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Biochemistry

Mechanism of mRNA capping by vaccinia virus guanylyltransferase: Characterization of an enzyme–guanylate intermediate

(covalent catalysis)

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ABSTRACT **Vaccinia virus RNA guanylyltransferase catalyzes the transfer of GMP from GTP to the 5′-triphosphate or diphosphate terminus of RNA to generate the cap structure G(5′)ppp(5′)N-. The guanylylation reaction consists of a series of at least two partial reactions:**

(*i*) GTP + E ⇌ E–pG + PP_i,

(*ii*) E–pG + (p)ppNpNpN- → GpppNpNpN- + E.

In the first of these, GTP reacts with capping enzyme in the absence of an RNA acceptor to form a covalent enzyme–guanylate intermediate. The GMP is linked to the M_r 95,000 subunit of the capping enzyme via a phosphoamide bond, as judged by the acid-labile, alkali-stable nature of the bond and by the susceptibility of the linkage to cleavage by hydroxylamine at pH 4.75. The isolated enzyme–guanylate complex is able to transfer the guanylate moiety to triphosphate-terminated poly(A) to yield the 5′ cap structure GpppA or to pyrophosphate to regenerate GTP. Both partial reactions of transguanylylation require a divalent cation.

The 5′-terminal structure of eukaryotic mRNAs consists of a blocked, methylated "cap," ^{m7}G(5′)ppp(5′)NpN. The biochemistry of cap formation has been most effectively studied by using virus core-associated or core-extracted capping enzyme systems (1–6). Modification of the 5′-triphosphate terminus of primary transcripts occurs by the following sequence of enzymatic reactions:

(*i*) $$\text{pppRNA} \xrightarrow[\text{triphosphatase}]{\text{RNA}} \text{ppRNA} + P_i$$

(*ii*) $$\text{ppRNA} + \text{Gp*p*p*} \underset{\text{transferase}}{\overset{\text{guanylyl-}}{\rightleftharpoons}} \text{Gp*pp RNA} + \text{p*p*}$$

(*iii*) $$\text{GpppRNA} + \textit{S}\text{-adenosylmethionine} \xrightarrow[\text{7-methyl-transferase}]{\text{guanine}} {}^{m7}\text{GpppRNA} + \textit{S}\text{-adenosylhomocysteine}$$

in which the RNA terminus is successively cleaved to a diphosphate-terminated RNA, guanylylated, and then methylated at the N7 position of guanine. A multifunctional capping enzyme complex that catalyzes all three of these reactions (individually or in concert) has been purified to apparent homogeneity from vaccinia virus cores (4, 5). The vaccinia capping enzyme has an apparent native M_r of 120,000 and a sedimentation coefficient of 6.5 and consists of two polypeptides of M_r 95,800 and 26,400 (4).

The transguanylylation reaction catalyzed by vaccinia capping enzyme involves a reversible transfer of GMP from GTP to an RNA acceptor, with elimination of pyrophosphate (2, 6). Though the stoichiometry, the cofactor requirements, and the donor and acceptor specificities of this reaction have been defined (2, 3), almost no information is available concerning the catalytic mechanism. Our recent demonstration that vaccinia capping enzyme catalyzes a GTP–$^{32}PP_i$ exchange reaction in the absence of an RNA cap acceptor suggested that transguanylylation occurs via a capping enzyme–guanylate intermediate (4). The present report substantiates the existence of the vaccinia capping enzyme–GMP complex and demonstrates its involvement as an intermediate in the transguanylylation reaction according to the following mechanism:

(*i*) E + Gp*pp ⇌ E–p*G + PP_i

(*ii*) E-p*G + ppRNA → E + G*pppRNA.

MATERIALS AND METHODS

Virus. Vaccinia virus (strain WR) was purified from infected HeLa cells by sedimentation through a sucrose cushion and two sucrose gradient sedimentations (7).

Capping Enzyme. Vaccinia capping enzyme [RNA guanylyltransferase–RNA (guanine-7-)methyltransferase complex] was purified from 300 A_{260} units of vaccinia virions as described (4). In this procedure the enzyme was solubilized from virus cores by treatment with deoxycholate followed by successive chromatography steps on columns of DEAE-cellulose, denatured DNA-cellulose, and phosphocellulose. Further purification was achieved by sedimentation in a glycerol gradient. Capping enzyme was assayed by its ability to catalyze a GTP–PP_i exchange reaction (4). Triphosphate-terminated poly(A) was prepared by using *Escherichia coli* RNA polymerase as described (4).

Materials. Radioactive nucleotides were purchased from Amersham. All other materials were obtained from described sources (4).

RESULTS

Evidence for a Capping Enzyme–Guanylate Complex. The formation of an enzyme–GMP complex as an initial intermediate in the transguanylylation reaction was suggested by the ability of the vaccinia capping enzyme to catalyze an exchange reaction between GTP and $^{32}PP_i$ in the absence of an RNA cap acceptor (4). The enzyme–GMP complex in vaccinia virions was demonstrated directly as follows (see legend to Fig. 1 for details). Permeabilized vaccinia virions were incubated at 37°C with [α-^{32}P]GTP in the presence of $MgCl_2$. After 5 min of incubation, the mixture was centrifuged to remove free GTP, and the viral cores were resuspended in a buffered solution containing 2% $NaDodSO_4$. The solubilized viral material was then subjected to electrophoresis in a polyacrylamide gel containing 0.1% $NaDodSO_4$. As shown in Fig. 1*a*, autoradiography of the gel revealed that ^{32}P from [α-^{32}P]GTP was associated with a single polypeptide migrating with an apparent M_r of 95,000. That

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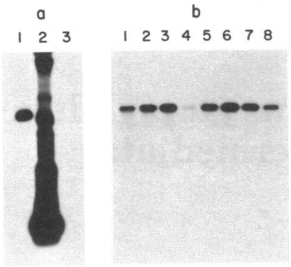

FIG. 1. Formation of protein–[^{32}P]guanine nucleotide complex by vaccinia virions and by purified vaccinia capping enzyme. (*a*) Vaccinia virions. Reaction mixtures (50 μl) containing 60 mM Tris·HCl (pH 8.1), 10 mM dithiothreitol, 4 mM $MgCl_2$, 0.05% Nonidet P-40, 0.36 A_{260} unit of purified vaccinia virus, and nucleoside [^{32}P]triphosphate as indicated were incubated for 5 min at 37°C. Reactions were terminated by addition of 4 μl of 0.1 M EDTA, and virions were recovered by centrifugation. The pelleted material was resuspended in 0.2 ml of 10 mM Tris·HCl (pH 8.1) and recentrifuged. The second pellet was dissolved in 25 μl of gel sample buffer [60 mM Tris·HCl, pH 6.8/2% (wt/vol) Na-DodSO$_4$/10% (vol/vol) glycerol] and applied to a 5–20% linear polyacrylamide slab gel (4) containing 0.1% NaDodSO$_4$. Electrophoresis was carried out at 140 V until the bromphenol blue marker had migrated completely out of the gel. An autoradiograph of the gel is shown. The labeled nucleotides included in the reactions were: lane 1, 5 μM [α-^{32}P]GTP (140 Ci/mmol, 1 Ci = 3.7 × 10^{10} becquerels); lane 2, 1 μM [γ-^{32}P]ATP (1000 Ci/mmol); lane 3, 5 μM [α-^{32}P]UTP (140 Ci/mmol). (*b*) Purified capping enzyme. Vaccinia capping enzyme was purified as described (4) and assayed for formation of protein–nucleotide complex at various stages of purification. Reaction mixtures contained 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$, 1 μM [α-^{32}P]dGTP (134 Ci/mmol), and capping enzyme fractions as indicated. The reaction mixture containing vaccinia virus was supplemented with 0.05% Nonidet P-40. After 5 min at 37°C, reactions were halted by addition of 4 μl of 0.1 M EDTA, 75 μl of cold 10% (wt/vol) trichloroacetic acid, and 5μg of bovine serum albumin. Acid-insoluble material was recovered by centrifugation. The acid-insoluble pellet was rinsed with ether, dried, and dissolved in gel sample buffer containing 1% 2-mercaptoethanol. Samples were heated at 100°C for 4 min prior to electrophoresis. An autoradiograph of the gel is shown. The reaction mixtures included: lane 1, 0.3 A_{260} unit of vaccinia virus containing ≈1 unit of GTP–PP$_i$ exchange activity (see legend to Fig. 2 for assay conditions); lane 2, 12 μl of DEAE-cellulose I enzyme preparation containing ≈1.5 units of GTP–PP$_i$ exchange activity; lane 3, 16 μl of DNA-cellulose enzyme preparation containing ≈1.2 units of GTP–PP$_i$ exchange activity; lanes 4–8, 16 μl each of phosphocellulose column fractions 16–20 containing approximately 0.19, 0.85, 0.79, and 0.43 unit of GTP–PP$_i$ exchange, respectively.

the ^{32}P-labeled material was indeed complexed with protein was verified by the quantitative degradation of this species when the solubilized viral material was treated with proteinase K prior to electrophoresis (data not shown).

Labeling of the M_r 95,000 protein with [α-^{32}P]GTP could not be attributed to redistribution of [^{32}P]phosphate and subsequent protein phosphorylation by the virion associated protein kinase. As shown in Fig. 1*a*, lane 2, incubation of virions with [γ-^{32}P]ATP and $MgCl_2$ yielded a completely different pattern of labeled polypeptides, with the predominant phosphorylated species being of low molecular weight.

Labeling of the M_r 95,000 protein occurred in the presence of [α-^{32}P]dGTP or [α-^{32}P]GTP, but did not occur when [α-^{32}P]UTP was included in the reaction mixture (Fig. 1*a*, lane 3). The apparent specificity of this reaction for guanine nucleotides and the similarity in size of the ^{32}P-labeled product to the large subunit of the vaccinia capping enzyme (4) tentatively suggested that the reaction product corresponded to the large capping enzyme subunit containing covalently bound [^{32}P]GMP. This will be confirmed in experiments presented below.

Requirements for Complex Formation in Vaccinia Virions. The formation of ^{32}P-labeled protein by vaccinia virions in the presence of [α-^{32}P]GTP or [α-^{32}P]dGTP required a divalent cation. Omission of $MgCl_2$ reduced the incorporation of ^{32}P from GTP and dGTP into the M_r 95,000 protein by 90% and 98%, respectively. When 6 mM EDTA was included in reaction mixtures lacking $MgCl_2$, complex formation was quantitatively inhibited. The labeled complex was formed rapidly; the amount of radioactivity incorporated into the M_r 95,000 protein was the same at 2, 5, and 10 min of incubation at 37°C. Complex formation was relatively independent of GTP concentration; increasing the [α-^{32}P]GTP concentration from 2 μM to 28 μM stimulated ^{32}P incorporation less than 25%. The amount of ^{32}P-labeled enzyme–GMP complex formed varied linearly with the concentration of vaccinia virions. Incubation of 0.09, 0.225, and 0.45 A_{260} unit of purified virions in a 50-μl reaction mixture containing 5 μM [α-^{32}P]GTP resulted in the incorporation of 1193, 2296, and 3799 cpm (Cerenkov) into the M_r 95,000 polypeptide.

Enzyme–GMP Complex Formation by Purified Capping Enzyme. To confirm that the incorporation of ^{32}P from [α-^{32}P]GTP and [α-^{32}P]dGTP into the M_r 95,000 protein was catalyzed solely by vaccinia guanylyltransferase, we purified the capping enzyme from viral cores. When the soluble enzyme fractions were assayed for their ability to form the protein–GMP complex, it was necessary to include a trichloroacetic acid precipitation step in order to recover the complex relatively free of contaminating nucleoside triphosphate. As shown in Fig. 1*b*, the protein nucleotide complex was stable to acid at 4°C. The appearance of a ^{32}P-labeled band migrating slightly faster than the M_r 95,000 protein was the result of heating the sample at

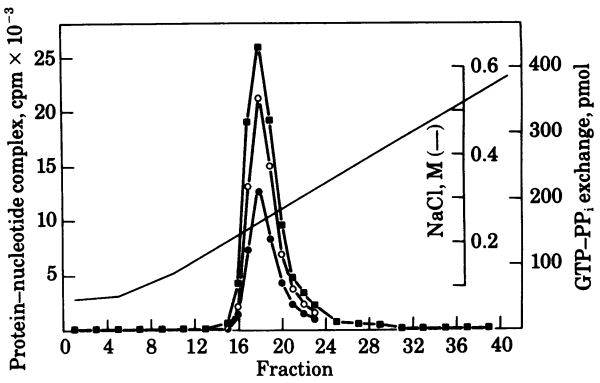

FIG. 2. Phosphocellulose chromatography. Vaccinia capping enzyme was purified through the DNA-cellulose step as described (4). Approximately 380 units (of GTP–PP$_i$ exchange activity) of this enzyme preparation was applied to a 3-ml column of phosphocellulose that had been equilibrated with buffer A (50 mM Tris·HCl, pH 8.2/1 mM EDTA/2.5 mM dithiothreitol/10% glycerol/0.1% Triton X-100) containing 50 mM NaCl. The column was washed with the same buffer and eluted with a 40-ml linear gradient of 0.065–0.6 M NaCl in buffer A. Fractions (1 ml) were collected and capping enzyme was assayed by GTP–PP$_i$ exchange (■). Reaction mixtures (50 μl) containing 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$, 0.3 mM GTP, 1 mM [^{32}P]PP$_i$ (15 cpm/pmol), and 6-μl aliquots of the column fractions were incubated at 37°C for 30 min. Incorporation of ^{32}P into acid-soluble, Norit-adsorbable material was determined as described (4). One unit of GTP-PP$_i$ exchange enzyme catalyzed 1 nmol of ^{32}P incorporation in 30 min at 37°C. ^{32}P radioactivity was assayed by Cerenkov spectrometry. Aliquots (16 μl) of fractions 15–23 were assayed for the ability to form protein–[^{32}P]nucleotide complex in the presence of either 1 μM [α-^{32}P]GTP (●) or 0.75 μM [α-^{32}P]dGTP (○). The specific activity of both nucleotides was 130 Ci/mmol. Reaction conditions, processing of the samples, and electrophoresis conditions were as described in the legend to Fig. 1*b*. The gels were autoradiographed; the M_r 95,000 protein was cut out and Cerenkov radioactivity was measured.

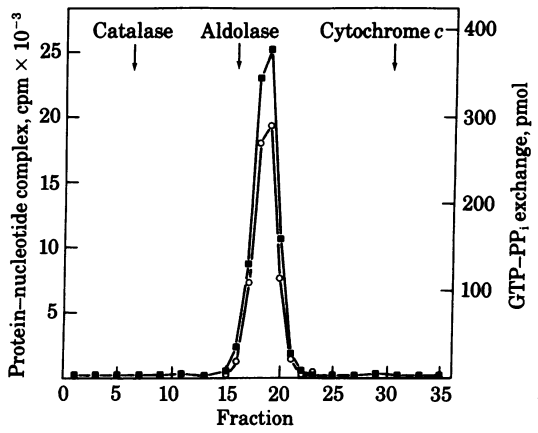

FIG. 3. Glycerol gradient centrifugation. An aliquot (0.7 ml) of phosphocellulose column fraction 19 was made 0.8 M with respect to NaCl and layered on an 1.6-ml linear gradient of 15–35% glycerol in buffer A containing 0.8 M NaCl. The gradient was centrifuged for 62 hr at 4°C in an SW41 rotor. Fractions (≈0.34 ml) were collected from the bottom of the tube. Aliquots (6 μl) of the gradient fractions were assayed for GTP–PP_i exchange activity (■) as described in the legend to Fig. 2, except that incubation was for 45 min. Aliquots (8 μl) of fractions 15–23 were assayed for formation of protein–nucleotide complex in the presence of 1 μM [α-^{32}P]GTP (410 Ci/mmol) as described in the legend to Fig. 2. The M_r 95,000 protein was cut out of the gel and Cerenkov radioactivity was measured (○). The sedimentation of marker proteins in a parallel gradient is indicated.

100°C prior to electrophoresis. This minor band (containing one-tenth the radioactivity present in the major band) was absent when the boiling step was omitted. The minor band was also evident in silver-stained gels of unlabeled capping enzyme preparations when the protein was boiled prior to electrophoresis (4).

The results in Fig. 1*b* indicate that the material with the ability to form the M_r 95,000 complex copurified with capping enzyme through successive chromatography steps on columns of DEAE-cellulose (lane 2), DNA-cellulose (lane 3), and phosphocellulose (lanes 4–8). Fig. 2 shows that the phosphocellulose elution profile of complex-forming activity (with both GTP and dGTP) was coincident with that of the guanylyltransferase (as assayed by GTP–PP_i exchange). Complex-forming activity remained physically associated with capping enzyme during sedimentation in a 15–35% (vol/vol) glycerol gradient containing 0.8 M NaCl (Fig. 3). The enzyme sedimented at 6.5 S relative to the indicated marker proteins.

Table 1. Requirements for formation of enzyme–[^{32}P]GMP complex by purified capping enzyme

Enzyme added, μl	Additions	^{32}P-Labeled M_r 95,000 protein, cpm
2	Complete	2,440
5	Complete	4,940
10	Complete	9,490
15	Complete	16,850
10	Omit $MgCl_2$	26
10	Omit $MgCl_2$, add $MnCl_2$	9,430
10	Omit $MgCl_2$, add $CaCl_2$	156

The complete reaction mixture (50 μl) contained 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$, 1 μM [α-^{32}P]GTP (48,000 Cerenkov cpm/pmol), and the indicated amounts of enzyme from phosphocellulose fraction 18 (Fig. 2). This enzyme fraction contained ≈72 units of GTP–PP_i exchange activity per ml. $MnCl_2$ and $CaCl_2$ were added in place of $MgCl_2$ at 1 mM concentration. After incubation for 5 min at 37°C, the samples were precipitated with trichloroacetic acid, processed, and analyzed by gel electrophoresis as described in the legend of Fig. 2. The ^{32}P-labeled M_r 95,000 protein was localized by autoradiography and cut out, and Cerenkov radioactivity was measured.

Characteristics of Complex Formation by Purified Enzyme. Formation of ^{32}P-labeled M_r 95,000 enzyme–guanylate complex by the purified capping enzyme (phosphocellulose fraction) occurred in the presence of [α-^{32}P]GTP but not in the presence of [β-^{32}P]GTP, suggesting that the complex was formed by transfer of GMP from GTP to the large subunit of capping enzyme with concomitant release of PP_i. As shown in Table 1, complex formation by purified enzyme required a divalent cation; no complex was formed in the absence of $MgCl_2$. Manganese promoted complex formation as effectively as magnesium; calcium, which was previously reported not to support the transguanylylation reaction (2), did not support formation of enzyme–GMP complex. The amount of enzyme–guanylate complex formed varied linearly with the concentration of enzyme (Table 1). If it is assumed that (*i*) no active guanylyltransferase molecules are guanylylated prior to the *in vitro* reaction with GTP, (*ii*) all active enzyme must be capable of complex formation, and (*iii*) there is one GMP molecule bound per M_r 95,000 subunit, then the concentration of active enzyme may be readily determined. From the data in Table 1, the concentration of active capping enzyme in phosphocellulose fraction 18 was found to be 22 μM. These assumptions were also applied to the determination of the num-

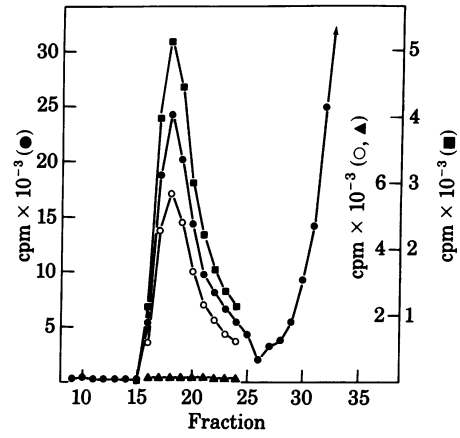

FIG. 4. Isolation of capping enzyme–[^{32}P]GMP complex by gel filtration. Release of [^{32}P]GMP from the isolated complex. Enzyme–GMP complex was prepared in a reaction mixture (50 μl) containing 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$, 1 μM [α-^{32}P]GTP, and 20 μl of phosphocellulose fraction 18. After 5 min at 37°C, the reaction was terminated by addition of 6 μl of 0.1 M EDTA. The sample was immediately applied to a 2.5-ml column of Sephadex G-50 that had been equilibrated with 50 mM NaCl in buffer A at 4°C. Three-drop fractions were collected, and Cerenkov radioactivity was measured (●). Aliquots (25 μl) of fractions 15–24 were prepared for electrophoresis by addition of 0.5 vol of a 3-fold concentrated solution of gel sample buffer containing 3% 2-mercaptoethanol. (The acid preparation step was omitted, as was the heating of the sample prior to electrophoresis.) The M_r 95,000 protein was cut out, and Cerenkov radioactivity was measured (■). Aliquots (20 μl) of fractions 16–24 were precipitated by addition of 0.1 ml of cold 10% trichloroacetic acid and 7 μg of bovine serum albumin. Insoluble material was collected by centrifugation, and the amount of radioactivity in the pellet (○) and the supernatant was determined by Cerenkov spectrometry. Ninety-one percent of the total radioactivity was recovered in the pellet. Incubation of 20 μl of the fractions for 10 min at 37°C in reaction mixtures (50 μl) containing 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$, and 0.1 mM PP_i resulted in quantitative release of ^{32}P from an acid-precipitable form (▲).

Table 2. Requirements for [^{32}P]guanylate release by isolated capping enzyme–GMP complex

Reaction conditions	Acid-insoluble ^{32}P, cpm
Control (no incubation)	7890
Complete	196
Omit $MgCl_2$ and PP_i	7820
Omit PP_i	6750
Omit $MgCl_2$	8070

Capping enzyme–[^{32}P]GMP complex was prepared and isolated free of [α-^{32}P]GTP as described in the legend of Fig. 4. Aliquots (20 μl) of the isolated [^{32}P]GMP-enzyme complex were either acid precipitated directly (control) or were incubated for 5 min in a 50-μl reaction mixture containing 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$ and 0.1 mM PP_i (complete reaction). Reaction components were varied as indicated. Reactions were terminated and acid-insoluble material was recovered as described in the legend of Fig. 4. Acid-insoluble radioactivity was determined by Cerenkov spectrometry. In the control sample, 92% of the total radioactivity was recovered in the trichloroacetic acid-precipitated pellet.

ber of active capping enzyme molecules per vaccinia virus particle. Assuming 1 A_{260} unit of purified virus was equivalent to 1.2×10^{10} particles (7), we found that there were 60–80 active capping enzyme molecules per particle.

Capping Enzyme–GMP Complex Is an Intermediate in Transguanylylation. In order to demonstrate that the capping enzyme–guanylate complex is a functional intermediate in guanylyl transfer, the complex was isolated free of GTP and shown to catalyze GMP transfer to an appropriate acceptor. ^{32}P-Labeled enzyme–GMP complex was generated by using purified enzyme and isolated by Sephadex G-50 gel filtration. As shown in Fig. 4, the enzyme–guanylate complex eluted with the void volume and was clearly separated from [α-^{32}P]GTP. The amount of labeled M_r 95,000 protein obtained after gel electrophoresis paralleled the total radioactivity profile about the void volume, and >90% of the radioactive material in these fractions could be recovered by precipitation with trichloroacetic acid. Incubation of the isolated complex with 4 mM $MgCl_2$ and 0.1 mM PP_i resulted in quantitative release of the radioactivity in an acid-soluble form. The acid-soluble material was completely adsorbable to Norit charcoal, thus ruling out the release of the ^{32}P label as inorganic phosphate or pyrophosphate. The Norit-adsorbed material was eluted with aqueous ethanolic ammonia and analyzed on polyethyleneimine-cellulose plates developed with 1.2 M LiCl. Autoradiography of the chromatograms revealed a major labeled product comigrating with GTP (comprising 93% of the total radioactivity) and a minor species comigrating with GDP (containing 7% of the total radioactivity). The small amount of [^{32}P]GDP was probably derived from [^{32}P]GTP via the action of the nucleoside triphosphate phosphohydrolase activity associated with the capping enzyme complex (4). As shown in Table 2, the release of ^{32}P label from the isolated capping enzyme–guanylate complex was dependent on the presence of a divalent cation ($MgCl_2$) and a suitable acceptor (i.e., pyrophosphate).

When isolated enzyme–guanylate complex was incubated with triphosphate-terminated poly(A) instead of PP_i, the release of [^{32}P]guanylate from the complex was accompanied by formation of capped poly(A). Digestion of the reaction product with nuclease P1 yielded ^{32}P-labeled GpppA, which was identified by thin-layer chromatography on polyethyleneimine-cellulose (Fig. 5). These data indicate that the enzyme–guanylate complex is an intermediate in the mRNA capping reaction and in the GTP–PP_i exchange reaction.

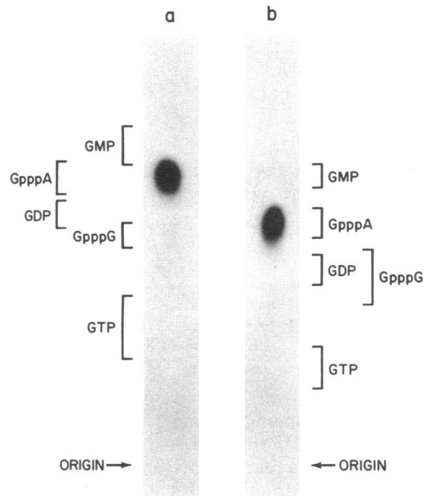

FIG. 5. RNA capping by isolated enzyme–[^{32}P]GMP complex. Enzyme–[^{32}P]GMP complex was prepared and isolated by gel filtration essentially as described in the legend of Fig. 4. A 50-μl aliquot of the isolated complex was incubated in a reaction mixture (0.1 ml) containing 60 mM Tris·HCl (pH 8.2), 4 mM dithiothreitol, 4 mM $MgCl_2$, and 25 pmol (of termini) of triphosphate-terminated poly(A). After 15 min at 37°C, the sample was precipitated with cold 10% trichloroacetic acid and the insoluble material was recovered by centrifugation (80% of the radioactivity was recovered in the pellet). The pellet was resuspended in 0.1 ml of 0.2 M Tris·HCl (pH 8.2), and protein was removed by two phenol extractions. The aqueous material was precipitated with ethanol, dried, and resuspended in H_2O. The sample was then digested for 90 min at 37°C with 40 μg of nuclease P1 in the presence of 25 mM sodium acetate, pH 6.0/2 mM $MgCl_2$. The digest was deproteinized by phenol extraction and aliquots of the sample were chromatographed on polyethyleneimine-cellulose plates developed with either 1.2 M LiCl (*a*) or 0.6 M $(NH_4)_2SO_4$ (*b*). The chromatograms were autoradiographed and the positions of marker nucleotides were located by UV illumination.

Table 3. Stability of the capping enzyme–GMP linkage

Exp.	Additions and incubation	Acid-insoluble ^{32}P, cpm
1	Control, 5 min, 100°C	1050
	0.1 M HCl, 5 min, 100°C	31
	0.1 M NaOH, 5 min, 100°C	923
2	Control, no additions, no incubation	3240
	3.86 M hydroxylamine (pH 4.75), 20 min, 37°C	39*
	4 M sodium acetate (pH 4.75), 20 min, 37°C	3170
	0.2 M hydroxylamine (pH 7.5), 20 min, 37°C	1920

Exp. 1. Enzyme–[^{32}P]guanylate complex was isolated by gel filtration and made 1% with respect to $NaDodSO_4$. Aliquots (10 μl) of this preparation received 0.2 ml of H_2O (control), 0.2 ml of 0.1 M HCl, or 0.2 ml of 0.1 M NaOH. The samples were heated for 5 min, placed on ice, and then treated with 2 ml of 10% trichloroacetic acid and 120 μg of denatured DNA. After the sample was kept on ice for 5 min, acid-insoluble material was collected on glass-fiber filters and radioactivity was measured in toluene-based scintillation fluid. *Exp. 2.* Aliquots (10 μl) of isolated enzyme–[^{32}P]GMP complex containing 1% $NaDodSO_4$ received 0.15 ml of the indicated reagents. After incubation for 20 min at 37°C, reactions were terminated by addition of 2 ml of 10% trichloroacetic acid. Acid-insoluble radioactivity was determined as in Exp. 1. The control sample was acid precipitated with no additions and no incubation at 37°C.

* Incubation for 5 min at 37°C in 3.86 M hydroxylamine (pH 4.75) reduced acid-insoluble radioactivity to 770 cpm.

Characterization of Capping Enzyme–GMP Linkage. The stability of the enzyme–[^{32}P]guanylate complex to acid precipitation at 4°C and to boiling in $NaDodSO_4$ at neutral pH (Fig. 1*b*) indicates that the guanylate residue is covalently attached to the large subunit of the enzyme. Studies of the nature of the protein–GMP linkage are shown in Table 3.

The [^{32}P]GMP–enzyme complex remained acid precipitable after boiling for 5 min in 0.1 M NaOH yet was quantitatively dissociated by boiling for 5 min in 0.1 M HCl. These properties (i.e., acid lability and alkaline stability) suggest a phosphoamide linkage between GMP and the protein (8). This was substantiated by cleavage of the enzyme–[^{32}P]GMP complex by hydroxylamine. Following the protocol of Gumport and Lehman (9), we found that the complex was quantitatively dissociated by 3.86 M hydroxylamine (pH 4.75) at 37°C, but was stable in the presence of 4 M sodium acetate at the same pH and temperature. Cleavage by neutral hydroxylamine was far less pronounced. The data tend to rule out a phosphoester or mixed anhydride linkage but strongly support a phosphoamide linkage—e.g., GMP (5′ → Nε)lysine or GMP (5′ → Nimid)histidine.

DISCUSSION

Vaccinia RNA guanylyltransferase catalyzes the transfer of GMP from GTP to the 5′-diphosphate terminus of RNA to yield the cap structure G(5′)ppp(5′)N. We have shown that the capping reaction occurs in the following sequence of at least two partial reactions:

$$(i) \qquad \text{Gppp} + \text{E} \rightleftharpoons \text{E–pG} + \text{PP}_i$$

$$(ii) \qquad \text{E–pG} + \text{(p)ppRNA} \rightharpoonup \text{E} + \text{GpppRNA}.$$

In reaction *i*, the capping enzyme reacts with GTP to form an enzyme–guanylate intermediate with concomitant release of PP_i. The intermediate consists of a GMP residue covalently linked to the M_r 95,000 capping enzyme subunit, apparently via a phosphoamide bond. Formation of the enzyme–guanylate complex requires GTP and a divalent cation, but does not require the presence of a cap acceptor. dGTP readily substitutes for GTP in formation of a stable enzyme–nucleotide complex, but UTP does not participate in this reaction. Because only GTP and dGTP are donors in the capping reaction (3), it appears that the donor nucleotide specificity of the vaccinia capping enzyme resides at the level of formation of the enzyme–nucleotide intermediate. Either magnesium or manganese (but not calcium) will satisfy the metal cofactor requirement in reaction *i*. A similar cofactor specificity has been shown for the complete capping reaction (2).

In reaction *ii*, the guanylylated enzyme will (in the absence of GTP) catalyze transfer of the GMP moiety to the 5′ terminus of RNA to form a GpppN cap structure. The ability of the isolated enzyme–GMP intermediate to cap triphosphate-terminated poly(A) suggests that the RNA triphosphatase activity of the capping enzyme (4, 5) remains associated with the guanylylated enzyme. The enzyme–GMP intermediate will also react with PP_i to regenerate GTP. Guanylyl transfer to either PP_i or RNA requires $MgCl_2$.

Previous studies (4, 5) have established that three classes of enzymatic reactions (γ-phosphate cleavage, transguanylylation, and transmethylation) are catalyzed by a vaccinia capping enzyme complex consisting of two major subunits of M_r 95,800 and 26,400 (4). However, none of the enzymatic functions had been assigned with certainty to any of the subunits. The demonstration that the M_r 95,000 subunit contains the site of covalent guanylylation clearly implicates this subunit in the transguanylylation reaction. Though the M_r 95,000 subunit is necessary for transguanylylation according to the proposed mechanism, it is not clear whether it is sufficient to catalyze guanylyl transfer in the absence of the M_r 26,000 protein nor is it apparent where the functional domains of the methyltransferase or RNA triphosphatase activities are localized. The ability to specifically label the active site on the M_r 95,000 subunit with [α-^{32}P]GTP should facilitate attempts to address these and other structure–function relationships.

The mechanism of the transguanylylation reaction within the vaccinia virus core is almost certainly the same as that of the purified enzyme, as shown by the ability of the cores to form the [^{32}P]GMP- or dGMP-labeled M_r 95,000 protein and to transfer the guanylate residue back to PP_i in the absence of GTP. It is also likely that a mechanism of capping involving a covalent enzyme–guanylate intermediate is operative in other capping systems (such as reovirus cores, HeLa cell nuclei, and rat liver nuclei) that catalyze transfer of GMP from GTP to RNA with displacement of PP_i (1, 10, 11). Like the vaccinia enzyme, the partially purified rat liver guanylyltransferase catalyzes $^{32}PP_i$ exchange with GTP (11). Further studies will be necessary to establish the generality of this mechanism.

A covalent enzyme–adenylate intermediate has been shown to participate in the DNA ligase reaction via a mechanism similar to that of transguanylylation (12). Significantly, in *E. coli* DNA ligase and the T4 DNA ligase, the AMP moiety is linked to the ε-amino group of a single lysine residue of the enzyme via a phosphoamide bond (9). The capping enzyme–guanylate linkage, too, seems to be a phosphoamide bond, though the amino acid residue has not been identified. It has been suggested by Shabarova (8) that enzyme–nucleotide phosphoramidates may be a general mechanistic feature of nucleotidyltransfer reactions.

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