Capping of vesicular stomatitis virus pre-mRNA is required for accurate selection of transcription stop-start sites and virus propagation

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SUPPLEMENTARY METHODS

In vitro transcription and product analyses

In vitro transcription (Figures 1 and S1) was performed at 30°C for 2 h in 25 µl of the standard transcription reaction mixtures including 100 µM [α-^{32}P]GTP (0.8–1 × 10^4 cpm/pmol), 1 mM each of the other three NTPs, 0.4 µg protein of N-RNA, 0.05 µg of P, and 0.1 µg of L (wild-type or mutant), as described previously (40). When indicated, poly(A) tails were removed from mRNAs by RNase H digestion in the presence of oligo(dT) as described (40). ^{32}P-Labeled transcripts were purified by phenol/chloroform extraction followed by ethanol precipitation, and dissolved in 15 µl of 80% formamide loading solution. Transcripts in 1.5-µl and 14.5-µl aliquots of the samples were resolved along with RNA size makers (*e.g.*, Ambion Century and Decade markers, Life Technologies) by 5% (for mRNAs) and 20% (for the leader RNA), respectively, urea-PAGE, and detected by autoradiography (4,40). Molar amounts of ^{32}P-labeled transcripts synthesized under the standard conditions were estimated as described previously (40). The wild-type L protein synthesized 21 ± 1 fmol leader RNA and 7.7 ± 0.9 fmol N mRNA (the mean ± standard deviation of three independent determinations).

For cap analyses (Figure S1D), *in vitro* transcription was carried out in 25-µl reaction mixtures including 100 µM [α-^{32}P]GTP (3.5 × 10^4 cpm/pmol), 1 mM each of the other three NTPs, 0.8 µg of N-RNA complex, 0.1 µg of P, and 0.2 µg of L (wild-type or mutant). ^{32}P-Labeled transcripts were purified and then digested with 0.5 units of nuclease P1 and 1 unit of calf intestine alkaline phosphatase (CIAP), as described (40). The digests were mixed with DEAE Sephacel (GE Healthcare) in the presence of 0.1 M triethylamine-bicarbonate buffer (pH 7.5). After washing the resin with the same buffer, ^{32}P-labeled cap structures were eluted from the resin with 1 M triethylamine-bicarbonate buffer, and analysed along with cap analogues by PEI-cellulose TLC (40).

For enzymatic sequencing of short transcripts (Figure 2C–G), *in vitro* transcription was performed in 50-µl reaction mixtures including 100 µM GTP (instead of [α-^{32}P]GTP), 1 mM each of the other three NTPs, 1.6 µg of N-RNA, 0.2 µg of P, and 0.4 µg of L (wild-type or HR-RH mutant). Transcripts from 6 reactions were combined and incubated in 20 µl of a capping buffer (Epicentre) with 1 µM [α-^{32}P]GTP (6.7 × 10^6 cpm/pmol), 10 units of vaccinia virus mRNA capping enzyme (Epicentre), and 0.3 units of yeast inorganic pyrophosphatase (Sigma) for 2 h at 37°C. The resulting cap-labelled RNAs were purified by phenol/chloroform extraction followed by ethanol precipitation, and then analysed by

electrophoresis in a 15% polyacrylamide sequencing gel containing 7 M urea followed by autoradiography. Major cap-labelled short RNAs were purified from the gel as described (40). Purified cap-labelled RNAs (1,500 cpm) were partially digested with RNase T1 or RNase A in 3 µl of a digestion buffer [20 mM sodium acetate buffer (pH 5.0), 1 mM EDTA, 7 M urea, 0.03% bromophenol blue (BPB), 0.03% xylene cyanol FF (XC)] in the presence of 5 µg of tRNA. To generate hydroxide cleavage ladders, the cap-labelled RNAs (1,500 cpm) were hydrolysed in the presence of 5 µg of tRNA in 3 µl of an alkaline solution [25 mM NaOH, 7 M urea, 0.03% BPB, 0.03% XC] at 90°C for 30 or 60 seconds. The resulting digests were analysed by 20% urea-PAGE.

For post-capping of transcripts with the VSV L protein (Figure 2H and I), unlabelled transcripts were synthesized with 0.1 µg of the HR-RH mutant in 25-µl reaction mixtures, purified as described above, and dissolved in 10 µl of H_2O. Two-microliter aliquots were incubated in 10 µl of the VSV capping reaction mixtures (40) with 0.25 µM [α-^{32}P]GDP (1.7×10^3 cpm/fmol) and 0.2 µg of the D714A L mutant (6) for 30°C for 2 h (Figure 2H). After the capping reaction, the reaction mixtures were incubated with 1 unit of CIAP at 37°C for 10 min. Cap-labelled RNAs were purified by phenol/chloroform extraction followed by ethanol precipitation, and analysed by 20% urea-PAGE (40). Similarly, 4 µl of the sample was subjected to a 20-µl VSV capping reaction with 0.25 µM [α-^{32}P]GDP (4.3×10^3 cpm/fmol) and 0.4 µg of the D714A L mutant (Figure 2I). ^{32}P-Labeled RNAs were purified by 20% urea-PAGE, and their nuclease P1-digests were analysed by PEI-cellulose TLC (40).

For Northern blot analyses (Figures 3, 4, 5A, and S3), unlabelled transcripts were synthesized with 0.1 µg of the wild-type or mutant L protein in 25-µl reaction mixtures as described above, and deadenylated with RNase H and oligo(dT) when indicated. RNAs were purified by phenol/chloroform extraction followed by ethanol precipitation, and dissolved in 10 µl of H_2O. Small RNAs in 2.5-µl aliquots were separated by 15% urea-PAGE, transferred to Hybond NX nylon membranes (GE Healthcare), and crosslinked to the membranes with 1-ethyl-3-[3-dimethylaminopropyl)carbodiimide (Thermo Scientific) as described by Pall and Hamilton (41). Long deadenylated RNAs in 2-µl aliquots were separated by 5% urea-PAGE, transferred to Immobilon NY+ membranes (Millipore), and UV-crosslinked, as described (40). When indicated, 20-fold diluted samples (corresponding to 0.1-µl aliquots) were analysed. Transcripts on the membranes were hybridized with 5′-end-^{32}P-labeled oligo-DNA probes complementary to the leader RNA or mRNAs (see Figure legends), and visualized by autoradiography, as described (40).

For 5′-RACE analysis (Figures 5B and S4), unlabelled transcripts were synthesized with 0.1 µg of the wild-type or HR-RH mutant L protein in 25-µl reaction mixtures as described above. One percent and twenty percent of the total volumes of the wild-type and HR-RH RNA samples, respectively, were subjected to 5′-RACE analyses (see Materials and Methods in the main text).

For primer extension analyses (Figures 5C and S5), unlabelled transcripts were synthesized with 0.1 µg of the wild-type or HR-RH mutant L protein in 25-µl reaction mixtures as described above. The reactions were stopped by adding 25 µl of an ice-cold buffer [40 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.1 M NaCl, and 0.2% Triton X-100]. To remove N-RNA from the reaction mixtures, they were

overlaid onto 500-µl glycerol cushions [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.6 M NaCl, 20% glycerol] in 0.8 ml ultraclear tubes (Beckman), and centrifuged in the Sorvall AH-650 rotor with adaptors at 150,000 × *g* (average centrifugal force) for 20 min at 4°C. Consequently, N-RNA was precipitated through the cushion, and transcripts remained at the top of the cushion. The top fractions containing transcripts were collected. Transcripts were purified from the top fractions by phenol/chloroform extraction followed by ethanol precipitation, and dissolved in 10 µl of H_2O. One-half-microliter aliquots were subjected to primer extension reactions with Maxima H Minus reverse transcriptase (see Materials and Methods in the main text). For Figure S5, transcripts in 3-µl aliquots of the samples were incubated with or without 1 unit of TAP at 37°C for 30 min, purified by phenol/chloroform extraction followed by ethanol precipitation, and dissolved in 3 µl of H_2O. One-half-microliter aliquots were subjected to primer extension reactions at 42°C for 30 min with 0.3 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs). DNA sequencing ladders were generated with 5′-end-^{32}P-labeled primers used for the primer extension analyses, the pVSVFL-2 plasmid encoding the full-length anti-genomic RNA of VSV (46), and Sequenase Version 2.0 DNA Sequencing Kit (USB). Primer-extended DNA products were analysed by 8% urea-PAGE.

Generation of recombinant VSVs

To construct plasmids encoding a full-length anti-genome with the wild-type or mutant L gene, the 5.7-kbp HpaI-HindIII region of the L gene in the pVSVFL-2 plasmid (46) was replaced with the corresponding HpaI-HindIII fragment derived from the pFastBac L-H plasmid encoding the wild-type or mutant L protein (6), or pBS-L(HR1-1) plasmid encoding the D1671V mutant L protein (8). Although the resulting plasmid with the wild-type L gene (pVSV-L WT) has two nucleotide changes from the original sequence of pVSVFL-2, one of which leads to one amino acid change from proline at position 87 to serine in the L protein, there was no apparent difference in growth phenotypes between recombinant viruses generated from pVSVFL-2 and pVSV-L WT (data not shown). BHK-21 cells (2 × 10^6 cells) were infected with vTF7-3 (recombinant vaccinia virus expressing T7 RNA polymerase) at a multiplicity of infection of 1, transfected with pVSV-L WT or its mutant plasmid (2 µg) along with the supporting plasmids, pBS-N (0.4 µg), pBS-P (1.2 µg), and pBS-L (0.2 µg), using the FuGENE HD transfection reagent (Roche Applied Science), and cultured in DMEM supplemented with 5% FBS at 30°C for 4 days in a CO_2 incubator. The culture supernatants were filtrated through 0.22-µm syringe filters to remove vaccinia virus as described (46), and added to fresh BHK-21 cells (5 × 10^6 cells). The cells were cultured at 30°C for 2 (for wild-type) or 3 (for other mutants) days. The culture supernatants were recovered and filtrated through 0.22-µm filters. To determine titers of recombinant viruses, 100-µl aliquots of the filtrates and their 10-fold serial dilutions were subjected to a standard plaque assay with CV-1 cells in 6-well culture plates. Plaques were developed under agarose overlays at 30°C for 4 days in a CO_2 incubator, and fixed cells were stained with crystal violet.

SUPPLEMENTARY FIGURES

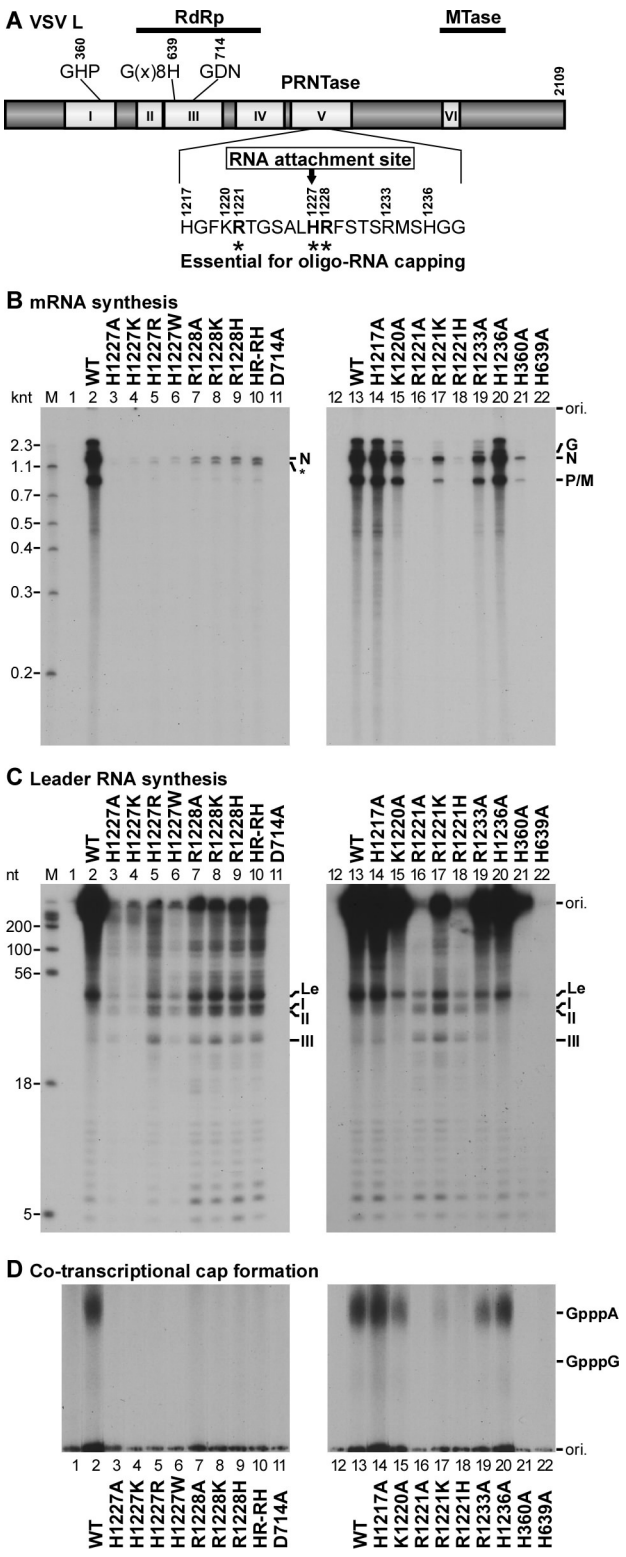

Figure S1. Cap-defective mutant L proteins cannot synthesize full-length mRNAs efficiently.

(A) The structure of the VSV L protein is schematically depicted with the six conserved regions (I–VI). Partial amino acid sequences are shown with the positions of the point mutations introduced in the recombinant VSV L protein. Amino acid residues required for *in vitro* oligo-RNA capping are marked by asterisks. RdRp, PRNTase, and MTase indicate RNA-dependent RNA polymerase, polyribonucleotidyltransferase, and methyltransferase, respectively. (B and C) The wild-type (WT) and mutant L proteins were subjected to transcription reactions reconstituted with the N-RNA template and the cofactor P protein. ^{32}P-Labeled transcripts were deadenylated with RNase H in the presence of oligo(dT), and analysed by 5% (B, for the mRNAs) or 20% urea-PAGE (C, for the leader RNA) followed by autoradiography. Lanes 1 and 12 indicate no L protein. The HR-RH mutant has H1227R and R1228H mutations. The positions of the viral mRNAs (G, N, and P/M), leader RNA (Le), and gel origins (ori.) are indicated. An unknown 1.2-knt RNA is marked with an asterisk (B). Unknown short transcripts are indicated by I, II, and III (C). M (marker) lanes show RNAs with indicated lengths. (D) Transcripts, synthesized with the WT or mutant L protein in the presence of [α-^{32}P]GTP, were digested with nuclease P1 and calf intestine alkaline phosphatase. The ^{32}P-labeled cap structures in the digests were purified using DEAE Sephacel, and analysed by PEI-cellulose TLC. Lanes 1 and 12 indicate no L protein. The positions of standard cap analogues are shown on the right.

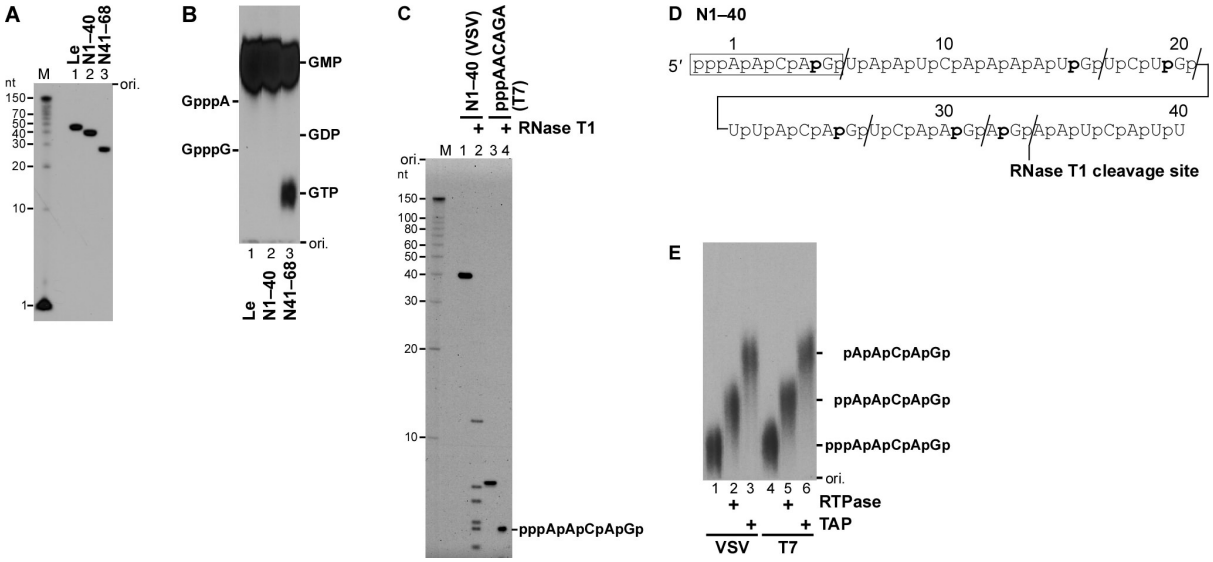

Figure S2. N1–40 and N41–68 RNAs synthesized by the HR-RH mutant L protein possess a 5′-triphosphate group.

(A and B) *In vitro* transcription was performed with the HR-RH mutant in the presence of [α-^{32}P]GTP. The leader (Le), N1–40, and N41–68 (containing a small amount of N1–28) RNAs were purified from transcripts by urea-PAGE, and re-analysed by 20% urea-PAGE followed by autoradiography (A). The purified RNAs were digested with nuclease P1 and the resulting ^{32}P-labeled guanine nucleotides were analysed along with unlabelled GTP, GDP, and GMP by PEI-cellulose TLC (B). (C–E) N1–40 RNA (see panel A, lane 2) and pppApApCpApGpA were synthesized in the presence of [α-^{32}P]GTP by the HR-RH mutant and T7 RNA polymerase (40), respectively, and purified. The purified RNAs (lanes 1 and 3) were completely digested with RNase T1 (lanes 2 and 4), and the resulting digests were analysed by electrophoresis on a 20% sequencing gel followed by autoradiography (C). M lane shows RNAs with indicated lengths. (D) The sequence of N1–40 RNA is shown with RNase T1 cleavage sites. ^{32}P-Labeled phosphate groups are indicated in bold letters. (E) The 5′-terminal fragment of N1–40 RNA (VSV synthesized) co-migrating with pppApApCpApGp generated from pppApApCpApGpA (T7 synthesized) was purified from the gel (see panel C). The purified RNAs were subjected to digestion with RNA 5′-triphosphatase (RTPase) of vaccinia virus capping enzyme or tobacco acid pyrophosphatase (TAP), as described previously (4). The resulting digests were analysed by PEI-cellulose TLC followed by autoradiography.

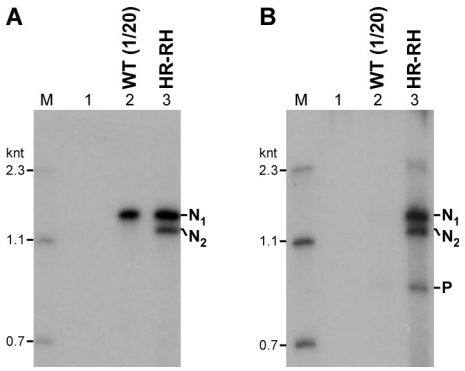

Figure S3. N_1 and N_2 RNAs synthesized by the HR-RH mutant have a 5′-tri- or di-phosphate group.

(A) Deadenylated RNAs, synthesized by either the wild-type (WT) or HR-RH mutant L protein (see Figure 4B, lanes 9 and 5), were analysed by Northern blotting with the ^{32}P-labeled (-)N401–428 probe. (B) The same samples used in panel A were subjected to *in vitro* capping with vaccinia virus capping enzyme in the presence of [α-^{32}P]GTP. The resulting capped RNAs were analysed by 5% urea-PAGE followed by autoradiography.

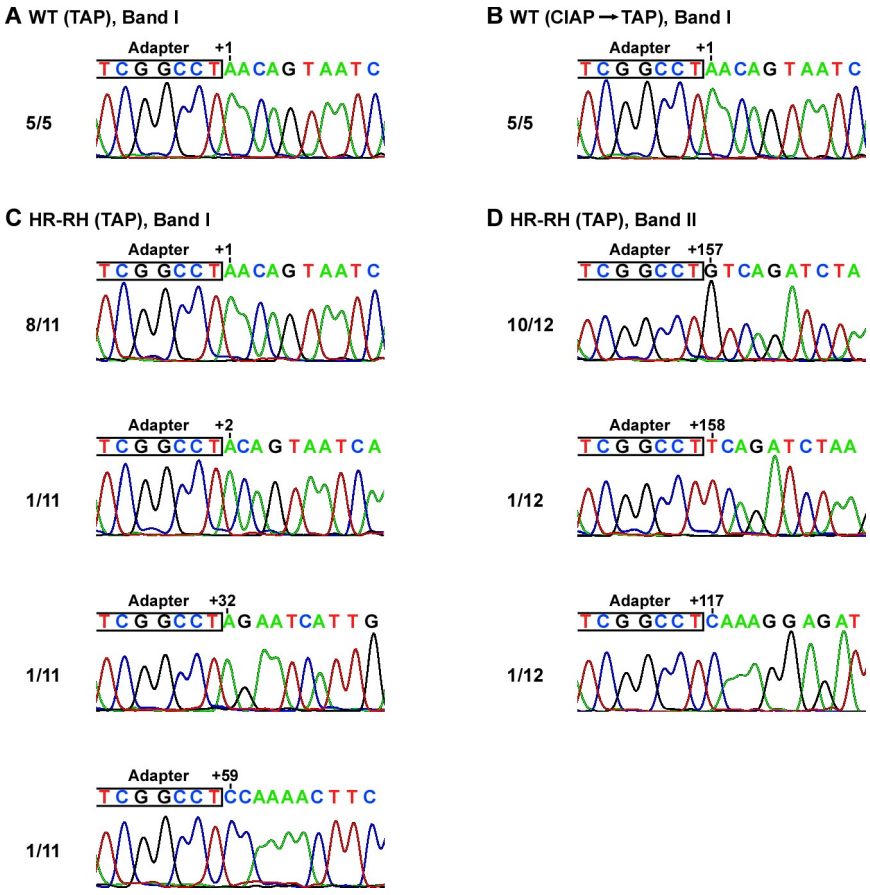

Figure S4. The 5′-terminal sequences of 3′-polyadenylated N transcripts synthesized by the wild-type or HR-RH mutant L protein.

As described in Figure 5B, transcripts, synthesized by the wild-type (WT, A and B) or HR-RH mutant (C and D) L protein, were pre-treated with or without calf intestine alkaline phosphatase (CIAP, B) followed by tobacco acid pyrophosphatase (TAP, A–D), and 5′-RACE products from 3′-polyadenylated N transcripts (see Figure 5B, bands I and II) were cloned and sequenced. Sequences of the adapter-ligated 5′-cDNA ends are shown with positions of their 5′-end residues in the *N* gene above electropherograms. Fractional numbers show the number of clones with the indicated sequences divided by the total number of clones sequenced.

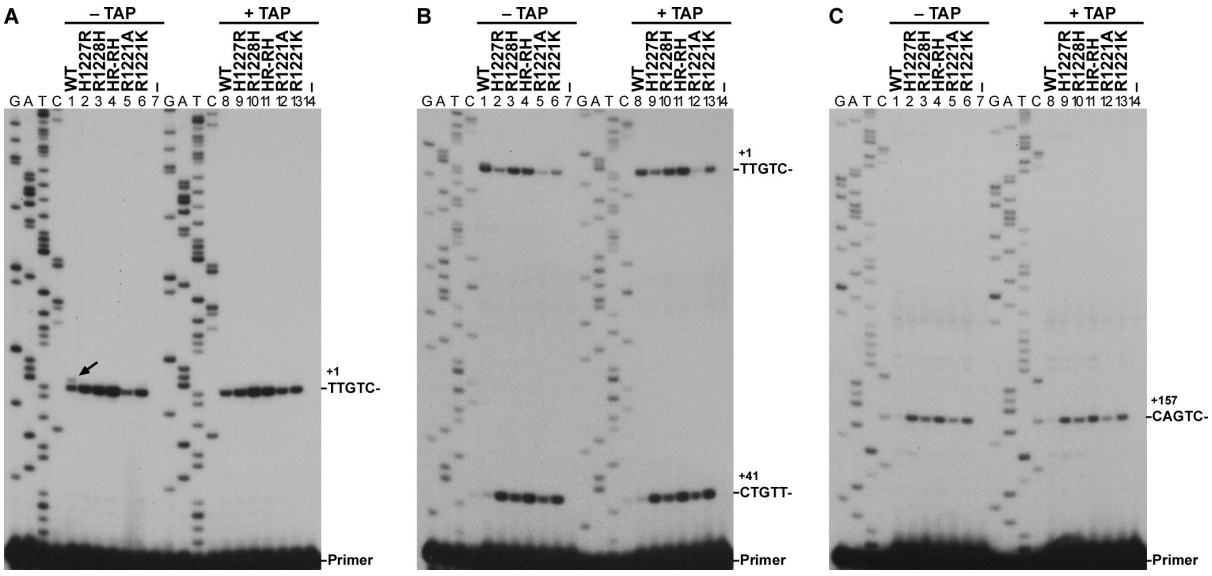

Figure S5. The cap defective mutant L proteins initiate transcription at positions +1, +41, and +157 of the *N* gene.

In vitro transcription was carried out with the wild-type (WT) or cap-defective mutant L protein. Lanes 7 and 14 indicate no L protein. Transcripts pre-incubated with (lanes 8–14) or without (lanes 1–7) TAP were subjected to reverse transcription in the presence of a 5′-end-^{32}P-labeled oligo-DNA primer complementary to positions +16 to +40 (A), +46 to +68 (B), or +168 to +188 (C) of N mRNA. Primer-extended DNA products were analysed along with sequencing ladders (G, A, T, and C) by 8% urea-PAGE. DNA sequences complementary to 5′-terminal sequences of transcripts are shown at the positions of primer-extended products. The one-nt longer product marked by an arrow was observed only when TAP-untreated transcripts synthesized by the WT L protein were used as templates, suggesting that the addition of an extra nucleotide to the 3′-cDNA end occurred due to the presence of the 5′-terminal cap structure as reported (58).

58. Davison, A.J. and Moss, B. (1989) Structure of vaccinia virus early promoters. J. Mol. Biol., 210, 749-769.

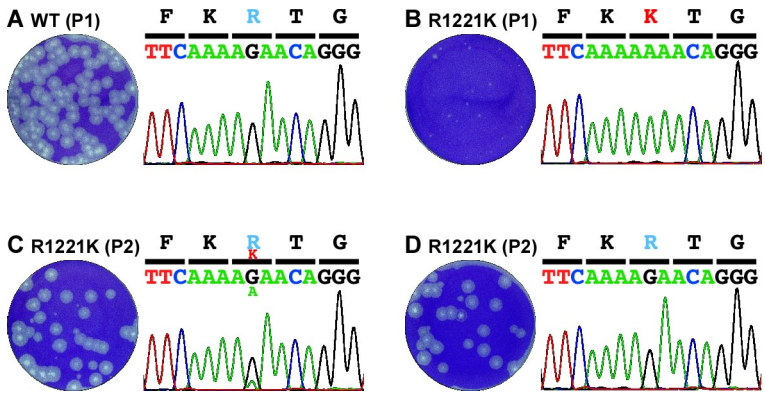

Figure S6. Characterization of the R1221K mutant VSV and its revertant.

BHK-21 cells (2×10^6 cells) were infected with the R1221K viruses [passage 1 (P1), B] at a multiplicity of infection of 0.001, and cultured at 30°C for 48 hours (C) or 37°C for 24 hours (D). Plaque phenotypes of the resulting passage 2 (P2) viruses in culture supernatants were compared with those of the wild-type (WT, A) and R1221K (P1) viruses. Viral genomic RNAs were extracted from their culture supernatants with TRIzol LS Reagent (Life Technologies) and treated with DNase I. First-strand cDNAs were synthesized from the RNAs with AccuScript Hi-Fi reverse transcriptase using random primers, and subjected to PCR with KOD Hot Start DNA polymerase to amplify four overlapping fragments that cover the entire *L* gene. The resulting PCR products were sequenced. Partial DNA sequences containing the 1221 codon are shown above electropherograms. There were no other nucleotide changes in the *L* gene during passaging the viruses.

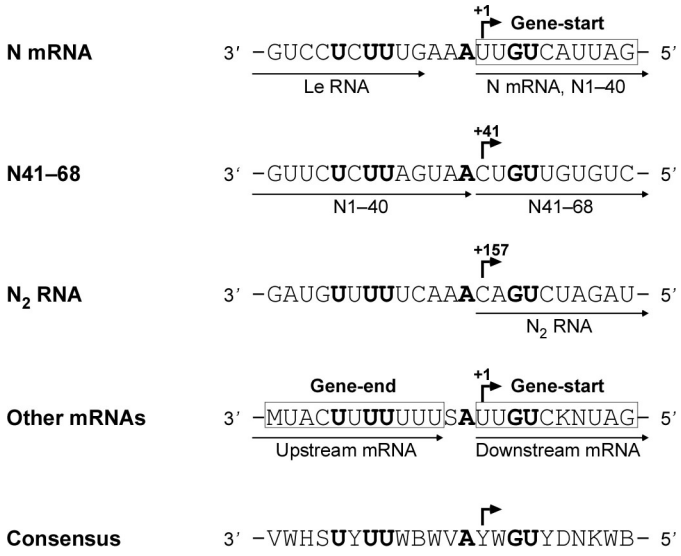

Figure S7. Local genomic RNA sequences surrounding internal transcription initiation sites.

Upstream and downstream sequences of internal transcription initiation sites for the indicated transcripts are compared with each other. The transcription initiation sites are marked by bent arrows with nucleotide positions within the *N* gene or other gene. The conserved gene-start and gene-end sequences are boxed. Arrows indicate template regions transcribed into 3′- and 5′-terminal sequences of upstream and downstream transcripts, respectively. Identical residues are shown in bold letters. The consensus sequence is shown at the bottom. M is A or C; S is G or C; K is U or G; N is any nucleotide; V is A, G, or C; W is U or A; H is C, U, or A; Y is C or U; B is G, C, or U; D is A, G, or U.