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In vitro capping and transcription of rhabdoviruses

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Abstract

The RNA-dependent RNA polymerase L protein of vesicular stomatitis virus (VSV), a prototypic nonsegmented negative strand (NNS) RNA virus classified into the *Rhabdoviridae* family, has been used to investigate the fundamental molecular mechanisms of NNS RNA viral mRNA synthesis and processing. *In vitro* studies on mRNA cap formation with the VSV L protein eventually led to the discovery of the unconventional mRNA capping pathway catalyzed by the guanosine 5'-triphosphatase and RNA:GDP polyribonucleotidyltransferase (PRNTase) activities. The PRNTase activity is a novel enzymatic activity, which transfers 5'-monophosphorylated (p-) RNA from 5'-triphosphorylated (ppp-) RNA to GDP to form 5'-capped RNA (GpppRNA) in a viral mRNA-start sequence-dependent manner. This unconventional capping (pRNA transfer) reaction with PRNTase can be experimentally distinguished from the conventional capping (GMP transfer) reaction with eukaryotic GTP:RNA guanylyltransferase (GTase) on the basis of the following differences in their substrate specificity for the cap formation: PRNTase uses GDP and pppRNA, but not ppRNA, whereas GTase employs GTP, but not GDP, and ppRNA. The pRNA transfer reaction with PRNTase proceeds through a covalent enzyme-pRNA intermediate with a phosphoamide bond. Hence, to prove the PRNTase activity, it is necessary to demonstrate the following consecutive steps separately: (1) the enzyme forms a covalent enzyme-pRNA intermediate, and (2) the intermediate transfers pRNA to GDP. This article describes the methods for *in vitro* transcription and capping with the recombinant VSV L protein, which permit detailed characterization of its enzymatic reactions and mapping of active sites of its enzymatic domains. It is expected that these systems are adaptable to rhabdoviruses and, by extension, other NNS RNA viruses belonging to different families.

Keywords

Rhabdovirus; Vesicular stomatitis virus; Transcription; mRNA capping; Cap structure; Polyribonucleotidyltransferase

1. Introduction

The *Rhabdoviridae* family includes a wide variety of bullet-shaped nonsegmented negative strand (NNS) RNA viruses that infect vertebrates, invertebrates, and plants (reviewed in Ref. [1]). The well-characterized viruses in this family are vesicular stomatitis virus (VSV,

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vesiculovirus) and rabies virus (lyssavirus); the latter is known to cause an acute deadly-infection in humans. The *Rhabdoviridae* family belongs to the *Mononegavirales* order consisting of a large and diverse group of NNS RNA viruses together with the *Paramyxoviridae* (e.g., measles, mumps, human parainfluenza, human respiratory syncytial, Nipah), *Filoviridae* (Ebola, Marburg), and *Bornaviridae* (Borna disease virus) families. Although these NNS RNA viruses manifest various morphological and biological characteristics, the structures of their genomes and multifunctional RNA-dependent RNA polymerase (RdRp) large (L) proteins are quite similar [2–4]. Therefore, their basic strategies for genome transcription and replication are expected to be similar.

Since Baltimore *et al.* [5] discovered the VSV-associated RdRp activity in 1970, VSV still remains a paradigm to study the molecular mechanisms of transcription and replication of NNS RNA viruses, especially *in vitro*. Earlier studies have also established VSV-associated unique mRNA processing activities including the poly(A) polymerase [6, 7], mRNA capping enzyme [8, 9], and cap methyltransferases [8, 10]. Since VSV is nonpathogenic to humans and possesses the highest RdRp activity *in vitro* among known NNS RNA viruses, it has served as a useful model for biochemical studies on NNS viral mRNA biogenesis over the last four decades. Although several *in vitro* transcription systems for other rhabdoviruses (e.g., Chandipura virus, Cocal, Piry [11]) and paramyxoviruses (e.g., Sendai [12, 13], measles [14, 15], human parainfluenza type 3 [16], human respiratory syncytial [17, 18]) have been developed, further understanding of the functions of these viral RdRps remained an obstacle due to their significantly low activity.

VSV possesses an 11-kilonucleotide (knt) negative strand RNA genome with five genes encoding nucleocapsid (N), phospho- (P), matrix (M), glyco- (G), and L proteins [1]. As illustrated in Fig. 1, the genomic RNA is tightly wrapped with the N proteins to form a helical nucleocapsid (N-RNA complex), which functions as a template for transcription as well as replication. The RdRp complex containing the catalytic L protein and its co-factor P protein interacts with the N-RNA complex to form a ribonucleoprotein (RNP) complex. *In vitro* studies on transcription have shown that the RdRp complex initiates transcription at the 3'-end of the genomic RNA within the N-RNA complex to sequentially synthesize the ~47-nucleotide (nt) uncapped leader (Le) RNA followed by five 5'-capped and 3'-polyadenylated mRNA species [8, 9, 19–24]. As depicted in Fig. 1, there is a gradient in transcript abundance in the following order: Le > N > P > M > G > L [19, 20].

The 5'-terminal cap structure (m⁷GpppN-, cap 0) of eukaryotic and viral mRNAs is composed of N⁷-methylguanosine (m⁷G) linked to the first nucleoside (N) of mRNA through the 5'-5' triphosphate (ppp) bridge. In all eukaryotic cells, this unique structure is formed on RNA polymerase II transcripts with cellular mRNA capping enzyme composed of RNA 5'-triphosphatase (RTPase) and GTP:RNA guanylyltransferase (GTase) followed by mRNA cap (guanine-N⁷)-methyltransferase (GN⁷-MTase), and plays pivotal roles in various aspects of mRNA metabolism including mRNA splicing, transport, translation, and stability (reviewed in Refs. [25–29]). In higher eukaryotes and their viruses, the cap 0 structure is further methylated at the 2'-O position of the first nucleoside to generate the cap 1 (m⁷GpppNm-) structure by mRNA cap (nucleoside-2'-O)-methyltransferase (2'-O-MTase). Recently, it has been suggested that 2'-O-methylation of the cap structure of some viral mRNAs is required to avoid anti-viral innate immunity triggered by non-cellular RNAs [30, 31].

Using *in vitro* transcription and RNA capping assays, biochemical mechanisms of mRNA cap formation by eukaryotic viruses have been directly solved. For nucleocytoplasmic large DNA viruses (e.g., vaccinia virus) and higher eukaryotic dsRNA viruses (e.g., reovirus), the cap 1 structure is formed by the same mechanism as in eukaryotic cells through the

sequential action of viral cap-forming enzymes: RTPase, GTase, cap G $\cap$ 7-MTase, and cap 2'-*O*-MTase [25, 27]. Recently, flaviviruses (*e.g.*, West Nile virus), positive strand RNA viruses, were found to follow the conventional pathway of the cap 1 formation [32, 33]. These viral GTases as well as cellular GTases transfer a GMP moiety of GTP to 5'-diphosphated RNA (ppN-RNA) through covalent enzyme-GMP intermediates to generate the Gp-ppN cap core structure [32, 34–36]. Interestingly, some viruses have evolved their unique mRNA capping enzymes. mRNA *N*7-methylguanylyltransferases (m⁷GTase) of alphavirus-like positive-strand RNA viruses [37, 38] and yeast L-A dsRNA virus [39] transfer m⁷GMP moieties from m⁷GTP (generated by viral GTP G $\cap$ 7-MTase) and cellular m⁷GpppN-RNA, respectively, to viral ppN-RNAs through covalent enzyme-m⁷GMP intermediates to yield the m⁷Gp-ppN cap 0 structure.

mRNAs of some NNS RNA viruses (VSV [8, 9], Newcastle disease virus [40], human respiratory syncytial virus [41], Sendai virus [12]) have been reported to carry a cap structure identical to that of eukaryotic mRNAs. However, earlier studies using an *in vitro* transcription system suggested that the cap biosynthetic pathway of VSV is distinctly different from that of eukaryotes and other unrelated viruses. For example, the VSV mRNA capping enzyme incorporates the GDP moiety of GTP into the Gpp-pA cap structure [8, 9], whereas cellular and other DNA and dsRNA viral mRNA capping enzymes incorporate the GMP moiety of GTP into the Gp-ppN cap structure [42–44]. Also, 2'-*O*-methylation of the VSV mRNA cap structure precedes G $\cap$ 7-methylation [10], in contrast to the order of cap methylation in higher eukaryotes and other viruses [33, 42–52].

Recent biochemical studies using newly developed *in vitro* assay systems with purified recombinant L proteins have revealed the unconventional mechanisms of mRNA cap formation by vesiculoviruses (VSV and Chandipura virus) [53–57]. As shown in Fig. 1 (inlet), the cap structure of the VSV mRNAs is formed by a series of the following enzymatic reactions catalyzed by the multifunctional L protein: (i) hydrolysis of GTP by the guanosine 5'-triphosphatase (GTPase) activity to generate GDP [53, 54], (iia and b) transfer of 5'-monophosphorylated (p-) RNA with the conserved vesiculoviral mRNA-start sequence (AACAG-) from 5'-triphosphorylated (ppp-) RNA to GDP by the RNA:GDP polyribonucleotidyltransferase (PRNTase) activity to form Gpp-pA-RNA [53, 55], and (iii and iv) methylation of the cap core structure (GpppA) at the ribose-2'-*O* position followed by the guanine-*N*7-position by the 2'-*O*- and G $\cap$ 7-MTase activities to produce m⁷GpppAm-RNA [58]. As highlighted in Fig. 1 (iia and b), PRNTase catalyzes a novel “pRNA transfer reaction”, which proceeds through a covalent enzyme-pRNA (L-pRNA) intermediate [53, 55]. In the L-pRNA intermediate, the 5'-phosphate group of the RNA is linked to the $\Lambda^{\text{e}2}$ position of the histidine residue at position 1,227 (H1227) in the VSV L protein via a phosphoamide bond [55]. H1227 is located within the active site HR (H1227-R1228) motif in the putative PRNTase domain, which is highly conserved in the L proteins of NNS RNA viruses [55, 57, 59, 60].

To analyze the precise functions of the L and P proteins in mRNA synthesis, several reconstituted transcription systems have been developed with native and/or recombinant VSV proteins [23, 53, 60–68]. Furthermore, to solve the molecular mechanism of VSV mRNA capping, an *in vitro* RNA capping system with an exogenously added RNA substrate has been established [53, 54]. This system has been used to identify active site amino acid residues of the putative mRNA capping domains in vesiculoviral L proteins [55, 56]. This article describes an *in vitro* VSV transcription system reconstituted with highly purified components (the N-RNA complex and the recombinant L and P proteins) as well as assay systems to measure the unconventional mRNA capping (PRNTase) activity of the VSV L protein. These *in vitro* systems represent a powerful approach to dissect the functions of the L protein.

2. Description of methods

2.1. Purification of the N-RNA complex, recombinant P, and L proteins

The Mudd-Summer strain of the VSV Indiana serotype (currently also known as vesicular stomatitis Indiana virus [VSIV]) is purified from culture supernatants of infected BHK-21 cells by precipitation followed by equilibrium banding on potassium tartrate-glycerol gradients using an ultracentrifuge, essentially as described previously [69]. The VSV RNP is purified from detergent-disrupted virions, and the N-RNA complex is prepared from the RNP by CsCl density gradient centrifugation, as described [53].

The recombinant VSV P protein is expressed as the N-terminal hexahistidine-tagged form in Sf21 insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen) and purified as described [53].

The recombinant VSV L protein is expressed as the C-terminal octahistidine-tagged form [54, 55] in Sf21 insect cells and purified according to the protocol of Ogino and Banerjee [53] with the modifications described below. Two 15-cm tissue culture dishes of Sf21 insect cells (1.2×10^7 cells/dish) are infected with recombinant baculovirus expressing the recombinant L protein (rAcNPV-L-H) at a multiplicity of infection of 1 plaque-forming unit per cell and cultured in Grace's insect cell culture medium supplemented with 10% fetal bovine serum at 27 °C for 3 days. The infected cells are homogenized in a hypotonic buffer, and a post-nuclear supernatant fraction is prepared, essentially as described previously [70]. The post-nuclear supernatant fraction is centrifuged at $10,000 \times g$ for 10 min, and the resulting pellet is resuspended in isotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 1 mM MgCl₂, 1 mM 1,4-dithiothreitol [DTT], 0.25 M sucrose) to obtain the P10 fraction (10 mg protein/ml, 0.4 ml). The P10 fraction can be stored at -80 °C. To further remove soluble proteins, the P10 fraction is diluted in 1 ml of isotonic buffer, and centrifuged at $15,000 \times g$ for 5 min. To solubilize the L protein, the resulting pellet is resuspended in 1 ml of TDG buffer (20 mM Tris-HCl [pH 8.0], 1 mM DTT, 10% [v/v] glycerol) containing 300 mM NaCl and 20 mM imidazole, and incubated for 30 min at 4 °C with rotation. The sample is centrifuged at $15,000 \times g$ for 5 min, and the resulting supernatant is collected. This supernatant is mixed with 50 μ l of 10% Triton X-100 followed by 40 μ l of a 50% slurry of nickel-nitrilotriacetic acid-agarose resin (Qiagen) preequilibrated with the above buffer, then incubated for 1 h at 4 °C with rotation. The resin is washed four times with 200 μ l of TDG buffer containing 300 mM NaCl, 20 mM imidazole, and 0.05 % Triton X-100, and the recombinant L protein is eluted from the resin four times with 20 μ l of TDG buffer containing 300 mM NaCl, 250 mM imidazole, and 0.05 % Triton X-100. The eluates are combined and dialyzed against TDG buffer containing 300 mM NaCl and 1 mM MgCl₂. After centrifugation of the dialysate at $15,000 \times g$ for 5 min, the resulting supernatant is collected and stored in small aliquots at -80 °C. Protein concentrations are determined by a Bradford protein assay (Bio-Rad) using bovine γ -globulin as a standard. Typically, 100–150 μ g of the recombinant L protein is obtained.

As shown in Fig. 2, the purified VSV RNP (lane 1), N-RNA complex (lane 2), recombinant P protein (r P, lane 3), and recombinant L protein (r L, lane 4) are resolved by electrophoresis in a 10% SDS-polyacrylamide gel with a low degree of crosslinking (acrylamide-bisacrylamide ratio of 30:0.4). This ratio is necessary to separate the P and N proteins. After electrophoresis, proteins in the gel are stained with Coomassie Brilliant Blue.

2.2. In vitro transcription of VSV

The *in vitro* transcription system of VSV has been successfully reconstituted with the highly purified N-RNA complex and recombinant L and P proteins [53, 63]. In this system, the recombinant L protein transcribes the genomic RNA within the N-RNA complex into the

leader RNA and 5'-capped and 3'-polyadenylated mRNAs. The following methods are adapted from Ogino and Banerjee [53].

2.2.1. *In vitro* transcription system reconstituted with purified factors—*In vitro*

transcription is performed according to the method described previously [53] with the modifications described below. A standard reaction mixture (25 μ l) contains 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM NaCl, 2 mM DTT, 0.2 mg/ml BSA, 1 mM each of ATP, CTP, and UTP, 100 μ M [α -³²P]GTP ($2\text{--}3 \times 10^3$ cpm/pmol), the N-RNA complex (0.4 μ g of protein), the recombinant P protein (0.05 μ g), and the recombinant L protein (0.1 μ g). Similarly, transcripts can be radiolabeled in the presence of 100 μ M [α -³²P]CTP ($2\text{--}3 \times 10^3$ cpm/pmol) or 100 μ M [α -³²P]UTP ($2\text{--}3 \times 10^3$ cpm/pmol). Unlabeled NTPs and [α -³²P]NTPs (3,000 Ci/mmol) are from Roche Applied Science and PerkinElmer, respectively. Note that commercially available [α -³²P]NTPs are diluted with unlabeled NTPs to give the desired specific radioactivities. After incubation of the reaction mixture for 2 h at 30 °C, the reaction is stopped by chilling on ice. To remove poly(A) tails from mRNAs, the reaction mixture is incubated with 0.3 units of RNase H (Roche Applied Science) in the presence of 100 ng of oligo(dT)₁₈ for 1 min at 37 °C. Poly(A) removal is optional, and is not required to analyze the leader RNA. After adding 290 μ l of RNA extraction buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% SDS, 0.2 M NaCl, 7 M urea) to the reaction mixture, ³²P-labeled RNAs are extracted by mixing with an equal volume (320 μ l) of phenol/chloroform/isoamyl alcohol (25:24:1, v/v). After centrifugation for 1 min at $15,000 \times g$, the aqueous layer is transferred to a new tube containing 1 μ l of 20 mg/ml glycogen (Roche Applied Science), and mixed with 2.5 volumes (800 μ l) of ethanol. The sample is placed on dry ice for 5 min, and centrifuged at $15,000 \times g$ for 10 min at 4 °C to precipitate RNAs with glycogen. To further remove unincorporated NTPs from RNAs, standard ethanol precipitation can be repeated two times. The resulting pellet is rinsed with cold 70% ethanol, dried in a SpeedVac concentrator (Savant), and dissolved in 10 μ l of 80% formamide loading solution (80% formamide, 10 mM EDTA, 0.02 % bromophenol blue [BPB], 0.01% xylene cyanol FF [XC]). To analyze ³²P-labeled mRNAs, a 1 μ l-aliquot of the sample is diluted in 9 μ l of the loading solution, denatured for 3 min at 95 °C, and electrophoresed along with marker RNAs for 2 h at 15 mA in a 5% polyacrylamide gel (8.2 $\times$ 6 cm, 1 mm thick) containing 7 M urea. The gel is dried in a vacuum gel dryer, and analyzed by autoradiography. To analyze ³²P-labeled leader RNA, a 9 μ l-aliquot of the sample is heat-denatured, and electrophoresed along with marker RNAs at 10 mA in a 15% polyacrylamide gel containing 7 M urea until the BPB dye reaches the bottom of the gel (~1 h). To generate ³²P-labeled marker RNAs (2,346, 1525, 1065, and 670 nt), pGEM Express Positive Control Template (Promega) and pGEM-3Z (Promega) digested with Ssp I are used as templates for *in vitro* transcription with bacteriophage RNA polymerases (Promega, USB, or Fermentas). The Decade RNA Marker System (Ambion) is used to prepare 5'-³²P-labeled small marker RNAs.

Fig. 3 shows typical results of *in vitro* transcription with the N-RNA complex, the recombinant P protein, and the recombinant L protein. When ³²P-labeled transcripts synthesized in a complete reaction are analyzed by 5% urea-PAGE, they migrate as a broad smear ranging from 0.8 to 2.7 knt (Fig. 3A, lane 1). Similarly, transcripts treated with RNase H in the absence of oligo(dT) migrate as a broad smear (lane 2). In contrast, if transcripts are treated with RNase H in the presence of oligo(dT) (lane 3), three discrete RNA bands with 1.3, 0.8, and 1.7 knt (representing N, P/M, and G mRNAs, respectively) are detected, indicating that all these RNAs are polyadenylated. In addition, a small amount of an unknown transcript with approximately 2.7 knt (marked by an arrow) is detected in the poly(A)⁺ samples (lanes 1 and 2) as well as in the poly(A)⁻ sample (lane 3). It should be noted that no detectable amount of the 6-knt L mRNA is synthesized in this reconstituted system similar to that with detergent disrupted VSV. On the other hand, Fig. 3B

demonstrates that the RNase H treatment does not digest the ~47-nt leader RNA lacking a poly(A) tail (compare lanes 2 and 3 to lane 1).

Comparing lanes 1–3 to lane 4 in Fig. 3C and D, all the N-RNA complex, the recombinant P protein (r P), and the recombinant wild-type L protein (r L WT) are required for synthesis of mRNAs and the leader RNA. In lane 5, the recombinant L protein having a D714A mutation (aspartic acid to alanine at position 714) in the GDN active site motif of the RdRp domain [55, 68] is used as a negative control.

Under the standard conditions, 3.8 ± 0.4 , 1.6 ± 0.1 , and 0.094 ± 0.008 pmol (the mean $\pm$ standard deviation of three independent determinations) of [α - 32 P]GMP were incorporated into the total mRNAs ranging from 0.8 to 2.7-knt, the 1.3-knt N mRNA, and the ~47-nt leader RNA, respectively. If the N mRNA and leader RNA contain 289–296 and 7 GMP residues, respectively, as reported for the Mudd-Summer strain (GenBank accession nos.: J02428 and EU849003), approximately 5.5 fmol N mRNA and 13 fmol leader RNA are synthesized, indicating that the molar ratio of the N mRNA to the leader RNA is approximately 1:2.4. This N-to-leader ratio is similar to that of 1:2.7 obtained by using an *in vitro* transcription system with detergent disrupted VSV [71].

2.2.2. Northern blot analysis of *in vitro* synthesized VSV transcripts—VSV

transcripts synthesized by the *in vitro* reconstituted transcription system are identified by Northern blotting, as described below. *In vitro* transcription is carried out in the presence of unlabeled GTP instead of [α - 32 P]GTP as described above (Section 2.2.1). After removal of poly(A) tails from mRNAs, transcripts are extracted with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol. The resulting pellet is washed with cold 70% ethanol, dried, and dissolved in 10 μ l of water (nuclease free). Two microliter aliquots of the sample are resolved along with 32 P-labeled marker RNAs by electrophoresis in a 5% (for mRNAs) or 15% (for leader RNA) polyacrylamide gel containing 7 M urea. RNAs in the polyacrylamide gel are transferred to an Immobilon NY+ membrane (Millipore) in cold 0.5 $\times$ TBE buffer at 40 V (for mRNAs) or 10 V (for leader RNA) for 14 h at 4 $^{\circ}$ C in a submarine electrophoretic transfer apparatus (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). After transfer, RNAs are UV-crosslinked to the membrane at 120 mJ/cm 2 using the UV Stratilinker 2400 (Stratagene) according to the manufacturer's protocol. Prehybridization and hybridization with 5'- 32 P-labeled antisense oligo-DNA probes are performed using ULTRAhyb-Oligo Hybridization Buffer (Ambion) according to the manufacturer's protocol. The following probes complementary to the nucleotide positions 401–428 of each mRNA are used to detect N, P, M, and G mRNAs, respectively: 5'-GTA GAT ACA AAG GCA ACC ATT TGT CAT C, 5'-AAA GCC ACT GGG ATT TCT GCT CTC CAC T, 5'-TGG TTG ACC TTG ATC TGC CAA TAC CGC T, and 5'-TGC ATA TCC ACA ACT TTG AGG AGG GAA G. To detect the leader RNA, a probe (5'-ATG ATA ATA ATG GTT TGT TTG TCT TCG T) complementary to the nucleotide position 1–28 of the leader RNA is used.

As shown in Fig. 4, when *in vitro* transcription is performed with the N-RNA complex and the recombinant P protein in the presence of the recombinant L protein (lane 2), but not in the absence the L protein (lane 1), 1.3-knt (A), 0.8-knt (B), 0.8-knt (C), and 1.7-knt (D) RNAs are specifically detected by the 32 P-labeled oligo DNA probes complementary to the N, P, M, and G mRNAs, respectively. Actual sizes of the N, P, M, and G mRNAs lacking poly(A) tails have been reported to be 1,326, 814, 831, and 1,665 nt, respectively (GenBank accession nos.: J02428 and EU849003). Fig. 4E (lane 2) demonstrates that the leader RNA is detected with the specific antisense probe by Northern blot analysis.

2.2.3. Analysis of the cap structure of *in vitro* synthesized VSV transcripts—

The 5'-cap structures of mRNAs are known to be resistant to nuclease P₁ and alkaline phosphatase. After digestion of *in vitro* synthesized VSV transcripts with nuclease P₁ and alkaline phosphatase, liberated cap structures are isolated and analyzed by the methods described below. *In vitro* transcription is carried out with 100 μ M [α -³²P]GTP ($3\text{--}4 \times 10^4$ cpm/pmol), 0.8 μ g of the N-RNA complex, 0.1 μ g of the recombinant P protein, and 0.2 μ g of the recombinant L protein in the absence or presence of 40 μ M S-adenosyl-L-methionine (AdoMet, Epicentre), as described above (Section 2.2.1). After incubation, RNAs are extracted with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol four times. The resulting pellet is rinsed with cold 70% ethanol, dried, and dissolved in 10 μ l of water. Then, 8 μ l of the sample is incubated for 1 h at 37 °C with 0.5 units of nuclease P₁ (Sigma-Aldrich) and 1 unit of calf intestine alkaline phosphatase (CIAP, Roche Applied Science) in 25 mM sodium acetate buffer (pH 5.5) (total sample volume = 10 μ l). The sample is mixed with 10 μ l of 96% formamide loading solution (96% formamide, 10 mM EDTA, 0.02% BPB, 0.01% XC), and heat-denatured. The sample is electrophoresed along with ³²P-labeled cap structures at 20 mA in a 20% polyacrylamide gel (17 $\times$ 13 cm, 0.75 mm thick) containing 7 M urea until the BPB dye migrates one-half to two-thirds of the length of the gel (~1 h 20 min). After electrophoresis, the buffer containing ³²P-labeled inorganic phosphate (P_i) in the lower reservoir of the electrophoresis apparatus should be discarded as radioactive waste. The gel is wrapped in plastic wrap, and exposed to X-ray film for autoradiography.

After autoradiography, a small block of the gel containing nuclease P₁ and CIAP-resistant ³²P-labeled materials (cap structures) is excised and chopped into pieces. The gel pieces obtained from three transcription reactions are put together in a microcentrifuge tube. Four hundred microliter of water are added, and the tube is rotated for 1 h. The tube is spun briefly, and the supernatant is collected. After repeating the elution, the first and second eluates are combined and mixed with 40 μ l of 1 M triethylamine-bicarbonate buffer (pH 7.5) (TEA-HCO₃). Then, the sample is incubated with 10 μ l of DEAE Sephacel (GE Healthcare, 50% slurry, pre-equilibrated with water) for 1 h. After washing the resin with 100 μ l of 50 mM TEA-HCO₃ twice, the bound materials are eluted from the resin three times with 20 μ l of 1 M TEA-HCO₃. The eluates are combined and dried using a SpeedVac concentrator. The dried materials are dissolved in 5 μ l of water.

The cap structures are analyzed by thin layer chromatography (TLC) on a polyethyleneimine (PEI) cellulose plate (20 $\times$ 20 cm, Sigma-Aldrich) followed by autoradiography. The origin is lightly drawn at 2 cm from the bottom of the plate with a soft pencil. The stationary phase of the TLC plate can be divided into 20 lanes with widths of 1 cm by scoring with a needle and straightedge. Scoring individual lanes prevents cross-contamination of multiple samples. Samples are spotted on the origin of the scored plate. After drying, the plate is soaked in methanol for 5 min and dried, as described by Randerath and Randerath [72]. The plate is developed along with external and/or internal standards with 0.4 M ammonium sulfate until the solvent front reaches within 0.5–1 cm of the top of the plate. After drying, the plate is wrapped in plastic wrap, and exposed to X-ray film.

For internal standards, some cap analogues (*e.g.*, GpppA and m⁷GpppA) are available from New England Biolabs. Samples are mixed with cap analogues (0.1–0.15 A₂₆₀ units) before spotting on the plate. After chromatography, the positions of standards on the plate are visualized under UV light at a wavelength of 254 nm. For external standards, ³²P-labeled cap structures (*e.g.*, GpppA, GpppAm, m⁷GpppA, and m⁷GpppAm) can be enzymatically synthesized. Briefly, to synthesize GpppA-RNA, any 5'-pppA-RNA is capped in the presence of [α -³²P]GTP by vaccinia virus mRNA capping enzyme having the RTPase, GTase, and G_{N7}-MTase activities using ScriptCap m⁷G Capping System (Epicentre,

currently available from Cellscript). Similarly, any 5'-pppRNA is capped and methylated in the presence of [α - 32 P]GTP and AdoMet by vaccinia virus mRNA capping enzyme to generate m⁷GpppA-RNA. If vaccinia virus 2'-O-MTase (ScriptCap 2'-O-Methyltransferase, Epicentre, currently available from Cellscript) is included in the capping and methylation reaction with vaccinia virus capping enzyme, m⁷GpppAm-RNA can be synthesized. To generate GpppAm-RNA, GpppA-RNA is methylated at the 2'-O position by vaccinia virus 2'-O-MTase. Finally, these capped RNAs are digested with nuclease P₁ to liberate 32 P-labeled cap structures.

As shown in Fig. 5A, digests of [α - 32 P]GMP-labeled VSV transcripts with nuclease P₁ and CIAP are directly analyzed by 20% urea-PAGE to detect cap structures. Under these conditions, the majority of 32 P-labeled phosphate groups in RNA chains are converted to [32 P]P_i, which is eluted from the polyacrylamide gel into the electrophoresis buffer in the lower reservoir. When *in vitro* transcription is performed without (lane 1) and with (lane 2) AdoMet (40 μ M, a saturating concentration), cap structures co-migrating with GpppA and m⁷GpppAm, respectively, are detected as major products on the polyacrylamide gel. Since m⁷GpppA and GpppAm co-migrate with GpppA and m⁷GpppAm, respectively, on the 20% polyacrylamide gel (data not shown), cap structures should be purified from the gel and reanalyzed along with GpppA, GpppAm, m⁷GpppA, and m⁷GpppAm by PEI-cellulose TLC to determine their methylation status. As demonstrated in Fig. 5B, the cap structures formed in the absence (lane 1) and presence (lane 2) of 40 μ M AdoMet co-migrate with GpppA and m⁷GpppAm, respectively, on the TLC plate. When *in vitro* transcription is carried out in the presence of a limited concentration (0.1 μ M) of AdoMet, a small amount of GpppAm is also formed (data not shown), as reported for transcripts synthesized from detergent-disrupted VSV *in vitro* [10].

2.3. In vitro RNA capping of VSV

The VSV mRNA cap structure is formed by the GTPase and PRNTase activities of the L protein, which can be independently measured by *in vitro* enzyme assays [53–55]. The PRNTase reaction is further divided into two steps: (1) covalent L-pRNA intermediate formation and (2) pRNA transfer from the L-pRNA intermediate to GDP. These two PRNTase reactions can also be assayed using two independent systems. Importantly, the PRNTase reaction exhibits strict RNA substrate specificity toward the 5'-triphosphorylated VSV mRNA start-sequence (*e.g.*, pppAACAG). The following methods measuring the unconventional RNA capping (PRNTase) activity are adapted from Ogino and Banerjee [53] and Ogino *et al.* [55].

2.3.1. Synthesis of 5'-triphosphorylated oligo-RNA substrates for *in vitro* capping

—Although chemical synthesis of 5'-triphosphorylated oligo-RNAs with desired sequences is not commercially available at present, some GTP- or ATP-initiated oligo-RNAs can be synthesized with T7 RNA polymerase using partially double-stranded oligo-DNAs as templates according to the methods described by Milligan and Uhlenbeck [73], Ogino and Banerjee [53], and Ogino *et al.* [55]. The following oligo-DNAs annealed to 5'-TAATACGACTCACTAT are used as templates to synthesize pppAAC, pppAACA, pppAACAG, and pppAACAGA, respectively: 5'-AAGTTAATAGTGAGTCGTATTA, 5'-AATGTTAATAGTGAGTCGTATTA, 5'-AACTGTTAATAGTGAGTCGTATTA (see Fig. 6A, [55]), and 5'-AATCTGTTAATAGTGAGTCGTATTA (template region underlined). A standard reaction mixture (40 μ l) contains 40 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 0.1 mg/ml BSA, 2 mM [γ - 32 P]ATP (10–30 cpm/pmol), 1 mM CTP, 1 mM GTP (not required to synthesize pppAAC and pppAACA), 0.1 μ M oligo-DNA template, and 1 unit/ μ l T7 polymerase (USB or Fermentas). Note that [γ - 32 P]ATP (3,000 Ci/mmol, PerkinElmer) is diluted with unlabeled ATP to give the desired specific

radioactivities. Similarly, transcripts can be radiolabeled in the presence of 1 mM [α - 32 P]CTP (10–30 cpm/pmol) or 1 mM [α - 32 P]GTP (10–30 cpm/pmol) [53]. pppACGAA and poly(A) ladders are synthesized as described previously [53]. After incubation of the transcription reaction mixture for 5 h at 37 °C, transcripts are extracted with 200 μ l each of RNA extraction buffer and phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and precipitated with five volumes (1 ml) of ethanol, as described above (Section 2.2.1). After repeating the ethanol precipitation, transcripts were separated by 20% urea-PAGE, as described above (Section 2.2.3). The gel is wrapped in plastic wrap, and exposed to X-ray film for ~30 min at room temperature. After autoradiography, a small block of the gel containing RNA is excised and chopped into pieces. To elute RNA from the gel pieces, they are incubated in 120 μ l of oligonucleotide elution solution (1 M ammonium acetate, 10 mM magnesium acetate, 0.1% SDS [74]) for 1 h at 37 °C with occasional mixing. RNA elution is repeated, and the first and second eluates are combined. RNA is precipitated with 1 ml ethanol, and washed with cold 70% ethanol. After drying the RNA pellet, it is dissolved in an appropriate volume of water. The amounts of 32 P-labeled RNAs are determined by measuring their radioactivities. Typically, yields of pppAAC, pppAACA, pppAACAG, and pppAACAGA are ~3.5, 2.1–3.1, 1.3–2.1, and 0.8–1.3 nmol, respectively.

After purification of oligo-RNAs, their sequences and homogeneity are confirmed by RNase digestion [53, 55]. Occasionally T7 polymerase synthesizes unexpected RNAs starting from different initiation sites [55, 75, 76] and/or having an additional nucleotide(s) at their 3'-ends [73] and/or in their internal chains [77]. Briefly, oligo-RNAs initiated with [γ - 32 P]ATP are completely digested with RNase T₁ or RNase A and their digests are analyzed by 20% urea-PAGE followed by autoradiography, as described by Ogino and Banerjee [53]. RNase T₁ and A specifically cleave GpN and (C/U)pN bonds, respectively, to leave 3'-terminal phosphorylated products via cyclic 2',3'-phosphate intermediates [78, 79]. Thus, the exact positions of internal G and C/U residues are located in oligo-RNAs. Fig. 6B provides an example of oligo-RNA digestion with RNases. pppApApC (lane 1), which migrates slightly faster than pppApApA (lane M), is resistant to RNases T₁ (lane 2) and A (lane 3). While pppApApCpA (lane 4) and pppApApCpApG (lane 7) are resistant to RNase T₁ (lanes 5 and 8), they are cleaved with RNase A into pppApApCp (lanes 6 and 9), which migrates faster than pppApApC (see lane 1). pppApApCpApGpA (lane 10) is digested with RNase T₁ (lane 11) and A (lane 12), releasing pppApApCpApGp and pppApApCp, respectively. It is important to note that electrophoretic mobilities of oligonucleotides largely depends on their base composition and number of terminal phosphate groups.

ppAACAG is generated from pppAACAG with the RTPase activity of vaccinia virus mRNA capping enzyme, which specifically removes the γ -phosphate group from pppRNA [80–82]. Briefly, 1.5 nmol of pppAACAG containing incorporated [α - 32 P]CMP or [α - 32 P]GMP (10–30 cpm/pmol) is incubated with 10 units (as GTase) of vaccinia virus mRNA capping enzyme (ScriptCap m7G Capping System, Epicentre) for 2 h at 37 °C in 60 μ l of a buffer (50 mM Tris-HCl [pH 8.0], 2 mM MgCl₂, 2 mM DTT, 0.1 mg/ml BSA), as described [55]. After the reaction, RNA is extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and recovered by ethanol-precipitation. The purity of ppAACAG is confirmed by 20% urea-PAGE and PEI-cellulose TLC as described previously [53].

To perform the L-pRNA intermediate formation assay (Section 2.3.4), 32 P-labeled pppAACAG with a high specific radioactivity (3.3 – 3.7×10^4 cpm/pmol) is synthesized in the presence of [α - 32 P]ATP (1.1 – 1.2×10^4 cpm/pmol), [α - 32 P]CTP (3.3 – 3.7×10^4 cpm/pmol), or [α - 32 P]GTP (3.3 – 3.7×10^4 cpm/pmol) and purified, as described [55].

2.3.2. Preparation of [α - 32 P]GDP—[α - 32 P]GDP is prepared from [α - 32 P]GTP with the nucleoside 5'-triphosphatase activity of vaccinia virus mRNA capping enzyme [80, 81, 83].

A reaction mixture (50 μ l) containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2 mM DTT, 0.1 mg/ml BSA, 1.3 μ M [α -³²P]GTP (3,000 Ci/mmol), and 10 units (as GTase) of vaccinia virus mRNA capping enzyme is incubated at 37 °C for 2 h. After the reaction, [α -³²P]GDP is extracted with an equal volume (50 μ l) of phenol/chloroform/isoamyl alcohol (25:24:1, v/v). After centrifugation, the aqueous layer is transferred to a new tube, and mixed with 200 μ l of DEAE Sephacel (GE Healthcare, 50% slurry, pre-equilibrated with water). After washing the resin with 200 μ l of 100 mM TEA-HCO₃ three times, [α -³²P]GDP is eluted from the resin four times with 400 μ l of 0.3 M TEA-HCO₃. The eluates are lyophilized using a SpeedVac concentrator. To remove residual TEA, the dried materials are dissolved in 150 μ l of water, and lyophilized again. Finally, [α -³²P]GDP is dissolved in an appropriate volume of water. Typically, a yield of [α -³²P]GDP is 40–50 pmol. The purity of [α -³²P]GDP is confirmed by PEI-cellulose TLC as described previously [53].

2.3.3. In vitro RNA capping system with the recombinant L protein—As illustrated in Fig. 7A, when pppAACAG and [α -³²P]GDP are incubated with the L protein, pAACAG is transferred from pppAACAG to GDP through a covalent L-(histidyl-*N*^ε2)-pAACAG intermediate to generate GpppAACAG [53, 55]. This entire PRNTase activity can be measured as described below.

The *in vitro* capping assay is performed in 10 μ l of the VSV capping buffer (25 mM MES-NaOH [pH 5.8], 0.5 mM MnCl₂, 2 mM DTT, 5% (v/v) glycerol, 0.1 mg/ml BSA) containing 0.25 μ M [α -³²P]GDP (30–50 cpm/fmol), 5 μ M pppAACAG (see Section 2.3.1 and below), and 40 ng of the recombinant L protein, as described [55]. Note that [α -³²P]GDP (Section 2.3.2.) is diluted with unlabeled GDP (Sigma-Aldrich) to give the desired specific radioactivities. pppAACAG and other oligo-RNAs initiated with [γ -³²P]ATP having low specific radioactivities (<30 cpm/pmol, Section 2.3.1) can be directly used as substrates, because their 5'-triphosphate groups are removed with CIAP after capping reactions as described below. If internally ³²P-labeled oligo-RNAs are used as substrates, it is better to wait until their specific radioactivities decay to lower levels (<5 cpm/pmol). After incubation of the capping reaction mixture for 2 h at 30 °C, the reaction is stopped by chilling on ice, and 1 μ l of 1 M Tris-HCl (pH 8.5) is added to the reaction mixture. Note that the L protein is not able to cap the oligo-RNA at higher pH (>8) [53]. The reaction mixture is incubated with 1 unit of CIAP at 37 °C for 10 min to hydrolyze free phosphate groups on the substrates. RNAs are extracted with phenol/chloroform/isoamyl alcohol, and precipitated with 5 volumes of ethanol twice. The resulting pellet is rinsed with cold 70% ethanol, dried, and dissolved in 10 μ l of water. An aliquot (5 μ l) of the sample is treated for 1 h at 37 °C with 0.15 units of nuclease P₁ in 25 mM sodium acetate buffer (pH 5.5) (total sample volume = 10 μ l). CIAP (0.5 units) can be optionally included in nuclease P₁ reaction mixtures, especially when internally ³²P-labeled oligo-RNAs are used as substrates. Then, the liberated cap structure (GpppA) is analyzed by PEI-cellulose TLC followed by autoradiography, as described above (Section 2.2.3). It is also important to demonstrate that the CIAP and nuclease P₁-resistant cap structure has –3 net charge and is sensitive to nucleotide pyrophosphatase (*e.g.*, tobacco acid pyrophosphatase), which cleaves the pyrophosphate linkage in the cap structure, as described previously [53, 54].

Fig. 7B shows a typical result of *in vitro* RNA capping with various RNA substrates by the recombinant wild-type L protein (r L WT). The substrate activities of pppAACAG (lane 3) and pppAACAGA (lane 4) to produce GpppA are significantly higher than that of pppAACAA (lane 2), suggesting that the recombinant L protein preferentially recognizes an RNA substrate longer than 4 nucleotides. As reported [53], pppACGAA (the leader RNA-start sequence, lane 5) as well as ppAACAG (lane 6) is inert as the substrate. Furthermore, previous studies show that the VSV L protein preferentially caps pppRNA started with the

ARCNG sequence (R = A/G), in which the A residue at position 1 and the pyrimidine residue (C > U) at position 3 are essential for the GpppA formation [53, 54]. As shown in lane 7, cap-deficient mutant L proteins (*e.g.*, H1227R [histidine to arginine at position 1,227]) [55] can be used as negative controls.

2.3.4. L-pRNA intermediate formation assay—The PRNTase reaction is divided into two steps: (1) covalent L-pRNA intermediate formation and (2) pRNA transfer to GDP. As depicted in Fig. 8A, the covalent L-pRNA intermediate can be detected using ³²P-labeled pppAACAG with a high specific radioactivity as a probe [53, 55]. Briefly, the recombinant L protein (0.2 μg) is incubated with 5 μM ³²P-labeled pppAACAG (3.3–3.7 × 10⁴ cpm/pmol, Section 2.3.1) for 15 min at 30 °C in 10 μl of the VSV capping buffer (Section 2.3.3). The reaction is stopped by adding 10 μl of 2× modified SDS-PAGE sample buffer (200 mM Tris-HCl [pH 8.0], 4 mM EDTA, 4% SDS, 0.02% BPB, 40% glycerol, 20 mM DTT). Since the phosphoamide bond in the L-pRNA is labile to higher temperature and lower pH [53], the sample should not be boiled in the standard Laemmli sample buffer. After stopping the reaction, the SDS-resistant L-pRNA intermediate is immediately resolved on 7.5% SDS-polyacrylamide gel (acrylamide-bisacrylamide ratio of 30:0.8, 17 × 13 cm, 0.75 mm thick) at 30 mA until the BPB dye migrates two-thirds of the length of the gel (~1 h 40 min). After electrophoresis, the lower part of the gel containing free RNA (the BPB dye front) is removed with a scalpel, and discarded as radioactive waste. After washing the gel with water, it is dried in a vacuum gel dryer, and analyzed by autoradiography.

Fig. 8B shows typical results of the L-pRNA intermediate formation with various RNA probes. Consistent with the RNA substrate specificity of the PRNTase activity (see Fig. 7B), the L protein efficiently forms L-pRNA intermediates with pppAACAG (Fig. 8B, lane 3) and pppAACAGA (lane 4), but not with pppAACA (lane 2), pppACGAA (lane 5), and ppAACAG (lane 6). Although the complex formation activities with pppAACA (lane 3) and pppACGAA (lane 5) appear to be slightly higher than the background level (lane 1, no L protein), it may be difficult to distinguish specific intermediate formation from non-specific binding at these low activity levels. The cap-deficient L proteins with mutations in the active site histidine residue in the PRNTase domain (*e.g.*, H1227R [see lane 7], H1227A) can be used as negative controls for this assay [55]. However, it should be noted that these cap-deficient mutant L proteins also show very weak complex formation activities with pppAACAG, despite the absence of the nucleophilic histidine residue required for the intermediate formation [55].

2.3.5. pRNA transfer assay—As shown in Fig. 9A, the pRNA transfer activity of the L protein can be measured by incubation of the purified L-pRNA intermediate with GDP [55]. As detailed in the protocol described by Ogino *et al.* [55], the L-pRNA intermediate is formed by incubation of the recombinant L protein with ³²P-labeled pppAACAG with a high specific radioactivity (3.3–3.7 × 10⁴ cpm/pmol) and purified by phosphocellulose (P11, Whatman) chromatography to remove free pppAACAG. The purified L-pRNA (pAACAG) intermediate (0.09 pmol) is incubated in 10 μl of the VSV capping buffer (Section 2.3.3) containing 0.25 μM GDP for 1 min at 30 °C. The reaction is stopped by adding 10 μl of 96% formamide loading solution. The sample is subsequently analyzed by 20% urea-PAGE followed by autoradiography (Section 2.2.3).

Fig. 9B shows a typical result of the RNA transfer reaction. The input L-pRNA intermediate is detected at the origin of the gel (lane 1). Although the purified L-pRNA intermediate is free from pppRNA, a very small amount of pRNA (a putative hydrolysis product of L-pRNA, marked by an arrow) is always co-purified with the intermediate [55]. When the L-pRNA intermediate is incubated without any RNA acceptors (lane 2), pRNA linked to the L protein is not released from the intermediate. In contrast, when the intermediate is incubated

with GDP, $78 \pm 5\%$ (the mean $\pm$ standard deviation of three independent determinations) of pRNA linked to the L protein is transferred to GDP (lane 3), generating the capped RNA (Gpp-pRNA). As reported previously [55], pRNA is not efficiently transferred from the intermediate to other NDPs such as ADP (see lane 4). The capped RNA released from the intermediate can be isolated from the polyacrylamide gel, and further characterized, as reported by Ogino *et al.* [55].

3. Concluding remarks

This article has described a robust *in vitro* transcription system with highly purified recombinant VSV L protein, which can transcribe the genomic RNA within the native N-RNA complex into the leader RNA and mRNAs in the presence of the recombinant P protein. As reported for *in vitro* transcription systems with detergent disrupted VSV [6–10], transcripts synthesized with the recombinant L protein are properly 5'-capped, methylated, and 3'-polyadenylated. Thus, this system is useful in identifying amino acid residues of the L protein as well as the P protein that are important for transcription and co-transcriptional mRNA processing and in analyzing detailed mechanisms of mRNA biogenesis. Furthermore, *in vitro* assay systems to analyze the sequential steps of VSV mRNA capping have been described. These systems have provided important insights into the unconventional mechanism of VSV mRNA capping and uncovered the active site for the capping enzyme domain in the L protein [53–55]. Information gained from studies on the VSV L protein has the potential to be applicable to L proteins of human pathogenic rhabdoviruses (*e.g.*, Chandipura, rabies, rabies-like bat lyssaviruses) and, by extension, other significant NNS RNA viruses. In fact, the Chandipura virus L protein has been shown to catalyze the unconventional capping reaction by using this *in vitro* capping assay [56]. On the other hand, the L protein of rinderpest virus belonging to the *Paramyxoviridae* family was recently suggested to form a covalent protein-GMP complex similar to the eukaryotic GTase-GMP intermediate [84]. However, so far, there is no direct evidence that the rinderpest L-GMP complex is a *bona fide* intermediate in the conventional capping reaction. Thus, it is essential to biochemically analyze capping mechanisms of other NNS RNA viruses belonging to different families using *in vitro* RNA capping assays. Further studies on mRNA capping with L proteins of rhabdoviruses and other NNS RNA viruses are extremely important for understanding how these viruses have evolved with their mRNA capping machineries to adapt to their host cells. Their unique enzymes responsible for viral mRNA capping are logical therapeutic targets for rational design of anti-viral agents.

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Abbreviations

CIAP	calf intestine alkaline phosphatase
GN7-MTase	(guanine- <i>N</i> 7)-methyltransferase
GTase	guanylyltransferase
GTPase	guanosine 5'-triphosphatase
NNS	nonsegmented negative strand
2'- <i>O</i> -MTase	(nucleoside-2'- <i>O</i>)-methyltransferase

RdRp	RNA-dependent RNA polymerase
RNP	ribonucleoprotein
PRNTase	polyribonucleotidyltransferase
RTPase	RNA 5'-triphosphatase
VSV	vesicular stomatitis virus.

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Fig. 1. Synthesis of VSV mRNAs with the cap 1 structure

The RdRp complex composed of the L and P proteins sequentially transcribes the genomic RNA wrapped with the N proteins (N-RNA template) into the leader (Le) RNA (~47 nt) and five 5'-capped and 3'-polyadenylated mRNAs. The cap 1 ($\text{m}^7\text{GpppAm-}$) structure is co-transcriptionally formed on the 5'-ends of mRNAs by the (i) guanosine 5'-triphosphatase, (iia and iib) RNA:GDP polyribonucleotidyltransferase (highlighted), (iii) mRNA cap (nucleoside-2'-*O*)-methyltransferase, and (iv) mRNA cap (guanine-*N*7)-methyltransferase activities. Moieties derived from GTP, RNA, and *S*-adenosyl-L-methionine (AdoMet) are shown in red, blue, and green, respectively. P_i and PP_i indicates inorganic phosphate and pyrophosphate, respectively. AdoHcy denotes *S*-adenosyl-L-homocysteine.

Fig. 2. Analysis of purified VSV transcription factors

The native VSV RNP (1.5 μ g protein), N-RNA complex (1 μ g), recombinant P protein (r P, 1 μ g), and recombinant L protein (r L, 1 μ g) were analyzed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining.

Fig. 3. *In vitro* VSV transcription system reconstituted with purified factors

(A and B) *In vitro* transcription was performed with the N-RNA complex, recombinant P protein, and recombinant L protein. Transcripts containing incorporated [α - 32 P]GMP (lane 1, no treatment) were incubated with RNase H in the absence (lane 2) or presence (lane 3) of oligo(dT), and analyzed by 5% (A) or 15% (B) urea-PAGE followed by autoradiography. Lanes M show 32 P-labeled marker RNAs with indicated lengths. The positions of the origin (ori.) and the VSV transcripts (N, P/M, G, and Le) are indicated. An arrow indicates an unidentified transcript. XC and BPB show the positions of xylene cyanol and bromophenol blue, respectively.

(C and D) *In vitro* transcription was carried out with the N-RNA complex, recombinant P protein (r P), recombinant wild-type L protein (r L WT), and/or recombinant D714A mutant L protein (r L D714A), as indicated. After poly(A) removal, transcripts containing incorporated [α - 32 P]GMP were analyzed by 5% (C) or 15% (D) urea-PAGE followed by autoradiography.

Fig. 4. Northern blot analysis of *in vitro* synthesized VSV transcripts

In vitro transcription was performed with the N-RNA complex and recombinant P protein in the absence (lane 1) or presence (lane 2) of the recombinant L protein (r L). After poly(A) removal, transcripts were separated by 5% urea-PAGE, and analyzed by Northern blotting with ^{32}P -labeled oligo-DNA probes complementary to the N (A), P (B), M (C), and G (D) mRNAs. Transcripts were separated by 15% urea-PAGE to analyze the leader (Le) RNA by Northern blotting (E). Lanes M show marker RNAs with indicated lengths.

Fig. 5. Identification of cap structures formed on *in vitro* synthesized VSV transcripts

(A) *In vitro* transcription was performed with the N-RNA complex, recombinant P protein, and recombinant wild-type L protein in the absence (lane 1) or presence (lane 2) of *S*-adenosyl-L-methionine (AdoMet). Transcripts containing incorporated [α - 32 P]GMP were digested with nuclease P₁ and CIAP, and the resulting digests were analyzed by 20% urea-PAGE followed by autoradiography. Arrowheads indicate major nuclease P₁ and CIAP-resistant products. The minor 32 P-labeled product (marked by an arrow) was identified as undigested GMP (not shown). Lanes M indicate the positions of 32 P-labeled GpppA and m⁷GpppAm.

(B) The major nuclease P₁ and CIAP-resistant products (see panel A, lanes 1 and 2) co-migrating with GpppA and m⁷GpppAm, respectively, were purified, and reanalyzed by PEI-cellulose TLC followed by autoradiography. The 32 P-labeled cap structures (GpppA, GpppAm, m⁷GpppA, and m⁷GpppAm) were enzymatically synthesized, and used as standards (lanes M).

Fig. 6. Synthesis of oligo-RNA substrates for the VSV mRNA capping enzyme

(A) [γ - 32 P]ATP-labeled oligo-RNAs (e.g., pppAACAG) were synthesized from unique synthetic DNA templates by T7 RNA polymerase.

(B) Indicated oligo-RNAs ([32 P]phosphate groups marked by asterisks) were purified and incubated in the absence (lanes 1, 4, 7, and 10) or presence of RNase T₁ or A, as indicated. The resulting digests were analyzed by 20% urea-PAGE followed by autoradiography. Lane M indicates [γ - 32 P]ATP-initiated poly(A) RNAs with indicated lengths. The positions of cleaved RNA products are shown on the right.

Fig. 7. *In vitro* VSV RNA capping assay with the recombinant L protein

(A) The PRNTase activity of the L protein can be measured using pppAACAG (blue) and [α - 32 P]GDP (red, a [32 P]phosphate group marked by an asterisk) as the substrates. The L protein transfers pRNA from pppRNA to GDP through a covalent L-pRNA intermediate to yield a capped RNA (GpppRNA). His indicates the histidine residue at position 1,227 (H1227) in the VSV L protein, which serves as a nucleophile forming the intermediate.

(B) The recombinant wild-type L protein (r L WT) or the H1227R mutant L protein (r L H1227R) was incubated with the indicated oligo-RNAs and [α - 32 P]GDP. Nuclease P₁ and CIAP-resistant products were analyzed by PEI-cellulose TLC followed by autoradiography. The positions of standard compounds are shown the left. The right panel shows the amounts of GpppA cap structure formed, which are determined by counting their 32 P radioactivities. The mean values of three independent experiments are shown with standard deviation indicated by error bars.

Fig. 8. L-pRNA intermediate formation assay with the recombinant L protein

(A) When the L protein is incubated with pppAACAG (blue, a [³²P]phosphate group marked by an asterisk), a covalent L-pRNA intermediate is formed.

(B) The recombinant wild-type L protein (r L WT) or the H1227R mutant L protein (r L H1227R) was incubated with the indicated oligo-RNAs containing incorporated [α -³²P]CMP. The resulting L-pRNA intermediates were analyzed by 7.5% SDS-PAGE followed by autoradiography. The right panel shows amounts of the L-pRNA intermediate formed, which are determined by counting their ³²P radioactivities. The mean values of three independent experiments are shown with standard deviation indicated by error bars.

Fig. 9. pRNA transfer assay with the purified L-pRNA intermediate

(A) When the purified L-pRNA (pAACAG, blue, [^{32}P]phosphate groups marked by asterisks) intermediate is incubated with GDP (red), pRNA is transferred from the intermediate to GDP, resulting in the release of a capped RNA (GpppRNA). (B) The purified L-pRNA (containing incorporated [α - ^{32}P]AMP) intermediate (lane 1, input) was incubated in the absence (lane 2) or presence of GDP (lane 3) or ADP (lane 4). After the reaction, released RNAs were analyzed by 20% urea-PAGE followed by autoradiography. The positions of the L-pRNA intermediate and GpppRNA are shown on the right. Lanes M indicate the positions of 5'-hydroxyl (HO-), capped (Gppp-), monophosphorylated (p-), and triphosphorylated (ppp-) AACAG RNAs. The position of free pAACAG co-purified with the L-pRNA intermediate is marked by an arrow.