RNA triphosphatase and guanylyl transferase activities are associated with the RNA polymerase protein L of rinderpest virus

M. Gopinath and M. S. Shaila

Correspondence
M. S. Shaila
shaila@mcbl.iisc.ernet.in
Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

Received 10 February 2009
Accepted 16 March 2009

INTRODUCTION

Following their synthesis in the nucleus, eukaryotic mRNAs undergo a series of post-transcriptional modifications such as the addition of a cap structure, splicing of introns and addition of a poly(A) tail. The eukaryotic cap structure found at the 5' end of mRNAs is crucial for their stability, translation and export from the nucleus to the cytosol (Furuichi & Shatkin, 2000). Capping of cellular mRNA is carried out by three independent enzymes localized to the nucleus, namely the RNA triphosphatase (RTPase), guanylyl transferase (GT), N7-Guanine and 2'-O-methyl transferase, to form an m7GpppAm-ended mRNA (Bisaillon & Lemay, 1997; Shuman, 2001) (Supplementary Fig. S1, available in JGV Online). Viruses that replicate in the host cell cytoplasm carry out post-transcriptional modifications on their mRNA, identical to their cellular mRNA counterparts, so that the viral mRNAs can be efficiently translated using host machinery. Viruses achieve this by encoding their own capping enzyme apparatus such as the vaccinia virus (VACV) D1/D12 subunit, baculovirus LEF-4 and reovirus l2 protein (Gross & Shuman, 1998a; Luongo et al., 2000; Shuman, 1990). In viruses such as reovirus and baculovirus, the same enzyme (l2 and LEF-4, respectively) catalyses both RTPase and mRNA GT activities, whereas VACV, West Nile virus and alpha virus encode separate RTPases (Benzaghou et al., 2006; Myette & Niles, 1996; Vasiljeva et al., 2000).

Rinderpest virus (RPV) is an important member of the genus Morbillivirus in the family Paramyxoviridae. The non-segmented negative-sense RNA genome of RPV is 15882 bases long (Baron & Barrett, 1995) and is tightly encapsidated by the nucleocapsid protein (N) (Ghosh et al., 1994). Transcription occurs in a sequential manner in the following order on the genome: 3'-le-N-P-M-F-H-L-tr-5'. The 52 nt leader RNA is not post-transcriptionally modified, whereas other mRNAs are capped, methylated and polyadenylated (Whelan et al., 2004). The two virus-encoded proteins, the large (L) protein (240 kDa) and the phosphoprotein (P), together constitute the viral RNA dependent RNA polymerase (RdRp) that transcribes the genomic RNA encapsidated by N protein (N-RNA) (Whelan et al., 2004). The L protein of RPV has been shown to be the catalytic subunit of RNA polymerase (Chattopadhyay et al., 2004). In negative-sense RNA viruses, L protein is believed to possess activities required...
for capping, methylation and polyadenylation of mRNAs (Banerjee, 1987). Purified ribonucleoprotein (RNP) complexes from vesicular stomatitis virus (VSV) and Sendai virus containing the N, L and P proteins along with the genomic RNA have been shown to catalyse capping as well as cap methylation activities in vitro (Galloway et al., 2008; Grdzeliashvili et al., 2006; Li et al., 2006; Ogino et al., 2005; Ogino & Banerjee, 2007, 2008). However, there is no direct demonstration of RTPase or GT activity associated with the RNP complex or L protein of any paramyxovirus. As a first step towards characterizing the capping and methylation activities associated with RPV L protein, we established previously an in vitro reconstituted system for viral transcription employing partially purified L–P complex expressed in insect cells (Gopinath & Shaila, 2008). In the present work, we describe a new enzymic activity exhibited by viral RNP as well as recombinant L protein, present as expressed in insect cells (Gopinath & Shaila, 2008). In the previously an step towards characterizing the capping and methylation RNP complex or L protein of any paramyxovirus. As a first demonstration of RTPase or GT activity associated with the Grdzelishvili by Ni-NTA affinity chromatography (Saikia was purified from insect cells infected with recombinant baculovirus was purified from insect cells co-infected with density-gradient centrifugation (Gopinath & Shaila, 2008). P protein was purified from insect cells infected with recombinant baculovirus by Ni-NTA affinity chromatography (Saikia et al., 2008). Purification of the recombinant deletion mutants of RPV L protein, LD1 and LD3 is described in the Supplementary Method (available in JGV Online).

**METHODS**

**Cells and viruses.** Vero cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS). *Spodoptera frugiperda* (SE21) insect cells were obtained from NCCS and maintained in TC-100 medium with 10% FCS. A tissue culture-adapted vaccine strain, RBOK, which is the second step, the transfer of a GMP moiety to an L–GMP covalent intermediate, was demonstrated and the second step, the transfer of a GMP moiety to an acceptor RNA, was also established.

**Antibodies.** Rabbit polyclonal antibodies against RPV P (Kaushik & Shaila, 2004), N (Mitra-Kaushik et al., 2001) and L (Raha et al., 2004) proteins were made previously in the laboratory. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Sigma.

**RTPase assay.** γ-[32P]-labelled RNA (25 nt) was synthesized using partially double-stranded synthetic DNA containing T7 RNA polymerase promoter. An oligonucleotide (5′-TAATACGACTCACTATAGGG-3′) corresponding to the T7 promoter sequence (−17 to −1) was annealed to the template oligonucleotide (5′-TCCAGCTCGA- TAGGATCTTGAATCCT-3′) at the promoter region (underlined). RNA was transcribed in vitro using T7 RNA polymerase in standard transcription buffer supplemented with 1 mM each of CTP, GTP and UTP, 50 μM ATP and 2 μCi [γ-32P]ATP to generate a 25 nt RNA containing the first 25 nt sequence of RPV N mRNA, specifically labelled at the 5′ end. The transcribed RNA was separated from unincorporated radioactive nucleotides by two rounds of gel filtration through Sephadex G50 columns followed by ethanol precipitation. The radiochemical purity of RNA was confirmed by TLC analysis. An RTPase assay was performed (5 μl) in 50 mM Tris/HCl, pH 7.4, 10 mM KCl, 5 mM MgCl₂, 2 mM DTT, 12.5 pmol labelled RNA substrate and the enzyme source (see legends to Figs 2 and 3). Reactions were incubated at 37 °C for 20 min (unless otherwise stated) and then terminated by the addition of 1 M formic acid and 0.5 M LiCl. As an alternative method, the reaction products were also resolved through 18% PAGE containing 7 M urea. Products were imaged and quantified by scanning the TLC plate/polyacrylamide gel using a phosphorimager (BAS-2000; Fujiﬁlm).

**L–GMP complex formation.** Reaction mixtures (10 μl) containing 50 mM Tris/HCl (pH 7.4), 2 mM DTT, 5 mM MgCl₂, 5 μCi [γ-32P]GTP and the enzyme source (see legends to Figs 2 and 3 for details) were incubated at 30 °C for 20 min. The reaction was halted by the addition of SDS loading buffer to 1 x final concentration. Samples were resolved by using 7% SDS-PAGE, after which gels were dried and analysed by phosphorimaging.

**Preparation of cap-labelled RNA, and the capping reaction.** Unlabelled, triphosphate-terminated RNA corresponding to the first 25 nt of RPV N mRNA was synthesized as described above. This was resolved using 18% urea-acrylamide gel electrophoresis, located by UV shadowing and eluted in buffer containing 0.5 M ammonium

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acetate, 0.1 % SDS and 1 mM EDTA at 42 °C overnight. The RNA was further purified by phenol:chloroform extraction and ethanol precipitation. The triphosphate-ended RNA was capped with VACV GT (Ambion) and 20 μCi [α-32P]GTP. Methylated, cap-labelled RNA was generated under identical conditions with 50 μM S-adenosyl methionine. The capped/cap-methylated RNA was gel purified as described above. The capping reaction [10 μl; containing 50 mM Tris/HCl (pH 7.4), 2 mM DTT, 5 mM MgCl2, 2 μg RNP and 5 pmol of either capped or cap-methylated RNA] was performed by incubation at 30 °C for 30 min. The reaction was stopped by the addition of SDS loading buffer and resolved using 7 % SDS-PAGE. Formation of the L–GMP covalent intermediate was visualized by phosphorimaging.

RESULTS

RPV viral mRNA is capped and methylated at the 5′ end

The RNP complex comprising the N-RNA, L, P and N proteins was isolated from purified virus and the presence of these proteins was verified by SDS-PAGE and immunoblotting. Silver staining showed that L protein, in addition to other viral proteins such as H, P, N and F, is present in very low amounts in both purified RPV and the RNP complex (Fig. 1a). The purity of the RNP complex was further confirmed by Western blotting with specific antibodies. Compared with the purified virions, RNP complex isolated from the virions lacks F and H proteins (Supplementary Fig. S3a) but contains N, P and L proteins [Fig. 1a (lane 2), b and c]. To determine the post-transcriptional modification of RPV viral mRNA in vitro, viral transcription was reconstituted in the presence of [α-32P]GTP and detergent-disrupted virions, RNP or recombinant L protein. The transcripts were purified and treated with TAP, which cleaves the pyrophosphate bond of the GpppN cap, liberating Gp or 7mGp if the cap is methylated (Shinshi et al., 1976). Treatment of mRNA synthesized by the virion and RNP with TAP resulted in the release of both Gp and 7mGp (Fig. 1d, lanes 2 and 4). In addition, a similar profile was obtained with the mRNA synthesized with recombinant L protein (Fig. 1e, lane 6), though the relative levels of GpppA and 7mGpppA were significantly different in mRNA synthesized by RNP and recombinant L protein. This suggests that RPV mRNAs possess both GpppA and 7mGpppA structures. The capping and methylation activities seen with the RNP complex are presumably not mediated by cellular capping machinery, since the RNP is obtained from purified virus. Control reactions with no protein or with purified glycerol gradient fractions from mock-infected cells lacking the L–P complex did not support any RNA synthesis (Gopinath & Shaila, 2008), and hence cap structure analysis was not performed.

L protein specifically cleaves the γ-phosphate of triphosphate-ended RNA

In order to test whether L protein possesses RTPase activity, a 25 nt triphosphate-ended RNA was synthesized and labelled at the γ-phosphate position. Incubation of this

with purified virus or RNP complex resulted in the release of inorganic phosphate from substrate RNA (Fig. 2, lanes 4 and 5). In addition, recombinant L protein partially purified from insect cells also showed RTPase activity...
Fig. 2. RTPase activity of L protein. [γ-32P]ATP-terminated RNA was incubated with 1 μg purified N-RNA (lane 1), P protein (lane 2), glycerol gradient fraction partially purified from non-recombinant baculovirus-infected insect cells (lane 3), purified RPV (lane 4), RNP complex (lane 5) or partially purified L–P complex (lane 6) at 37 °C for 20 min. The reaction mixture was analysed by TLC on PEI-cellulose plates. Pi was detected by phosphorimaging. Ori, origin of spotting.

Characteristics of RTPase activity

To gain additional insights into the L protein-associated RTPase activity, its kinetic properties were investigated. The velocity of the reaction linearly increased with time and reached saturation within 15 min (Fig. 3a); the rate of the reaction did not increase after 40 min. When incubated...
for longer periods, the optimal temperature for RTPase activity was 37°C (Fig. 3b). RNA triphosphatase activity was also seen in the absence of Mg²⁺, although the activity increased moderately with the addition of Mg²⁺ (Fig. 3c). However, a reaction carried out in the presence of 20 mM EDTA showed no RTPase activity, indicating the requirement of a metal ion for the reaction; the activity in the absence of additional Mg²⁺ indicates that L protein may contain endogenous Mg²⁺ ions bound to the protein. The RTPase activity showed a wide pH range with an optimum between pH 7.5 and 8.8 (Fig. 3d).

**L protein forms a covalent complex with GTP**

In order to test whether L protein also possesses GT activity for formation of the reaction intermediate, the L–GMP complex was examined. Incubation of either purified virion or the RNP complex with [α-³²P]GTP resulted in formation of L–GMP covalent complex which migrated to its original position on SDS-PAGE, which is similar to the positive control VACV GT (Fig. 4a, lanes 2, 3 and 4). Formation of the L–GMP covalent intermediate was dependent on input RNP concentration (Fig. 4c). The L–GMP covalent intermediate was also observed with the partially purified recombinant L protein (Fig. 4b, lanes 4–6). A control reaction containing the purified glycerol gradient fraction from mock-infected insect cells did not show the presence of labelled protein at the position of the L protein, although an intensely radiolabelled protein band of 72 kDa was seen, which may represent the insect cell GT (Fig. 4b, lanes 1–3). Since the L–P complex partially purified from insect cells also contains cellular GT, it was not employed for further biochemical characterization.

Sequence alignment of L proteins of negative-sense RNA viruses has identified six regions of conserved motifs separated by regions of low conservation (Supplementary Fig. S2, upper panel) (Poch et al., 1990). However, alignment of L proteins within the morbillivirus genus yielded three conserved domains that are separated by two highly variable hinge regions (McIlhatton et al., 1997), namely LD1 (aa 1–606), domain II (LD2; aa 650–1694) and LD3 (aa 1717–2183) (Supplementary Fig. S2b). These domains may function independently of each other, a prediction that is supported by the insertion of a green fluorescent protein open reading frame within the Hinge region of RPV L protein without affecting the polymerase function (Brown et al., 2005). In order to dissect the functions of individual domains in viral mRNA capping, both LD1 and LD3 of RPV L protein were expressed and purified using a recombinant baculovirus expression system (Supplementary Fig. S5). Incubation of purified LD3 with [α-³²P]GTP resulted in the formation of a radioactively labelled covalent complex which was dependent on input protein concentration (Fig. 4d, lanes 1–5); LD1 did not produce a covalent intermediate (Fig. 4d, lane 6). This provided convincing evidence for L/LD3-mediated covalent complex formation, characteristic of GT. Further biochemical characterization was carried out with RNP complex as the source of L protein. Addition of a molar excess of cold GTP to the reaction inhibited the covalent intermediate formation, whereas addition of other NTPs failed to inhibit the reaction, indicating that L protein forms a covalent complex only with the GTP moiety of GTP which is characteristic of GTs (Fig. 5a). The L–nucleotide intermediate was stable over a pH range of 6.0–8.8 (Fig. 5b) and maximal covalent intermediate formation was observed between 37 and 50°C (Fig. 5c).
characterization of the LD3–nucleotide covalent complex showed similar properties (data not shown).

L protein represents an RPV mRNA capping enzyme

The inability of L protein to form a covalent intermediate at an acidic pH range suggested the formation of phosphoamide linkage between one of the lysine residues of the protein and the phospho group of the GMP moiety, as has been reported for the VACV GT–GMP complex (Shuman & Hurwits, 1981). To investigate the linkage between the phospho group and L protein, aliquots of the L–GMP complex were incubated under acidic and alkaline conditions. The L–GMP complex formed was stable in both neutral and alkaline pH (Fig. 6a, lanes 1 and 4), whereas acidic conditions (0.1 M HCl and 4 M NH2OH) resulted in hydrolysis of the linkage between the protein and the radioactive nucleotide (Fig. 6a, lanes 2 and 3), indicating that L protein forms a phosphoamide linkage with the GMP moiety of GTP using one of its lysine residues, similar to the VACV capping enzyme (Shuman & Hurwits, 1981). The presence of PPi completely abrogated the L–GMP complex formation, whereas addition of inorganic pyrophosphatase rescued the inhibition (Fig. 6b, lanes 1–3). The additional band migrating at 72 kDa (lane 3) was found to be due to a contaminating GT present in the commercial preparation of the pyrophosphatase, as the labelled protein appeared when pyrophosphatase alone was incubated with [α-32P]GTP (Fig. 6b, lane 4). The second step of the GT reaction, with regard to the transfer of the GMP moiety to a diphosphate-ended acceptor RNA, was tested by an alternative method, as described for baculovirus GT LEF4 (Gross & Shuman, 1998b). Cap-labelled (Gp*ppA) or cap-labelled/methylated (7mGp*ppA) RNAs of 25 nt were prepared by VACV GT (Fig. 6c, lanes 1 and 2). Incubation of RNP complex with cap-labelled RNA resulted in the generation of L–GMP complex (Fig. 6d, lane 4), indicating that the GMP moiety was transferred from the capped RNA to L protein. A similar reaction performed with cap-labelled/methylated RNA did not yield L–GMP complex due to the irreversible nature of the reaction once the cap is methylated (Fig. 6d, lane 2). L protein was not detected in control reactions containing no protein (Fig. 6d, lanes 1 and 3).

RPV L protein forms a covalent complex with the GMP moiety

In order to analyse the nature of the nucleotide covalently bound to L protein, LD3–nucleotide covalent complex was eluted from SDS-PAGE gels and bound nucleotide was released from the protein by acid hydrolysis. Treatment of the radioactively labelled LD3–nucleotide complex with HCl or hydroxylamine liberated the bound nucleotide which co-migrated with the authentic GMP marker in TLC, suggesting that RPV LD3 forms a covalent intermediate with the GMP moiety of GTP (Fig. 7, lanes 3 and 4). Similar analysis could not be performed with the full-length L protein in the RNP complex, due to the high molecular mass of the L–GMP complex, which makes it difficult to recover from the gel. Neither LD1 nor LD3 showed any RTPase activity. Unlike the full-length L protein in the RNP complex, LD3 lacks the ability to transfer bound GMP to a diphosphate- or triphosphate-ended acceptor RNA (data not shown). These results indicate that LD1 does not play any role in the post-transcriptional modification of viral mRNA, whereas LD3 of RPV L protein partly plays a modular role in forming the covalent complex with the GMP moiety of GTP, which is the first step of GT activity.

DISCUSSION

Generation of capped cellular mRNA consists of two steps. First, the triphosphate end of the RNA is cleaved into Pi and a diphosphate terminated RNA. In the second reaction, a GMP moiety from GTP is transferred to the diphosphate-ended RNA to form a capped RNA. In this
report, a stepwise analysis was performed to demonstrate that each of these two activities is associated with the L protein of RPV. The use of purified virus-derived RNP complex eliminates the possibility of cellular triphosphatase contamination. We previously showed that the purified RNP complex, comprising the RNA polymerase as well as the recombinant L–P complex, is transcriptionally active (Ghosh et al., 1995). In both cases, production of full-length transcripts of viral mRNA had been demonstrated. In the present study, we have shown that the in vitro mRNA synthesized by RNP complex or the recombinant L–P complex is capped and methylated at the 5′ end (Fig. 1), although the presence of 2′-O-methylation of mRNA has not been tested. RTPase activity of L protein, the first step in viral mRNA capping, was tested in vitro using synthetic γ-32P-labelled RNA substrate containing sequences corresponding to viral genes. Viral mRNA 5′ cis sequences were used as the substrate because in many negative-sense RNA viruses, the capping machinery of these viruses requires specific cis-acting signals in the RNA (Ogino et al., 2005; Ogino & Banerjee, 2007; Wang et al., 2007). Under optimal conditions, L protein in the RNP complex, as well as L protein co-expressed with P protein in insect cells, efficiently catalyses the removal of γ-phosphate from the triphosphate-ended RNA (Fig. 2).

Baculoviruses encode two RTPases, namely BVP, a metal-independent RTPase, and LEF-4, a metal-ion-dependent RTPase (Gross & Shuman, 1998a, b). A mock-purified glycerol gradient preparation from non-recombinant baculovirus-infected cells did not show RTPase activity, ruling out the presence of baculovirus triphosphatases in the L–P complex fraction (Supplementary Fig. S3, lanes 10–12). Further evidence for L-mediated RTPase activity was obtained by the metal-dependency of the reaction, whereas the cellular RTPase reaction is metal-independent (Changela et al., 2001).

The second enzymatic activity required for mRNA capping has been well characterized in many viral GTs such as VACV D1, baculovirus LEF4 and reovirus λ2 protein (Gross & Shuman, 1998b; Luongo et al., 2000; Shuman, 1990). However, in non-segmented negative-sense RNA viruses, GT activity has only been demonstrated for VSV L protein (Ogino & Banerjee, 2007). In the present work, we have demonstrated, for the first time for any paramyxovirus, that L protein is able to cap viral mRNA. Purified RNP complex is free of any cellular GT, shown by the presence of a single species of radioactively labelled protein which migrated at the same molecular mass as L protein (Fig. 4a).

Fig. 6. Characteristics of the GT activity of L protein. (a) RPV L protein forms a phosphoamide linkage with the GMP moiety of GTP. An aliquot (4 μl) of the L–GMP complex (formed as in Fig. 4a) was incubated with the indicated solutions for 45 min at 37 °C. After the incubation, the reaction was adjusted to neutral pH by adding either HCl or NaOH. The reaction mix was resolved using 7% SDS-PAGE and visualized by phosphorimaging. (b) L–GMP complex was formed in the absence (−) or presence (+) of 100 μM inorganic pyrophosphate (PPi; lanes 1 and 2) and 0.1 U inorganic pyrophosphatase (PPase; lane 3). Lane 4, reaction mix containing 0.1 U inorganic pyrophosphatase and 5 μCi [α-32P]GTP. (c) Synthesis of cap labelled/ methylated RNA. Triphosphate-ended RNA was capped (GpppA) or capped and methylated (7′GpppA) in the presence of [α-32P]GTP and S-adenosyl methionine, using VACV GT. Labelled RNA was gel purified, resolved using 18% urea-acrylamide gel electrophoresis and analysed by phosphorimaging. (d) GMP transfer from capped viral RNA (25 nt) to L protein. Capped (GpppA) or cap methylated (7′GpppA) 25 nt RNA (5 pmol) was incubated in the absence (−) or presence (+) of 2 μg RNP complex. L–GMP complex was visualized by analysing the reaction mix using 7% SDS-PAGE and phosphorimaging.
LD3, may carry out co-transcriptional capping of viral mRNA. Hence, we mediated capping activity, though LD3 alone lacked the capability. LD3 alone was able to form the covalent complex with (aa 1717–2183) were expressed and purified to homogeneity. In order to delineate the region in RPV L protein that is responsible for GT activity, both LD1 (aa 1–606) and LD3 (aa 1717–2183) were expressed and purified to homogeneity. LD3 alone was able to form the covalent complex with the GMP moiety of GTP. An enzyme–GMP complex assay was carried out and the enzyme–nucleotide complex was electroeluted from the gel in Tris-glycine running buffer containing 0.1 % SDS. The eluted complex was dialysed against 10 mM Tris/HCl, pH 7.4, concentrated and treated with either water, 0.5 M NaOH, 0.5 M HCl or 4 M NH$_2$OH. Aliquots of the reaction product were spotted on PEI-TLC plates and developed with 0.45 M (NH$_4$)$_2$SO$_4$.

In order to delineate the region in RPV L protein that is responsible for GT activity, both LD1 (aa 1–606) and LD3 (aa 1717–2183) were expressed and purified to homogeneity. LD3 alone was able to form the covalent complex with GMP, providing further support for the L protein-mediated capping activity, though LD3 alone lacked the ability to transfer GMP to acceptor RNA. Hence, we speculate that LD2 of RPV L protein, in conjunction with LD3, may carry out co-transcriptional capping of viral mRNA in a modular fashion. In support of our view, Li et al. (2008), defined a new motif (GxxT)p(HR) within LD2 that is conserved in all negative-sense RNA viruses, which is crucial for viral mRNA capping.

All known eukaryotes and some known viral mRNA capping enzymes have been shown to transfer a GMP moiety to the diphosphate-ended acceptor RNA to generate a cap structure. VSV, a prototype model for negative-sense RNA viruses, forms a covalent complex with the substrate RNA rather than the nucleotide and uses GDP instead of GMP for capping by a polyribo nucleotidyl transferase activity (Ogino & Banerjee, 2007). In addition, it also forms an unusual tetra-phosphate cap structure on viral mRNA (GppppG) (Ogino & Banerjee, 2008). In contrast, the GT activity of RPV L protein is similar to cellular GT, since L forms an L–GMP covalent intermediate (Fig. 7). Thus, RPV L protein likely represents a lineage in negative-sense RNA viruses that is distinct from VSV capping enzyme. Furthermore, the present work suggests that RPV L protein may belong to the metal-dependent family of triphosphatases, contrary to its cellular counterparts which are metal-independent.

**REFERENCES**


