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Biochemistry

Interaction and mutual stabilization of the two subunits of vaccinia virus mRNA capping enzyme coexpressed in *Escherichia coli*

[RNA triphosphatase/RNA guanylyltransferase/RNA (guanine-N^7-)-methyltransferase/recombinant protein]

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ABSTRACT **The genes D1 and D12, predicted to encode the 95- and 31-kDa subunits of the vaccinia virus mRNA capping enzyme, were coexpressed from the same plasmid in *Escherichia coli*. Induction with low concentrations of isopropyl β-D-thiogalactoside was necessary to obtain soluble enzyme. The active heterodimer was purified by column chromatography and was shown to have both RNA guanylyltransferase and mRNA (guanine-N^7-)-methyltransferase activities. Formation of the m^{7}G(5′)pppG cap structure was verified by enzyme digestion and thin-layer chromatography. Each subunit was also expressed individually in *E. coli*. Without the large subunit, the small one was very unstable in some bacterial strains and could only be detected by pulse labeling with radioactive amino acids. The individually expressed large subunit contained the guanylyltransferase domain, but the activity from *E. coli* was <2% of that obtained with both subunits. Two other products of the D1 open reading frame were formed: a 55-kDa subfragment with the GMP binding site and a 38-kDa C-terminal fragment that started at amino acid 498. Expression of this heterodimeric enzyme in *E. coli* may facilitate the analysis of its functional domains and provide a useful reagent for the specific 5′ labeling of uncapped or capped RNA and for enhancing RNA translatability in eukaryotic systems.**

The 5′-terminal cap structure m^{7}G(5′)pppN- is a characteristic of eukaryotic mRNAs that is required for translation and stability. The cap is formed by posttranscriptional modification of the nascent mRNA by an enzyme with RNA guanylyltransferase and mRNA (guanine-N^7-)-methyltransferase activities that was first isolated from vaccinia virus (1). This multifunctional enzyme can remove the terminal γ-phosphate from an RNA chain, transfer the GMP moiety of GTP to the now diphosphate-ended RNA, and then transfer a methyl group from *S*-adenosylmethionine (AdoMet) to position 7 of the added guanosine (2–4). An enzyme-guanylate intermediate, in which a GMP residue is covalently attached to the large subunit of the enzyme via a phosphoramidate bond to a lysine ε amino group, was demonstrated (5, 6).

The poxviruses, of which vaccinia virus is the prototype, are unique in their ability to replicate and express their genes in the cytoplasm of infected cells (7). All of the enzymes and factors necessary for the formation of capped and polyadenylylated mRNA are contained within the infectious virus particle. The vaccinia virus capping enzyme is a 127-kDa protein composed of two subunits of 95 and 31 kDa (1) that are thought to be encoded by the D1 and D12 open reading frames (ORFs), respectively (8, 9). By expression of these two ORFs in *Escherichia coli*, we have confirmed the identity of the vaccinia virus capping enzyme genes. Furthermore, the large subunit alone was shown to have guanylyltransferase activity. Coexpression of both ORFs to form the heterodimer was needed, however, for stability and high activity. The recombinant protein should be a valuable reagent for specifically labeling the 5′ ends of mRNA and enhancing their translatability.

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MATERIALS AND METHODS

Construction of Plasmids. *Nde* I sites were created at the 5′ ends of the vaccinia virus D12 and D1 ORFs, which were then individually cloned between the *Nde* I and *Bam*HI sites of plasmid pET3-c (10) so that the ORFs were flanked by bacteriophage T7 promoter and termination sequences. The resulting plasmids were called pETD12 and pETD1. A *Bgl* II/*Bam*HI fragment, containing the bacteriophage T7 φ10 promoter, a ribosome binding site, and the complete D12 ORF of plasmid pETD12, was ligated to *Bgl* II cleaved pETD1, generating plasmid pETD12D1. The latter contains the D12 and D1 ORFs in tandem, with T7 promoters for each and a single termination sequence after the D1 ORF. *E. coli* BL21(DE3) (10), which contains an inducible bacteriophage T7 RNA polymerase gene, was transformed by the plasmids and used as the expression host.

Expression and Purification of the Capping Enzyme. Overnight cultures (4 ml) of transformed bacteria were inoculated into 4 liters of Superbroth (Quality Biologicals, Gaithersburg, MD) and after 2 hr at 37°C, isopropyl β-D-thiogalactoside (IPTG) was added. The bacteria were incubated for an additional 2 hr, collected by centrifugation, resuspended in 200 ml of buffer A [50 mM Tris·HCl, pH 8.0/2 mM dithiothreitol/1 mM EDTA/0.01% Nonidet P-40/5% (vol/vol) glycerol/50 mM NaCl/1 mM phenylmethylsulfonyl fluoride], and broken by passage through a French pressure cell. After centrifugation at 40,000 rpm in a type 60 Ti rotor (Beckman) for 30 min, the supernatant (40 ml) was applied to a DEAE-cellulose column (20 mm × 30 cm) equilibrated with buffer A. The flow-through fractions were applied to a phosphocellulose column (10 mm × 20 cm), and eluted with a 100-ml 0.05–1 M NaCl gradient in buffer A. Fractions with capping enzyme were pooled and applied to successive columns (10 mm × 20 cm) of single-stranded DNA-agarose and heparin-agarose and a HPLC TSK-300 column (7.5 mm × 30 cm) in buffer A. Protein concentration was determined by the Bio-Rad assay with bovine serum albumin used as the standard.

Assay for Enzyme–GMP Complex Formation. Reaction mixtures (15 μl) contained 4 μl of enzyme, 2 μCi of [α-^{32}P]-GTP (3000 Ci/mmol; 1 Ci = 37 GBq), and 1.5 μl of reaction buffer (50 mM Tris·HCl, pH 8.0/2 mM dithiothreitol, 50 mM $MgCl_2$). After 15 min at 37°C, 3 μl was applied to a SDS/10–15% PAGE system. Radioactivity was quantified with a

Abbreviations: AdoMet, *S*-adenosylmethionine; IPTG, isopropyl β-D-thiogalactoside; ORF, open reading frame.

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Betascope model 603 (Betagen, Waltham, MA) and enzyme units are expressed as pmol of GMP incorporated.

RNA Guanylyltransferase Assay. Reaction mixtures (15 μl) containing 0.1 μg of RNA, 4 μl of enzyme, 4 μCi of [α-^{32}P]GTP, 1.5 μl of reaction buffer (see above), 0.5 μl of RNasin (Promega), and 1 μl of 10 mM AdoMet were incubated for 20 min at 37°C. Radioactive RNA was detected by a DE-81 filter binding assay (1) and/or autoradiography after electrophoresis on 5% denaturing polyacrylamide gels. For analysis of cap structures, the RNA was digested with 5 μg of nuclease P1 in a 100-μl reaction mixture containing 30 mM sodium acetate (pH 5.3) and 0.4 mM $ZnSO_4$ for 30 min at 37°C. In some cases, the samples were also digested with 0.5 unit of alkaline phosphatase. Chromatography was on polyethylenimine-cellulose thin-layer sheets with 0.7 M LiCl.

RNA (guanine-N^7-)-methyltransferase Assay. The 50-μl reaction mixture contained 1 mM dithiothreitol, 50 mM Tris·HCl (pH 8.0), 10 mM GTP, 1 μCi of *S*-adenosyl [*methyl*-^{3}H]methionine ([^{3}H]AdoMet), and 4 μl of enzyme. After 30 min at 37°C, 45 μl was applied to a DE-81 filter. Unincorporated [^{3}H]AdoMet was removed by successively washing the filters with 25 mM ammonium formate, water, and absolute ethanol (11). A second assay involved the coupled transfer of GTP and AdoMet (2) as described above for RNA guanylyltransferase.

RESULTS

Expression of the Vaccinia Virus D1 and D12 ORFs in *E. coli*. The DNA segments containing the D1 and D12 ORFs were placed between bacteriophage T7 φ10 promoter and terminator sequences and the resulting plasmids were called pETD1 and pETD12, respectively. To coexpress the two ORFs, a third plasmid with both ORFs was constructed. This plasmid, called pETD12D1, contained the two ORFs D12 and D1 in tandem with separate promoters for each but only a single transcriptional terminator after the D1 ORF.

Each plasmid was transferred into *E. coli* BL21(DE3), which has an inducible bacteriophage T7 RNA polymerase. After IPTG induction of cells transformed with pETD12, the expected 32-kDa product was not detected by Coomassie blue staining after SDS/PAGE (Fig. 1*A*, lane b) but a pulse-labeled [^{35}S]methionine-labeled band of that size, as well as smaller ones, was detected (Fig. 1*B*, lane f). The 93-kDa product of the D1 ORF, in contrast, was expressed in high yield after IPTG induction of cells transformed with pETD1 (Fig. 1*A*, lane c). An additional induced polypeptide of 38 kDa was also noted. When D12 and D1 were coexpressed in cells transformed with pETD12D1 and induced with IPTG, a 32-kDa polypeptide as well as the 93- and 38-kDa polypeptides accumulated (Fig. 1*C*, lane k), suggesting interaction and stabilization of the capping enzyme subunits.

Purification of Guanylyltransferase from Cells Coexpressing the D12 and D1 ORFs. Incubation of capping enzyme with [α-^{32}P]GTP results in the formation of a covalent [α-^{32}P]-GMP-polypeptide (95 kDa) intermediate (5). Initially, we tried to detect such activity in crude lysates of *E. coli* that were transformed with pETD12D1 and induced with 0.4 mM IPTG. Nearly all of the induced polypeptides were insoluble, however, and only a faint radioactively labeled 93-kDa band was detected by PAGE. Paradoxically, the labeled 93-kDa band was more readily visualized when the cells were not induced, evidently because the small amount of basally expressed protein was soluble. We therefore tested a range of IPTG concentrations and found that 5 μM IPTG was optimal for production of active, soluble enzyme.

The soluble enzyme was purified by conventional column chromatography and assayed by [α-^{32}P]GMP complex formation (Fig. 2 *A–C*). The chromatographic properties of the recombinant enzyme were similar to those of the enzyme previously isolated from virus particles (1, 4). The labeled 93-kDa band comigrated with the purified capping enzyme from vaccinia virions (Fig. 2*B Inset*). In addition, when the purified enzymes from *E. coli* and vaccinia virions were incubated with [α-^{32}P]GTP, the labeled proteins sedimented at similar rates on parallel sucrose gradients (Fig. 2*E*).

Approximately 11% of the enzyme activity was recovered through the heparin-agarose step (Table 1). The final specific activity was 5000 units per mg of protein. Since GMP forms a covalent bond with the 93-kDa polypeptide, it was possible to calculate the pmol of labeled polypeptide. Using a molecular mass of 96,708 (12), we estimated that there is 0.48 mg of the large capping enzyme subunit per mg of purified protein.

The three induced bands of 93, 38, and 32 kDa, originally detected in the crude lysates (Fig. 1), were present in the purified enzyme (Fig. 3, lane e). After transfer to a membrane, N-terminal sequencing of the 93- and 32-kDa bands gave MXANVVSSXTIATYIXALAK and MDEIVKNIL/REGTHVLLPFYE, which corresponded to the properly

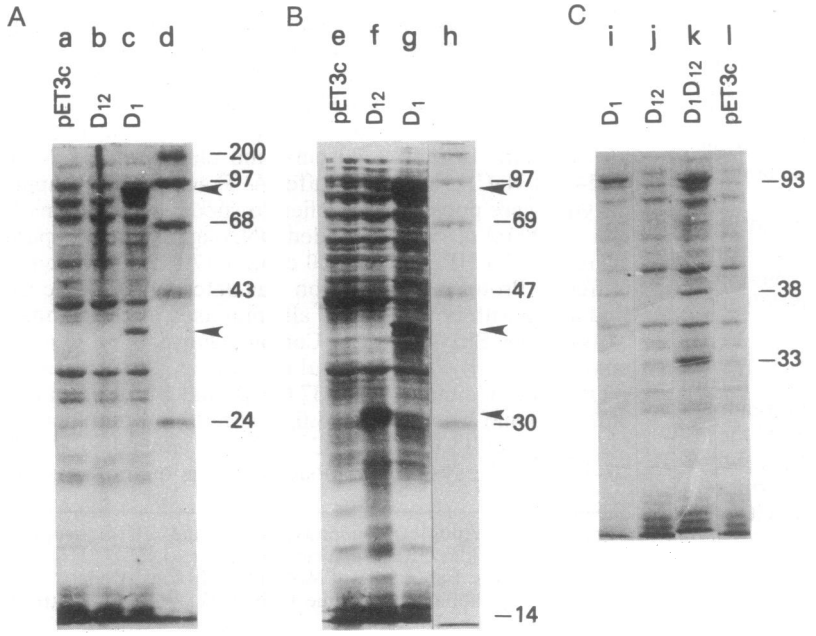

FIG. 1. Expression of vaccinia virus capping enzyme subunits in *E. coli*. Cells were transformed with the vector plasmid pET3c or with the same vector containing the D12, D1, or D12 and D1 ORFs and induced with 0.4 mM IPTG. Lysates were analyzed by SDS/PAGE. (*A* and *C*) Coomassie blue stain. (*B*) Lysates from *E. coli* labeled with [^{35}S]methionine were analyzed by SDS/PAGE and autoradiographed. Numbers on the right in *A* and *B* indicate the size of marker proteins in kDa. Arrowheads point to the induced 93- and 38-kDa polypeptides in *A* (lane c) and *B* (lane g) and to the 32-kDa polypeptide in *B* (lane f). Numbers on the right in *C* indicate the estimated sizes (kDa) of induced proteins.

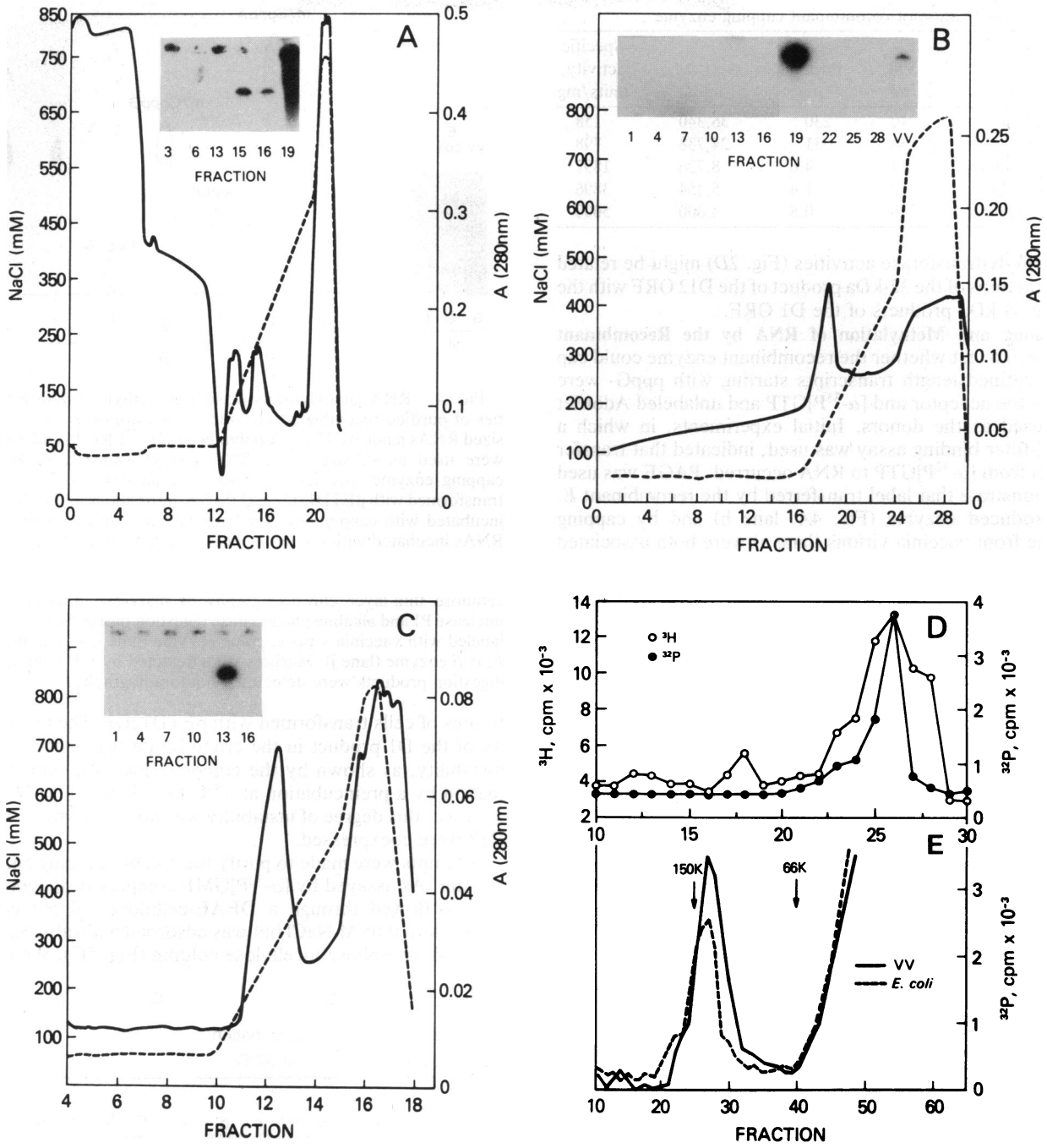

FIG. 2. Purification of the recombinant capping enzyme. Lysates of *E. coli* harboring pETD12D1 were clarified by ultracentrifugation, passed through a DEAE-cellulose column and chromatographed on successive columns of phosphocellulose (*A*), single-stranded DNA-agarose (*B*), heparin-agarose (*C*), and HPLC TSK-300 (*D*) and sedimented on a 5–20% sucrose gradient (*E*). (*A–C*) Dashed lines represent salt concentrations and solid lines indicate UV absorbance. Fractions were incubated with [α-^{32}P]GTP and analyzed by SDS/PAGE and autoradiography (*Insets*). The labeled band was estimated to be 93 kDa by comparison with marker proteins (not shown). Capping enzyme from vaccinia virions was assayed in parallel (*B*, lane vv). Fractions from the TSK column (*D*) also were assayed for methyltransferase activity by a GTP filter binding method using [^{3}H]AdoMet as a substrate. The radioactivity for the GMP binding (●) and methyltransferase (○) assays were determined and plotted. (*E*) Capping enzymes purified from *E. coli* and vaccinia virus were labeled with [α-^{32}P]GTP and sedimented on parallel 5–20% sucrose gradients for 35 hr at 60,000 × *g*.

initiated products of the D1 and D12 ORFs, respectively. The 38-kDa band, however, gave MKYINSEDYYGNQHNIIVEH, which corresponded to the sequence of the D1 ORF starting with the methionine residue at codon 498. Since a polypeptide that starts at amino acid 498 and ends at the D1 termination codon would be 38.2 kDa, the observed 38-kDa band probably represents the entire C-terminal fragment.

Association of Methyltransferase and Guanylyltransferase Activities. Vaccinia virus capping enzyme contains both guanylyltransferase and (guanine-N^7)-methyltransferase activities. Although capped RNA is the true substrate, the enzyme can transfer the methyl group from [^{3}H]AdoMet to GTP, providing a convenient DEAE-filter binding assay (11). The GTP-dependent methyltransferase activity present in the pooled guanylyltransferase fractions from the DNA-agarose and heparin-agarose columns were ≈6500 and ≈11,500 units/mg, indicating that the two activities copurified. The small difference in the TSK column elution profiles of the methyl-

Table 1. Purification of recombinant capping enzyme

Step	Vol, ml	Protein, mg	Activity, units	Specific activity, units/mg
Supernatant	40	230	36,340	158
DEAE-cellulose	30	31	24,738	798
Phosphocellulose	14	4.6	8,726	1897
DNA-agarose	12	1.4	5,454	3896
Heparin-agarose	16	0.8	4,000	5000

and guanylyltransferase activities (Fig. 2*D*) might be related to interactions of the 32-kDa product of the D12 ORF with the 93- and 38-kDa products of the D1 ORF.

Capping and Methylation of RNA by the Recombinant Enzyme. To test whether the recombinant enzyme could cap RNA, defined length transcripts starting with pppG- were used as the acceptor and [α-^{32}P]GTP and unlabeled AdoMet were used as the donors. Initial experiments, in which a DEAE-filter binding assay was used, indicated that transfer of label from [α-^{32}P]GTP to RNA occurred. PAGE was used to demonstrate that label transferred by the recombinant *E. coli* produced enzyme (Fig. 4*A*, lane b) and by capping enzyme from vaccinia virions (lane a) were both associated with intact RNAs. These labeled RNA bands were not seen when dephosphorylated small RNA of bacteriophage ϕ29 (13, 14) was used as a control (lane c).

To further analyze the labeling of the bacteriophage T7 RNA, a sample was purified, digested with nuclease P1 and alkaline phosphatase, and analyzed by polyethylenimine-cellulose thin-layer chromatography. The labeled material comigrated with an authentic m^{7}G(5′)pppG standard, indicating that successive enzymatic reactions had occurred (Fig. 4*B*, lanes i and j). The absence of labeled G(5′)pppG indicated complete methylation.

D1 ORF Product Alone Has Transguanylylation Activity. The cloning and expression of the vaccinia virus capping enzyme subunits in *E. coli* provides an opportunity to identify functional domains. As a first step, we wished to determine whether the product of the D1 ORF has guanylyltransferase activity by itself or whether the product of the D12 ORF was required. Accordingly, lysates of *E. coli* that were transformed with pETD1 were incubated with [α-^{32}P]GTP and analyzed by SDS/PAGE and autoradiography. A labeled 93-kDa band was detected (Fig. 5*A*). As before, we found that induction with low IPTG concentrations gave best results. Nevertheless, the activity was <2% of that obtained with

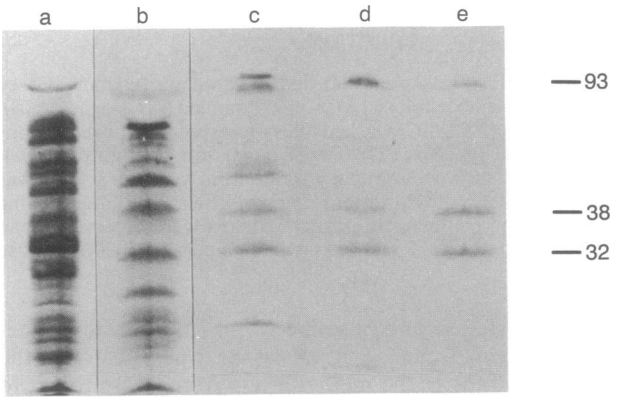

FIG. 3. Analysis of purified protein fractions by SDS/PAGE and Coomassie blue staining. Samples (3 μl) of the peak active fractions from Fig. 4 were loaded in each lane. Lanes: a, total lysate; b, DEAE-cellulose flow-through; c–e, fraction 15 from Fig. 2*A*, fraction 19 from Fig. 2*B*, and fraction 13 from Fig. 2*C*, respectively. Numbers on right indicate size in kDa.

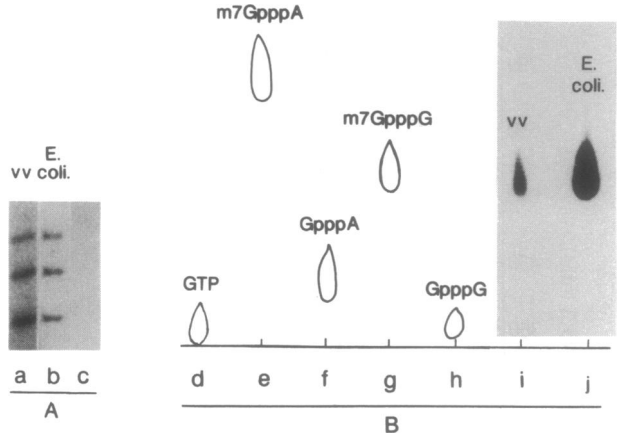

FIG. 4. RNA guanylyltransferase and methyltransferase activities of purified recombinant enzyme. A mixture of *in vitro* synthesized RNAs made by T7 RNA polymerase, [α-^{32}P]GTP, and AdoMet were used as substrates for RNA guanylyltransferase. Purified capping enzyme was derived from vaccinia virus (vv) or *E. coli* transformed with pETD12D1. (*A*) PAGE autoradiograph of T7 RNAs incubated with capping enzyme from vaccinia virions (lane a), T7 RNAs incubated with recombinant *E. coli* capping enzyme (lane b), or bacteriophage ϕ29 dephosphorylated small RNA incubated with recombinant *E. coli* capping enzyme (lane c). (*B*) Polyethylenimine-cellulose thin-layer chromatography of markers (lanes d–h) and nuclease P1 and alkaline phosphatase digestion products of T7 RNAs labeled with vaccinia virus capping enzyme (lane i) or recombinant *E. coli* enzyme (lane j). Markers were detected by UV light and the digestion products were detected by autoradiography.

lysates of cells transformed with pETD12D1. The low activity of the D1 product in the crude lysate was partly due to instability, as shown by the complete loss of complex formation by a preincubation at 37°C for 15 min (Fig. 5*B*). By contrast, this degree of instability was not seen when D1 and D12 were coexpressed.

Attempts were made to purify the soluble capping enzyme subunit. As assayed by [α-^{32}P]GMP complex formation, the protein flowed through a DEAE-cellulose column equilibrated with 0.05 M NaCl but was adsorbed and subsequently eluted from a phosphocellulose column (Fig. 5*C*). We noted

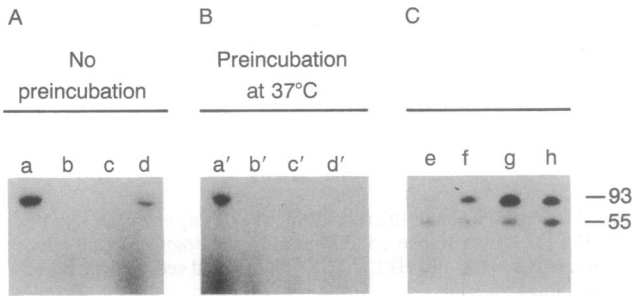

FIG. 5. *E. coli* expressed D1 ORF forms a protein–GMP complex. *E. coli* transformed with pETD12D1 (lanes a and a′), pETD12 (lanes b and b′), pET3-c (lanes c and c′), and pETD1 (lanes d and d′) were induced with 5 μM IPTG. The lysates were incubated directly (*A*) or after 20 min at 37°C (*B*) with [α-^{32}P]GTP. The proteins were analyzed by SDS/PAGE and autoradiographs were made. Larger amounts of lysate from pETD1-transformed cells than from pETD12D1-transformed cells were applied to the gel to see the bands with the same autoradiographic exposure time. (*C*) Lysate from *E. coli* transformed with pETD1 was centrifuged and the supernatant was passed through a DE-52-cellulose column and then applied to a phosphocellulose column and eluted with a linear 0.1–0.15 M NaCl gradient. Fractions were incubated with [α-^{32}P]GTP and analyzed by SDS/PAGE and autoradiography. Lanes e–h, successive alternate fractions with guanylyltransferase activity. Numbers on right indicate the estimated size in kDa of the radioactive bands.

a second [α-^{32}P]GMP complex of 55 kDa that did not precisely coelute with the major 93-kDa protein. Since this 55-kDa band and the previously noted 38-kDa polypeptide seen on Coomassie blue-stained gels (Fig. 1*A*, lane c) together add up to 93 kDa, they could be products of a single cleavage.

DISCUSSION

The vaccinia virus capping enzyme differs from the corresponding enzymes isolated from cellular sources in that it exists as a heterodimer with associated RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-N^7-)-methyltransferase activities. Our studies indicated that coexpression of the two subunits from a single plasmid resulted in the formation of an active heterodimer and suggested that this structure is necessary for stability in some strains of *E. coli*. Production of active soluble enzyme required a careful titration with IPTG to avoid aggregation. Expression of the enzyme did not appear to be highly toxic to *E. coli;* whether capping of host transcripts occurred was not investigated. The synthesis of vaccinia virus capping enzyme in bacteria eliminates the need to work with an infectious human pathogen. Based on previous recoveries of capping enzyme from vaccinia virus-infected HeLa cells (1), we estimated that <1/10th of that volume of *E. coli* was required to equal that.

Since the large subunit of capping enzyme forms a covalent complex with GMP, it was possible to determine the minimal number of active molecules in a preparation. We estimated that after heparin-agarose chromatography, 1 mg of protein contained 0.48 mg of large subunit, indicating a high degree of activity and purity. In addition to the expected 32-kDa small subunit, the preparation also contained a 38-kDa polypeptide shown by direct sequencing to be the C-terminal segment of the D1 ORF. The fragment starts with a methionine at amino acid 498 so that it could be a product of initiation within the ORF. However, a Shine–Dalgarno ribosome binding consensus sequence was not identified in the appropriate position. An alternative is that the 38-kDa fragment was formed by proteolytic cleavage either in *E. coli* or during isolation.

Since previous attempts in this laboratory to dissociate the vaccinia virus heterodimeric capping enzyme into active subunits were unsuccessful, it was of interest to express the individual genes in *E. coli*. Synthesis and partial purification of the large subunit was achieved and it was shown to form the 95-kDa protein–GMP complex, originally described as a capping intermediate by Shuman and Hurwitz (5). Evidence that the large subunit produced in *E. coli* forms a protein–GMP complex also has been obtained by E. Niles (personal communication). Shuman (15) reported that a 4.2S tryptic fragment of the virion-derived enzyme, which contained a 59-kDa segment of the large subunit, retained RNA guanylyltransferase activity. Our finding of a labeled 55-kDa polypeptide–GMP complex from D1-transformed *E. coli* may indicate cleavage at a protease susceptible site. Since the full-length product of the D1 ORF has a predicted mass of 96,708 Da, the 55-kDa polypeptide could be the N-terminal partner of the 38-kDa C-terminal fragment mentioned above. Possibly, protease cleavage separates the N-terminal active guanylyltransferase domain from the C-terminal small subunit binding domain. Although the D12 product was very unstable when expressed by itself in *E. coli* BL2(DE3), we obtained greater stability in *E. coli* HMS174(DE3), which should facilitate analysis of the activities of the small subunit of capping enzyme.

The specificity of the vaccinia virus capping enzyme for di- or triphosphate-ended RNA molecules and the availability of high specific activity [α-^{32}P]GTP and [^{3}H]AdoMet make it a valuable and unique labeling reagent (16–18). The ease of chemical removal of the cap structure from eukaryotic or viral RNAs leaving a triphosphate end makes labeling of the latter convenient and specific. The vaccinia capping enzyme has been used for identification and differentiation of the original ends of RNAs from those formed by processing or degradation, genomic mapping of transcription initiation sites either by blotting or by nuclease S1 methods, limited RNA sequencing, modifying *in vitro* synthesized RNAs to enhance translation in eukaryotic systems, studying the role of the cap structure in ribosome binding, and priming of influenza transcription. The ability to make large amounts of the recombinant vaccinia virus capping enzyme in *E. coli* should greatly extend the practical uses of this reagent.

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