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Unique capping activity of the recombinant RNA polymerase (L) of vesicular stomatitis virus: association of cellular capping enzyme with the L protein☆

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Abstract

Vesicular stomatitis virus (VSV), a prototype of non-segmented negative strand RNA viruses, packages an RNA-dependent RNA polymerase (L) which, together with an associated phosphoprotein (P), transcribes the genome RNA, in vitro and in vivo, into mRNAs that are capped at the 5′-ends. However, unlike cellular guanlylyltransferase (GT), the RNA polymerase incorporates GDP in the capped structure, as $Gp^{\alpha}p^{\beta}$–$p^{\alpha}A$. In an effort to characterize the capping activity of the RNA polymerase, we have purified recombinant L (rL) protein expressed in insect cells. The rL, like the virion L polymerase, also caps transcribed mRNAs with identical unique cap structure. Interestingly, the purified rL is found to be tightly bound to the GT of the insect cell during all stages of purification. VSV grown in baby hamster kidney cells also packages cellular GT of the murine cell, suggesting that VSV L protein or its associated proteins may have a strong affinity for the cellular GT. The GT bound to rL, however, formed E–GMP complex, whereas no such complex was detected with the rL protein. It appears that the L protein may contain the putative active site for the unique capping reaction or the tightly bound cellular GT may by some unknown mechanism participate in the unique capping reaction.

Keywords: Recombinant VSV RNA polymerase/capping

It has been a puzzling fact for many years that the virion-associated RNA polymerase consisting of the (L) large subunit of the RNA-dependent RNA polymerase (252 kDa) and the (P) phosphoprotein (29 kDa) of VSV [1] would efficiently cap the transcribed mRNAs in vitro but a definitive conclusion could not be made with regard to the identification of the capping enzyme. This was further complicated by the fact that the 5′–5′ triphosphate linkage in the cap structure of VSV mRNAs is unique since the α- and β-phosphates of GTP (i.e., GDP) and the α-phosphate of the penultimate base A are incorporated, e.g., $Gp^{\alpha}p^{\beta}$–$p^{\alpha}A$ [2,3] in contrast to reported cellular and other viral capping reactions where only the α-phosphate of GTP (i.e., GMP) is incorporated into the cap structure Gp^{α}–$p^{\beta}p^{\alpha}X$ [4,5]. The conventional wisdom predicted that a virus such as VSV which replicates in the cytoplasm and is negative stranded must manifest this unique GT activity by a virus encoded protein, e.g., the L protein. Initial attempts to purify the capping activity from the ribonucleoprotein (RNP) failed due to the paucity of L and P proteins recovered from the virus because approximately 50 molecules of L protein and 500 molecules of P protein constitute the RNA polymerase within the virion [6]. Availability of recombinant L protein (rL) using baculovirus expression system provided for the first time an opportunity to study its biochemical properties [7]. Here we provide evidence that the rL, like virion-associated L protein, also manifests unique capping activity. However, it remains tightly bound to the cellular capping enzyme. The precise role of the RNA polymerase-bound cellular GT in the unique capping reaction remains unknown.

☆ *Abbreviations:* VSV, vesicular stomatitis virus; RNP, ribonucleoprotein; rL, recombinant L protein expressed in Sf21 cells; GT, guanylyltransferase.

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Materials and methods

Cell culture and viruses. Sf21 and BHK-21 cells were grown as described previously [7]. VSV (Indiana serotype) was grown in BHK-21 cells and the released virus was purified [8,9]. The recombinant L expressed in Sf21 cells was purified as detailed elsewhere [7]. Briefly, Sf21 cells were infected with recombinant baculovirus (containing L gene). The harvested cells were lysed in a buffer containing 25 mM Tris–HCl (pH 7.5), 1.0 mM DTT, 0.1% Triton X-100, and 2.0 mM EDTA. The soluble supernatant was loaded onto a phosphocellulose column and the pool-II fraction [7] was subjected to 0–40% ammonium sulphate fractionation. The 40% fraction, called PC/40%, was dialyzed and used for subsequent reactions.

E–GMP complex formation and purification. The enzyme was first incubated with [α-^{32}P]GTP in a reaction mixture containing 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM DTT at 30 °C for 15 min. ^{32}P-labeled proteins were then separated from the unincorporated precursor nucleotide by passing through a Sephadex G50 nick spin column. Radiolabeled protein samples were then analyzed in a SDS–polyacrylamide gel (PAGE). Covalently labeled E–GMP complex was visualized by autoradiography.

Immunoprecipitation of L associated GT activity. The peptide antibody specific to the NH_2-terminal sequence of L protein [10] was used to immunoprecipitate the L protein using PC/40% fraction. The immunoprecipitate recovered after Protein A–sepharose treatment as the secondary antibody was washed extensively with a buffer containing 50 mM Hepes (pH 7.4), 250 mM NaCl, and 0.1% NP40. The precipitate was finally washed with a 25 mM Tris–HCl (pH 7.5) buffer containing 5 mM $MgCl_2$ and 1 mM DTT and assayed for E–GMP complex forming activity by SDS–PAGE electrophoresis.

In vitro transcription of VSV mRNAs. In vitro transcription reactions were carried out in a standard reaction mixture containing either Triton X-100 disrupted whole virus [2], or RNP [11] or N-RNA template free of L and P proteins [9]. To synthesize non-radioactive transcripts, 1.0 mM of each NTP was used. Cold GTP of 0.05 mM was used when the transcription reactions were carried out either in presence of [α-^{32}P]GTP or [β-^{32}P]GTP. Incubation was carried out at 30 °C for 4 h. Synthesized RNAs were then extracted with an equal volume of phenol–chloroform mixture followed by ethanol precipitation. Precipitated RNA was dissolved in 50 μl 20 mM Tris–HCl, pH 8.0 and passed through a Sephadex G-50 nick spin column to remove any unincorporated nucleotides. Samples were again extracted with phenol–chloroform mixture and ethanol precipitated. The precipitates were dissolved in 50 μl 20 mM Tris–HCl, pH 8.0 buffer and incubated with 20 U/ml of bacterial alkaline phosphatase. Samples were repurified through spin column followed by phenol extraction and ethanol precipitation.

For cap structure analyses, labeled RNA products were incubated with P1 nuclease followed by nucleotide pyrophosphatase as detailed earlier [2]. Digested products were chromatographed on a thin layer polyethyleneimine–cellulose plate developed with 0.4 M ammonium sulphate.

Results and discussion

Reconstituted VSV transcripts are uniquely capped

To investigate whether rL protein has the ability to cap transcribed mRNAs, similar to that observed for purified virus [2,3] we used purified rL from the insect cells in an in vitro transcription reconstitution reaction using purified viral N-RNA (devoid of L and P proteins) as template and exogeneously added *Escherichia coli* expressed P protein which was phosphorylated [9] by the endogenous CKII present in the rL fraction or by the addition of recombinant CKII. Unlabeled mRNAs with approximately 1 pmol of 5′-termini were synthesized and purified. To test whether the 5′-ends are capped the mRNAs were treated with purified vaccinia GT in the presence of [α-^{32}P]GTP which will score the uncapped polyphosphorylated mRNAs, if any. As shown in Fig. 1A (lane 1), the RNA synthesis in the reconstitution reaction was efficient as shown by the synthesis of VSV-specific [α-^{32}P]GTP-labeled mRNAs by urea–PAGE electrophorosis. The unlabeled mRNAs, however, could not be capped by vaccinia GT (Fig. 1A, lane2), whereas the 5′-polyphosphorylated RNA products, used as control, could be fully capped (Fig. 1B, lane 1), indicating that the transcribed mRNAs are all blocked at their 5′-termini. Furthermore, the 5′-termini were not labeled by polynucleotide kinase in the presence of [γ-^{32}P]ATP prior treatment with alkaline phosphatase (Fig. 1A, lane 3), confirming that the mRNAs are indeed

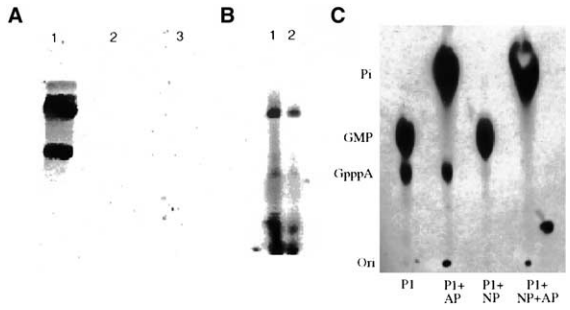

Fig. 1. Reconstituted VSV transcripts are all capped. In vitro transcription reconstitution was carried out using Sf21 expressed rL protein, N-RNA template, and bacterially expressed P protein. (A) Transcription reactions were done either in presence of [α-^{32}P]GTP (lane 1) or cold GTP (lanes 2 and 3) along with other three unlabeled NTPs. For each of lane 2 and 3, 50 equivalent reactions were pooled to synthesize at least 0.6 pmol of 5′-end. Cold transcripts were then either incubated with purified vaccinia GT and [α-^{32}P]GTP (lane 2) or treated with alkaline phosphatase followed by incubation with polynucleotide kinase and [γ-^{32}P]ATP (lane 3). (B) Unlabeled 5′-triphosphate containing RNA (60 nt long) synthesized by T7 RNA polymerase from a T7 promoter driven plasmid was incubated with purified vaccinia GT and [α-^{32}P]GTP (lane 1) or treated with alkaline phosphatase followed by incubation with polynucleotide kinase and [γ-^{32}P]ATP (lane 2). One pmol 5′-end of the in vitro synthesized T7 RNA was used for each reaction. (C) ^{32}P-GMP labeled VSV transcripts were synthesized by in vitro transcription reconstitution followed by purification through spin column for removal of unincorporated precursors. Samples were then treated with P1 nuclease and chromatographed on PI–cellulose plate. The radiolabeled spot migrating with the unlabeled marker GpppA was eluted. Eluted samples were then treated with either P1 nuclease (P1) or P1 nuclease followed by alkaline phosphatase treatment (P1 + AP) or P1 nuclease followed by nucleotide pyrophosphatase treatment (P1 + NP) or P1 nuclease followed by nucleotide pyrophosphatase treatment and subsequently treated with alkaline phosphatase (P1 + NP + AP) and were chromatographed on PI–cellulose plate. Position of migration of cold GpppA, GMP, and inorganic phosphates was marked at the left-hand side. 'Ori' represents the origin.

fully capped. In a separate series of experiments we analyzed directly the capped structure by the standard enzymatic procedure [4] using [α-^{32}P]GMP labeled mRNAs (Fig. 1C). The α-^{32}P-GMP labeled RNA released the 5′ GpppA structure upon treatment with nuclease P1. As expected, the capped structure subsequently released ^{32}Pi when treated with nucleotide pyrophosphatase and alkaline phosphatase [2,3]. However, the genesis of the three phosphates in the cap structure was not immediately apparent from these studies.

To address the intriguing question whether indeed the rL incorporates α- and β-phosphates of GTP into the capped structure of VSV mRNAs, β-labeled GTP was used in the in vitro transcription reconstitution reaction using rL or Triton-disrupted VSV or purified RNP (both obtained from BHK cells). As shown in Fig. 2, the β phosphate of GTP was incorporated in all three cases into the cap structure, similar to that determined previously for latter two cases [2,3]. For both Triton-disrupted VSV or purified RNP, ^{32}Pi was released when the isolated capped structure was treated with P1 nuclease and nucleotide pyrophosphatase, thus, confirming the structure to be Gpp*–pA, where asterisk is the ^{32}P label. Interestingly, for the rL protein, the Pi was also released upon similar treatment confirming the capped structure to be also Gpp*–pA. A few minor spots appeared in the rL protein lane, the identity of which are not known at this time. Thus, it seems that the rL can cap the 5′-ends of transcribed RNA where the α- and β-phosphates of GTP were incorporated into the cap structure.

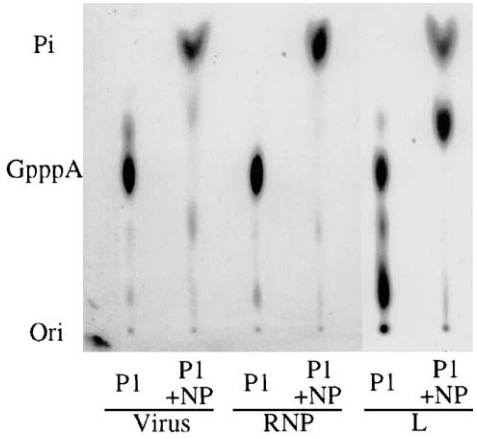

Fig. 2. Incorporation of [β-^{32}P]GTP in the cap structures of either VSV transcripts. Transcription reconstitution was carried out using either total virus (VSV) or RNP complex isolated from purified virion of VSV (RNP) or rL protein (L) in presence of [β-^{32}P]GTP. Transcripts were then passed three times through spin column followed by ethanol precipitation each time. Radiolabeled transcripts are then digested with either P1 nuclease alone (P1) or P1 nuclease and nucleotide pyrophosphatase (P1 + NP) and were chromatographed on PI–cellulose plate. Position of migration of cold GpppA and inorganic phosphates was marked at the left-hand side. 'Ori' represents the origin.

Association of cellular GT with the L protein

Since the rL fraction is capable of forming the uniquely capped transcripts in vitro, we then proceeded to characterize the formation of covalent complex of enzyme–GMP (E–GMP) (or possibly E–GDP) by the rL fraction which is the required intermediate of the conventional capping reaction [12]. The purified rL protein was incubated in the presence of [α-^{32}P]GTP and the presumptive intermediate was analyzed by SDS–PAGE followed by autoradiography. Surprisingly, a distinct labeled band migrating at 73 kDa was clearly seen in the gel (Fig. 3A, lane 1) whereas no labeled band was detected with the L protein. Interestingly, the labeled band migrated similar to that reported for insect cell GT [13]. The radioactivity in the 73 kDa protein complex was found to be resistant to calf intestinal alkaline phosphatase treatment and also covalently linked to the cellular GT via phosphoramidate linkage as shown by resistance to hydrolysis by alkali (not shown). These results coupled with its molecular weight, the 73 kDa protein appears to be insect cell GT.

To test whether the cellular GT is indeed bound to the L protein, immunoprecipitation reaction was carried out by anti-L antibody [10]. As shown in Fig. 3A, the labeled intermediate was quantitatively precipitated by L antibody (lane 2) but not by control anti-P antibody

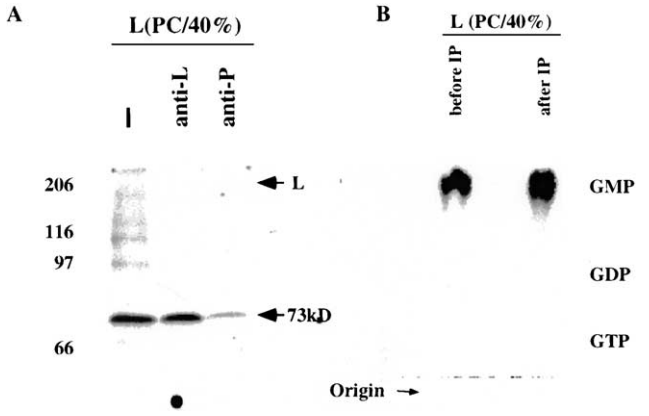

Fig. 3. Presence of E–GMP complex forming activity in the rL fraction. (A) The PC/40% fraction containing rL was incubated with [α-^{32}P]GTP to assay for E–GMP complex forming activity before (-) or after immunoprecipitation with either L antibody (anti-L) or P antibody (anti-P), as described in "Materials and methods" and analyzed in a 10% SDS–PAGE. Numbers on the left-hand side represent the position of migration of a known molecular weight protein marker. The arrows indicate the position of migration of L protein detected by Coomassie Blue staining and the 73 kDa radiolabel band. (B) TLC analysis of the radiolabeled E–guanosin complex. The ^{32}P-labeled band migrating at the position of 73 kDa, before IP or after IP, was electroeluted from the SDS–PAGE. The samples were then incubated in the presence of 0.15 N HCl at 85 °C for 5 min. Acid hydrolyzed samples were then analyzed by TLC. 'Ori' represents the origin. Position of migration of cold GTP, GDP, and GMP was marked at the right-hand side.

(lane 3) indicating that the GT activity is tightly bound to the L protein.

To test whether the GT bound rL formed the conventional E–GMP or possibly an E–GDP complex, acid hydrolysis of the complex was carried out followed by TLC. As shown in Fig. 3B, the label released after hydrolysis migrated with GMP indicating that the enzyme–guanosine nucleotide complex was indeed E–GMP.

To further study the association of rL protein with insect cell GT, we followed the presence of GT in the L protein fractions in various steps during the purification procedure. As shown in Fig. 4, throughout the purification steps including phosphocellulose chromatography, 40% ammonium sulphate fractionation, Sephacryl S-300 gel filtration, and DE52 column chromatography, the cellular GT, identified by E–GMP complex forming activity (Fig. 4C), coeluted with the rL protein as revealed by immuno blotting with anti-L antibody (Fig. 4B). The silver stained gel profile of the peak fraction of each purification step is shown in Fig. 4A. Although, the L protein is clearly discernible, no distinct band at the 73 kDa position is visible by silver staining. However, the ratio of the intensity of the western blotted L band (Fig. 4B) and the radioactivity in E–GMP complex (Fig. 4C) remained relatively constant during each step of purification suggesting that the cellular GT is a stoichiometric component of the L protein. It is noteworthy, that the cellular GT at the later stages of purification produced two radioactive bands probably representing the cleavage products of the polypeptide usually observed for cellular GT [13]. It should be noted that the capping reactions were carried out primarily with the PC/40% AS fraction because the subsequent fractions lost transcription activity significantly, possibly due to removal of some putative host factor(s).

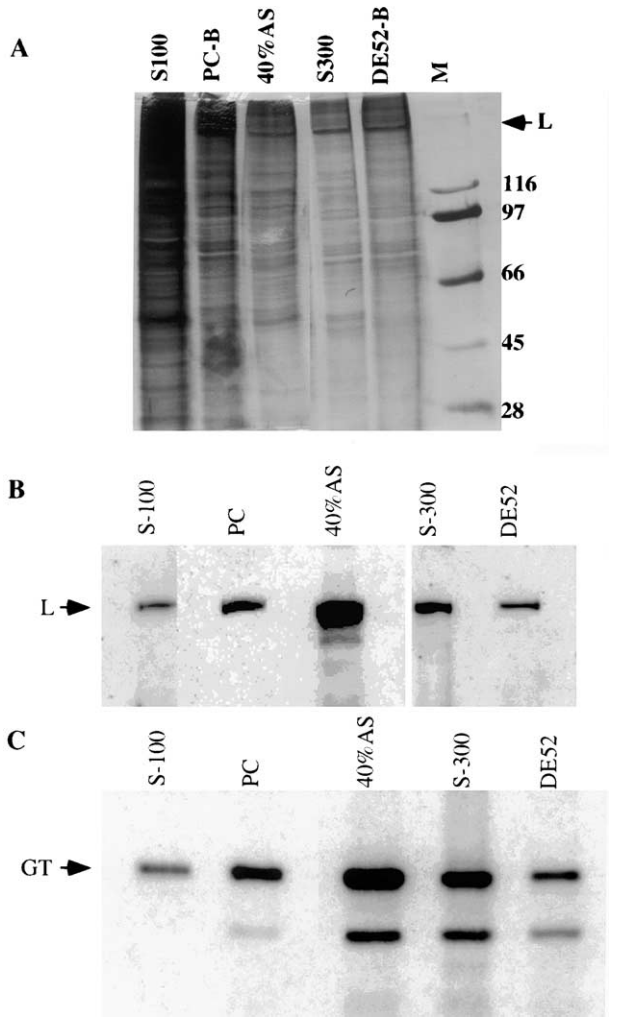

Fig. 4. Coelution of E–GMP complex forming activity with the rL protein. (A) The silver stained gel profile of the peak fraction of each purification step (marked on the top of each lane) is shown. 'M' represents the known molecular weight protein marker and the numbers on the right-hand side represents the position of migration of known molecular weight protein marker. (B and C) Coelution of the L protein and cellular GT activity during L purification. The peak fractions containing L protein from different column chromatographic steps were checked (B) either by western blot with anti-L antibody or (C) were assayed for E–GMP complex forming activity. The arrow in the upper panel indicates the position of migration of L protein detected by immunoblotting with anti-L antibody. The arrow in the lower panel shows the position of 73 kDa E–GMP complex forming activity of cellular GT. Source of each peak fraction was mentioned on the top of each panel.

Packaging of cellular GT in the purified virions grown in mammalian cells

The fact that the rL was purified from insect cells and the insect cell GT seemed to be tightly associated with the L protein prompted us to take a closer look at the real situation where the virus is grown in mammalian cells. Since purified VSV grown in BHK cells efficiently caps transcribed RNAs [2,3], the virion-associated L protein, by extension of the above results, must also associate with the BHK cell GT. To test this prediction, RNP from purified VSV grown in BHK cells was analyzed both for the presence of GT by western blot analysis using mouse anti-GT antibody and the formation of the E–guanosine nucleotide complex. As shown in Fig. 5A, the RNP reacted with anti-mouse GT antibody and also formed an E–guanosine nuclecotide complex, this time migrating at 68 kDa (Fig. 5B), the reported molecular weight of mouse GT [14]. The recombinant mouse GT and BHK 21 cell extract served as controls for this analysis. These results further underscore the point that the cellular GT must be packaged within the virions presumably bound to the L protein during assembly of the virus, prior to the budding process.

The results embodied in this communication, thus, indicate that recombinant L protein expressed in insect

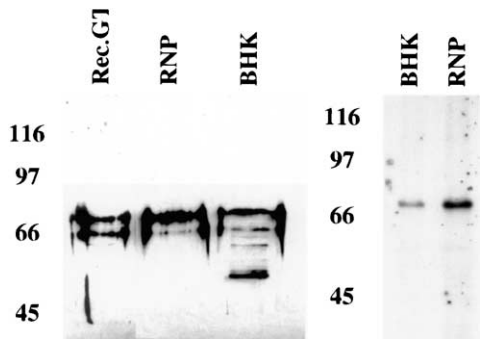

Fig. 5. Presence of cellular GT in VSV grown in BHK-21 cells. (A) RNP complex isolated from the purified virion of VSV grown in BHK-21 cells were immunoblotted against the anti-mouse GT antibody. Recombinant mouse GT (Rec. GT) and whole cell extract of uninfected BHK-21 cells (BHK) were used as positive controls. (B) E–GMP complex forming activity was assayed either with RNP or with uninfected BHK-21 cell extract and analyzed in a 10% SDS–PAGE followed by autoradiography.

cells carries out the unique capping reaction by which the α- and β-phosphates of GTP are incorporated into the 5′ blocked structure of VSV mRNAs similar to that observed for the virion-associated L protein [3]. It was further shown that, in addition to the translation elongation factors, EF-1 (αβγ) [8] the rL is tightly associated with the insect cell GT or mammalian GT when isolated from BHK cells as part of the transcribing RNP. These findings underscore the point that cellular GT either from invertebrate or vertebrate cells must have specific and strong affinity for the VSV L protein and associate with it in the cytoplasm presumably during its synthesis. Alternatively, GT may bind to one of the constituents of the elongation factor, probably the EF-1α, which has the strongest affinity for the L protein [8]. Further studies are clearly needed to gain insight into this specific interactive process. The cogent questions remain whether the L protein or the cellular GT or both participate in the capping reaction. The fact that GT bound to both rL and the virion-associated GT formed the E–GMP complex strongly suggest that the bound GT may not directly carry out the GDP transfer reaction leading to the formation of the unique VSV cap structure. A possibility exists that GT bound to EF-1α, a GDP binding protein, may manifest the unique capping activity. Alternatively, the L protein may contain the active site of the GT activity and transfers GDP to the 5′-termini of the RNA without the formation of an E–guanosine nucleotide complex. An alternate novel mechanism has recently been hypothesized that posits that the capping reaction is mediated by the L protein in which the nascent RNA transcripts undergo forward slippage followed by polymerase mediated attack of GDP across the ApA dinucleotide leading to the formation of the unique cap structure [15]. Future experiments would certainly be directed to understand the possible mechanism of this unique capping reaction and the role played by the bound cellular GT, if any.

Acknowledgments

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