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Expression and Characterization of an RNA Capping Enzyme Encoded by *Chlorella* Virus PBCV-1

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We report that the A103R protein of *Chlorella* virus PBCV-1 is an mRNA capping enzyme that catalyzes the transfer of GMP from GTP to the 5′ diphosphate end of RNA. This is a two-step reaction in which the enzyme first condenses with GTP to form a covalent enzyme-GMP intermediate and then transfers the GMP to an RNA acceptor to form a GpppN cap. Purified recombinant A103R is a 38-kDa monomer that lacks RNA (guanine-7-) methyltransferase activity. With respect to its size, amino acid sequence, and biochemical properties, A103R is more closely related to the yeast RNA guanylyltransferases than it is to the multifunctional capping enzymes coded for by other large DNA viruses—the poxviruses and African swine fever virus. We surmise that in order to cap its transcripts, PBCV-1 must either encode additional 5′ processing activities or else rely on the host alga to provide these functions.

Animal viruses have played a pivotal role in defining the structure of the mRNA cap and the biochemistry of cap formation (1). Ensinger and colleagues (2) showed in 1975 that the capping of vaccinia virus mRNAs occurs by a series of three enzymatic reactions in which the 5′ triphosphate terminus of the transcript first cleaves to a diphosphate-terminated RNA by RNA triphosphatase and then is capped with GMP by RNA guanylyltransferase and methylated at the N-7 position of guanine by RNA (guanine-7-) methyltransferase. This same pathway is used by reovirus and all cellular capping systems that have been examined to date (1, 17), although exceptions to this scheme have been noted for certain RNA viruses (1, 20).

An enzyme that catalyzes all three steps in cap formation has been purified from vaccinia virus particles (12, 21, 24). The capping enzyme is a heterodimer of 95- and 33-kDa subunits encoded by the viral D1 and D12 genes, respectively. There are three distinct catalytic sites within the 844-amino-acid D1 subunit. The RNA triphosphatase and RNA guanylyltransferase active sites are located within an amino-terminal 545-amino-acid module (13). The methyltransferase active site resides within a 305-amino-acid module at the carboxyl terminus of D1 (11). The D12 subunit, which is catalytically inert, serves as a stimulatory factor for the methyltransferase (11).

Several other DNA viruses encode homologs of the vaccinia virus D1 protein. Shope fibroma virus (SFV) encodes an 836-amino-acid polypeptide that is 60% identical to vaccinia virus D1 (22). SFV and vaccinia virus, which are both poxviruses, replicate within the cytoplasm of infected cells. African swine fever virus (ASFV) encodes an 868-amino-acid protein that is 21% identical to D1 (14). ASFV is an icosahedral virus that has both nuclear and cytoplasmic stages in its replication cycle. Guanylyltransferase activity has been demonstrated for the SFV and ASFV gene products (14, 22), but little is known about the subunit structures of the native SFV and ASFV enzymes or their associated activities. It is presumed that the SFV and ASFV guanylyltransferases possess triphosphatase and methyltransferase activities.

Cellular transcripts are capped by the same series of reactions as vaccinia virus mRNAs (17). However, the cellular guanylyltransferase and methyltransferase reactions are catalyzed by two distinct enzymes encoded by separate genes (10, 15). The 50-kDa yeast cap methyltransferase includes a 205-amino-acid segment with sequence similarity to the methyltransferase domain of vaccinia virus D1 (10). The cellular gene encoding RNA guanylyltransferase has been identified in two organisms—*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (15, 19). The 402-amino-acid *S. pombe* capping enzyme is 38% identical to the guanylyltransferase from *S. cerevisiae* (19). The sequence similarity of the two yeast guanylyltransferases and the D1-like proteins coded for by the DNA animal viruses is limited to a set of six short collinear motifs that are likely to constitute the nucleotidyl transferase active site (19, 20).

The theme that emerges from a comparison of the available set of cap synthesis genes is that capping enzymes coded for by animal DNA viruses are large multifunctional polypeptides which consist of modular domains whereas the cellular capping enzymes are smaller and more functionally discrete. Sequence comparisons suggest that the DNA virus-encoded and cellular enzymes are divergent branches on a phylogenetic tree. We were intrigued, therefore, by the recent finding of a potential RNA guanylyltransferase homolog coded for by *Paramecium bursaria Chlorella* virus-1 (PBCV-1) (7). PBCV-1 is the prototype of a family of large polyhedral DNA viruses that replicate in certain unicellular eukaryotic *Chlorella*-like green algae (23). The PBCV-1 genome, like the genomes of the poxviruses and ASFV, is a linear double-stranded DNA molecule with inverted terminal repeats and covalently closed hairpin telomeres. The sequence of 55% of the 330-kbp PBCV-1 genome has been reported (7–9); the virus is estimated to encode ~340 genes.

PBCV-1 gene A103R encodes a putative polypeptide which includes each of the six motifs shared among the cellular and DNA virus-encoded guanylyltransferases discussed above. The predicted 330-amino-acid A103R protein displays 24 to 25% amino acid identity overall with the *S. cerevisiae* and *S. pombe* guanylyltransferases (7). Remarkably, there is little sequence conservation with the guanylyltransferase domains of the poxvirus capping enzymes exclusive of the six short motifs. Potentially interesting questions about the evolution of the capping

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enzymes are raised, and these questions can be addressed once the biochemical properties of the PBCV-1 A103R protein have been defined.

In order to determine which activities, if any, are associated with the PBCV-1 A103R protein, we expressed A103R in *Escherichia coli* and purified the recombinant protein to apparent homogeneity. We demonstrate that A103R is indeed an RNA guanylyltransferase that catalyzes the transfer of GMP from GTP to the 5′ diphosphate end of an RNA acceptor to form the cap structure GpppN. A103R does not catalyze cap methylation. In this regard, and with respect to its donor specificity, the *Chlorella* virus enzyme is more closely related to the cellular guanylyltransferases than it is to the capping enzymes coded for by other DNA viruses.

MATERIALS AND METHODS

T7-based vector for expression of PBCV-1 capping enzyme in bacteria. The PBCV-1 A103R gene was amplified from a plasmid template containing a viral genomic DNA fragment by PCR (7). Oligonucleotide primers complementary to the 5′ and 3′ ends of the gene were designed to introduce *Nde*I restriction sites at the translation initiation codon and immediately 3′ of the translation stop codon. The sequence of the 5′ flanking primer was 5′-ACATAATTATTACATATGGTTCCTCCCACAATCAAC and that of the 3′ flanking primer was 5′-ATAAACGTGACATCATATGTTAGTAACAACCATA. PCR was carried out with *Pfu* DNA polymerase (Stratagene). The PCR product was digested with *Nde*I and then inserted into the *Nde*I site of T7-based expression plasmid pET3c (Novagen). The resulting plasmid, pET-A103R, was transformed into *E. coli* BL21(DE3).

Expression and purification of recombinant A103R protein. A 500-ml culture of *E. coli* BL21(DE3)/pET-A103R was grown at 37°C in Luria-Bertani medium containing 0.1 mg of ampicillin per ml until the A_{600} reached 0.5. The culture was adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation was continued at 37°C for 4 h. Cells were harvested by centrifugation, and the pellet was stored at −80°C. All subsequent procedures were performed at 4°C. Thawed bacteria were resuspended in 100 ml of buffer A (50 mM Tris HCl [pH 7.5], 10% sucrose) containing 0.15 M NaCl. The sample was sonicated for 30 s and then adjusted to 0.1% Triton X-100. The suspension was frozen on dry ice and then allowed to thaw at 4°C. Sonication was then repeated. A second round of freezing, thawing, and sonication ensued. The lysate was separated into soluble and insoluble fractions by centrifugation for 45 min at 18,000 rpm with a Sorvall SS34 rotor. The soluble extract (63 mg of protein) containing recombinant A103R protein was adjusted to 50 mM NaCl by the addition of 2 volumes of buffer A and then applied to a 35-ml column of DEAE-cellulose that had been equilibrated with buffer A containing 50 mM NaCl. The flowthrough fraction (25 mg of protein) was applied to a 15-ml column of phosphocellulose that had been equilibrated in buffer A containing 50 mM NaCl. The column was washed with the same buffer and then eluted stepwise with buffer B (50 mM Tris HCl [pH 8.0], 10% glycerol) containing 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M NaCl. A103R was retained on the column and was recovered predominantly in the 0.4 M fraction (5 mg of protein). Enzyme fractions were stored at −80°C and thawed on ice just prior to use. The protein concentration of the phosphocellulose enzyme preparation was determined by UV absorbance according to the following formula: protein (in milligrams per milliliter) = $1.55(A_{280}) - 0.76(A_{260})$.

Enzyme-GMP (EpG) complex formation. Standard reaction mixtures (20 μl) containing 50 mM Tris HCl (pH 8.5), 5 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 μM [α-^{32}P]GTP, and enzyme were incubated for 5 min at 37°C, and then the reaction was halted by the addition of sodium dodecyl sulfate (SDS) (1% final concentration). The samples were electrophoresed through a 12% polyacrylamide gel containing 0.1% SDS. Label transfer to the 38-kDa A103R polypeptide was visualized by autoradiographic exposure of the dried gel and was quantitated by scanning the gel with a FUJIX BAS1000 Bio-Imaging Analyzer.

Glycerol gradient sedimentation. An aliquot (40 μg) of the phosphocellulose A103R preparation was applied to a 4.8-ml, 15 to 30% glycerol gradient containing 50 mM Tris HCl (pH 8.0) and 0.5 M NaCl. The gradient was centrifuged at 50,000 rpm for 24 h at 4°C with a Beckman SW50 rotor. Fractions (0.22 ml) were collected from the bottom of the tube. Aliquots of every other fraction were assayed for EpG formation activity. The polypeptide composition of the gradient fraction was examined by SDS-polyacrylamide gel electrophoresis (PAGE). Marker proteins, vaccinia virus capping enzyme, bovine serum albumin, and cytochrome *c* were sedimented in a parallel gradient.

Isolation of capping enzyme-[^{32}P]GMP complex by gel filtration. Reaction mixtures (50 μl) containing 50 mM Tris HCl (pH 8), 5 mM DTT, 5 mM $MgCl_2$, 1 μM [α-^{32}P]GTP, and either 1 μg of A103R protein or 2.5 μg of vaccinia virus capping enzyme (D1-D12 hetrodimer) were incubated for 5 min at 37°C. The samples were adjusted to 20 mM EDTA and 10% glycerol. Native EpG complex was resolved from free GTP by gel filtration through a 1-ml column of Sephadex G-50 that had been equilibrated with buffer B containing 50 mM NaCl; gel filtration was performed at 4°C. Three-drop fractions (~120 μl) were collected serially; the elution profile was determined by Cerenkov counting of each fraction.

Preparation of RNA substrates. γ-^{32}P-labeled triphosphate-terminated poly(A) was synthesized as described previously (21) and then converted to 5′ diphosphate-terminated poly(A) by treatment with vaccinia virus capping enzyme. The RNA triphosphatase reaction mixture (0.2 ml) containing 50 mM Tris HCl (pH 8.0), 5 mM DTT, 5 mM $MgCl_2$, 500 pmol of γ-^{32}P-labeled triphosphate-terminated poly(A), and 50 pmol of purified recombinant vaccinia virus capping enzyme was incubated for 1 h at 37°C. The quantitative release of $^{32}P_i$ from poly(A) was verified by polyethyleneimine-cellulose thin-layer chromatography. The RNA product was then recovered by two rounds of precipitation with 10% trichloroacetic acid. The poly(A) was resuspended in 0.1 M Tris HCl, pH 8.0, and then extracted with phenol-chloroform, ethanol precipitated, and resuspended in 100 μl of 10 mM Tris HCl (pH 8.0)–1 mM EDTA.

Cap-labeled poly(A) [G**p**ppA(pA)n] and methylated cap-labeled poly(A) [m7G**p**ppA(pA)n] were prepared by the transfer of [^{32}P]GMP from [α-^{32}P]GTP to triphosphate-terminated poly(A) by using purified vaccinia virus capping enzyme. Reaction mixtures (50 μl) contained 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 5 mM DTT, 50 pmol of triphosphate-terminated poly(A), 4 μM [α-^{32}P]GTP, and 0.5 μg of purified recombinant vaccinia virus capping enzyme (D1-D12 heterodimer) and were supplemented with either 10 μM *S*-adenosylhomocysteine [for preparation of m7G**p**ppA(pA)n] or 50 μM *S*-adenosylmethionine (AdoMet) [for preparation of m7G**p**ppA(pA)n]. After incubation for 30 min at 37°C, unincorporated GTP was removed by multiple rounds of trichloroacetic acid precipitation. The RNA was extracted with phenol-chloroform and recovered by ethanol precipitation. Recovery of labeled RNA was assessed by scintillation counting. The molar concentration of cap-labeled RNA was calculated according to the specific activity of the input [α-^{32}P]GTP donor.

RESULTS

Expression of the PBCV-1 A103R protein in bacteria. The amino acid sequence similarity of the A103R open reading frame of *Chlorella* virus PBCV-1 and the RNA capping enzymes of *S. cerevisiae* and *S. pombe* suggested that A103R might possess guanylyltransferase activity. To test this possibility, we expressed the A103R protein in bacteria under the transcriptional control of a bacteriophage T7 promoter. The pET-A103R expression plasmid was introduced into *E. coli* BL21(DE3), a strain that contains the T7 RNA polymerase gene under the control of a *lac*UV5 promoter. A 38-kDa polypeptide doublet corresponding to A103R was detectable by SDS-PAGE in whole-cell extracts of IPTG-induced bacteria (Fig. 1A, lane 1). This doublet was not present when bacteria containing the pET vector alone were induced with IPTG (data not shown). After centrifugal separation of the crude lysate, A103R protein was recovered in the soluble supernatant fraction (Fig. 1A, lane 2).

Recombinant A103R forms a covalent protein-GMP complex in vitro. The mRNA guanylyltransferase reaction entails two sequential nucleotidyl transfer steps (18). In the first step, nucleophilic attack on the α-phosphate of GTP by the enzyme results in the liberation of PP_i and formation of a covalent EpG intermediate. Hence, guanylyltransferase activity can be detected with high sensitivity and specificity, even in crude extracts, by label transfer from [α-^{32}P]GTP to the enzyme. In order to assay guanylyltransferase activity of the expressed A103R protein, we incubated either whole-cell or soluble extracts of IPTG-induced BL21(DE3)/pET-A103R cells in the presence of [α-^{32}P]GTP and a divalent cation. This incubation resulted in the formation of an SDS-stable nucleotidyl-protein adduct that migrated as a single 38-kDa species during SDS-PAGE (Fig. 1B, lanes 1 and 2). Labeling of this polypeptide was not detected in extracts prepared from bacteria that lacked the A103R gene (data not shown). We conclude that the expressed A103R protein is active in transguanylylation.

Purification of recombinant PBCV-1 guanylyltransferase. The A103R protein was purified from soluble bacterial extract by ion-exchange chromatography. The A103R polypeptide doublet did not bind DEAE-cellulose at low ionic strength (50 mM NaCl). SDS-PAGE analysis of the DEAE flowthrough

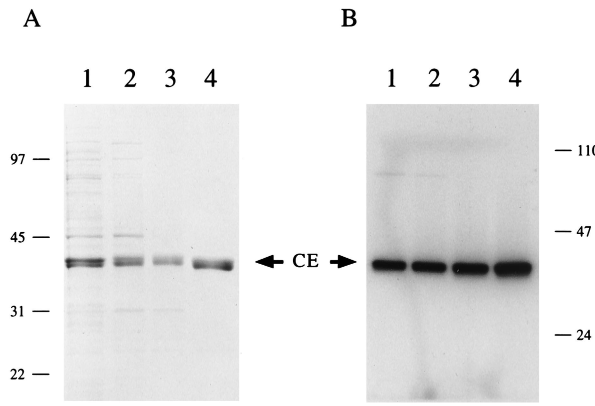

FIG. 1. Expression, purification, and guanylyltransferase activity of the PBCV-1 A103R protein. (A) The polypeptide compositions of recombinant A103R protein at sequential stages of purification were analyzed by SDS-PAGE. Lane 1, whole cell lysate of IPTG-induced BL21(DE3)/pET-A103R; lane 2, soluble lysate fraction; lane 3, DEAE-cellulose flowthrough fraction; lane 4, 0.4 M NaCl phosphocellulose eluate. The gel was fixed and stained with Coomassie blue dye. The positions and sizes (in kilodaltons) of the coelectrophoresed marker polypeptides are shown on the left. The position of the recombinant capping enzyme polypeptide doublet (CE) is indicated on the right. (B) Guanylyltransferase activity. The reaction mixtures (20 μl) contained 50 mM Tris HCl (pH 8.0), 5 mM DTT, 5 mM $MgCl_2$, 1 μM [α-^{32}P]GTP, and recombinant A103R protein at various stages of purification. Lane 1, whole-cell lysate; lane 2, soluble lysate; lane 3, DEAE-cellulose flowthrough; lane 4, 0.4 M NaCl phosphocellulose eluate. The reaction products were resolved by SDS-PAGE. An autoradiograph of the dried gel is shown. The enzyme-[^{32}P]GMP complex is denoted by the arrow on the left. The positions and sizes (in kilodaltons) of prestained marker polypeptides are indicated on the right.

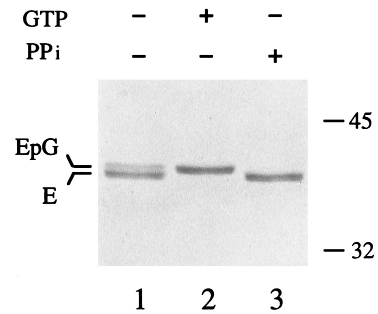

FIG. 2. Unguanylylated and GMP-bound A103R proteins are interconvertible in vitro. An aliquot (0.4 μg) of the phosphocellulose A103R protein was incubated for 10 min at 37°C in a reaction mixture containing 50 mM Tris HCl (pH 8.5), 5 mM $MgCl_2$, and 5 mM DTT (lane 1) supplemented with either 1 mM GTP (lane 2) or 1 mM $NaPP_i$ (lane 3). The reactions were then adjusted to 25 mM EDTA and denatured in 1% SDS. The samples were analyzed by SDS-PAGE. A Coomassie blue-stained gel is shown. The positions of the protein-GMP complex (EpG) and the unguanylylated A103R protein (E) are indicated on the left. The positions of 45- and 32-kDa marker proteins are denoted on the right.

fraction (Fig. 1A, lane 3) showed that most of the polypeptides greater than 40 kDa in size were eliminated at this step. A103R adsorbed to phosphocellulose and was recovered during step elution with 0.4 M NaCl (Fig. 1A, lane 4). The phosphocellulose preparation was virtually homogeneous with respect to the 38-kDa A103R doublet. Approximately 5 mg of purified recombinant A103R protein was obtained from a 500-ml culture of IPTG-induced bacteria.

The guanylyltransferase activity profile, assayed by formation of a 38-kDa protein-GMP complex, coincided with the relative abundance of the A103R protein during the DEAE-cellulose and phosphocellulose purification steps (Fig. 1B, lanes 3 and 4, and other data not shown). When the phosphocellulose fraction was centrifuged through a 15 to 30% glycerol gradient in 0.5 M NaCl, a single peak of guanylyltransferase activity that coincided with the 38-kDa A103R doublet was detected (data not shown). We estimated a sedimentation coefficient of 3.2S relative to marker proteins sedimented in a parallel gradient (data not shown). This result suggested that the PBCV-1 guanylyltransferase is a monomer of the A103R protein.

Purified PBCV-1 guanylyltransferase is a mixture of free and GMP-bound A103R protein. The A103R protein appeared as a polypeptide doublet after being analyzed by SDS-PAGE; this was the case at every stage of purification (Fig. 1A). The faster-migrating species predominated in the phosphocellulose preparation (Fig. 2, lane 1). We hypothesized that the two polypeptides represented the free or unguanylylated enzyme (E) and the guanylylated enzyme (EpG). If this is true, then the two forms might be interconvertible in vitro. Indeed, the faster-migrating species was converted quantitatively into the slower-migrating species by incubation of the enzyme preparation in the presence of 1 mM GTP and magnesium (Fig. 2, lane 2). This maneuver is predicted to drive the reaction equilibrium toward EpG formation. Conversely, all of the slower-migrating polypeptide was shifted to the more rapidly migrating free protein after the enzyme was incubated with 1 mM PP_i and magnesium (Fig. 2, lane 3). It is well established in other capping systems that incubation of EpG in the presence of PP_i liberates GTP by reversal of the guanylylation reaction (16, 18, 26). The results of this experiment illuminate two important properties of the recombinant PBCV-1 guanylyltransferase: (i) the reaction of GTP with guanylyltransferase is freely reversible, and (ii) essentially all of the protein in the purified A103R preparation is catalytically competent in nucleotidyl transfer.

Characterization of the enzyme-guanylate formation reaction. The amount of EpG complex formed during a 5-min incubation at 37°C in the presence of 1 μM [α-^{32}P]GTP was proportional to the amount of added A103R protein (Fig. 3A). We estimated, on the basis of the molar amount of GMP label-transfer versus the molar amount of A103R added, that ~50% of the protein was converted to EpG. This value is slightly less than the ~70% of the A103R molecules estimated by SDS-PAGE to be initially in the unguanylylated form. Because the results in Fig. 2 show clearly that all of the free enzyme can be converted to EpG in vitro, we suspect that the underestimate of the molar fraction of reactive enzyme in the experiment illustrated in Fig. 3A results from an overestimate of the A103R protein concentration. A kinetic analysis of EpG formation indicated that the reaction was completed within 20 s at either 25 or 37°C. The level of EpG remained unchanged up to 10 min (data not shown).

EpG complex formation depended on a divalent cation cofactor. This requirement was satisfied by either magnesium or manganese. The yield of EpG was proportional to the magnesium concentration from 0.1 to 1 mM and was maximal at 2 to 5 mM (Fig. 3B). Although manganese was a more effective cofactor than magnesium at concentrations below 0.5 mM, similar levels of EpG formation were seen at 1 to 5 mM of either cation (Fig. 3B). Neither calcium, cobalt, copper, nor zinc supported EpG formation when present at a 5 mM concentration (data not shown). PP_i, a reaction product, inhibited EpG formation. In a standard guanylyltransferase reaction containing 1 μM [α-^{32}P]GTP and 5 mM $MgCl_2$, EpG formation was reduced by half at 50 μM PP_i; >90% inhibition was observed at 0.2 mM PP_i (Fig. 3C). P_i had no effect on EpG formation at a 5 mM concentration (data not shown). Activity

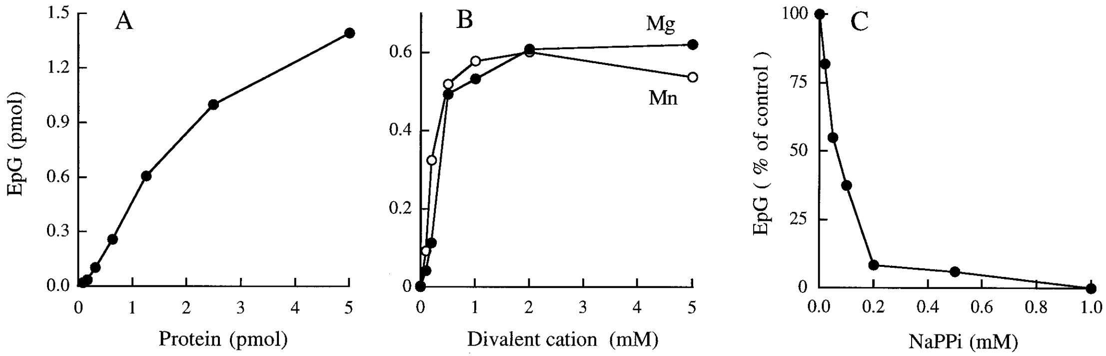

FIG. 3. Characterization of the guanylyltransferase activity of A103R. (A) Protein titration. The reaction mixtures (20 μl) contained 50 mM Tris HCl (pH 8.5), 5 mM DTT, 5 mM $MgCl_2$, 1 μM [α-^{32}P]GTP, and A103R (phosphocellulose fraction). The extent of EpG formation (in picomoles) is plotted as a function of input protein. The molarity of A103R was calculated from the protein concentration (in milligrams per milliliter, determined by UV absorbance), assuming 100% purity and a molecular weight of 38,000. (B) Divalent cation requirement. The reaction mixtures (20 μl) contained 50 mM Tris HCl (pH 8.5), 5 mM DTT, 1 μM [α-^{32}P]GTP, 50 ng of A103R (phosphocellulose fraction), and a divalent cation (either $MgCl_2$ or $MnCl_2$) as indicated. The extent of EpG formation (in picomoles; *y* axis) is plotted as a function of divalent cation concentration. (C) Inhibition by PP_i. The reaction mixtures (20 μl) contained 50 mM Tris HCl (pH 8.5), 5 mM DTT, 5 mM $MgCl_2$, 1 μM [α-^{32}P]GTP, 50 ng of A103R, and $NaPP_i$ as indicated. The yield of EpG (expressed relative to the amount of EpG formed in a control reaction lacking $NaPP_i$) is plotted as a function of PP_i concentration.

in 50 mM Tris HCl buffer was optimal at pH 8.0 to 9.0; the amount of EpG formed at pH 6.0 to 6.5 was ~50% of the amount formed at pH 8.5 (data not shown).

Nucleotide specificity. The A103R protein reacted specifically with [α-^{32}P]GTP (Fig. 4). The yield of EpG increased as a function of GTP concentration and reached saturation at 1 μM [α-^{32}P]GTP (Fig. 5A). Half-saturation was achieved at ~0.2 μM GTP (Fig. 5A). There was no label transfer to the 38-kDa polypeptide in the presence of [γ-^{32}P]GTP (Fig. 4). Similarly, [γ-^{32}P]ATP failed to label the protein. Trace amounts of protein-nucleoside monophosphate were formed in a reaction mixture containing [α-^{32}P]ATP in lieu of GTP. We suspect that this outcome actually represents an attack by the enzyme on [α-^{32}P]ITP, which arises by spontaneous deamination of ATP. ITP is an effective substrate for capping by the HeLa cell guanylyltransferase (25). Other ribonucleoside triphosphates—[α-^{32}P]CTP and [α-^{32}P]UTP—were inert in nucleotidyl transfer (Fig. 4).

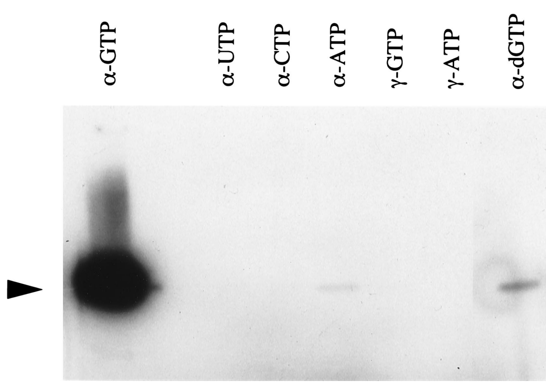

FIG. 4. Nucleotide specificity. The reaction mixtures contained 50 mM Tris HCl (pH 8.5), 5 mM DTT, 5 mM $MgCl_2$, 50 ng of A103R, and 0.17 μM of ^{32}P-labeled nucleoside triphosphate as indicated (except for [γ-^{32}P]GTP, which was included at 0.1 μM). Incubation was for 5 min at 37°C. The reaction products were resolved by SDS-PAGE. An autoradiograph of the dried gel is shown. The position of the 38-kDa enzyme-nucleotide complex is indicated by the arrowhead on the left. The specific activities of the nucleotides were as follows: [α-^{32}P]GTP, 9.6 × 10^5 cpm/pmol; [α-^{32}P]UTP, 8.4 × 10^5 cpm/pmol; [α-^{32}P]CTP, 1.0 × 10^6 cpm/pmol; [α-^{32}P]ATP, 1.0 × 10^6 cpm/pmol; [γ-^{32}P]GTP, 3.1 × 10^6 cpm/pmol; [γ-^{32}P]ATP, 5.5 × 10^5 cpm/pmol; and [α-^{32}P]dGTP, 3.6 × 10^5 cpm/pmol.

[α-^{32}P]dGTP was an extremely poor donor for enzyme-guanylate formation by A103R compared with [α-^{32}P]GTP (Fig. 4 and 5A), indicating that the PBCV-1 enzyme discriminates between ribose and deoxyribose sugars. We estimate from the nucleoside triphosphate titration experiment in Fig. 5A that

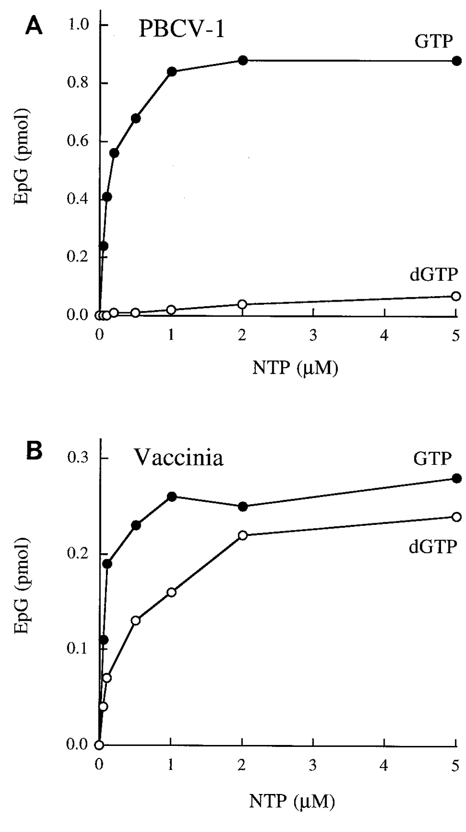

FIG. 5. Nucleotide sugar specificity in EpG formation. The reaction mixtures (20 μl) contained 50 mM Tris HCl (pH 8.5), 5 mM $MgCl_2$, 5 mM DTT, [α-^{32}P]GTP or [α-^{32}P]dGTP as indicated, and either 50 ng of PBCV-1 guanylyltransferase (A) or 125 ng of purified vaccinia virus capping enzyme (B). Incubation was for 5 min at 37°C. The extent of EpG formation is plotted as a function of nucleoside triphosphate (NTP) concentration.

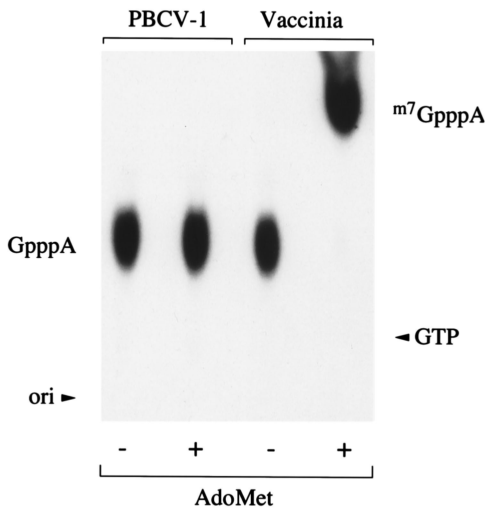

FIG. 6. The A103R EpG complex is an intermediate in RNA capping. The PBCV-1 and vaccinia virus enzyme-[^{32}P]GMP complexes were prepared and isolated by gel filtration as described in Materials and Methods. Aliquots (10 μl) of the gel-filtered complexes were incubated for 5 min at 37°C in reaction mixtures (200 μl) containing 50 mM Tris HCl (pH 8.0), 2 mM $MgCl_2$, 5 mM DTT, and 50 pmol of diphosphate-terminated poly(A), with (+) or without (−) 50 μM AdoMet. The mixtures were then extracted once with phenol and once with chloroform-isoamyl alcohol (24:1). RNA was recovered from the aqueous phase by ethanol precipitation and resuspended in 30 μl of 10 mM Tris HCl (pH 8.0)–1 mM EDTA. Aliquots (4 μl) were digested in 30 mM sodium acetate (pH 5.2) with 5 μg of nuclease P1 (Boehringer Mannheim) for 60 min at 37°C. The digests were analyzed by thin-layer chromatography on polyethyleneimine-cellulose plates developed with 0.45 M ammonium sulfate. An autoradiogram of the thin-layer chromatography plate is shown. The location of the origin (ori) and the positions of GTP, GpppA, and m7GpppA are noted.

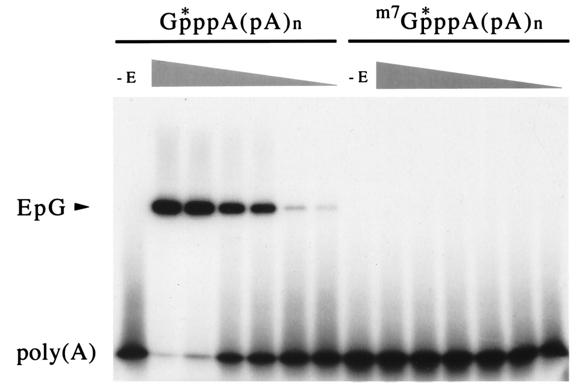

FIG. 7. GMP transfer to A103R from capped poly(A). The reaction mixtures (10 μl) contained 50 mM Tris HCl (pH 8.5), 5 mM DTT, 5 mM $MgCl_2$, 10 fmol of cap-labeled poly(A) [G**p**ppA(pA)n] or methylated cap-labeled poly(A) [m7G**p**ppA(pA)n] as indicated above the lanes, and either 400, 200, 40, 20, 4, or 2 fmol of the phosphocellulose fraction of A103R (proceeding from left to right within each titration series). Control reactions lacking A103R are shown in lanes -E. After incubation for 15 min at 37°C, the reactions were terminated by adding SDS to 1%, and the samples were analyzed by SDS-PAGE. An autoradiograph of the dried gel is shown. The positions of the EpG complex and the cap-labeled poly(A) substrate are indicated on the left.

GTP is 300-fold more effective than dGTP in EpG formation. This level of sugar specificity by the PBCV-1 guanylyltransferase is in marked contrast to that of the vaccinia virus guanylyltransferase, which readily utilizes dGTP in EpG formation (Fig. 5B). Rather, the PBCV-1 enzyme resembles the human capping enzyme in its extreme preference for GTP over dGTP (25, 26).

RNA capping by PBCV-1 guanylyltransferase. Purified A103R was incubated with [α-^{32}P]GTP, and the enzyme-[^{32}P]GMP complex was isolated by gel filtration. The A103R-[^{32}P]GMP complex was then incubated with diphosphate-terminated poly(A) in the presence of magnesium. A parallel reaction performed with purified vaccinia virus capping enzyme-[^{32}P]GMP complex served as a positive control for cap formation. The products of the capping reaction were extracted with phenol-chloroform to remove the radiolabeled enzyme, and the RNA acceptor was recovered by ethanol precipitation. The RNA samples were digested with nuclease P1 and then were analyzed by polyethyleneimine-cellulose thin-layer chromatography. Nuclease P1 digestion of the PBCV-1 guanylyltransferase reaction product liberated a single radioactive species corresponding to cap dinucleotide G**p**ppA (Fig. 6). The mobility of this species was identical to that of cap dinucleotide synthesized by the vaccinia virus capping enzyme and was clearly distinct from that of free GTP (Fig. 6). Formation of the GpppA dinucleotide depended on digestion of the capping reaction product with nuclease P1; in undigested samples, the label remained at the position of the origin during thin-layer chromatography, as was expected for a polynucleotide (data not shown). These results substantiate A103R as an RNA capping enzyme.

In the same experiment, we tested whether A103R has an associated RNA (guanine-7-) methyltransferase activity. This test was done by analyzing the cap structures synthesized in the presence of AdoMet. The vaccinia virus capping enzyme again served as a positive control. Inclusion of AdoMet in the vaccinia virus capping reaction resulted in the quantitative methylation of all capped ends, as was evinced by the release of the more rapidly migrating m7GpppA dinucleotide after digestion with nuclease P1 (Fig. 6). However, inclusion of AdoMet in the PBCV-1 capping reaction elicited no detectable methylation of the RNA cap (Fig. 6).

Transfer of GMP from the RNA cap to A103R. To confirm that the A103R protein-GMP complex is an intermediate in cap synthesis, we tested whether GMP could be transferred from the RNA cap to the A103R protein via reversal of the capping reaction. [α-^{32}P]GMP-labeled capped poly(A) was synthesized with the vaccinia virus capping enzyme, [α-^{32}P]GTP, and triphosphate-terminated poly(A). Methylated cap-labeled poly(A) was synthesized in a parallel reaction containing AdoMet. The capped poly(A) products were recovered free of [α-^{32}P]GTP by multiple rounds of precipitation with trichloroacetic acid and then with ethanol; the radiochemical purity of the capped RNA was confirmed by thin-layer chromatography (data not shown). The PBCV-1 guanylyltransferase was incubated with cap-labeled poly(A) [G**p**ppA(pA)n] in the presence of magnesium, and the reaction products were analyzed by SDS-PAGE. The cap-labeled poly(A) migrated near the bottom of the gel (Fig. 7, lane -E). Inclusion of the A103R protein in the reaction resulted in label transfer to the 38-kDa A103R polypeptide. The extent of GMP transfer from the cap to the protein was proportional to the amount of A103R added (Fig. 7) and was completely dependent on the inclusion of magnesium (data not shown). Nearly all of the GMP was donated back to A103R under conditions of enzyme excess (Fig. 7). Transfer of half the input label to protein was achieved at ~5 nM A103R. These results indicate that the second step of the guanylyltransferase reaction is freely reversible and that the PBCV-1 enzyme binds avidly to the capped RNA product. After the PBCV-1 guanylyltransferase was incubated with methylated cap-labeled poly(A) [m7G**p**ppA-(pA)n] in the presence of magnesium, no transfer of m7GMP from RNA to protein was detected, even in enzyme excess (Fig. 7). Thus, cap methylation renders the guanylyltransferase reaction irreversible.

DISCUSSION

The PBCV-1 A103R gene encoding a putative mRNA guanylyltransferase was identified during sequencing of the viral DNA genome (7). We have now shown that A103R is an RNA capping enzyme. This result was achieved by expressing the PBCV-1 protein in bacteria and purifying the protein to homogeneity. A103R, like other DNA virus and cellular capping enzymes, catalyzes the transfer of GMP from GTP to the diphosphate end of RNA to form a GpppN cap structure. Our experiments indicate that this transfer occurs in two steps involving condensation of the enzyme with GTP to form a covalent EpG intermediate and then transfer of GMP from A103R to the RNA acceptor to form the cap. Although release of PP_i as a reaction product in the first step was not demonstrated directly in this study, the failure to detect label transfer to A103R from [γ-^{32}P]GTP, plus the inhibition of EpG formation by PP_i, is consistent with this reaction scheme. Note that EpG formation does not require the presence of an RNA cap acceptor and that GMP transfer to RNA from EpG occurs in the absence of GTP. These results, together with the demonstration of the transfer of GMP from the capped RNA product to A103R, establish that EpG is a true catalytic intermediate. Thus, the PBCV-1 capping enzyme adheres to the same mechanism of covalent nucleotidyl transfer as the vaccinia virus (18), reovirus (3), and cellular (4, 16, 26) guanylyltransferases.

In size and amino acid sequence, the A103R protein most closely resembles the guanylyltransferases of *S. cerevisiae* and *S. pombe* and is more distantly related to the capping enzymes coded for by the poxviruses and ASFV. We find that the biochemical properties of A103R are also more akin to those of the cellular guanylyltransferases. First, A103R, like the human capping enzyme, has a strong preference for GTP over dGTP in EpG formation (26). Although a direct comparison of GTP and dGTP in EpG formation has not been reported for the yeast guanylyltransferase, the finding that excess unlabeled GTP, but not dGTP, inhibits yeast enzyme-[^{32}P]GMP formation in vitro (4) suggests that the yeast enzyme displays the same nucleotide sugar selectivity as the human and PBCV-1 proteins. In contrast, the vaccinia virus enzyme utilizes either GTP or dGTP as a cap donor. (Note that differences between virus-encoded and host-encoded capping enzymes in substrate specificity may be useful in designing drugs that selectively block capping of virus-encoded mRNAs.)

A second distinction between the vaccinia virus and PBCV-1 capping enzymes is the lack of an associated methyltransferase activity. Whereas the native vaccinia virus enzyme catalyzed quantitative methylation of the newly incorporated cap guanosine in the presence of AdoMet, there was no detectable cap methylation by recombinant A103R. We note that the vaccinia virus D1 subunit alone has much weaker methyltransferase activity than the D1-D12 heterodimer (11). Although it is possible that A103R might require a stimulatory subunit to catalyze cap methylation, we believe that this is unlikely, because the A103R protein bears no resemblance to either the yeast cap methyltransferase or the methyltransferase catalytic domain of the vaccinia virus capping enzyme (10, 11). In its lack of intrinsic methyltransferase activity, A103R again resembles the yeast and mammalian guanylyltransferases. This resemblance raises some interesting issues about PBCV-1 mRNA synthesis in vivo. For example, if PBCV-1 mRNAs contain a standard m7GpppN cap, then the virus must either encode a separate cap methyltransferase or else rely on the host to provide this function. No homolog of the known cap methyltransferases has been uncovered in the 55% of the PBCV-1 genome DNA sequence already reported (7–9), and no homolog is encoded within the remainder of the PBCV-1 genome (6).

RNA triphosphatase activity is intrinsic to vaccinia virus D1. Biochemical studies of the cellular enzymes suggest that the guanylyltransferase and triphosphatase activities isolated from rat liver and from brine shrimp reside within a single polypeptide (27, 28). However, in *S. cerevisiae*, the RNA triphosphatase activity associated with the guanylyltransferase during its isolation from yeast extracts actually resides within a polypeptide subunit distinct from the guanylyltransferase (5). To address whether the recombinant PBCV-1 A103R protein possessed RNA triphosphatase activity, we assayed for the release of $^{32}P_i$ from γ-^{32}P-poly(A). We detected a very low level of phosphate release by the phosphocellulose A103R preparation that was independent of a divalent cation cofactor. However, this activity did not cosediment precisely with A103R during glycerol gradient centrifugation. In addition, we were able to resolve the PBCV-1 guanylyltransferase from the phosphate-releasing activity by adsorbing the phosphocellulose A103R protein to an SP5PW column and eluting the protein with a linear salt gradient. We conclude that the phosphatase was a bacterial contaminant and surmise that the *Chlorella* virus guanylyltransferase does not have an intrinsic RNA triphosphatase activity. The implication, as discussed above for cap methylation, is that γ-phosphate cleavage of PBCV-1 transcripts is performed either by a separate virus-encoded enzyme or by an activity provided by the host.

In conclusion, it is remarkable that PBCV-1 should encode an mRNA capping enzyme that is structurally and functionally more similar to the monofunctional yeast RNA guanylyltransferases than to the multifunctional capping enzymes coded for by other large DNA viruses. It is conceivable that this similarity of A103R to cellular guanylyltransferases is dictated by a unique virus-host dynamic, whereby capping of PBCV-1 mRNAs entails the interaction of a virus-encoded component (the guanylyltransferase) with triphosphatase and methyltransferases encoded by the host.

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