The Vaccinia Virus mRNA (Guanine-\(N^\gamma\)-)-methyltransferase Requires Both Subunits of the mRNA Capping Enzyme for Activity*

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Plasmid vectors capable of expressing the large and small subunits of the vaccinia virus mRNA capping enzyme were constructed and used to transform Escherichia coli. Conditions for the induction of the dimeric enzyme were optimized. The individual subunits lacked methyltransferase activity when assayed alone. However, mixing the D1R and D12L subunits permitted reconstitution of the methyltransferase activity, and this appearance in activity accompanied the association of the subunits. In contrast, mixing the D12L subunit with the D1R-60K proteolytic fragment failed to yield methyltransferase activity or result in a physical association of the two proteins. These results demonstrate that the methyltransferase active site resides in the carboxy-terminal portion of the D1R subunit. Furthermore, since the mRNA triphosphatase and guanyltransferase active sites reside in the NH\(_2\)-terminal domain of the D1R subunit, and the methyltransferase activity is found in the carboxy-terminal portion of this subunit and D12L, there must be at least two separate active sites in this enzyme.

Vaccinia virus, the prototypic poxvirus, possesses a double-stranded DNA genome of 191,686 base pairs capable of encoding approximately 200 proteins. Poxviruses replicate within the cytoplasm of the infected cell and must encode the enzymes necessary for viral gene expression and DNA replication (for review, see Ref. 2). Encapsidated within the virion core are the enzymes required for early gene expression: a multisuunit RNA polymerase (3, 4), early gene transcription initiation (5, 6) and termination factors (7), poly(A) polymerase (8, 9), and the mRNA capping enzyme (10). The availability of these enzymes and the ability to manipulate their coding sequences allow for a unique opportunity to study the mechanism of transcriptional regulation and mRNA processing outside the confines of the nucleus.

Most eukaryotic mRNA are capped at the 5' and polyadenylated at the 3' end. The role of these modifications in mRNA stability, processing, and translational efficiency (11, 12) have been studied. The cap 0 structure of mRNA, \(\text{m}^7\text{G}^'\text{pppN(pN)}_n\), is the product of three sequential reactions as follows,

1. \(\text{ppN(pN)}_n + \text{AdoMet} \rightarrow \text{ppN(pN)}_n \rightarrow \text{ppN(pN)}_n + \text{P}_i\)
2. \(\text{ppN(pN)}_n + \text{GTP} \rightarrow \text{G}^'\text{pppN(pN)}_n + \text{PP}_i\)
3. \(\text{G}^'\text{pppN(pN)}_n + \text{AdoMet} \rightarrow \text{m}^7\text{G}^'\text{pppN(pN)}_n + \text{AdoHcy}\)

requiring three enzymatic activities: mRNA triphosphatase (13, 14), mRNA guanyltransferase, and mRNA (guanine-\(N^\gamma\)-)-methyltransferase (10), respectively. In vaccinia, these enzyme activities are contained within a 130,000 molecular weight heterodimeric protein possessing 97- and 33-kDa subunits (10) called the capping enzyme. In addition, this complex contains nucleoside triphosphate phosphohydrolase activity (ATPase/GTase) (15), acts as a factor required for initiation of intermediate gene transcription (16), and is the viral early gene transcription termination factor (7). Therefore, the capping enzyme plays a pivotal role in regulating viral gene expression at both the transcription and mRNA processing levels.

Identification of the functional domains required for each of the activities in the cap formation pathway would provide insight into the mechanism of cap synthesis. The formation of a GMP intermediate during the guanyltransferase reaction permitted this activity to be mapped to a 58-kDa proteolytic fragment of the large subunit (17–19). mRNA triphosphatase activity was shown to map to this domain as well (19). Methyltransferase activity, however, did not fractionate with the guanyltransferase activity (17, 19), demonstrating the potential for separate functional units. Since antisera specific for the small subunit revealed that D12L protein co-sedimented with methyltransferase activity, it was proposed that the small subunit possessed the methyltransferase active site and that the tight association of the subunits linked the functional domains. The localization of the large and small subunits to genes D1R (20) and D12L (21) of the vaccinia HindIII D restriction fragment (22) allowed for overexpression of the subunits in Escherichia coli (18, 19). However, the large scale purification of the capping enzyme in a soluble ribonuclease-free form is essential for resolution of the domain structure of the enzyme and for conducting an investigation of both early gene transcription termination and intermediate gene transcription initiation.

This report describes the expression and purification of the vaccinia capping enzyme from recombinant E. coli. Various induction conditions were tested in order to increase the yield

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1 The abbreviations used are: AdoMet, S-adenosyl-L-methionine; IPTG, isopropyl-1-thio-\(\beta\)-D-galactopyranoside; AdoHcy, S-adenosyl-L-homocysteine; PEI, polyethyleneimine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
of soluble protein including decreased growth temperature, addition of ethanol, and choice of BL21(DE3) pLysS. Capping enzyme was purified to near homogeneity and shown to be ribonuclease free. Using either partially purified D1R or D12L, we demonstrated, by mixing experiments, that the methyltransferase activity requires both subunits. Furthermore, we showed that the D12L subunit must associate with the carboxyl terminus of D1R to reconstitute activity.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**

pET3a DI2L-8—Gene DI2L lies between 14,350 and 13,487 in the vaccinia HindIII D fragment (22). Plasmid 722, which contains sequence from 12,838 to 16,060 (22), was digested with HpaII at 14,323 and BamHI at 12,838 and the 1,435-base pair fragment isolated. The 5’ region of DI2L from 14,550 to 14,223 was synthesized as a double-stranded DNA linker possessing an NdeI site at the left end corresponding to the ATG of DI2L and an HpaII site at the right end. A three way ligation into pET3a (23), opened at the NdeI and BamHI sites, resulted in the insertion of the entire DI2L coding sequence downstream from the bacteriophage T7 gene 10 promoter.

pET3a DIR-63—Gene DIR lies between 103 and 2,637 in the HindIII D fragment (22). The left end of gene DIR was reconstructed by isolating a fragment containing a portion of the 5’ end of gene DIR, from the FokI site at 118 to the BamHI site at 514 from plasmid 774a, which contains HindIII D fragment sequence from 1 to 514. A donor of −70°C for large scale propagation of the capped mRNA, to the sequence from 103 to 118, containing an NdeI site at the appropriate ATG and a FokI site at the right end. The vector pET3a was cleaved with NdeI and BamHI, and a three way ligation was carried out yielding the plasmid pET3a D1 which contains the correct 5’ end of gene DIR linked to the bacteriophage T7 gene 10 promoter. The bulk of gene DIR was isolated from a derivative of the plasmid 813 which contains sequence from 1 to 4778 (18). A SmaI site at position 3,714 in 813 was converted to a BglII site yielding the corresponding DNA fragment with 5’ site at position 3,714 in 813Bg after digestion with XbaI at 363 and BglII at 3714, and the 1,435-base pair isolated. The resulting fusion protein contains the carboxyl-terminal 225 amino acids of DIR. The pET3a DIR ΔCOO-217-derived protein was used as an antigen for the production of antisera against the NH2 terminus of DIR. Immunoblot analysis was carried out as described in the supplier’s instructions (Bio-Rad and Millipore).

**Enzyme Activity Assays**

**mRNA (Guanine-N7-) methyltransferase**—Assays based on Refs. 27 and 18 contained 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 5 to 20 mM 5’-[methyl-3H]adenosyl-1-methionine (0.5-2 Ci/mmol), 10 mM GTP, and enzyme. Reactions were incubated at 37 °C for 30 min and an aliquot spotted onto DEAE paper. Samples were washed four times with 25 mM ammonium formate, one time with water, and two times with ethanol. The samples were then quantified by scintillation counting.

**mRNA Guanylytransferase**—The formation of the enzyme-GMP intermediate was followed using assays as described (19). Each contained 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 5 mM MgCl2, 1-2 μCi of [γ-32P]ATP (10 Ci/mmol), and enzyme fractions.4 Reactions were incubated at 37 °C for 5 min, an aliquot spotted onto Polygram 300 PEI/UV25 thin layer chromatography sheets, and developed in 0.7 M LiCl and 2% acetic acid (19, 29), autoradiographed, and quantified by Cerenkov counting.

**Enzyme Purification**

**Co-expressed Subunits**—Purification through the phosphocellulose column was based on protocols by Martin et al. (10) and Shuman (19). Four hundred grams of BL21(DE3) pLysS pET3a DIR/DI2L-8 were thawed in 800 ml of lysing buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (v/v), 10 mM β-mercaptoethanol, 50 mM NaCl, 0.1% sodium deoxycholic acid, 0.01% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. After lysis appeared complete (Phase I), 1 M NaCl (1 ml of cells) and MgCl2 to 10 mM were added. After elimination of the viscosity, the lysate was homogenized for 1 min at 0 °C in a Waring blender, and insoluble material was removed by centrifugation at 10,000 × g for 15 min. The pellet was resuspended, rehomogenized, and recentrifuged. The supernatants were pooled, and polyethylene glycol was added to a 20% (v/v) solution, stirred for 15 min, and centrifuged at 10,000 × g for 15 min. The resulting supernatant was centrifuged at 100,000,000 × g for 1 h. Ammonium sulfate was added to the S100 fraction to 45% saturation, stirred for 30 min at 4 °C, and the sample centrifuged at 10,000 × g for 10 min. The pellet was resuspended with a final minimal volume of buffer A (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-

1 J. Myette, personal communication.
mRNA (Guanine-N\textsuperscript{7}-) methyltransferase

Induction of soluble methyltransferase activity by IPTG and ethanol

Methyltransferase activity was measured in a soluble extract derived from 1 liter of cells induced for 48 h at 15–20 °C under various conditions.

| IPTG (µM) | Ethanol % | Total protein (mg) | Total incorporation (nmol) | Specific activity (nmol/mg) |
|----------|-----------|--------------------|---------------------------|---------------------------|
| A        | 0         | 0                  | 441                       | 10.8                      |
|          | 10        | 0                  | 369                       | 71.4                      |
|          | 50        | 0                  | 370                       | 269.3                    |
|          | 100       | 0                  | 456                       | 508.3                    |
|          | 400       | 0                  | 592                       | 726.8                    |

Table 1: Induction of soluble methyltransferase activity by IPTG and ethanol

Protein concentrations were determined by the Bio-Rad Protein Assay using bovine serum albumin as a standard.

Nanomoles of methyl groups transferred to GTP in 30 min at 20 µM AdoMet.

Specific activity in nanomoles of methyl groups transferred to GTP/mg of protein.

![Fig. 1. Chromatographic purification of the vaccinia mRNA capping enzyme.](image-url)

Fig. 1. Chromatographic purification of the vaccinia mRNA capping enzyme. A, enzyme purified through phosphocellulose was applied to a hydroxylapatite column and eluted with a 10–500 mM NaHPO\(_4\) gradient. Fractions were assayed for methyltransferase activity as described under “Experimental Procedures” and is expressed in picomoles of \(^*\)GTP formed in 30 min at 5 µM AdoMet. UV absorbance at 280 is indicated (○). B, the ability to form an enzyme-GMP complex was analyzed by incubation of fractions with [\(^32\)P]GTP followed by SDS-PAGE and autoradiography. PC pool I contains the phosphocellulose fractions applied to the hydroxyapatite column. PC pool II contains the side fractions from the phosphocellulose column that were not purified further. The location of D1R and D1R-60K was based on molecular weight standards (not shown). C, fractions 59–69 of the hydroxyapatite column were pooled, concentrated, and applied to a 2.5 x 50-cm Sephacryl S-200 HR column equilibrated with buffer A. The flow-through containing the capping enzyme was applied to a 10.000 X g centrifugation step. The supernatant was brought to 20% saturation with ammonium sulfate. The subsequent (Bio-Rad Bio-Gel HT) column which was developed with a linear gradient of 10–500 mM NaHPO\(_4\) in HT buffer (100 ml x 100 ml). Capping enzyme was assayed as described above, and fractions containing the capping enzyme were pooled, concentrated, and applied to a 2.5 x 50-cm Sephacryl S-200 HR column equilibrated with buffer A. Methyltransferase, ATPase, and guanylyltransferase activities were assayed and fractions containing activity were stored at -70 °C. D, the ability to form an enzyme-GMP complex was analyzed by incubation of fractions with [\(^32\)P]GTP followed by SDS-PAGE and autoradiography.
A, and dialyzed overnight against the same buffer (2 × 4 liters). The dialysate was applied to a 4 × 20-cm DEAE-cellulose (Whatman DE-52) column and the location of D12L in the flow-through determined by immunoblot analysis.

D1R-60K—BL21(DE3) pLysS pET3a D1R/D12L-3 was induced by addition of 400 μM IPTG and grown for 48 h at 22 °C. Lysis of 241 g of cells and purification through the dialysis of the ammonium sulfate pellet was as described above for the co-expressed subunits.

The dialysate was centrifuged at 10,000 × g, and the supernatant applied to a 4 × 20-cm DEAE-cellulose (Whatman DE-52) column and the location of D12L in the flow-through determined by immunoblot analysis.

FIG. 2. Analysis of Sephacryl S200 HR fractions by SDS-PAGE and Western blot. Aliquots of fractions 47-61 of the Sephacryl S200 HR column were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (A) or subjected to immunoblot analysis using antisera directed against the NH2 terminus of D1R, D12L, and molecular weight standards are indicated. PI, preimmune protein; D1R-GOK, D1R-GOK, were mixed with D12L and allowed to incubate on ice. After 30 min, methyltransferase assays were performed as described above.

**Peptide Sequencing**

A fraction containing D1R-60K, the proteolytic fragment of D1R, was concentrated, separated by SDS-PAGE, electroblotted in CAPS buffer onto ProBlott membrane (Applied Biosystems), and stained with Coomassie Brilliant Blue. The NH2-terminal amino acid sequence of D1R-60K was determined by the University Peptide Sequencing Facility (SUNY Buffalo).

**Reconstitution of Methyltransferase Activity**

Varying amounts of either the partially purified D1R or the proteolytic fragment, D1R-60K, were mixed with D12L and allowed to incubate on ice. After 30 min, methyltransferase assays were performed as described above.

**Subunit Association**

Partially purified D12L and D1R were mixed and after 30 min on ice, and the sample was applied to a 1.5 × 40-cm Sephacryl S-200 (Pharmacia) column. The elution profile of D12L was determined by immunoblot analysis and the profile of D1R determined by detection of the enzyme-GMP intermediate. D1R, D12L, partially purified capping enzyme, as well as molecular weight markers were applied separately to the column to determine their elution profiles.

**RESULTS**

**Induction**—The plasmids, pET3a D1R-63, pET3a D12L-8, and pET3a D1R/D12L-3, which express the D1R, D12L, and both D1R and D12L proteins, respectively, were used to transform E. coli strains HMS174(DE3), HMS174(DE3) pLysS, BL21(DE3), and BL21(DE3) pLysS and induced to overexpress the subunits of the vaccinia virus capping enzyme. Consistent with the results of Guo and Moss (18) and Shuman (19), addition of 400 μM IPTG at 37 °C resulted in the synthesis of insoluble protein. Alternative induction conditions were tested in an attempt to increase the yield of soluble capping enzyme, including lowering the induction temperature (24), altering the concentration of IPTG, including lowering the induction temperature (24), altering the concentration of IPTG, the addition of ethanol (25), and varying the time course of induction. Enzyme was partially purified through the diazied ammonium sulfate fraction and assayed for methyltransferase activity.

**TABLE II**

| Fraction                  | Volume (ml) | Protein (mg) | ATPase (%) | Enzyme-GMP (%) | MTase (%) |
|---------------------------|-------------|--------------|------------|----------------|-----------|
| Whole cell extract        | 1,200       | 28,800       |            |                |           |
| S10                       | 1,370       | 28,770       |            |                |           |
| S100                      | 1,200       | 19,200       |            |                |           |
| 45% ammonium sulfate      | 180         | 6,840        |            |                |           |
| DEAE flow-through         | 444         | 533          | 142,080    | 57.72          | 3,241     |
| Phosphocellulose pool     | 39          | 41           | 36,270     | 15.60          | 1,197     |
| Hydroxylapatite pool      | 47          | 9.4          | 7          | 12.69          | 503       |
| S200 HR fraction 50       | 2.75        | 1.51         | 2,613      | 2.56           | 77        |
| S200 HR fraction 58       | 2.75        | 0.04         |            |                | 35        |

*a* Protein concentrations were determined by the Bio-Rad Protein Assay using bovine serum albumin as a standard.

*b* Total nanomoles of P1 released per 5 min at 5 mM ATP.

*c* Total nanomoles of enzyme-GMP complex formed in 10 min at 10 μM GTP.

*d* Total nanomoles of methyl groups transferred to GTP in 5 min at 20 μM AdoMet.

*e* Inhibited by P1 in the elution buffer.
could be harvested. A combination of low growth temperature, temperature induction was attempted with these strains to increase the D1R subunit expression. However, when a low temperature was used, the D1R subunit remained primarily insoluble under all induction conditions tested, and the soluble protein present was highly susceptible to proteolysis in the absence of D12L.

Enzyme Purification—Capping enzyme was purified from BL21(DE3) pLysS pET3a D1R/D12L-3 induced by the addition of 400 μM IPTG in the presence of 2.5% ethanol for 24 h at 15–20 °C. Four-hundred grams of induced cells were lysed, treated with DNase I, and insoluble material removed by centrifugation at 10,000 × g for 15 min. The cleavage of D1R into a prominent 60-kDa peptide observed by Guo and Moss (18) and Shuman (19) is greatly inhibited by the presence of phenylmethylsulfonyl fluoride in the lysis buffer; other protease inhibitors were not tested. Following centrifugation, PEI was added to 0.05%, and the nucleic acid precipitate was removed by centrifugation. The PEI supernatant was centrifuged at 100,000 × g and the S100 fraction treated with ammonium sulfate to 45% saturation. The 45% ammonium sulfate fraction was dialyzed and applied to DEAE-cellulose. The flow-through fraction, containing the capping enzyme, was applied to a phosphocellulose column. The methyltransferase and guanylyltransferase activities co-elute at 0.17 M NaCl, as reported previously (10, 18, 19).

Fractions from the phosphocellulose column were pooled, applied to hydroxylapatite, and eluted with a NaHPO₄ gradient (Fig. 1, A and B). The elution profile of this column revealed the separation of two peaks of guanylyltransferase activity. The full size D1R (Fig. 1B) co-eluted with D12L (not shown) and methyltransferase activity at approximately 300 mM NaHPO₄. D1R-60K, the proteolytic product of D1R, was well resolved from the full size capping enzyme, eluting at 170 mM NaHPO₄ (Fig. 1B). Methyltransferase activity appears to peak two or three fractions ahead of the major guanylyltransferase activity. This may be due to a partial separation of two methyltransferase components present in the activity peak (see below).

Fractions 59–69, which contained the full size capping enzyme, were pooled, concentrated, and applied to a Sephacryl S200 HR sizing column. The resulting profile exhibited two methyltransferase activity peaks (Fig. 1C). ATPase and guanylyltransferase activities (Fig. 1, C and D) co-eluted with the first methyltransferase activity peak. SDS-PAGE analysis (Fig. 2A) revealed the presence of both the D1R subunit and the D12L subunit, demonstrating that the first peak contains full size capping enzyme. An analysis of the second broader methyltransferase component revealed a lack of ATPase activity (Fig. 1C) and very low guanylyltransferase activity which consisted of a minor amount of D1R and its proteolytic product D1R-60K (Fig. 1D). Coomassie Brilliant Blue staining of SDS-PAGE gels revealed the presence of D12L in both methyltransferase peaks (Fig. 2A). In addition to D12L, the second methyltransferase component contains a family of slower migrating bands of 30–40 kDa as seen in the Coomassie Brilliant Blue-stained gel. In order to determine the relationship of this family of proteins to the D1R subunit of the capping enzyme, antisera directed against either the NH₂ terminus or carboxyl terminus of D1R was generated. Immunoblot analysis revealed that the non-D12L proteins present in the second methyltransferase peak were derived from the carboxyl terminus of D1R (Fig. 2C). NH₂-terminal proteolytic fragments found preferentially in the first activity peak (Fig. 2B) are due to nicking of the large subunit in such a way that the products remain in the enzyme complex and are released after denaturation in SDS. These results demonstrate that the methyltransferase domain is separable from the mRNA triphosphatase, guanylyltransferase, and ATPase active sites and that this domain must lie either within the D12L subunit alone, the carboxyl terminus of D1R, or, alternatively, be shared between these two proteins (17, 30).

Capping enzyme produced in the E. coli expression system was purified to near homogeneity through the scheme described above (Table II and Fig. 3). DEAE-cellulose chromatography resulted in a 13-fold purification of the 45% am-
monium sulfate pellet. Methyltransferase activity appeared to increase as endogenous AdoMet and potential inhibitors were removed by this resin. The phosphocellulose column resulted in a 3.5-fold increase in the specific activity of guanylyltransferase and a 5-fold increase in methyltransferase specific activity. There was a significant decrease in total activity as compared with the DEAE-cellulose pool, since, based on SDS-PAGE analysis, only 60% of the phosphocellulose column peak was pooled and carried through the next step. The elution of the capping enzyme from the hydroxylapatite column resulted in a significant increase in purity of the enzyme leaving a few minor contaminating proteins (Fig. 3). The high NaHPO₄ required to elute the enzyme inhibits ATPase activity and partially inhibits the guanylyltransferase. The Sephacryl S200 HR column fractionated the hydroxylapatite pool into two general families of proteins (Figs. 2A and 3). Fractions from this column were not pooled, and aliquots were stored at -70 °C. Fractions 50 and 58 were assayed for methyltransferase activity. The specific activity did not significantly increase following elution from this column, since few contaminating proteins are removed. ATPase and enzyme-GMP formation activity were detected in fraction 50 but not fraction 58 (Fig. 1, C and D). Minor contamination observed in Fig. 3, lane 50, are proteolytic products of the D1R protein (Fig. 2C). Approximately 23% of the capping enzyme in fraction 50 is in an enzyme-GMP complex under standard assay conditions based on a protein concentration determined by the Bio-Rad Protein Assay.

Since ribonuclease contamination in the capping enzyme preparation would make detailed analysis of enzymatic activity difficult, the hydroxylapatite pool as well as fractions 50 and 58 from the Sephacryl S200 HR column were tested for ribonuclease activity. Enzyme was added to a 294-base 32P-labeled RNA under assay conditions that mimicked the guanylyltransferase reaction, and samples were removed at various times and analyzed by gel electrophoresis. Over the time course of the assay, ribonuclease activity was not detected in the hydroxylapatite pool or in the subsequent Sephacryl S200 HR fractions (Fig. 4).

The localization of the guanylyltransferase and mRNA triphosphatase to D1R was independently determined by Guo and Moss (18) and Shuman (17, 19). Shuman and Morham (31), through the use of carboxyl-terminal deletions, further mapped these activities to the amino two-thirds of the large subunit. The mRNA triphosphatase must also map to this fragment, since the guanylyltransferase assays contained triphosphate terminated poly(A) RNA as a substrate. We found that the purified proteolytic product, D1R-60K, contains the ATPase and GTPase activities as well (not shown). The proteolytic fragment, D1R-60K, was separated from the capping enzyme on both hydroxylapatite (Fig. 1B) and heparin-agarose (Fig. 5A). In order to localize the 60-kDa proteolytic product within D1R, a fraction from a heparin-agarose column containing D1R-60K was concentrated, separated on SDS-PAGE, transferred to a ProBlott membrane, and the NH₂-terminal sequence determined. The sequence, MDANVVSSST, corresponds to the first 10 amino acids of D1R, demonstrating that the guanylyltransferase, mRNA triphosphatase, and ATPase domains lie within the NH₂-terminal 60 kDa of the large subunit.

**Reconstitution of Methyltransferase Activity**—In order to determine which subunit of the capping enzyme is required for methyltransferase activity, a plasmid encoding each subunit was introduced into BL21(DE3) pLysS and induced at 22 °C. D12L was fractionated to approximately 20% purity (Fig. 5A) and appeared stable throughout the purification, unlike the subunit induced at 37 °C in BL21(DE3) (18). Cells induced to synthesize D1R did not yield high quantities of soluble enzyme. Furthermore, D1R was more susceptible to proteolysis in the absence of D12L and so proved difficult to purify to any great extent (Fig 5A). The proteolytic fragment of D1R, D1R-60K, was separated from the full size capping enzyme by passage over a heparin-agarose column and is approximately 10% pure (Fig. 5A). Each extract was assayed for the ability to form an enzyme-GMP complex (Fig. 5B). As expected, fractions containing D12L alone lacked guanylyltransferase activity, whereas samples containing D1R formed an enzyme-GMP complex. Fractions 35 and 37 derived from
the heparin-agarose column lacked full size capping enzyme but contained active D1R-60K. Comparing partially purified D1R expressed alone and D1R-60K fractions, there is at least 20 times less guanyltransferase activity in the D1R preparation. However, impurities in the D1R preparation may have decreased the enzyme-GMP formation activity of the fraction.

Methyltransferase assays performed using D1R, D1R-60K, or D12L alone lack methyltransferase activity. In order to determine if methyltransferase activity could be reconstituted by mixing the subunits, assays containing varying amounts of D1R and D12L were performed (Fig. 5C). As the level of D12L was increased from 0.0012 to 0.12 μg, at different D1R levels, an increase in the incorporation of methyl groups into "GTP was seen. This increase was dependent on the presence of GTP (Fig. 5C), and ATP could not substitute as an acceptor (not shown). Therefore, both the D1R and D12L subunits are required for the vaccinia virus methyltransferase activity. In order to determine if reconstitution of methyltransferase activity required the carboxyl terminus of the D1R subunit, the addition of D12L to the NH2 terminus of D1R, the D1R-60K fragment, was tested. Mixing experiments using aliquots of D12L and D1R-60K were performed (Fig. 5D). Methyltransferase activity was not detected after mixing D1R-60K with D12L, demonstrating that the COOH-terminal region of D1R is required to reconstitute methyltransferase activity. These results are consistent with the presence of D1R carboxyl-terminal fragments in the second methyltransferase peak of the Sephacryl S200 HR column (Fig. 2C) and confirm the observations of Cong and Shuman (30).

In order to determine if a physical association of the two subunits is required for reconstitution of methyltransferase activity, the partially purified subunits were mixed and applied to a Sephacryl S200 sizing column (Fig. 6). Co-expressed capping enzyme subunits yielded a profile in which guanylyltransferase and methyltransferase co-eluted. An extract containing D1R in the absence of D12L eluted primarily as a high molecular weight aggregate with a trailing shoulder of enzyme-GMP complex formation activity. When extracts containing D12L were applied to the column, the small subunit peaked in fractions 31 and 32 as determined by immunoblot analysis (not shown). Extracts of D1R, containing its proteolytic fragment, D1R-60K, and D12L expressed separately, and applied to the column resulted in a shift in the elution profile of both D1R and D12L. A fraction of the D1R aggregate dissociated and the enzyme-GMP complex formation now eluted in the region of the native capping enzyme. A portion of D12L eluted as a higher molecular weight species at the position of the co-expressed capping enzyme. Methyltransferase activity was now detectable and eluted similarly to the native capping enzyme (Fig. 6B). Therefore, the D1R and D12L subunits must associate to reconstitute methyltransferase activity. The elution of the D1R-60K fragment was unchanged by the presence of D12L (Fig. 6C), providing further evidence that the NH2-terminal domain of D1R does not bind to the D12L subunit.

**DISCUSSION**

In order to purify enough of the vaccinia virus mRNA capping enzyme to carry out physical studies, it was necessary to overexpress the subunits in soluble form well above the levels found in virions or in virus-infected cells. Expression vectors were constructed which direct the synthesis of capping enzyme subunits (CE), a crude preparation of D1R/D1R-60K, partially purified D12L, or a mixture of D1R/D1R-60K and D12L. A, D1R enzyme-GMP complex formation was assayed and presented as picomoles of "GTP formed in 30 min at 5 μM AdoMet. O, D1R/D1R-60K; •, D1R/D1R-60K + D12L; A, partially purified co-expressed subunits (CE) control. B, methyltransferase activity was assayed and presented as picomoles of guanyltransferase activity of the fraction. C, D1R-60K enzyme-GMP formation was assayed as described above. O, D1R/D1R-60K; •, D1R/D1R-60K + D12L; A, partially purified co-expressed subunits (CE). D1R aggregate dissociated and the enzyme-GMP complex formation now eluted in the region of the native capping enzyme. A portion of D12L eluted as a higher molecular weight species at the position of the co-expressed capping enzyme. Methyltransferase activity was now detectable and eluted similarly to the native capping enzyme (Fig. 6B). Therefore, the D1R and D12L subunits must associate to reconstitute methyltransferase activity. The elution of the D1R-60K fragment was unchanged by the presence of D12L (Fig. 6C), providing further evidence that the NH2-terminal domain of D1R does not bind to the D12L subunit.
zyme that was 90% pure and ribonuclease-free. Chromatography of the hydroxylapatite pool on Sephacyr S200 HR resolved two peaks of methyltransferase activity. The first peak was shown by SDS-PAGE analysis to contain full size capping enzyme, exhibiting both the ATPase and guanylyltransferase activities. The second peak contained the small subunit, D12L, in addition to a family of proteins shown by immunoblot analysis to be carboxyl-terminal fragments of the large subunit D1R. The number of proteolytic fragments in this methyltransferase fraction varied between preparations of enzyme.

A 60-kDa proteolytic fragment of D1R formed in E. coli is capable of catalyzing the mRNA triphosphatase and guanylyltransferase reactions (18, 19). Shuman and Morham (31) localized this fragment through deletion analysis to the amino-terminal two-thirds of D1R. We sequenced the NH₂-terminal portion of the 60-kDa fragment and demonstrated that it was derived from the NH₂ terminus of the large subunit. Additionally, we localized the ATPase/GTPase activity to this 60-kDa domain (not shown).

The partially purified D1R and D12L subunits individually lack methyltransferase activity. Through mixing experiments both subunits were shown to be required to reconstitute methyltransferase activity. In order to determine if association of the subunits was necessary for activity, a mixture of D1R and D12L was applied to a Sephacyr S200 column. Methyltransferase activity was found only in fractions which contained the associated subunits. In contrast, when D1R-60K, the NH₂-terminal fragment of D1R, was mixed with D12L, it both failed to reconstitute activity and physically associate with the small subunit. These results demonstrate that the reconstitution of the methyltransferase activity requires a physical association between the D12L subunit and the carboxyl-terminal domain of the D1R subunit. This model is supported by the co-elution of carboxyl-terminal fragments of D1R in the second peak of methyltransferase observed after Sephacyr S200 HR chromatography. Furthermore, the results of Cong and Shuman (30) are in full agreement with these conclusions.

A model of the functional domain structure of the mRNA capping enzyme based on prior experiments (17, 19, 31) and these results is presented (Fig. 7). The carboxyl terminus of D1R associates with D12L forming a dimeric protein complex capable of catalyzing the first three reactions in the capping pathway. In E. coli, a protease preferentially cleaves D1R releasing an NH₂-terminal 60-kDa domain and a family of 30–40-kDa COOH-terminal fragments. The NH₂-terminal domain of D1R, D1R-60K, contains the GTPase, ATPase, mRNA triphosphatase, and guanylyltransferase active sites. The D1R COOH-terminal domain associated with the D12L subunit contains the methyltransferase active site.

The presence of multiple domains possessing enzyme activities required for cap formation establishes the presence of at least two active sites required for mRNA capping and at least three different sites capable of binding GTP for the GTPase, guanylyltransferase, and methyltransferase activities. The arrangement of the mRNA triphosphatase and guanylyltransferase activities within a single complex active site or multiple sites is currently being investigated. The methyltransferase activity is clearly carried out at a different location. We are currently attempting to identify the subunits involved in AdoMet and GTP binding at this site in order to determine the role of each subunit in methyltransferase activity.

This arrangement raises several fundamental questions related to the number of mRNA binding sites on the enzyme and the need to move the 5' end of the mRNA product of the guanylyltransferase reaction to the methyltransferase active site. Currently, we are trying to further localize the functional domains of the catalytic activities of this remarkable enzyme as well as understand its role in both viral early gene transcription termination and intermediate gene expression.

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