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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 guanylyltansferase was achieved by ammonium sulfate precipitation and chromatography using phosphocellulose and SP5PW 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 capping, permitting quantitative modification of RNA 5′ ends. Guanylyltransferase sedimented as a single component of 6.5 S during further purification in a glycerol gradient; this S value is identical with that of the heterodimeric capping enzyme from vaccinia virions. Electrophoretic analysis showed a major polypeptide of M_r 95,000 cosedimenting with the guanylyltransferase. RNA triphosphatase activity cosedimented exactly with guanylyltransferase. Methyltransferase activity was associated with guanylyltransferase and was also present in less rapidly sedimenting fractions. The methyltransferase activity profile correlated with the presence of a M_r 31,000 polypeptide. These results indicate that the D1 and D12 gene products are together sufficient to catalyze all three enzymatic steps in cap synthesis. A model for the domain structure of this enzyme is proposed.

Capping of the 5′ terminus of vaccinia virus mRNA occurs by a series of three reactions catalyzed, respectively, by RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7)-methyltransferase (1). These three enzymes are components of a heterodimeric capping enzyme complex containing subunit polypeptides of M_r 95,000 and M_r 31,000 (2–4). In addition to its role in 5′ RNA modification, the vaccinia capping enzyme is involved in correct 3′ end formation of vaccinia early mRNAs by acting as a transcription termination factor for the vaccinia DNA-dependent RNA polymerase (5).

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 capping 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 ε-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

pET-D1—pUC-D, a plasmid containing the entire genomic *Hin*dIII D fragment of vaccinia WR in pUC13 (a gift of Dr. Michael Merchlinsky, National Institutes of Health), was cleaved with endonucleases *Nsi*I (at nucleotide 2676 of the D-fragment sequence (7)) and *Pst*I (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 *Hin*dIII and *Sal*I and then inserted into M13mp18 (that had been cut with *Hin*dIII and *Sal*I) 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 *Nde*I restriction site at the translation initiation codon of the D1 ORF, and (2) eliminate an internal *Nde*I 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. Marians and R. Digate, Sloan-Kettering Institute). Phage plaques arising from transformation of the replicative form into *E.*

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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 *Hin*dIII/*Sal*I restriction fragment containing the mutated D1 ORF was cloned into pUC19 (cleaved with *Hin*dIII and *Sal*I) to generate pD1-Nde. A 2.8-kilobase pair *Nde*I restriction fragment of pD1-Nde that contained the D1 ORF was then cloned into the *Nde*I 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.

pET-D12—A D12 ORF-containing fragment spanning nucleotides 13416–15178 of the *Hin*dIII D genomic fragment (7) was excised from pUC-D by cleavage with *Nsi*I and then cloned into the *Pst*I restriction site of pUC19 to generate pUC-D12#5. The viral DNA insert was excised from the polylinker of pUC-D12#5 with *Bam*HI and *Hin*dIII and cloned into M13mp18 (that had been restricted with *Bam*HI and *Hin*dIII) to generate M13-D12-5/3. Uracil-substituted phage DNA was prepared as above, and oligonucleotide-directed mutagenesis was used to create an *Nde*I restriction site at the translation initiation codon of the D12 ORF. A *Hin*dIII/*Bam*HI restriction fragment containing the mutated D12 gene was excised from replicative form phage DNA and cloned into pUC18 to generate pUC-D12-Nde. A 0.93-kilobase pair *Nde*I/*Bam*HI fragment of pUC-D12-Nde that contained the D12 ORF was then cloned into the T7-based vector pET3c (that had been restricted with *Nde*I and *Bam*HI) to generate pET-D12.

pET D1/D12—The D12 expression cassette (including the T7 promoter, ribosome binding site, D12 ORF, and T7 transcription terminator) was excised from pET-D12 by restriction with *Bgl*II and *Hin*dIII nucleases. The recessed ends of the fragment were made blunt by treatment with Klenow DNA polymerase, and the cassette was then cloned into the *Sma*I restriction site of pET-D1. This procedure yielded the plasmid, pET -D1/D12, containing D1 and D12 expression cassettes in tandem and in the same transcriptional orientation (Fig. 1). It is predicted that the D1 gene product will be translated from a dicistronic mRNA initiating at the leftward T7 promoter and terminating downstream of the D12 gene, while the D12 protein will be translated from two mRNAs, the dicistronic mRNA containing D1 and D12 in tandem and a monocistronic mRNA initiating at the rightward T7 promoter and coterminating with the dicistronic RNA (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 λCE6 as described (14, 15), or, in the case of BL21(DE3), by addition of IPTG[1] to 0.4 mM (14, 15). Preparation of lysates from small scale cultures was performed as described (15). Lysates were separated 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 ^{32}P-labeled enzyme-guanylate complex (8). Reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.17 μM [α-^{32}P]GTP (1000–3000 Ci/mmol), and enzyme were incubated for 5 min at 37 °C. Reactions were halted by the addition of SDS, and samples were analyzed by electrophoresis through 10% polyacrylamide gels containing 0.1% SDS. Electrophoresis was at 50 mA until the bromphenol blue dye had migrated out of the gel. Gels were dried and labeled polypeptides visualized by autoradiography. *E*pG formation activity was quantitated by cutting out gel slices containing the labeled polypeptide(s) and counting Cerenkov radioactivity. Activity was determined within a linear range of *E*pG formation *versus* added enzyme fraction. One unit of activity formed 1 pmol of protein-guanylate complex in the standard assay. RNA capping (requiring the action of both RNA triphosphatase and RNA guanylyltransferase) was measured as the incorporation of label from [α-^{32}P]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 $^{32}P_i$ from γ-^{32}P-poly(A) as described (16). RNA (guanine-7)-methyltransferase was assayed by conversion of cap-labeled poly(A) (GpppAp(A)$_n$) to methylated capped poly(A) (^{m7}GpppAp(A)$_n$) in the presence of AdoMet, as described (16). Cap-labeled poly(A) was prepared using 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 A_{600} = 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 Sorvall SS34 rotor. Nucleic acid was depleted from the supernatant by dropwise addition of a polymin P solution (1% (w/v)) with rapid stirring to 0.05% final concentration. Insoluble material was removed by centrifugation for 30 min at 20,000 rpm. The polymin P 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, 0.1% Triton X-100). The 20% ammonium sulfate supernatant was adjusted to 40% saturation; the 20–40% ammonium sulfate precipitate was collected by centrifugation as above and resuspended in 20 ml of buffer A. 40 to 60% ammonium sulfate and 60 to 80% ammonium sulfate precipitates were prepared sequentially in similar fashion and pellets resuspended in 20 ml of buffer A.

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-eluted serially with 0.5 M NaCl in buffer A and 1.0 M NaCl in buffer A. Guanylyltransferase activity was retained on the column and eluted in the 0.5 M NaCl step (data not shown). The phosphocellulose preparation (45 ml) was dialyzed against buffer A (500 ml, with two changes) and applied to a SP5PW column (8 × 75 mm) that had been equilibrated with buffer A. The column was washed with buffer A and developed with a 50-ml gradient of 0–1.0 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 λCE6, and the cellular polypeptide composition was analyzed electrophoretically at various times after infection. In the case of BL21pET-D1, phage infection resulted in the time-dependent accumulation of a prominent M_r 95,000 polypeptide as well as the parallel induction (at lower level) of a second protein of M_r 39,000 (the latter being presumed to be a proteolytic product of the M_r 95,000 polypeptide). The

[1] The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; AdoMet, *S*-adenosylmethionine.

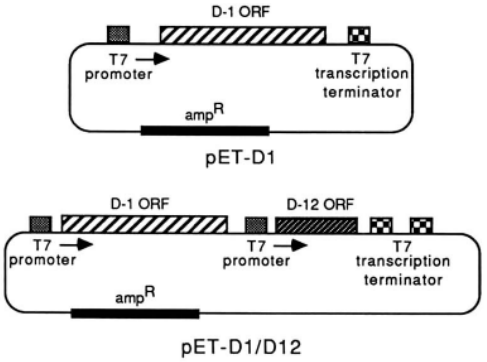

FIG. 1. **T7-based plasmids for the expression of vaccinia capping enzyme subunits.** Construction of pET-D1 and pET-D1/D12 is described under "Experimental Procedures." The figure depicts the salient functional elements of the plasmids (not drawn to scale). The direction of transcription from the T7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

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_r 39,000 species than M_r 95,000 protein upon phage infection. In the case of cells bearing pET-D12, λCE6 infection resulted in the time-dependent appearance of a single M_r 31,000 polypeptide in either BL21 or HMS174, although the extent of accumulation appeared higher in HMS174.[2] 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_r 95,000 and the M_r 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_r 95,000 and M_r 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 ^{32}P-labeled M_r 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 ^{32}P-labeled M_r 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 polymin P supernatant fraction (containing soluble protein depleted of nucleic acid) with [α-^{32}P]GTP and $MgCl_2$ resulted in the formation of a M_r 95,000 guanylylated polypeptide, as well as two minor labeled species of lower molecular weight (Fig. 2). The electrophoretic mobility of the M_r 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 polymin P supernatant contained most of the guanylyltransferase activity. This fraction catalyzed formation of a major M_r 95,000 protein-GMP complex and a minor labeled polypeptide of M_r 60,000 (Fig. 2). Residual M_r 95,000 *E*pG-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 SP5PW. M_r 95,000 *E*pG formation activity was retained on the SP5PW column and eluted at 0.16 M NaCl (Fig. 3; fraction 22, *top panel*). The minor M_r 60,000 *E*pG-forming activity eluted at lower salt and was partially resolved from the M_r 95,000 species (Fig. 3; fractions 18 and 20, *top panel*). Since the formation of *E*pG (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 triphosphate-terminated poly(A) acceptor was assayed across the column. A peak of GMP incorporation eluted in parallel with the *E*pG-forming activity (Fig. 3, *middle panel*). The broad appearance of the guanylyltransferase peak was attributable to assay conditions

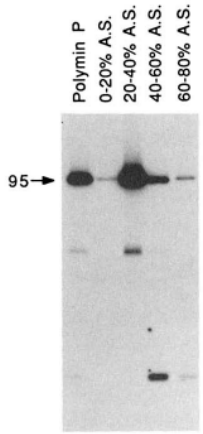

FIG. 2. **Initial fractionation of RNA guanylyltransferase activity from BL21(DE3)pET-D1/D12.** Aliquots (1 μl) of the indicated fractions were assayed for protein-guanylate complex formation as described under "Experimental Procedures." An autoradiograph of the protein gel is shown. The position of the labeled M_r 95,000 capping enzyme subunit is indicated by the *arrow*. The protein concentrations of the fractions were: polymin 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).

[2] M. Spector and S. Shuman, unpublished work.

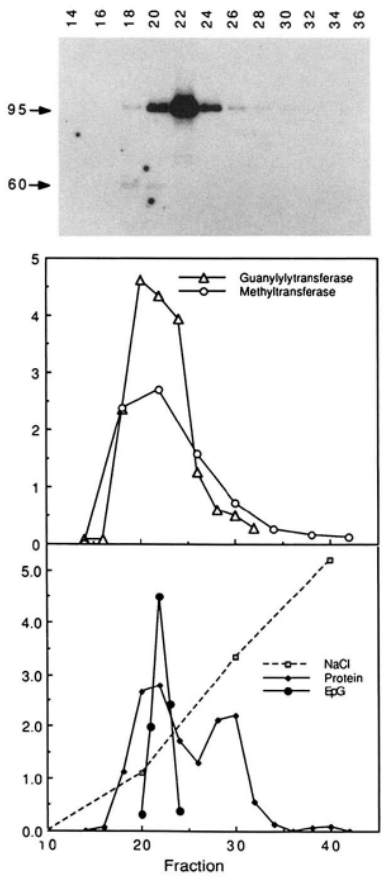

FIG. 3. **SP5PW chromatography.** The dialyzed phosphocellulose fraction was chromatographed on SP5PW as described in the text. Fractions (1 ml) were collected during salt gradient elution. *Top panel,* aliquots (1.5 μl) of the indicated fractions were assayed for protein-guanylate complex formation. An autoradiograph of the protein gel is shown. The position of the labeled M_r 95,000 capping enzyme subunit is indicated by the *arrow. Middle panel,* guanylyltransferase reaction mixtures (20 μl) contained 50 mM Tris-HCl, pH 7.5, 1.25 mM $MgCl_2$, 5 mM dithiothreitol, 25 μM [α-^{32}P]GTP, 11 pmol (of ends) of triphosphate-terminated poly(A), and 1.5 μl of the indicated column fractions. After incubation for 30 min at 37 °C, reactions were halted by the addition of 5% trichloroacetic acid. Acid-insoluble material was collected by filtration and counted in liquid scintillation fluid. Activity is plotted as GMP incorporation into acid-insoluble material (picomoles). Methyltransferase reaction mixtures (10 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 50 μM AdoMet, 60 fmol of cap-labeled poly(A), and 1 μl of a 20-fold dilution (in buffer A) of the indicated column fractions. After incubation for 10 min at 37 °C, the reaction mixtures were made 50 mM in sodium acetate, pH 5.5, and incubated for an additional 60 min at 37 °C with 5 μg of nuclease P1. The digests were spotted on polyethyleneimine cellulose TLC plates that were then developed with 0.45 M ammonium sulfate. Labeled dinucleotides corresponding to ^{m7}GpppA and GpppA were detected by autoradiography, then cut out and counted in liquid scintillation fluid. Activity is plotted as the amount (cpm $\times$ $10^{-3.3}$) of labeled ^{m7}GpppA synthesized. The activity of fraction 22 represents quantitative methylation of the input RNA substrate. *Bottom panel,* NaCl concentration was determined using a Radiometer conductivity meter and is plotted as M ($\times$ 10^{-1}). Protein concentration is plotted as (μg/ml) $\times$ 10^{-2}. *E*pG is plotted as the concentration (nM $\times$ 10^{-2}) of active guanylyltransferase in the indicated fractions, as determined by titration of enzyme-guanylate formation.

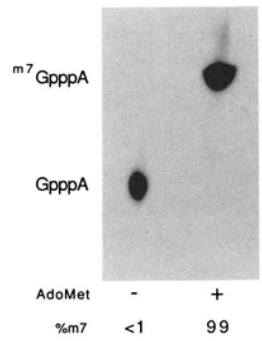

FIG. 4. **Analysis of cap structures synthesized by large and small subunits coexpressed in *E. coli*.** Control reaction mixtures (20 μl) contained 50 mM Tris-HCl pH 8.0, 1.25 mM $MgCl_2$, 5 mM dithiothreitol, 2.7 μM [α-^{32}P]GTP, 38 pmol (of ends) of triphosphate-terminated poly(A), and 3 μl of SP5PW 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 ^{m7}GpppA/(^{m7}GpppA + GpppA)) was determined by cutting out the labeled species and counting in liquid scintillation fluid and is indicated below each lane.

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. Triphosphate-terminated poly(A) was capped with [α-^{32}P]GTP and the nature of the cap dinucleotide was determined by thin layer chromatography after digestion with nuclease P1. Under standard conditions, the SP5PW fraction synthesized the unmethylated cap GpppA. Inclusion of AdoMet in the reaction resulted in the synthesis of the methylated cap ^{m7}GpppA; the efficiency of the methylation reaction was underscored by the finding that 99% of the capped product was ^{m7}GpppA. The cap products made by the SP5PW fraction were identical chromatographically to the dinucleotides synthesized under the same conditions by capping enzyme purified from vaccinia virions (not shown).

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 M NaCl. M_r 95,000 *E*pG-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.* Methyltransferase 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).

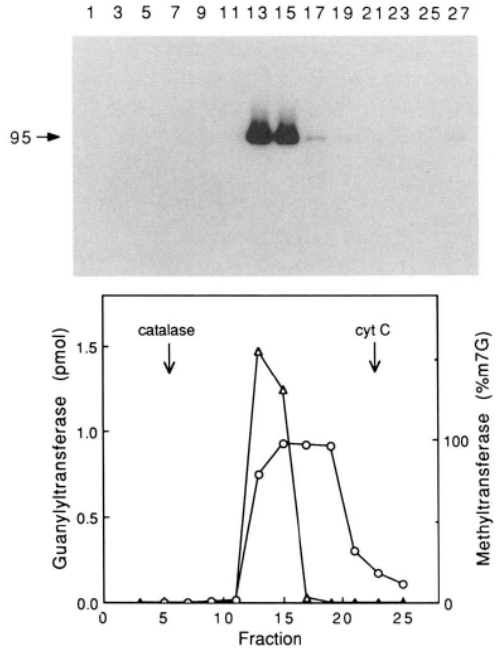

FIG. 5. **Glycerol gradient sedimentation.** Sedimentation of SP5PW fraction 22 in a 15–30% glycerol gradient is described in the text. Fractions (0.185 ml) were collected from the bottom of the tube. *Top panel,* aliquots (1 μl) of the indicated fractions were assayed for protein-guanylate complex formation. An autoradiograph of the protein gel is shown. The position of the labeled M_r 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 8 min at 37 °C. Activity is plotted as GMP incorporation into acid-insoluble material (picomoles, *open triangles*). Methyltransferase reaction mixtures were constituted as described in the legend to Fig. 3 and contained 1 μl of a 20-fold dilution (in buffer A) of the indicated gradient fractions. Incubation was for 5 min at 37 °C. After nuclease digestion and chromatographic separation, labeled dinucleotides corresponding to ^{m7}GpppA and GpppA were detected by autoradiography, then cut out and counted in liquid scintillation fluid. Activity is plotted as the percent of the input RNA substrate converted to methylated form (*open circles*). The positions of marker proteins (that had been sedimented in a parallel gradient) are indicated by the *arrows*.

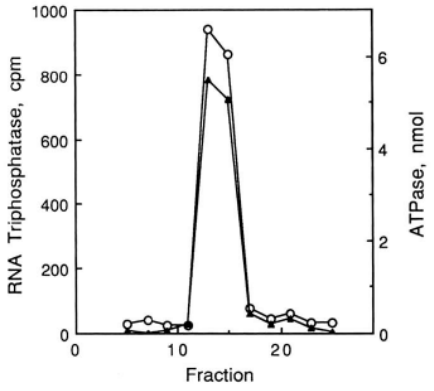

FIG. 6. **γ-Phosphate cleavage activity associated with guanylyltransferase after sedimentation in a glycerol gradient.** RNA triphosphatase reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 25 pmol (of ends) γ-^{32}P-poly(A) and enzyme (1 μl of a 50-fold diluted sample of the indicated glycerol gradient fractions) were incubated for 5 min at 37 °C. The samples were then spotted on polyethyleneimine cellulose TLC plates that were developed with 0.75 M potassium phosphate, pH 3.5. Reaction products corresponding to $^{32}P_i$ were located by autoradiography, then cut out and counted in liquid scintillation fluid. The activity profile is indicated by the *open circles*. The activity of peak fraction 13 in this experiment corresponds to cleavage of 15% of the input RNA ends. ATPase reaction mixtures (50 μl) containing 40 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM [γ-^{32}P]ATP, and 5 μl of the indicated glycerol gradient fractions were incubated for 30 min at 37 °C. Release of $^{32}P_i$ was quantitated as described (3); the activity profile is indicated by the *solid triangles*.

TABLE I

γ-Phosphate cleavage by expressed capping enzyme

Experiment 1. Complete RNA triphosphatase reactions were constituted as described in Fig. 6 and included 1 μl of a 10-fold diluted sample of glycerol gradient fraction 14. Divalent cations (chloride salt) were substituted for magnesium as indicated at 5 mM concentration. The activity of the complete reaction represents cleavage of 95% of the input RNA triphosphate ends. Experiment 2. Complete ATPase reactions contained 60 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 1 mM [γ^{32}P]ATP, and 5 μl of glycerol gradient fraction 14. Incubation was for 30 min at 37 °C. Divalent cations (chloride salt) were substituted for magnesium as indicated at 4 mM concentration.

Experiment and additions	RNA triphosphatase
	cpm $^{32}P_i$ released
1. Complete	10,150
Omit magnesium	0
Omit magnesium, add manganese	852
Omit magnesium, add calcium	30
	ATPase
	nmol $^{32}P_i$ released
2. Complete	5.8
Omit magnesium	0
Omit magnesium, add manganese	6.6
Omit magnesium, add calcium	<0.1
Omit magesium, add cobalt	17.5

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 required a divalent cation; 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

was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). RNA triphosphatase, ATPase, *E*pG formation, and RNA guanylyltransferase activity profiles all correlated with the presence of a major polypeptide of M_r 95,000 (indicated by *) in fractions 13–15. This M_r 95,000 polypeptide comigrated with the D1 gene product expressed inducibly in *E. coli* (not shown). A polypeptide of M_r 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 less 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 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 *E*pG assay permits direct determination of the molar concentration of active enzyme molecules, assuming a stoichiometry of one GMP residue bound per M_r 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 activity relative to the polymin P supernatant is probably attributable to the removal of interfering activities, particularly bacterial GTPases. The amount of active purified enzyme obtained at this step from 1 liter of bacteria is comparable to the amount of guanylyltransferase purified from 1100 A_{260} 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 homogeneous heterodimeric enzyme of M_r 127,000. The implication that the glycerol fraction is therefore 32% pure is not out of line with the electrophoretic analysis of polypeptide composition.

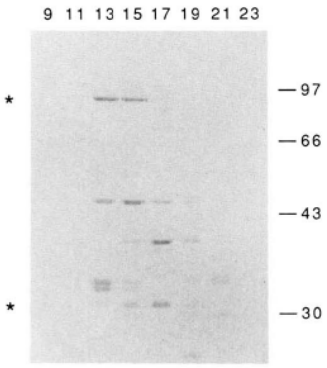

FIG. 7. **Polypeptide composition of glycerol gradient fractions.** Aliquots (35 μl) of the indicated glycerol gradient were made 1% in sodium dodecyl sulfate and electrophoresed through a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Protein was visualized by staining with Coomassie Blue dye. A photograph of the stained gel is shown. The positions and sizes (in kilodaltons) of coelectrophoresed marker proteins are indicated on the *right*. The polypeptides comigrating with the D1 and D12 gene products are indicated by the *asterisks* (*) on the *left*.

Quantitative RNA Capping by Expressed Capping Enzyme—A time course of GMP incorpration 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 the 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_r 95,000 and M_r 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

TABLE II

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

Guanylyltransferase assays were performed as described under "Experimental Procedures." Enzyme was assayed at three dilutions to establish linearity. The values reported for SP5PW pool and glycerol pool fractions represent the sum of the activity and protein present in the individual fractions (assayed separately) that comprise the pool.

Fraction	Capping enzyme		Protein		Specific activity	Yield
	nM	*pmol*	*μg/ml*	*mg*	*pmol enzyme/μg protein*	%
Polymin P	6.6	396	4110	246	1.61	100
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)		887		0.82	1081	224
Fraction 22	449		280		1603	
Glycerol gradient						
Pool (13–15)		58.7		0.028	2075	131[a]
Fraction 14	166		64		2580	

[a] The yield at the glycerol step is a normalized value based on the amount of enzyme applied to the gradient.

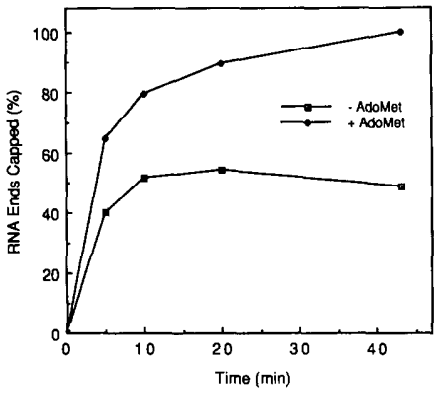

FIG. 8. **Effect of AdoMet on guanylyltransferase activity.** Reaction mixtures (0.1 ml) containing 50 mM Tris-HCl pH 7.5, 1.25 mM $MgCl_2$, 5 mM dithiothreitol, 25 μM [α-^{32}P]GTP, 57 pmol (of ends) of triphosphate-terminated poly(A), 8 μl of SP5PW fraction 23 (1.9 pmol of enzyme), and (where indicated) 50 μM AdoMet were incubated at 37 °C. Aliquots were removed at the indicated time points and assayed for incorporation of radioactivity into acid-insoluble material. Activity (expressed as the percent of input RNA ends that were guanylylated) is plotted as a function of time.

and small enzyme subunits appear to form a heterodimeric complex in the prokaryotic millieu, 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 catalyzes 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_r 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_r 31,000 subunit, which is itself sufficient to catalyze cap methylation; (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 pathogen. 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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Catalytic activity of vaccinia mRNA capping enzyme subunits coexpressed in Escherichia coli.

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