followed by T4 PNK prior to Exo treatment had no detectable effect (Fig. 6B, lane 6), indicating that these were not 5′ pppRNA. The RNA synthesized by C1120A displayed a similar sensitivity, confirming that these mRNAs were also fully capped (Fig. 6B, lanes 7 to 12). In contrast, RNA synthesized by G1154A, T1157A, H1227A, and R1228A showed an altered sensitivity profile. In each case, RNA was resistant to Exo digestion (Fig. 6B, lanes 14, 20, 26, and 32), indicating that they were not 5′ pRNA. Treatment of RNA with TAP, followed by AP, T4 PNK, and Exo resulted in the total degradation of all RNA (Fig. 6B, lanes 16, 22, 28, and 34), indicating that they were either capped or 5′ pppRNA. Omission of TAP from these reactions led to degradation of the majority of the RNA produced by G1154A, T1157A, H1227A, and R1228A
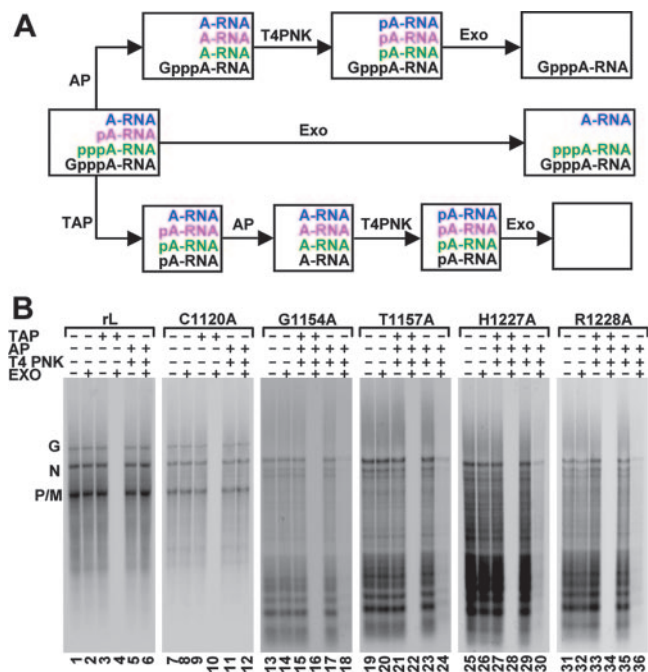
FIG. 6. Prematurely terminated transcripts contain 5′ triphosphate. (A) Schematic of the strategy to determine the status of the 5′ termini following sequential treatments with AP, T4 PNK, terminator Exo, and TAP. (B) Transcription reactions performed as in Fig. 4B, treated with the indicated enzymes, analyzed on acid-agarose gels, and detected by PhosphorImager. Migration of the viral N, P, M, and G mRNAs is shown.

(Fig. 6B, lanes 18, 24, 30, and 36), consistent with the presence of a 5′ pppRNA. However, we cannot formally eliminate the possibility that some of these transcripts lacked 5′ phosphates as they too would be resistant to exonuclease cleavage. These experiments indicate that these polymerases are unable to process the 5′ end of the RNA. Notably, a product that comigrates with full-length N mRNA was faintly visible for each of these mutants, consistent with a low level of synthesis of a capped full-length transcript (Fig. 6B, lanes 18, 24, 30, and 36).

Dissection of mRNA capping and polymerase processivity defects. The above experiments show that some polymerases with substitutions in CRV were defective in cap formation and

polymerase processivity. Thus, it was formally possible that transcripts were uncapped because they failed to reach the necessary length during their synthesis. To discriminate between these possibilities, we adapted a *trans* capping assay. For this assay, 5-nt-long triphosphate-initiated transcripts that correspond in sequence to either the VSV leader RNA pppApCpGpApA or the VSV mRNA pppApApCpApG were generated by T7 RNA polymerase in the presence of [α-^{32}P]GTP. These transcripts were analyzed by electrophoresis on 20% polyacrylamide gels, the 5-nt products were excised and purified, and their identities were confirmed by cleavage with RNase T$_1$ (Fig. 7A, lanes 1 to 6). RNase T$_1$ cleaves 3′ of G residues and cleaves the leader RNA oligonucleotide into a radioactive 3-mer and an unlabeled 2-mer (Fig. 7A, lanes 2 and 3). In contrast, the mRNA oligonucleotide was resistant to cleavage, indicating that it is the correct size and sequence (Fig. 7A, lanes 5 and 6). Unlabeled RNA, synthesized in an identical fashion, was incubated with highly purified wt L protein and [α-^{32}P]GTP. This resulted in the *trans* capping of the mRNA transcript (Fig. 7B, lanes 3 and 4). To confirm that this RNA was capped, the products were treated with TAP, which specifically removed the mRNA cap, resulting in the appearance of a Gp product (Fig. 7B, lane 5). Consistent with the presence of a 5′ cap, the RNA was resistant to Exo degradation (Fig. 7B, lane 6). In contrast to the ability of wt L to cap 5-nt transcripts corresponding to the mRNA, the leader transcript was not capped (Fig. 7B, lanes 10 and 11). This is consistent with the reported sequence-specific requirement for cap formation (29, 42). Using this assay, we examined whether polymerases with defects in processivity as well as cap formation were able to cap in *trans*. Consistent with their inability to generate capped RNA during in vitro transcription reactions, G1154A, T1157A, H1227A, and R1228A were defective in *trans* cap formation (Fig. 7C, lanes 1 to 6). A low level of *trans* capping activity was observed for G1154A and T1157A, which is consistent with the Exo digestion experiments. These data support and extend the results of direct cap analysis of the products of in vitro transcription reactions by demonstrating that G1154A, T1157A, H1227A, and R1228A are defective in cap formation independent of their defects in processivity.

Earlier work demonstrated that the VSV capping enzyme could efficiently use dGTP during cap formation (35). We thus also examined the ability of purified L to transfer RNA onto
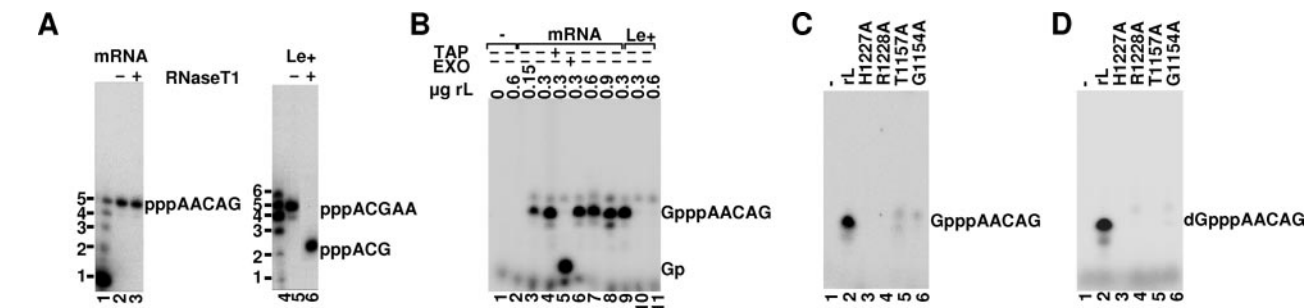

FIG. 7. Defects in cap formation are independent of defects in processivity. (A) Five nucleotides of RNA corresponding to the 5′ end of Le or mRNA was synthesized by T7 RNA polymerase and purified by 20% PAGE. Purified RNAs and their T$_1$ digestion products are shown along with size markers. (B) Unlabeled 5-nt RNAs were incubated with purified L and [α-^{32}P]GTP prior to electrophoresis by 20% PAGE. Where indicated, products of this reaction were treated with TAP or Exo. rL, recombinant L. (C) As for panel B, except using the indicated mutants. (D) As for panel C, except using dGTP as an acceptor.

dGDP as an acceptor. Consistent with this earlier study, and confirming that the activity studied here reflects VSV mRNA capping, wt L efficiently used dGTP in this reaction (Fig. 7D, lane 2), whereas G1154A, T1157A, H1227A, and R1228A were unable to use dGTP (Fig. 7D, lanes 3 to 6). These data thus show that a motif, GXXT[n]HR, present within CRV of L protein plays a central role in mRNA cap formation.

DISCUSSION

Using genetic and biochemical approaches, we determined the role of CRV of the L protein of VSV in RNA synthesis. We reconstituted RNA synthesis in vitro using highly purified polymerase components and found that all amino acid changes to CRV of L either abolished or diminished RNA synthesis. The majority of the polymerases that synthesized RNA produced transcripts that initiated at the correct position, were capped and appeared full length. However, four substitutions, G1154A, T1157A, H1227A, and R1228A, resulted in the synthesis of uncapped RNAs that terminated prematurely. These data suggest a model for RNA synthesis in which addition of the mRNA cap plays a key role in controlling polymerase processivity. Coupled with the fact that the mRNA capping machinery requires specific signals in the RNA (29, 42) and that mRNA cap methylation is accomplished by two activities that share a common SAM binding site (22), these data also show that the entire capping apparatus of these viruses is distinct to that employed by other systems. Furthermore, these experiments define a motif, GxxT[n]HR, that is essential for VSV mRNA cap formation. These residues are strictly conserved among the L proteins of all NNS RNA viruses, suggesting that they cap their mRNAs via a polyribonucleotidyltransferase.

Identification of CRV of L protein as the capping enzyme. Prior to the present study, little information was available regarding the function of CRV of L protein. This region was suggested as a potential metal binding region based on the conservation of Cys and His residues and was suggested to play a critical structural role for L (33). In this report, we demonstrated that substitutions to CRV diminished or prevented RNA synthesis (Fig. 4). Despite this, polymerases that synthesized RNA still responded correctly to the *cis*-acting signals that control polymerase initiation (Fig. 5). The most striking effect of substitutions to CRV was on mRNA cap formation (Fig. 5). Four polymerases were found to be defective in cap formation (Fig. 5) and synthesized transcripts with 5′ triphosphate (Fig. 6). These transcripts were truncated ranging in size from 100 nt up to the full-length N mRNA (Fig. 4 and 6). The defect in cap formation was not simply a consequence of a defect in polymerase processivity (Fig. 7). Substitutions G1154A, T1157A, H1227A, and R1228A resulted in polymerases that were unable to cap short VSV RNAs in *trans*. Further analysis showed that these defects in cap formation were accompanied by defective RTPase activities of polymerase (Fig. 6). These data show that CRV plays a critical role for full function of the L protein and was required for cap formation.

For two of the *Paramyxoviridae*, SeV and human RSV, the effect of amino acid changes in this region of L has been examined. For SeV, clusters of charged-to-alanine substitutions were introduced in CRV of L, which resulted in defects in RNA synthesis (10). Some of these changes differentially affected transcription versus replication, specifically inhibiting transcription. However, the effect of these alterations on mRNA cap formation was not analyzed. For human RSV, an inhibitor that resulted in the synthesis of short uncapped transcripts was used to select for resistant mutants (23). Resistant mutants contained substitutions in CRV of L, specifically at E1269D, I1381S, and L1421F. These mutants were four- to eightfold less sensitive to the inhibitor. Although these data are consistent with CRV functioning as capping enzyme, they are also consistent with this region of L serving a critical processivity function. A sequence alignment between a portion of this region of L protein and 41 amino acids of a nucleoside diphosphate (NDP) kinase led these investigators to suggest that this region of L might play a role in nucleotide binding.

An unusual motif in CRV of L protein essential for mRNA cap formation. Sequence alignments of CRV of NNS RNA virus L proteins identified conserved residues (Fig. 2). In this study, we found that the four underlined residues present within the conserved sequence $\underline{G}Sx\underline{T}xe[X_{27-28}]W[X_{37-39}]\underline{HR}$ were essential for mRNA cap formation. The mechanism of cap formation in VSV was recently shown to involve a novel polyribonucleotidyltransferase activity (29). This activity involves the sequence specific recognition of RNA by L protein and its transfer onto a GDP acceptor. Given the apparent conservation of the residues essential for this activity across NNS RNA virus L proteins, we suggest that this represents a signature motif for this polyribonucleotidyltransferase activity. Intriguingly, this motif is even present in the polymerase of Borna disease virus, although the conservation does not extend to include H1294. A search of the current protein sequence database using a hidden Markov model based upon this motif did not reveal other members of this family of enzymes.

Relationship of NNS RNA virus capping enzyme to other capping enzymes. Conventional mRNA cap structures are formed by the action of two enzymes, an RTPase and an RNA GTase. RTPases fall into two groups that differ in their requirement for a divalent cation. The metal dependent enzymes found in protozoa, fungi and viruses have been shown to remove the γ phosphate from both RNA and NTPs (19). These enzymes were recently recognized as being part of a triphosphate tunnel metalloenzyme superfamily, reflecting the structural properties of these enzymes and their dependence upon divalent cations (19). A signature of these enzymes are two glutamate-containing motifs that serve as a metal binding site and are essential for catalysis. One interpretation of the effects of CRV substitutions is that the VSV L protein behaves in a similar way to the triphosphate tunnel metalloenzyme superfamily, although there are no obvious counterparts to the glutamate motifs present in L. However, this region shares features common to many metal ion binding proteins, including a large number of Cys and His residues that might fulfill this role. Metal-independent RTPase enzymes are found in plants and metazoans and employ a cysteine phosphatase-like mechanism for catalysis (9, 40). These enzymes contain a signature HCXXXXXR[S/T] motif and are typically incapable of hydrolyzing NTP as a substrate. A number of viral RTPases have been described and many serve as metal-dependent enzymes capable of hydrolyzing γ phosphates from NTP and RNA.

However, several of these enzymes are distinctly different to the triphosphate tunnel metalloenzymes. These include the NS3 protein of Kunjin virus, which contains signature Walker A and B motifs that play key roles in its RTPase activities (26), and the rotavirus NSP2 protein which employs a histidine triad for γ phosphate hydrolysis (41). Inspection of the VSV L protein sequence indicates that the sequence lacks any such motifs.

RNA GTases typically contain a signature Kx[D/N]G motif that plays a central role in the reaction. The lysine residue forms the covalent adduct with Gp, prior to its transfer onto the diphosphate RNA acceptor (17, 18). As with RTPases, viruses have evolved GTases that use distinct motifs, such as the HxH motif present in alphavirus capping machines that transfer 7^{m}Gp through a covalent histidine Gp intermediate (3). In addition, Kx[V/L/I]S has been suggested to serve as the GTase active site for reovirus and rotavirus (24, 25). Consistent with the novel mechanism involved in cap formation for VSV, none of these motifs are present or required in L.

Evolution of the NNS RNA virus capping machinery. The evolutionary origin of a polyribonucleotidyltransferase that mediates cap formation is unknown. In contrast, CRIII, which catalyzes ribonucleotide polymerization, shares homology with all other template-dependent polynucleotide polymerases (34). Although there are differences between the MTase domain and other known MTases, it shares significant homology with known 2′-O MTases (7, 12, 15, 21). Thus, the polymerase and methylase domains share a relationship to other enzymes that catalyze similar activities, whereas the capping enzyme has no apparent homologues. Distinct flavors of cap formation have been defined in other parasites of eukaryotes; however, they all involve transfer of guanylate onto the RNA chain, rather than vice versa. The evolutionary origin of the polyribonucleotidyl transferase activity required for formation of the cap structure is thus unclear. It is striking that this enzyme functions in a sequence-specific manner (29, 42), and the constraints associated with this may have led to selection against this type of cap-forming activity, leaving this activity confined to the NNS RNA virus L proteins. Alternatively, the enzyme could perhaps have evolved through adapting an existing activity.

A model linking cap formation to polymerase processivity. In this study, we have shown that polymerases defective in mRNA cap formation synthesized short transcripts in vitro. Prior work showed that mutation of the conserved elements of a VSV gene start sequence led to synthesis of short transcripts that were not reactive with an antibody against trimethyl-guanosine (39). This led to the development of a model in which 5′ processing is linked to polymerase processivity. However, it was formally possible that these elements serve as a processivity signal independent of cap formation and that transcripts must be a minimum length in order to gain access to the capping machinery. Here we separated these events to study cap formation independent of processivity through the use of a *trans* capping assay. Our data provide strong support for such a model (Fig. 8) in which cap formation leads to polymerase becoming fully processive so that it now elongates until it encounters a gene-end termination and polyadenylation signal. Failure of polymerase to add the cap leads to frequent intragenic termination and accumulation of uncapped RNA.
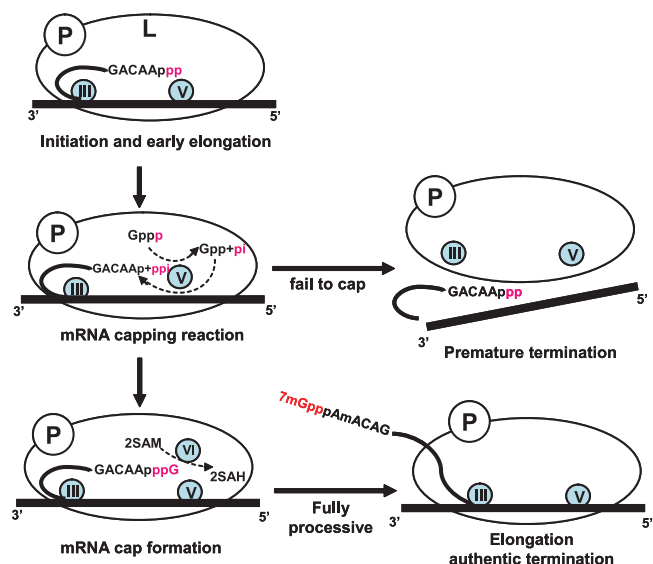
In summary, we show that a key function of CRV of L

FIG. 8. Model for mRNA synthesis in NNS RNA viruses. See text for details.

protein is to cap viral mRNA and we define a motif that is absolutely essential for this activity. Given that this reaction is catalyzed by a novel polyribonucleotidyltransferase activity, our data suggest that such enzymes are characterized by a GxxT[n]$_{64}$HR motif. Our data also support that this activity is conserved among all NNS RNA viruses for which L gene sequence is available. By directly defining the critical and conserved amino acids required for the essential polyribonucleoti-dyltransferase, these data support the notion that the mRNA capping enzymes of NNS RNA viruses are attractive targets for antiviral therapy.

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