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Eukaryotic mRNA capping enzyme-guanylate covalent intermediate

(mRNA guanylyltransferase/HeLa cell/phosphoamide/covalent catalysis)

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ABSTRACT **Incubation of HeLa cell mRNA guanylyltransferase (GTP:mRNA guanylyltransferase, EC 2.7.7.50) with [α-^{32}P]GTP and a divalent cation in the absence of an RNA acceptor results in the formation of a covalent enzyme-guanylate complex. The complex, after purification by phosphocellulose chromatography, can transfer its bound GMP moiety to pyrophosphate, regenerating GTP, or to the 5′-diphosphate end of poly(A), forming a cap structure G(5′)pppA(pA)$_n$. The GMP-polypeptide has a molecular weight of 65,000 and is stable to heating in the presence of sodium dodecyl sulfate. On the basis of the alkali-stable and acid-labile nature of the bond and its susceptibility to nucleophilic attack by hydroxylamine at low pH, the GMP-polypeptide linkage appears to be a phosphoamide bond. After digestion with trypsin, a single GMP-peptide was resolved by two-dimensional electrophoresis and chromatography.**

Capping and methylation are important early steps in eukaryotic mRNA processing and are required for formation of stable translationally active messages (see ref. 1 for review). Enzymes that catalyze the following reactions, in which pppN-N- represents the 5′ end of nascent eukaryotic mRNA, and AdoMet and AdoHcy represent *S*-adenosylmethionine and *S*-adenosylhomocysteine, respectively, have been identified:

(*i*) pppN-N- → ppN-N- + P_i

(*ii*) GTP + ppN-N- ⇄ G(5′)pppN-N- + PP_i

(*iii*) AdoMet + G(5′)pppN-N- → m^7G(5′)pppN-N- + AdoHcy

(*iv*) AdoMet + m^7G(5′)pppN-N- → m^7G(5′)pppNm-N- + AdoHcy

(*v*) AdoMet + m^7G(5′)pppNm-N → m^7G(5′)pppNm-Nm- + AdoHcy.

A mRNA guanylyltransferase (EC 2.7.7.50) or capping enzyme that catalyzes step *ii* was isolated from HeLa cell (2–4) and rat liver (5) nuclei and from wheat germ (6). Although their substrate specificities were defined, eukaryotic capping enzymes have been only partially purified and their catalytic properties have not yet been fully investigated. Significantly, however, rat liver capping enzyme preparations were found to catalyze a second reaction, GTP–PP_i exchange (5). A RNA triphosphatase that catalyzes step *i* was separated from capping enzyme at an early stage in the purification of the latter, accounting for the diphosphate end specificity (3, 4). Separate methyltransferases that catalyze steps *iii–v* (7, 8) and an additional one that methylates adenine residues of cap I structures (9) have also been isolated from HeLa cells.

Several viruses that replicate in the cytoplasm of infected cells, including cytoplasmic polyhedrosis virus, vaccinia virus, reovirus, and vesicular stomatitis virus, have independent capping and methylating systems (see ref. 1 for review). A multifunctional enzyme complex that contains subunits of 95,000 and 37,000 daltons and that catalyzes steps *i*, *ii*, and *iii* above has been purified from vaccinia virus to near homogeneity and extensively characterized (10–14). This viral enzyme complex was shown to carry out GTP–PP_i exchange (15) and to form a covalent GMP intermediate upon incubation with GTP (16).

We now demonstrate that a eukaryotic mRNA guanylyltransferase obtained from HeLa cells, which differs from the vaccinia virus enzyme in several notable respects, including smaller size and absence of associated triphosphatase and methyltransferase activities, also forms a covalent GMP complex. The stable enzyme intermediate quantitatively transfers its GMP residue to the 5′ end of RNA to form a cap structure or to PP_i to form GTP. A 65,000-dalton GMP-polypeptide was resolved by polyacrylamide gel electrophoresis, indicating that the HeLa cell enzyme contains a single subunit. Its active site was identified by tryptic peptide analysis and the nature of the covalent GMP linkage was determined. A similar wheat germ RNA guanylyltransferase-GMP intermediate has also been found (J. Keith, personal communication), suggesting a common catalytic mechanism for eukaryotic and some viral capping enzymes.

MATERIALS AND METHODS

Enzyme Purification. RNA guanylyltransferase was purified by ion-exchange and affinity chromatography from 50 liters of logarithmically growing HeLa cells as described (3). One enzyme unit catalyzes transfer of 1 pmol of GMP from GTP to poly(A) in 30 min (3).

Demonstration of Enzyme-GMP Covalent Intermediate. Reaction conditions were adapted from those previously used for assaying RNA guanylyltransferase (4). Approximately 0.5 unit of enzyme was incubated with 2 μM [α-^{32}P]GTP (400 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels)/25 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) (pH 7.25)/2.5 mM $MnCl_2$/4 mM dithiothreitol/0.02% Triton X-100 in a total volume of 50 μl. After incubation at 37°C for 5 min, the reaction was terminated by addition of 0.1 ml of 10 mM EDTA containing 0.1 mg of bovine serum albumin. Protein was precipitated at 0°C for 10 min in 7.5% trichloroacetic acid and then washed twice with ice-cold 70% (vol/vol) ethanol and twice with diethyl ether. The final pellet was dissolved in 40 mM Tris/glycine (pH

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Abbreviations: Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid; PEI, polyethyleneimine.

8.8)/2% sodium dodecyl sulfate/1% 2-mercaptoethanol/0.02% bromophenol blue. After heating for 3 min at 95°C, electrophoresis was performed with a discontinuous buffer system containing 0.1% sodium dodecyl sulfate in a 15% polyacrylamide slab gel alongside radioactive protein molecular weight markers (17). The gel was then dried and autoradiographed. Radioactive bands were eluted and measured by liquid scintillation spectrophotometry. Under these conditions, 5 fmol of enzyme-GMP complex was formed.

Purification of Active Enzyme-GMP Intermediate. Approximately 5–10 units of purified RNA guanylyltransferase was incubated with 2.5 μM [α-^{32}P]GTP as described above except the reaction volumes were increased to 200 μl. After 5 min at 37°C, 0.5 ml of 25 mM Tris·HCl (pH 7.25)/0.05 M NaCl/2.5 mM EDTA/0.1% Triton X-100 containing 200 μg of bovine serum albumin was added to stop the reaction. Capping enzyme was bound to a phosphocellulose column (0.7 × 1.0 cm) equilibrated with 25 mM Tris·HCl (pH 7.25)/0.05 M NaCl/0.1 mM EDTA/0.1% Triton X-100. After the column had been washed with at least 20 bed volumes of equilibration buffer, the protein was eluted with 3 bed volumes of buffer containing 0.5 M NaCl. The eluate was diluted to reduce the NaCl concentration to 0.1 M and the phosphocellulose chromatography step was repeated to remove all traces of [α-^{32}P]GTP.

Tryptic Peptide Analysis. Phosphocellulose-purified [^{32}P]GMP-enzyme was precipitated with trichloroacetic acid with 0.25 mg of bovine serum albumin carrier and then washed twice with 70% ethanol and twice with ether. The pellet was dissolved in 0.2 ml of 0.05 M NH_4HCO_3 and digested with 15 μg of trypsin at 37°C for 90 min. An additional 15 μg of trypsin was added and the incubation was continued for 2 more hr. The hydrolysate was dried by lyophilization and dissolved in 10 μl of pyridine/acetic acid/water (2.5:2.5:95, vol/vol) pH 4.5 electrophoresis buffer. The insoluble residue was removed by centrifugation, leaving greater than 75% of the radioactive material in the supernatant, which was then spotted on a 20 × 20 cm thin-layer cellulose plate and electrophoresed at 500 V for 90 min. The plate was air dried, turned 90°, and chromatographed in 1-butanol/acetic acid/pyridine/water (75:15:60:60, vol/vol).

Sources of Materials. Nucleotides and dinucleoside triphosphate cap markers were purchased from P-L Biochemicals. Nuclease P1, calf intestinal alkaline phosphatase, and tosylamidophenylethyl chloromethyl ketone-treated trypsin were obtained from Accurate Chemicals, Boehringer Mannheim, and Worthington, respectively. Phosphocellulose (P-11), Sephadex G-75, and polyethyleneimine (PEI) and cellulose thin-layer plates were supplied by Whatman, Pharmacia, and Brinkmann, respectively. Radioactive chemicals were from Amersham.

RESULTS

Demonstration of Enzyme-Guanylate Complex. Pooled enzyme fractions obtained during various stages of purification of the HeLa cell RNA guanylyltransferase were incubated with [α-^{32}P]GTP under conditions suitable for capping, except that no RNA acceptor was present. Unincorporated GTP was removed by precipitation with trichloroacetic acid and the protein pellet was dissolved in a buffer containing sodium dodecyl sulfate and mercaptoethanol. After heating at 95°C, the samples were subjected to polyacrylamide gel electrophoresis. Autoradiographs of the dried gel indicated the presence of a single labeled band from all pooled enzyme fractions (Fig. 1). By comparison with radioactive protein markers, the molecular weight of the GMP-polypeptide was estimated to be 65,000. As a control, the vaccinia virus RNA guanylyltransferase-GMP intermediate (16) was electrophoresed in parallel; it had a molecular weight of approximately 95,000 (Fig. 1). Further experiments indicated that maximal amounts of GMP-enzyme complex

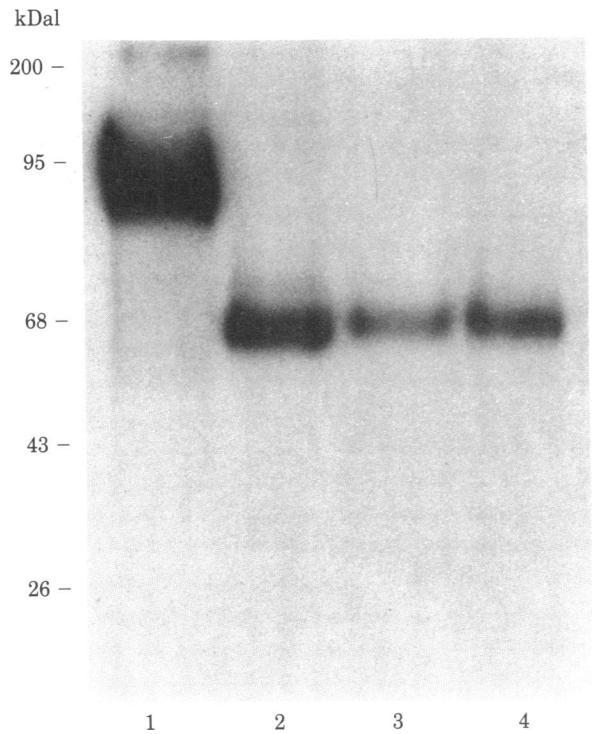

FIG. 1. Identification of capping enzyme-guanylate complex by polyacrylamide gel electrophoresis. Pooled HeLa cell RNA guanylyltransferase fractions after successive purification on phosphocellulose (0.83 unit, 1.2 μg of protein, track 2), cibacron blue-agarose (0.26 unit, 0.2 μg of protein, track 3), and GTP-agarose (0.21 unit, 0.1 μg of protein, track 4) columns were incubated with [α-^{32}P]GTP. Purified vaccinia virus capping enzyme (5 units) was used as a control (track 1). After precipitation with trichloroacetic acid, the material was heated at 95°C in sodium dodecyl sulfate and mercaptoethanol and analyzed by polyacrylamide gel electrophoresis. An autoradiograph is shown. The numbers on the left indicate the molecular masses in kilodaltons of radioactively labeled marker proteins.

formed within 5 min at 37°C and that either Mn^{2+} or Mg^{2+} was required.

Nucleotide Specificity. Previous studies indicated that the HeLa cell RNA guanylyltransferase could not form cap structures with ATP, CTP, UTP, dGTP, or m^7GTP as donors; only GTP and ITP were effective (4). Attempts to form a covalent enzyme intermediate by using various [α-^{32}P]NTPs indicated a similar specificity. Only with [α-^{32}P]GTP was an intense radioactively labeled polypeptide band detected (Fig. 2). A faint radioactive band was seen with [α-^{32}P]dGTP; however, this product was not further characterized. A very faint band was also obtained when [α-^{32}P]ATP was used. The molecular weight of the AMP-labeled polypeptide was greater than that of the GMP-labeled polypeptide, however, indicating a different enzyme activity, possibly a DNA or RNA ligase (18). In this regard, it is important to point out that the HeLa cell RNA guanylyltransferase has not been purified to homogeneity.

Tryptic Peptide Analysis. As a further step in the physical characterization of the GMP-enzyme intermediate, tryptic peptide analysis was performed. The GMP-peptide migrated cathodally during electrophoresis at pH 4.5 and anodally at pH 6.5. One major radioactive spot was detected when electrophoresis at either pH was followed by chromatography in the second dimension. A pH 4.5 pattern is shown in Fig. 3. Preliminary experiments indicate the GMP-peptide obtained by trypsin digestion of the vaccinia virus RNA guanylyltransferase-guanylate intermediate did not comigrate with the HeLa cell GMP-peptide (data not shown).

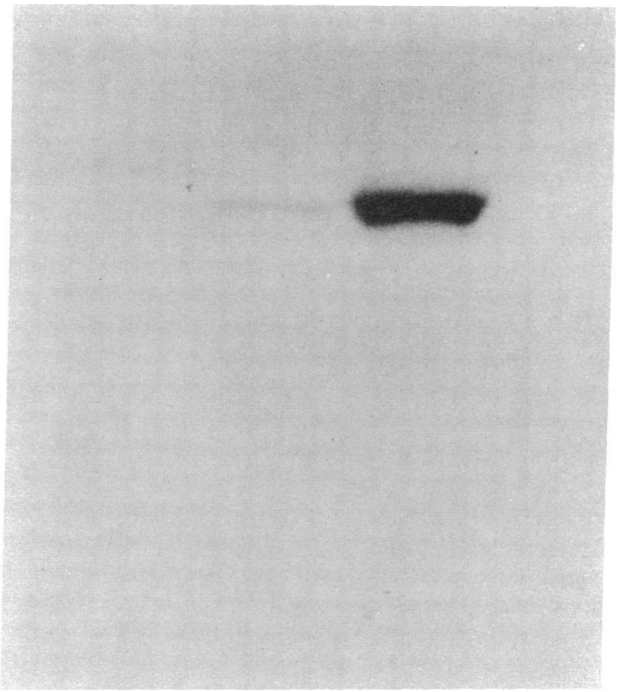

FIG. 2. Nucleotide specificity for formation of covalent capping enzyme complex. HeLa cell mRNA guanylyltransferase (1 unit) was incubated with the indicated α-^{32}P-labeled NTP or dNTP at 2 μM and the sample was analyzed by polyacrylamide gel electrophoresis as in Fig. 1. The numbers below the autoradiograph are the amounts of radioactive material recovered from the labeled protein band in the case of ATP, dGTP, and GTP. Because no labeled band was seen with UTP, a section of the gel corresponding in position to the enzyme-GMP complex was eluted and its radioactivity was measured.

Chemical Nature of the GMP Linkage. To determine the nature of the GMP linkage, purified ^{32}P-labeled GMP-polypeptide was subjected to the various treatments indicated in Table 1, and trichloroacetic acid-insoluble and -soluble radioactive materials were measured. Its resistance to alkali and sensitivity to strong acid and nucleophilic attack by hydroxylamine at low pH suggested a phosphoamide bond. Similar results were obtained with vaccinia virus enzyme-GMP intermediate (16).

Reversal of GMP-Enzyme Intermediate Formation. Active GMP-enzyme intermediate free of detectable GTP was obtained by chromatography two times on small phosphocellulose columns. When the purified GMP-polypeptide was incubated with pyrophosphate and Mn^{2+}, virtually quantitative conversion of the ^{32}P label to a trichloroacetic acid-soluble form was obtained (Table 2). This conversion did not occur in the absence of pyrophosphate or divalent cation. The acid-soluble component was shown to be GTP by thin-layer chromatography on PEI-cellulose (Fig. 4). Trace amounts of GDP were also detected. Quantitative reversal could be demonstrated with as little as 4 μM pyrophosphate.

Transfer of GMP to a Polyribonucleotide Acceptor. To prove that the covalent GMP-polypeptide is a true RNA guanylyltransferase intermediate, transfer of the GMP to a suitable polyribonucleotide acceptor was necessary. Accordingly, phosphocellulose purified GMP-enzyme was incubated with a 5′-diphosphate-terminated poly(A) and Mn^{2+}. After extraction with chloroform/phenol and precipitation of the aqueous layer with ethanol, more than 80% of the radioactively labeled material was recovered, suggesting transfer to the polynucleotide. This was confirmed by digesting the labeled material with nuclease P1 and alkaline phosphatase. The expected product, G(5′)pppA, was identified by PEI-cellulose thin-layer chromatography (Fig. 5). Careful quantitation indicated that at least 75% of the GMP in the enzyme intermediate had been transferred to form the cap structure.

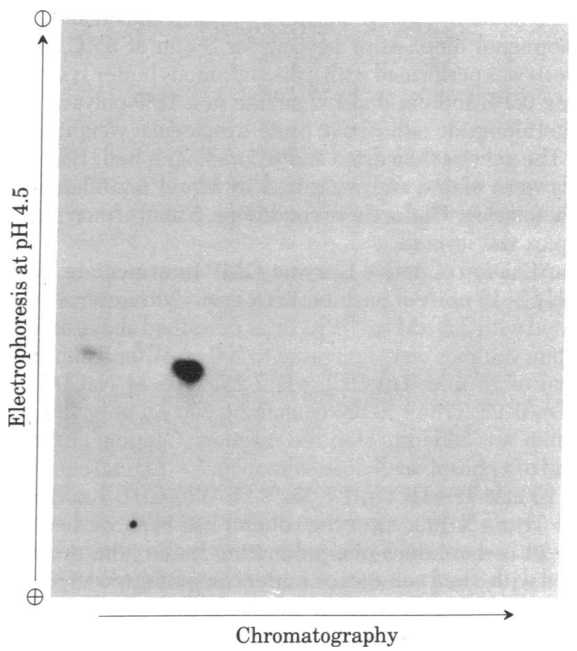

FIG. 3. Two-dimensional tryptic peptide analysis of capping enzyme-GMP complex. After purification by two rounds of phosphocellulose chromatography, the ^{32}P-labeled capping enzyme-guanylate complex was digested with trypsin and applied to a 20 × 20 cm thin-layer cellulose plate. Electrophoresis at pH 4.5 was followed by chromatography in the second dimension. The small autoradiographic spot near the left corner of the plate corresponds to the origin.

Formation of GMP-Enzyme Intermediate from Capped Poly(A). We have shown that an active enzyme intermediate was formed by incubating RNA guanylyltransferase with [α-^{32}P]GTP. Moreover, this first step was reversed by addition of pyrophosphate. However, when a polynucleotide acceptor was added, GMP was transferred to form a cap structure. To see if this second step could be reversed, we incubated vaccinia virus RNA guanylyltransferase and HeLa cell RNA guanylyltransferase with G(5′)*pppA(pA)$_n$ and then analyzed the products by polyacrylamide gel electrophoresis. With vaccinia virus enzyme, formation of a 95,000-dalton covalent enzyme intermediate was readily seen, whereas only a faint band of 65,000 daltons was detected with the HeLa cell enzyme.

DISCUSSION

Previous studies with HeLa cell mRNA guanylyltransferase established that the net reaction consists of transfer of a GMP residue from GTP to the 5′-diphosphate end of RNA or oligonucleotide acceptor (4). The present study reveals that the transfer occurs via a stable covalent enzyme-guanylate intermediate. This intermediate was identified by incubating partially purified capping enzyme preparations with [α-^{32}P]GTP in the absence of a RNA acceptor. The complex, after removal of unbound GTP by repeated phosphocellulose chromatography, could transfer its GMP residue either to PP_i, forming GTP, or to the 5′-diphosphate end of poly(A), forming a cap structure. These partial reactions are indicated below (E, enzyme):

$$(i) \quad \text{GTP} + \text{E} \rightleftarrows \text{E-pG} + PP_i$$

$$(ii) \quad \text{E-pG} + \text{ppA(pA)}_n \rightarrow \text{E} + \text{G(5′)pppA(pA)}_n.$$

Table 1. Chemical reactivity of guanylyltransferase-GMP complex

Treatment*	Acid-insoluble ^{32}P cpm	Acid-soluble ^{32}P cpm
H_2O, 10 min at 70°C	1583	58
0.1 M HCl, 10 min at 70°C	258	1756
0.1 M NaOH, 10 min at 70°C	1787	68
3.86 M NH_2OH (pH 4.75), 20 min at 37°C	32	2385
3.2 M NaOAc (pH 4.5), 20 min at 37°C	2500	84
0.2 M NH_2OH (pH 7.5), 20 min at 37°C	2025	185

* Guanylyltransferase was incubated under standard conditions with 2 μM [α-^{32}P]GTP for 5 min. Reaction was terminated by addition of EDTA (10 mM) and bovine serum albumin (100 μg) followed by three successive precipitations with ice-cold trichloroacetic acid (7.5%). The acid-insoluble pellet was washed twice with cold 70% ethanol and then ether, dissolved, and chromatographed on a Sephadex G-75 column. Radioactive material eluting in the void volume was pooled and monitored by denaturing $NaDodSO_4$/polyacrylamide gel electrophoresis under conditions permitting retention of any P_i and GMP in the gel. The gel was vacuum dried on DEAE-paper and the radioactivity was visualized by autoradiography. Under these conditions, a single radioactive enzyme-GMP band was visualized free of any contaminating nucleotide or orthophosphate. Aliquots (10 μl) of purified GMP-enzyme complex were incubated with 200 μl of the various reagents as shown in the table above. After incubation, the enzyme was precipitated from 10% trichloroacetic acid in a final volume of 0.5 ml after addition of 50 μg of bovine serum albumin. Acid-soluble supernatants were extracted with ether; acid-insoluble pellets were washed with 70% ethanol and then ether and finally dissolved in 0.5 ml of 0.02 M Tris·HCl (pH 7.5)/0.5% sodium dodecyl sulfate. The radioactivities of both fractions were measured by liquid scintillation spectrometry after addition of 10 ml of Aquasol.

Reaction *i* is specific for GTP, although dGTP may be used to a slight extent. A divalent cation, either Mn^{2+} or Mg^{2+}, is required for forward and backward reactions. The complete reversal of step *i* by micromolar concentrations of PP_i accounts for the previously described inhibition of the complete reaction by this compound (4) and for GTP–PP_i exchange (5). Although transfer of the GMP moiety from the enzyme complex to RNA is quantitative, the reverse reactions could barely be demonstrated. This difficulty in reversal of step *i* accounts for our inability to reverse the complete reaction (4).

Sucrose gradient sedimentation of the HeLa cell capping enzyme suggested a molecular weight of about 49,000 (3). Because the enzyme-guanylate complex migrated during sodium dodecyl sulfate/polyacrylamide gel electrophoresis as a 65,000-dalton polypeptide, the HeLa cell mRNA guanylytransferase must contain only one subunit. The active site of the enzyme was identified by peptide analysis. A single GMP-peptide was resolved by two-dimensional electrophoresis and chromatography. Although the GMP-bound amino acid was not determined, the chemical reactivity strongly suggested a phosphoamide linkage similar to that found with *Escherichia coli* and T4 DNA ligase (18). In those cases, an adenylate residue is connected to the ϵ-amino group of lysine. Indeed, the capping enzyme nucleotidyl transfer reaction is quite similar to that catalyzed by DNA ligase. By contrast, phosphodiester bonds link DNA to tyrosine residues of topoisomerases (19, 20), poliovirus RNA to tyrosine of the Vpg protein (21, 22), and adenovirus (23) and phage ϕ29 (24) DNA to serine residues of terminal proteins.

Significantly, covalent guanylate complexes have been found for vaccinia virus (16) and wheat germ (J. Keith, personal communication) as well as HeLa cell capping enzymes, indicating a common catalytic mechanism for modifying animal, plant, and

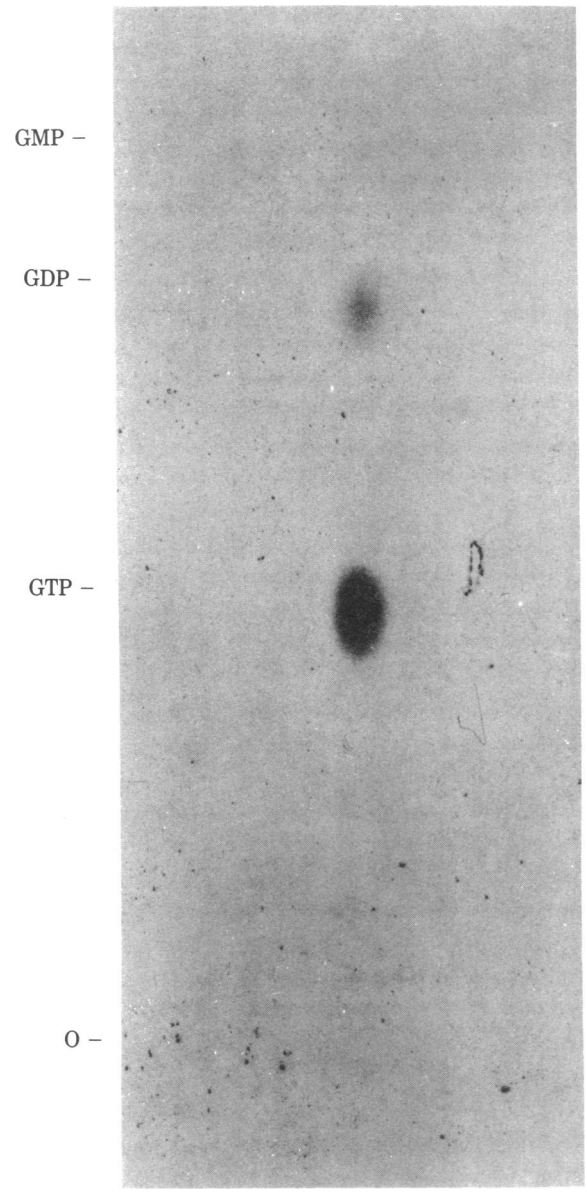

FIG. 4. Pyrophosphorolysis of capping enzyme-guanylate complex. Purified capping enzyme-guanylate complex (3000 cpm Cerenkov) was incubated at 37°C for 10 min in a "complete" 50-μl reaction mixture containing 25 mM Tes (pH 7.25), 2.5 mM $MnCl_2$, 2.5 mM dithiothreitol, 0.1 mM EDTA, and 0.02 mM sodium pyrophosphate. The reaction was terminated by addition of EDTA and sodium dodecyl sulfate. After coprecipitation with 100 μg of serum albumin, the trichloroacetic acid-soluble material was extracted with ether three times, diluted 1:5 with 10 mM triethylammonium bicarbonate (pH 7.5), and applied to a DEAE-cellulose column. After washing, bound material was eluted with 1 M triethylammonium bicarbonate. This eluate, containing 95% of the applied acid-soluble material, was dried and applied to a PEI-cellulose thin-layer plate with appropriate unlabeled nucleotide markers. After chromatography in 1.6 M LiCl, the plate was autoradiographed and examined under UV illumination. O, origin.

Table 2. Pyrophosphorolysis of capping enzyme-guanylate complex

Conditions	Acid-soluble ^{32}P cpm
Complete reaction	2280
$-PP_i$	260
$-MnCl_2$	480
+EDTA at 3 mM	186

Purified HeLa cell capping enzyme-GMP complex was incubated as described in the legend to Fig. 4.

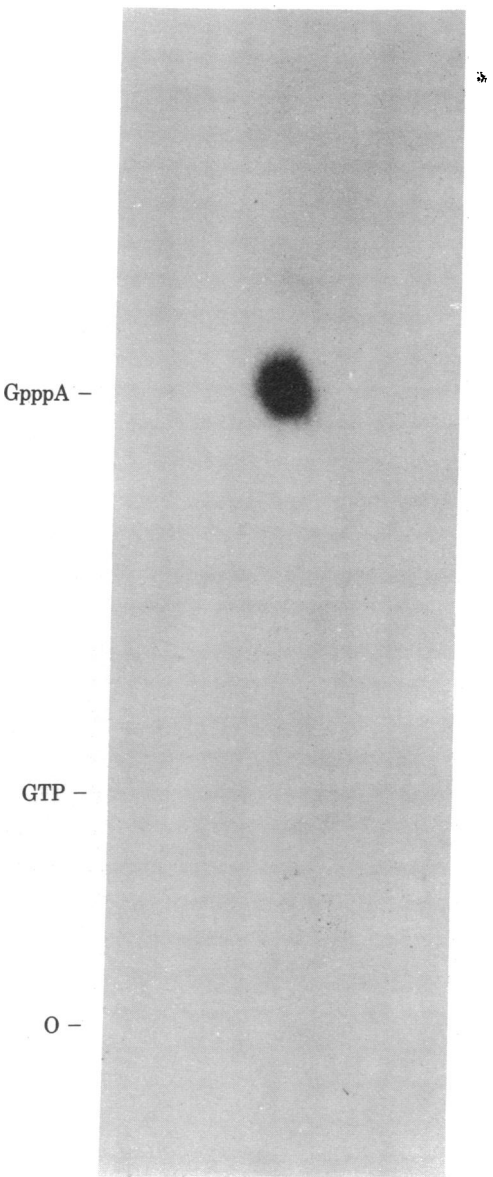

FIG. 5. Transfer of GMP from capping enzyme-guanylate complex to a polyribonucleotide acceptor. Purified capping enzyme-[^{32}P]guanylate complex (4000 cpm Cerenkov) was incubated with 10 pmol of 5'-diphosphate-terminated poly(A) in 50 μl of 25 mM Tes (pH 7.25)/2.5 mM $MnCl_2$/4 mM dithiothreitol at 37°C for 30 min. After deproteinization by phenol/chloroform (1:1) extractions, the poly(A) in the aqueous phase was coprecipitated with 50 μg of commercial poly(A) by addition of ethanol. The pellets were washed twice with 70% ethanol, dried, and digested with nuclease P1 and calf alkaline phosphatase. The hydrolysate and appropriate nucleotide markers were applied to a PEI-cellulose thin-layer plate and chromatographed with 1.6 M LiCl. An autoradiograph is shown.

vaccinia virus mRNAs. The possibility that the mRNA guanylyltransferase subunit of the vaccinia capping enzyme complex is derived from the cellular enzyme was eliminated in the present study. First, the HeLa cell GMP-polypeptide has a molecular weight of 65,000, whereas the GMP-polypeptide of vaccinia virus grown in HeLa cells has a molecular weight of 95,000 (16). Second, the GMP-peptides obtained by trypsin digestion of the two enzymes were resolved from each other. Other differences between cellular and viral enzymes were also noted. Shuman and Hurwitz (16) reported that dGTP was almost as effective as GTP in forming the vaccinia enzyme covalent nucleotide intermediate. By contrast, only traces of the intermediate were formed with dGTP and the HeLa cell enzyme.

Although the HeLa cell RNA guanylyltransferase has been purified as a single-subunit enzyme, it seems possible that it interacts with other proteins involved in the synthesis and modification of mRNA. In this regard, Bajszár and coworkers (25) reported that 30S ribonucleoprotein particles from rat liver contain RNA guanylyltransferase and methyltransferase activities. On the basis of *in vivo* data that capping occurs soon after initiation of RNA synthesis (26, 27), *in vitro* data that efficient capping occurs in crude extracts that initiate transcription from defined templates (28), and analogy with cytoplasmic polyhedrosis virus (29), Darnell and coworkers suggested an association of capping enzymes with initiation complexes for RNA polymerase II transcription (26). Our preliminary experiments indicate no capping enzyme activity associated with purified calf thymus RNA polymerase. The capping enzyme-GMP intermediate, however, may provide a tracer to follow possible interactions with RNA polymerase or other proteins in purified or crude systems.

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