Catalytic Activity of Vaccinia mRNA Capping Enzyme Subunits Coexpressed in Escherichia coli*

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RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7)-methyltransferase activities are associated with the vaccinia virus mRNA capping enzyme, a heterodimeric protein containing polypeptides of $M_r 95,000$ and $M_r 31,000$. The genes encoding the large and small subunits (corresponding to the D1 and the D12 ORFs, respectively, of the viral genome) were coexpressed in Escherichia coli BL21(DE3) under the control of a bacteriophage T7 promoter. Guanylyltransferase activity (assayed as the formation of a covalent enzyme-guanylate complex) was detected in soluble lysates of these bacteria. A 1000-fold purification of the guanylyltransferase was achieved by ammonium sulfate precipitation and chromatography using phosphocellulose and SP-Sepharose columns. Partially purified guanylyltransferase synthesized GpppA caps when provided with 5'-triphosphate-terminated poly(A) as a cap acceptor. In the presence of AdoMet the enzyme catalyzed concomitant cap methylation with 99% efficiency. Inclusion of $S$-adenosyl methionine increased both the rate and extent of RNA cap formation with 99% efficiency. In the presence of AdoMet the enzyme catalyzed concomitant cap methylation with 99% efficiency. Inclusion of $S$-adenosyl methionine increased both the rate and extent of RNA cap formation with 99% efficiency. Inclusion of $S$-adenosyl methionine increased both the rate and extent of RNA cap formation with 99% efficiency.

Attempts to analyze the domain structure of vaccinia capping enzyme have been frustrated previously by the inability to dissociate the subunits with preservation of catalytic activity. Recently, the identification of the viral genes encoding the $M_r 95,000$ and $M_r 31,000$ polypeptides has made feasible a molecular genetic approach to the problem. The gene encoding the large enzyme subunit has been mapped to the D1 ORF (open reading frame) of the viral genome (6, 7). The large subunit participates in the transguanylation step in the cap synthesis pathway by forming an enzyme-guanylate intermediate (8). The intermediate consists of a GMP residue attached covalently to the $M_r 95,000$ enzyme subunit via a phosphoamide bond to the $\epsilon$-amino group of a single lysine (8–10). It is not clear, though, whether the large subunit is sufficient to catalyze GMP transfer, or if participation of the small subunit is required as well. The gene encoding the small subunit has been mapped to the D12 ORF of the vaccinia genome (11), yet no biochemical function has been assigned to this polypeptide.

The present report demonstrates the synthesis of active vaccinia virus mRNA capping enzyme in Escherichia coli as a consequence of coexpression of the D1 and D12 ORFs. The enzyme has been purified 1000-fold and shown to catalyze all three steps in cap formation. A separate paper (17) describes the heterologous expression of the large subunit alone and the assignment of specific enzymatic properties to that polypeptide per se.

EXPERIMENTAL PROCEDURES

Construction of Plasmids

det-D1—pUC-D, a plasmid containing the entire genomic HindIII D fragment of vaccinia WR in pUC13 (a gift of Dr. Michael Merckinovsky, National Institutes of Health), was cleaved with endonucleases NciI (at nucleotide 9676 of the D1 fragment sequence (7)) and PstI (in the plasmid polylinker) and then religated to generate pUC-D1. A fragment containing the D1 ORF was excised from pUC-D1 by cleavage with HindIII and Sall and then inserted into M13mp18 (that had been cut with HindIII and Sall) in order to generate phage M13-D1. Uracil-substituted single-stranded DNA was isolated from M13-D1 that had been grown in E. coli CJ236 (dut+, ung-). The D1 gene was then altered by site-directed mutagenesis (12) so as to (1) create an Ndel restriction site at the translation initiation codon of the D1 ORF, and (2) eliminate an internal Ndel restriction site (at nucleotide 713 of the D1 sequence) while preserving the coding information at the amino acid level. This was performed by hybridizing to the uracil-substituted M13-D1 DNA two oligonucleotides designed to effect these sequence alterations and then converting the doubly primed circles to replicative form by the action of the DNA polymerase III system and DNA ligase purified from E. coli (provided generously by Drs. K. Marinas and R. Digest, Sloan-Kettering Institute). Phase plaques arising from transformation of the replicative form into E. coli transformants were isolated,

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coli JM105 were amplified. Single-stranded DNA was isolated from phage supernatants, and the presence of the two oligonucleotide-directed mutations was assayed by dideoxy sequencing. Replicative form DNA was isolated from cells infected with appropriately mutated phage, and a HindIII/SalI restriction fragment containing the mutated D1 ORF was cloned into pUC19 (cleaved with HindIII and SalI) to generate pD1-Nde. A 2.8-kilobase pair NdeI restriction fragment of pD1-Nde that contained the HindIII site of the D1 ORF was then cloned into the NdeI restriction site of the T7-based expression vector pET3c (13) to generate pET-D1. A schematic illustration of pET-D1 is shown in Fig. 1.

Expression of D1 and D12 ORFs in E. coli
pET-D1, pET-D12, and pET-D1/D12 were used to transform E. coli strains BL21, BL21(DE3), and HMS174 (14). Gene expression was induced either by infection of the plasmid-bearing BL21 or HMS174 bacteria with AcE6 as described (14, 15), or, in the case of BL21(DE3), by addition of IPTG to 0.4 mM (14, 15). Preparation of lysates from small scale cultures was performed as described (15). Lysates were induced by centrifugation into insoluble and soluble protein fractions. The polypeptide composition of these crude fractions was analyzed by electrophoresis through 10% polyacrylamide gels containing 0.1% SDS, with visualization of protein by staining with Coomassie Blue dye.

Enzyme Assays
RNA guanylyltransferase activity was assayed in crude bacterial extracts and throughout purification by the formation of [γ-32P]GTP into acid-insoluble material in the presence of triphosphate-terminated poly(A) as a cap acceptor. Preparation of cap acceptor RNA was performed as described (3). Reaction conditions are specified in the figure legends. RNA triphosphatase was assayed as the release of 32P, from γ-32P-poly(A) as described (16). RNA (guanine-7)-methyltransferase activity was assayed by conversion of cap-labeled poly(A) (GpppAp(A)) to methylated capped poly(A) ([Gppp]Ap(A)) in the presence of AdoMet, as described (16). Cap-labeled poly(A) was prepared using capping enzyme purified from vaccinia virions (16). Reaction conditions are specified in the figure legends.

Enzyme Purification: Coexpressed Subunits
A 1-liter culture of BL21(DE3)pET-D1/D12 was grown in LB medium with 100 µg/ml ampicillin until A600 = 1.0, then cells were harvested by centrifugation and stored at −80 °C. All subsequent procedures were performed at 4 °C. Cell lysis was achieved by treatment of thawed, resuspended cells (volume = 60 ml) with lysozyme and Triton X-100 as described (15). Insoluble material was removed by centrifugation at 20,000 rpm for 60 min in a Sorval SS34 rotor. Nucleic acid was depleted from the supernatant by dropwise addition of 2 mM F solution (that had been equilibrated with 0.5 M NaCl) to prepare a 0.05 M NaCl final concentration. Insoluble material was removed by centrifugation for 30 min at 20,000 rpm. The polyvinyl pyrrolidone supernatant was adjusted to 20% saturation by the addition of solid ammonium sulfate. Precipitate was recovered by centrifugation for 30 min at 20,000 rpm and the 0–20% ammonium sulfate pellet resuspended in 10 ml of buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 1% polyvinyl alcohol) to a final concentration of 20% ammonium sulfate precipitate was collected by centrifugation and resuspended in 20 ml of buffer A to a final concentration of 60% ammonium sulfate and centrifuged for 30 min at 20,000 rpm.

The 20–40% ammonium sulfate preparation, containing most of the guanylyltransferase activity, was diluted 2.5 fold with buffer A and then applied to a 20-ml column of phosphocellulose that had been equilibrated with buffer A. After washing with buffer A the column was step-chelated serially with 0.5 M NaCl in buffer A and 1.0 M NaCl in buffer A. Guanylyltransferase activity was eluted on the column and eluted in the 0.5 M NaCl step (data not shown). The phosphocellulose preparation (4 ml) was dialyzed against buffer A (500 ml, with two changes) and applied to a SP-SF column (8 X 75 mm) that had been equilibrated with buffer A. The column was washed with buffer A and developed with a 50-ml gradient of 0.5 to 1.0 M NaCl in buffer A using a Waters 650 chromatography system.

Materials
Phosphocellulose P11 was obtained from Whatman. All radionucleotides were purchased from Amersham Corp. Restriction endonucleases and other enzymes used in cloning were purchased from either New England BioLabs or Bethesda Research Labs. AdoMet and nuclease P1 were purchased from Boehringer. Reagents for protein determination were a product of Bio-Rad.

RESULTS
Expression of Capping Enzyme Subunits in E. coli—The bacteriophage T7-based system of Studier and Moffatt (14) was used to achieve expression of capping enzyme subunits in E. coli. The vaccinia D1 and D12 ORFs were cloned separately into the vector pET3c (13) to generate plasmids pET-D1 and pET-D12, and together in the same plasmid to generate pET-D1/D12 (Fig. 1). Initial experiments (not shown) were designed to demonstrate the synthesis of the D1 and D12 proteins upon provision of T7 RNA polymerase. Accordingly, E. coli BL21 carrying pET-D1 or pET-D12 were infected with AcE6, and the cellular polypeptide composition was analyzed electrophoretically at various times after infection. In the case of BL21(pET-D1), phage infection resulted in the time-dependent accumulation of a prominent Mf, 95,000 polypeptide as well as the parallel induction (at lower level) of a second protein of Mf, 39,000 (the latter being presumed to be a proteolytic product of the M, 95,000 polypeptide). The

1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SD5, sodium dodecyl sulfate; AdoMet, S-adenosylmethionine.
relative amounts of the two induced polypeptides was influenced by the bacterial host strain, eg. HMS174 (a strain that contains the lon and ompT proteases that are lacking in BL21) accumulated proportionately more M, 39,000 species than M, 95,000 protein upon phage infection. In the case of cells bearing pET-D12, ACE6 infection resulted in the time-dependent appearance of a single M, 31,000 polypeptide in either BL21 or HMS174, although the extent of accumulation appeared higher in HMS174. When T7 RNA polymerase was provided instead by IPTG induction of E. coli BL21(DE3) carrying pET-D1 or pET-D12, the pattern of inducible protein accumulation was the same as that found with phage infection. Cells bearing plasmid pETD1/D12 were induced to accumulate both the M, 95,000 and the M, 31,000 polypeptides when T7 RNA polymerase was provided by either method. All subsequent studies described in this and the succeeding report (17) involve coexpression of D1 and D12 ORFs from pET-D1/D12 or expression of D1 ORF alone from pET-D1.

**Solubility and Activity of Coexpressed Subunits**—Electrophoretic analysis of soluble and insoluble fractions derived from lysates of λCE6-infected BL21pETD1/D12 cells or IPTG-induced BL21(DE3)pET-D1/D12 cells revealed that the D1 and D12 polypeptides were detectable only in the insoluble pellet (not shown). The M, 95,000 and M, 31,000 proteins could be solubilized readily in either 8 M urea or 5 M guanidine HCl; however, both proteins precipitated upon removal of either denaturant by dialysis. Other manipulations, such as phage infection or IPTG induction at lowered temperatures (either 30 °C or 25 °C), failed to enhance the solubility of the expressed proteins. Guanylyltransferase activity was assayed in crude extracts by the formation of covalent protein-GMP complex, this being a highly sensitive and specific method for detection of capping enzyme. Formation of a 32P-labeled M, 95,000 polypeptide was mediated by insoluble protein from cells induced to express the D1 ORF, but not by the soluble protein fraction. Thus, the ability to bind GMP covalently was retained in some part by the large subunit even in insoluble form (see accompanying article (17)). Crude extracts of bacteria that did not carry the plasmid-borne vaccinia D1 ORF did not catalyze formation of the 32P-labeled M, 95,000 polypeptide (not shown).

Additional studies showed that BL21(DE3)pET-D1/D12 cells accumulated appreciable amounts of soluble guanylyltransferase activity without IPTG induction. This can be attributed to the basal level of T7 RNA polymerase expression from the lacUV5 promoter in the DE3 prophage. This permitted the purification of guanylyltransferase from the soluble fraction, as described under “Experimental Procedures” and summarized below.

**Enzyme Purification and Characterization: Coexpressed Subunits**—Incubation of the polyanion P supernatant fraction (containing soluble protein depleted of nucleic acid) with [α-32P]GTP and MgCl₂ resulted in the formation of a M, 95,000 guanylylated polypeptide, as well as two minor labeled species of lower molecular weight (Fig. 2). The electrophoretic mobility of the M, 95,000 labeled protein was identical with that of the enzyme-GMP intermediate formed by capping enzyme purified from vaccinia virions (not shown). The 20–40% ammonium sulfate fraction derived from the polyanion P supernatant contained most of the guanylyltransferase activity. This fraction catalyzed formation of a major M, 95,000 protein-GMP complex and a minor labeled polypeptide of M, 60,000 (Fig. 2). Residual M, 95,000 EpG-forming activity was recovered in the 40–60% and 60–80% ammonium sulfate fractions. The apparent resolution of the more rapidly migrating labeled polypeptides during ammonium sulfate fractionation (Fig. 2) may reflect the separation of endogenous proteases responsible for the formation of these smaller species.

The 20–40% ammonium sulfate fraction was purified further by sequential chromatography on columns of phosphocellulose and SP-SephW. M, 95,000 EpG formation activity was retained on the SP-SepW column and eluted at 0.16 M NaCl (Fig. 3; fraction 22, top panel). The minor M, 60,000 EpG-forming activity eluted at lower salt and was partially resolved from the M, 95,000 species (Fig. 3; fractions 18 and 20, top panel). Since the formation of EpG (an obligate step in mRNA capping) is a less stringent assay of guanylyltransferase than is the ability to transfer the GMP moiety to the 5' end of RNA, capping activity with a tripolyphosphate-terminated poly(A) acceptor was assayed across the column. A peak of GMP incorporation eluted in parallel with the EpG-forming activity (Fig. 3, middle panel). The broad appearance of the guanylyltransferase peak was attributable to assay conditions. Additional studies showed that BL21(DE3)pET-D1/D12 cells accumulated appreciable amounts of soluble guanylyltransferase activity without IPTG induction. This can be attributed to the basal level of T7 RNA polymerase expression from the lacUV5 promoter in the DE3 prophage. This permitted the purification of guanylyltransferase from the soluble fraction, as described under “Experimental Procedures” and summarized below.

**Expression of Vaccinia mRNA Capping Enzyme**

![FIG. 1. T7-based plasmids for the expression of vaccinia capping enzyme subunits. Construction of pET-D1 and pET-D1/D12 is described under "Experimental Procedures." The figure depicts the salient functional elements of the plasmids (not drawn to scale). The direction of transcription from the T7 promoter is indicated by the arrows.](image)

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![FIG. 2. Initial fractionation of RNA guanylyltransferase activity from BL21(DE3)pET-D1/D12. Aliquots (1 μl) of the indicated fractions were assayed for protein-guanylate complex formation as described under "Experimental Procedures." An autoradiograph of the protein gel is shown. The position of the labeled M, 95,000 capping enzyme subunit is indicated by the arrow. The protein concentrations of the fractions were: polyanion P supernatant, 4.11 mg/ml; 0–20% ammonium sulfate, (A.S.), 0.19 mg/ml; 20–40% ammonium sulfate, 1.69 mg/ml; 40–60% ammonium sulfate, 7.58 mg/ml; 60–80% ammonium sulfate, 2.55 mg/ml. Protein concentration was measured by dye binding using bovine serum albumin as a standard (23).](image)
Expression of Vaccinia mRNA Capping Enzyme

that reflected yield rather than rate of reaction.

Analysis of the products of the capping reaction by the E. coli enzyme is shown in Fig. 4. Control reaction mixtures (20 µl) contained 50 mM Tris-HCl pH 8.0, 1.25 mM MgCl₂, 5 mM dithiothreitol, 2.7 µM [α-³²P]GTP, 38 pmol (of ends) of triphosphate-terminated poly(A), and 3 µl of SP5PW fraction 23 (Fig. 3). A parallel set of reactions included 50 µM AdoMet. After incubation for 30 min at 37 °C, reactions were halted by the addition of 10% trichloroacetic acid. The RNA product was recovered by two cycles of precipitation with trichloroacetic acid, followed by sequential extractions with phenol-chloroform and chloroform, and a final ethanol precipitation step. The labeled RNA pellet was resuspended in 30 µl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Aliquots (3.5 µl) were digested with 5 µg of nuclease P1 prior to analysis by thin layer chromatography on polyethyleneimine cellulose plates developed with 0.45 M ammonium sulfate. An autoradiographic exposure of the chromatogram is shown. The positions of methylated and unmethylated caps are indicated on the left. Omission (−) or inclusion (+) of AdoMet in the reactions is indicated below the figure. The extent of methylation of the cap (expressed as m⁷GpppA/(m⁷GpppA + GpppA)) was determined by cutting out the labeled species and counting in liquid scintillation fluid and is indicated below each lane.

Cap methylation by native vaccinia capping enzyme is not obligately coupled to cap synthesis (4) and can be assayed with high sensitivity by the AdoMet-dependent conversion of 5’-guanylylated poly(A) to methylated capped poly(A), as described (16). Assay of RNA (guanine-7)-methyltransferase activity across the SP5PW column showed that this activity eluted broadly, but in parallel with RNA guanylyltransferase activity (Fig. 3, middle panel), suggesting that capping and methylating activities may be associated physically. In order to test this possibility, an aliquot of the peak SP5PW fraction 22 was centrifuged through a 15–30% glycerol gradient in buffer A containing 0.5 mM NaCl. M, 95,000 EpG-forming activity sedimented as a single component of 6.5 S relative to marker proteins that were sedimented in a parallel gradient (Fig. 5, top panel). The ability to cap triphosphate-terminated poly(A) cosedimented at 6.5 S. This sedimentation constant of the expressed guanylyltransferase was identical with the S value of the heterodimeric capping enzyme purified from vaccinia virions (3) and was higher than the 5.5 S value obtained for monomeric large subunit per se (17), suggesting that the coexpressed large and small subunits might form a heterodimeric complex in E. coli. Methylytransferase activity remained associated with guanylyltransferase during sedimentation (in gradient fractions 13–15), but the activity profile was diffuse and peaked in less rapidly sedimenting fractions that contained little guanylyltransferase (Fig. 5).
Capping of triphosphate-terminated RNA requires both RNA γ-phosphate cleavage and GMP transfer to the resulting diphosphate RNA end. RNA triphosphatase activity is intrinsic to the native capping enzyme from vaccinia virions, as is the ability to cleave the γ-phosphate of purine nucleoside triphosphates (3). Assay of the glycerol gradient fractions for RNA triphosphatase and ATPase (Fig. 6) revealed single peaks of enzyme activity at 6.5 S that were coincident with each other and with the activity profile of the guanylyltransferase. Characterization of the RNA triphosphatase and ATPase activities associated with the expressed capping enzyme is presented in Table I. RNA triphosphate cleavage required magnesium. Manganese was able to activate the triphosphatase partially, while calcium could not satisfy the divalent cation requirement. ATPase activity was activated by magnesium, manganese, and cobalt activated the ATPase, while calcium did not support activity. These features correspond exactly to the reaction requirements reported for the capping enzyme complex purified from vaccinia virions (3), suggesting that the triphosphate phosphohydrolase activity of the glycerol gradient fraction can be attributed to the expressed guanylyltransferase. Two prokaryotic enzymes that specifically cleave the γ-phosphate from RNA (and that also have NTPase activity) have been identified in E. coli (24). One of the bacterial enzymes, referred to as "alkaline RNA triphosphatase," is active without specificity for the 5'–RNA NTP base. The alkaline triphosphatase does not require magnesium for activity, however, thus ruling out the possibility that this enzyme is contributing to the RNA triphosphatase present in the capping enzyme preparation. The second bacterial enzyme, "ATP-terminated RNA triphosphatase," is substrate-specific as indicated. The sedimentation properties and cation specificity of this enzyme have not been reported. The polypeptide composition of glycerol gradient fractions

![Figure 6. γ-Phosphate cleavage activity associated with guanylyltransferase after sedimentation in a glycerol gradient. RNA triphosphatase reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 5 mM diithiothreitol, 25 pmol (of ends) γ-32P-poly(A) and enzyme (1 μl of a 50-fold diluted sample of the indicated glycerol gradient fractions) were incubated for 5 min at 37°C. Release of 32P, as quantitated as described (3), the activity profile is indicated by the solid triangles.](https://example.com/diagram.png)

![Figure 5. Glycerol gradient sedimentation. Sedimentation of SP5PW fraction 22 in a 15–30% glycerol gradient is described in the text. Fractions (0.185 ml) were collected from the bottom of the tube. Top panel, aliquots (1 μl) of the indicated fractions were assayed for protein–guanylate complex formation. An autoradiograph of the protein gel is shown. The position of the labeled M, 95,000 capping enzyme subunit is indicated by the arrow. Bottom panel, aliquots (1 μl) of the gradient fractions were assayed for guanylyltransferase activity as described in the legend to Fig. 3, except that the incubation was for 5 min at 37°C. Activity is plotted as the percent of the input RNA substrate converted to acid-insoluble material (picomoles, open circles).](https://example.com/diagram.png)
was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). RNA triphosphatase, ATPase, EpG, formation, and RNA guanylyltransferase activity profiles all correlated with the presence of a major polypeptide of M, 95,000 (indicated by *) in fractions 13–15. This M, 95,000 polypeptide comigrated with the D1 gene product expressed inducibly in E. coli (not shown). A polypeptide of M, 31,000 (that comigrated with the D12 gene product expressed inducibly in E. coli) was also detected in the peak fractions 13–15 (Fig. 7, lower *). This polypeptide was present as well in rapidly sedimenting fractions and appeared to correlate with methyltransferase activity. These data are consistent with the expressed capping enzyme purified from E. coli being a heterodimer of the D1 and D12 gene products. The existence of free methyltransferase activity and the implications for the domain structure of the capping enzyme are discussed below.

The purification of RNA guanylyltransferase from E. coli BL21(DE3)pET-D1/D12 is summarized in Table II. The EpG assay permits direct determination of the molar concentration of active enzyme molecules, assuming a stoichiometry of one GMP residue bound per M, 95,000 polypeptide. The enzyme was purified 1000-fold at the SP5PW step (peak fraction) with a yield at this step of 224%. The apparent increase in yield attributable to the removal of interfering activities, particularly bacterial GTPases. The amount of active enzyme obtained at this step from 1 liter of bacteria is comparable to the amount of guanylyltransferase purified from 1100 A260 of purified vaccinia virions (roughly, the yield of virus obtained from infecting 10–12 liters of HeLa suspension cells) (5). At the glycerol gradient step, the expressed guanylyltransferase had been purified 1300-fold. The specific activity of the peak glycerol fraction was 2580 pmol/mg of protein, compared with a theoretical maximum of 7874 for fully active homogenous enzyme of M, 127,000. The implication that the glycerol fraction is therefore 32% pure is not out of line with the electrophoretic analysis of polypeptide composition.

Quantitative RNA Capping by Expressed Capping Enzyme—A time course of GMP incorporation into a 5'-triphosphate-terminated poly(A) cap acceptor is shown in Fig. 8. Under conditions of enzyme excess, the expressed capping enzyme (SP5PW fraction) capped one-half of the input RNA ends. Inclusion of 50 μM AdoMet in the reaction increased the rate and extent of the reaction and resulted in the quantitative modification of all 5' ends. The effect of AdoMet on RNA modification by the expressed enzyme mimics that observed with the enzyme purified from virions (18). This is attributable to the fact that cap methylation renders the RNA terminus resistant to pyrophosphorolysis of the capped product (19) and to transfer of the guanylate moiety back to the enzyme large subunit (20), and therefore serves to drive the reaction to completion at equilibrium. The ability to cap quantitatively suggests that the SP5PW preparation does not contain high levels of (1) nonspecific phosphatases that would convert the 5' ends to 5'-monophosphate or 5'-hydroxyl forms that are not cap acceptors for the vaccinia enzyme, or (2) nucleotidyl pyrophosphatases that would degrade the triphosphate cap bridge after cap formation. The exact levels of these activities in the enzyme preparation have not been determined directly, however.

DISCUSSION

The capping enzyme purified from infectious vaccinia virus particles contains polypeptides of M, 95,000 and M, 31,000 that are encoded, respectively, by the D1 and D12 viral genes. The present study proves that these two gene products, when coexpressed in a heterologous system, are sufficient to mediate all three enzymatic reactions (RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7)-methyltransferase) leading to the formation of "cap zero" 5' ends. The enzymatic and physical properties of the recombinant guanylyltransferase purified from E. coli are consistent with those of the enzyme obtained from virions. In particular, the large

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**TABLE II**

Purification of RNA guanylyltransferase from E. coli BL21(DE3)pET-D1/D12

| Fraction       | Capping enzyme | Protein | Specific activity | Yield |
|----------------|----------------|---------|------------------|-------|
| Polymatin P    | nm             | pmol    | μg/ml            | mg    | pmol enzyme/μg protein | %     |
| 20–40% ammonium sulfate | 11.1           | 222     | 1700             | 33.9  | 6.53               | 56    |
| Phosphocellulose | 11.5           | 517     | 157              | 7.1   | 73.2               | 130   |
| SP5PW Pool (21–23) | 6.6            | 396     | 4110             | 246   | 1.61               | 100   |
| Fraction 22    | 449            | 887     | 0.82             | 1081  | 224                |       |
| Glycerol gradient | 166            | 58.7    | 0.028            | 2075  | 131*               |       |

* The yield at the glycerol step is a normalized value based on the amount of enzyme applied to the gradient.
and small enzyme subunits appear to form a heterodimeric complex in the prokaryotic milieu, much as they do during the early phase of the vaccinia growth cycle when they are newly synthesized (21) and during their encapsidation into the virus core. The recombinant heterodimeric guanylyltransferase expresses cap methylation, yet clearly not all methyltransferase expressed in E. coli is associated physically with guanylyltransferase (Fig. 5). A plausible explanation for this finding is implicit in the following model of the organization of functional domains within the capping enzyme complex. I draw upon a previous model (16) and propose that (1) the RNA triphosphatase and RNA guanylyltransferase domains reside on the $M_2$, 95,000 subunit and that this protein per se is sufficient to catalyze γ-phosphate cleavage and nucleotidyl transfer; (2) the methyltransferase domain resides on the $M_2$, 31,000 subunit, which is itself sufficient to cap methyltransferase; (3) association of the two subunits with 1:1 stoichiometry is an inherent property of the subunits and requires no other viral factors. The existence of free methyltransferase is thereby accounted for by a molar excess of the small subunit relative to the large in E. coli BL21(DE3)pET-D1/D12. This excess is precisely what is expected based on the nature of the coexpression plasmid pET-D1/D12. D12 expression is driven from two T7 promoters while D1 expression is directed by only one promoter (Fig. 1). Therefore, all other factors being equal (e.g. mRNA stability, translation efficiency, protein solubility, protein stability, etc.), the small subunit should accumulate to a higher steady-state level than the large subunit.

Testing of various aspects of this proposal should be feasible through a molecular genetic approach involving expression of individual subunits in E. coli. The accompanying paper (17) describes the catalytic properties of the large capping enzyme subunit and substantiates the domain structure of this protein discussed above.

Finally, the vaccinia mRNA capping enzyme has been a valuable reagent for the manipulation of the 5' terminal structure of RNA (22). Purification of the enzyme from large quantities of virions necessitates a facility for cell culture and exposure of personnel to infectious pathogens. The ability to purify the capping enzyme from bacteria (in good amounts and with high yield) using the relatively simple procedure presented above may prove useful for studies of the role of the 5' RNA cap in mRNA biogenesis and function.

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