

## Modification of the 5' End of mRNA

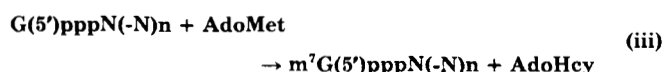
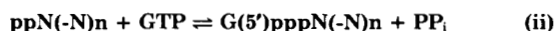
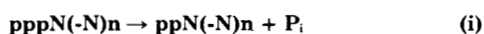
ASSOCIATION OF RNA TRIPHOSPHATASE WITH THE RNA GUANYLYLTRANSFERASE-RNA (GUANINE-7-) METHYLTRANSFERASE COMPLEX FROM VACCINIA VIRUS\*

(Received for publication, July 20, 1979)

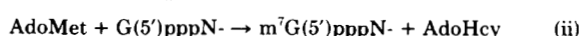
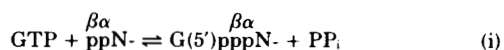
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**Purified RNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex from vaccinia virus contains RNA triphosphatase activity. The latter activity, which removes the  $\gamma$ -phosphate from triphosphate-ended polyribonucleotides, co-chromatographed with the capping and methylating enzyme complex on seven different ion exchange or affinity columns and co-sedimented with the complex on a glycerol gradient. On a molar basis, the RNA triphosphatase was about 100 times more active than the associated RNA guanylyltransferase. When the purified enzyme complex was incubated with poly(A) containing a 5'-triphosphate, removal of the  $\gamma$ -phosphate preceded capping. Furthermore, there was no significant difference in the rate or extent of capping 5'-diphosphate- or 5'-triphosphate-ended poly(A). Physical association of the three enzymatic activities appears to be an efficient mechanism for carrying out the following successive steps in cap formation:**



A cap structure, consisting of a 7-methylguanosine residue connected from the 5' position through a triphosphate bridge to the 5' position of 2'-O-methyladenosine or 2'-O-methylguanosine, is present at the end of vaccinia virus mRNA synthesized *in vitro* (1) and in infected cells (2). An  $M_r = 127,000$  enzyme complex, purified from vaccinia virus cores, was previously found to contain both RNA guanylyltransferase and RNA (guanine-7-)methyltransferase activities (3-5). Subsequently, an additional enzyme involved in cap formation, RNA (nucleoside-2'-)methyltransferase, was purified from vaccinia virus (6). Based on the properties of these enzymes, as well as on transcriptional studies carried out using vaccinia virus cores and specifically labeled ribonucleoside triphosphates (7), the following sequence of reactions was proposed:



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Steps (i) and (ii) are carried out by the RNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex, while Step (iii) is catalyzed by the RNA (nucleoside-2'-)methyltransferase. In this scheme, ppN- represents an incomplete or nascent RNA chain that had its terminal phosphate removed. Although an enzyme that specifically cleaves the  $\gamma$ -phosphate from polyribonucleotides was isolated by Tutas and Paoletti (8), purified preparations of RNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex were also found to contain significant RNA triphosphatase activity (5). Whether the latter activity was a minor contaminant or a part of the capping-methyltransferase complex, however, was not determined. The present experiments were designed to investigate the physical and functional relationship between RNA triphosphatase and RNA guanylyltransferase.

### EXPERIMENTAL PROCEDURES

**Virus**—Vaccinia virus (strain WR) was purified from infected HeLa cells by sedimentation through a sucrose cushion and two subsequent sucrose gradient sedimentations (9). Radioactive virus was prepared using  $1.5 \times 10^9$  cells in 3 liters of Eagle's medium which contained one-tenth of the standard amount of each amino acid and was supplemented with 3 mCi of [ $^{35}\text{S}$ ]methionine and 5% dialyzed horse serum. The specific activity of the purified virus was  $9.8 \times 10^6$  cpm/mg (1  $A_{260\text{ nm}}$  of virus contains 64  $\mu\text{g}$  of protein). For enzyme purification, radioactive virus was mixed with unlabeled virus prior to the preparation of cores. Protein estimations were made by liquid scintillation counting, assuming that all proteins are uniformly labeled with [ $^{35}\text{S}$ ]methionine.

**Enzyme Purification**—Approximately 90 mg of purified vaccinia virus was treated with dithiothreitol and the detergent Nonidet P-40 to remove the viral envelope. Cores were isolated by sedimentation through a sucrose cushion and were then treated with deoxycholate-containing buffer to extract soluble enzymes. Insoluble structural proteins were removed by high speed centrifugation, and the supernatant was passed through a DEAE-cellulose column to remove nucleic acids. At this point, we departed from our previously published procedure (3, 10) by diluting the enzyme so as to contain 0.05 M Tris-HCl (pH 8.4), 0.1% Triton X-100, 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 0.05 M NaCl and then passing it through a DEAE-Bio-Gel column (1  $\times$  5 cm) that had been equilibrated with this buffer. Succeeding column purifications were carried out as described in the appropriate figure legends.

**Enzyme Assays**—RNA guanylyltransferase activity was measured essentially as described by Martin *et al.* (3). The reaction mixture (50  $\mu\text{l}$ ) contained 25 mM Tris-HCl (pH 7.9), 2.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 25  $\mu\text{M}$  [ $8\text{-}^3\text{H}$ ]GTP (10 Ci/mmol), and 40 pmol of 5'-diphosphate-ended poly(A). After 30 min at 37°C, 40  $\mu\text{l}$  of the reaction mixture was spotted on DE81 filters. Unincorporated GTP was removed by washing three times with 5% Na<sub>2</sub>HPO<sub>4</sub> and then successively with water, ethanol, and ether. The filters were then dried and counted in a toluene-based scintillation fluid.

RNA (guanine-7-)methyltransferase was assayed as previously described (6) except that 250  $\mu\text{M}$  G(5')pppG was used as the methyl acceptor.

RNA triphosphatase was assayed according to Tutas and Paoletti

(8), with some modifications. Reaction mixtures for monitoring column fractions (25  $\mu$ l) containing 50 mM Tris-HCl (pH 8.4), 2.5 mM  $MgCl_2$ , 2 mM dithiothreitol, and 1 pmol of  $\gamma$ - $^{32}P$ -labeled poly(A) (2,500 cpm/pmol) were incubated with enzyme at 37°C for 5 min. The reaction was terminated by addition of 5  $\mu$ l of 50 mM EDTA, and the sample was spotted on PEI thin layer sheets and chromatographed in 0.75 M  $KH_2PO_4$  (pH 3.4). P<sub>i</sub> was located by radioautography, and the spot was cut out and counted. For accurate quantitation of RNA triphosphatase, 5 pmol of  $\gamma$ - $^{32}P$ -labeled poly(A) (500 cpm/pmol) was used, enzyme preparations were diluted 5-fold with Buffer A, and a 5-min time course of incorporation was determined. Enzyme activity was computed from the linear portion of the reaction, usually at 2 to 3 min.

**Preparation of Substrates**—Poly(A) with a labeled 5'-triphosphate end (11, 12) was synthesized in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 2 mM  $MnCl_2$ , 2 mM dithiothreitol, 1.6  $A_{260}$  units of denatured calf thymus DNA/ml, 16 units of *Escherichia coli* RNA polymerase holoenzyme/ml, and 0.3 mM ATP. Either [ $\gamma$ - $^{32}P$ ]ATP (500 or 2,500 cpm/pmol) or [ $\beta$ - $^{32}P$ ]ATP (100 or 10,000 cpm/pmol) prepared as described by Furuichi and Shatkin (13) was used. Incubations were for 1 h at 37°C. After precipitation with trichloroacetic acid, the material was dissolved in 50 mM Tris-HCl (pH 7.0), 5 mM  $MgCl_2$ , and 1 mM dithiothreitol and digested with pancreatic DNase I (0.1 mg/mg of DNA) at 37°C for 1 h. After repeated phenol/chloroform (1:1) and ether extractions, the RNA was precipitated with ethanol and then reprecipitated several times with trichloroacetic acid or passed through a Sephadex G-75 column, or both.

To remove the  $\gamma$ -phosphate from poly(A) preparations, the RNA triphosphatase activity (8) present in the DEAE-cellulose flow-through of soluble extracts of vaccinia virus was used. One nanomole of  $\beta$ - or  $\gamma$ - $^{32}P$ -labeled poly(A) was incubated in 2 ml of 0.05 M Tris-HCl (pH 8.5), 5 mM  $MgCl_2$ , 2 mM dithiothreitol, with 6,500 units of RNA triphosphatase for 30 min at 37°C. After repeated phenol/chloroform and ether extractions, the poly(A) was precipitated with ethanol.

The 5' ends of poly(A) preparations were analyzed by KOH or RNase T<sub>2</sub> digestions, or both, and thin layer chromatography on polyethyleneimine cellulose, using 0.75 M  $KH_2PO_4$  (pH 3.4). Adenosine tetraphosphate, triphosphate, diphosphate, and monophosphate markers were used. After radioautography, the labeled materials were eluted and counted.

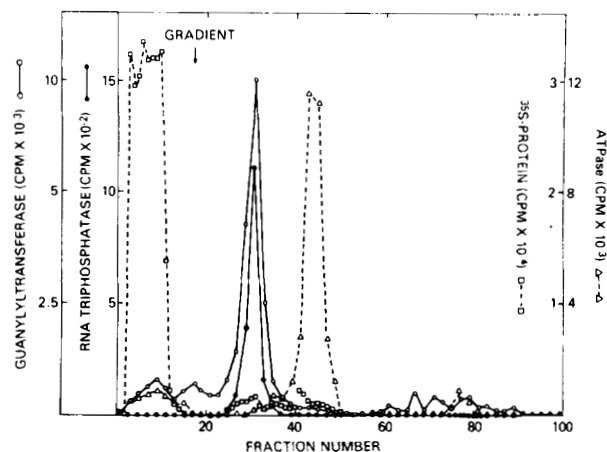
G(5')pppG, used for RNA (guanine-7)-methyltransferase assays, was synthesized chemically in our laboratory by S. Martin, using a modification of the Hoard and Ott procedure (14).

Triphosphate- and diphosphate-ended dinucleotides such as pppApGp and ppApGp were synthesized chemically by J. Tomasz (Institute of Biophysics, Biology Research Center, Hungarian Academy of Sciences), and samples were kindly provided by Dr. A. Shatkin (Roche Institute, Nutley, N. J.).

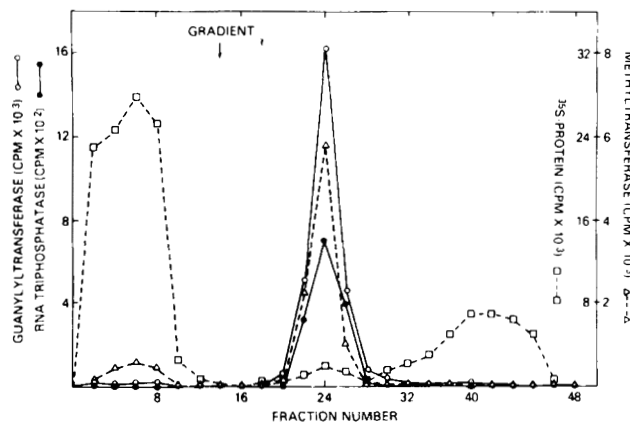
**Materials**—CM-Sephadex and quaternary aminoethyl (QAE)-Sephadex were purchased from Pharmacia Fine Chemicals. DEAE-Bio-Gel is a product of Bio-Rad Corp. Heparin-agarose was made by B. M. Baroudy of this laboratory using CNBr-activated Sepharose 4B. DNA cellulose was prepared according to the procedure of Alberts and Herrick (15). DEAE-cellulose (DE52), DEAE-cellulose filters (DE81), and phosphocellulose (P-11) were obtained from Whatman. Boehringer Mannheim supplied calf intestinal alkaline phosphatase, and P-L Biochemicals provided ADP-agarose and GTP-agarose. Radioactive compounds were purchased from Amersham/Searle Corp. Nonidet P-40 was a gift of Shell Oil Co. (United Kingdom).

## RESULTS<sup>1</sup>

**Association of RNA Triphosphatase with RNA Guanylyltransferase-RNA (Guanine-7)-Methyltransferase Complex**—Enzymes were extracted from purified vaccinia virus particles by deoxycholate treatment as previously described (10). Insoluble structural proteins were removed by centrifugation, and the supernatant was passed through a DEAE-cellulose column to remove nucleic acids. The effluent was diluted and



**FIG. 1. DNA-cellulose chromatography.** The effluent from a DEAE-Bio-Gel column was diluted to contain 0.05 M Tris-HCl (pH 8.4), 0.05 M NaCl, 2 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol and was applied to a column (1  $\times$  15 cm) of cellulose containing 0.1 mg/ml of bound single-stranded DNA. After washing the column, a 640-ml gradient from 0.05 M NaCl to 0.4 M NaCl in the above buffer was applied at 25 ml/h. RNA guanylyltransferase, RNA triphosphatase, and DNA-dependent ATPase activities were measured, and samples were taken for counting [ $^{35}S$ ]methionine.



**FIG. 2. CM-Sephadex chromatography.** The effluent from a DEAE-Bio-Gel column (13.4 mg of protein) was dialyzed overnight at 4°C against 150 volumes of 0.05 M sodium acetate (pH 6.0), 0.1 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol and then loaded on a column (1  $\times$  15 cm) of CM-Sephadex equilibrated with the same buffer. The column was washed, and a 160-ml linear gradient from 0.1 M to 0.6 M NaCl in starting buffer was applied at a flow rate of 10 ml/h. Fractions (4 ml) were collected, and enzyme assays were performed.

passed through a DEAE-Bio-Gel column that retained the viral DNA-dependent RNA polymerase.<sup>2</sup> The effluent from the DEAE-Bio-Gel column was applied directly to a denatured DNA-cellulose column, which was developed with a linear salt gradient. Analysis of the column fractions (Fig. 1) indicated that RNA triphosphatase and RNA guanylyltransferase, as well as RNA (guanine-7)-methyltransferase (not shown), co-eluted. DNA-dependent ATPase (previously (10) referred to as nucleoside triphosphate phosphohydrolase I) eluted as a separate peak, indicating that it is distinct from the RNA triphosphatase (Fig. 1). Some of the virus used for enzyme preparation had been labeled *in vivo* with [ $^{35}S$ ]methionine to facilitate protein determinations. As shown in Fig. 1, by comparison of enzyme activity and labeled protein, a considerable purification of the enzyme complex was obtained in this step.

<sup>2</sup> B. Baroudy and B. Moss, manuscript in preparation.

<sup>1</sup> Portions of this paper (including "Results" (Figs. 3 to 6) and "Discussion" (Fig. 13)) are presented in miniprint following this paper. Miniprint can be easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-1461, cite author(s), and include a check or money order for \$1.05 per set of photocopies.

Co-chromatography of RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7-)methyltransferase was also obtained on CM-Sephadex (Fig. 2). As shown in the accompanying miniprint supplement, co-chromatography of all three activities also occurred on columns of QAE-Sephadex (Fig. 3), heparin-agarose (Fig. 4), phosphocellulose (Fig. 5), ADP-agarose, and GTP-agarose (Fig. 6) that were developed with linear salt gradients. In addition, RNA guanylyltransferase that had been purified on columns of poly(U)-agarose and poly(A)-agarose was previously found to contain RNA triphosphatase activity (5).

A summary of the results obtained using several of the above columns in succession is presented in Table I. A significant feature is that the ratio of RNA triphosphatase to RNA guanylyltransferase was not significantly altered after successive column purification steps. The low levels of enzyme activity in the initial fractions apparently resulted from prolonged storage in deoxycholate, since analysis of these pooled fractions was delayed until the purification was completed.

Association of RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7-)methyltransferase was further

TABLE I  
Co-purification of RNA guanylyltransferase and RNA triphosphatase

A unit of RNA guanylyltransferase or RNA triphosphatase is the amount of enzyme that will cap or hydrolyze the triphosphate ends of 1 pmol of triphosphate-ended poly(A) in 30 min at 37°C.

Fraction	Protein	RNA triphosphatase	Guanylyltransferase	Triphosphatase/guanylyltransferase
	mg	units	units	
Deoxycholate-treated virus	94.0	24,104	12,413	
High speed supernatant	21.1	52,775	11,952	
DEAE-cellulose	24.0	1,088,804	18,937	57.5
DEAE-Bio-Gel	13.4	1,716,521	26,392	65.0
CM-Sephadex	0.45	222,965	5,160	44.0
QAE-Sephadex A-50	0.19	414,156	4,029	102.0
Heparin-agarose	0.064	282,240	5,600	50.0
Phosphocellulose	0.032	152,880	2,090	73.0

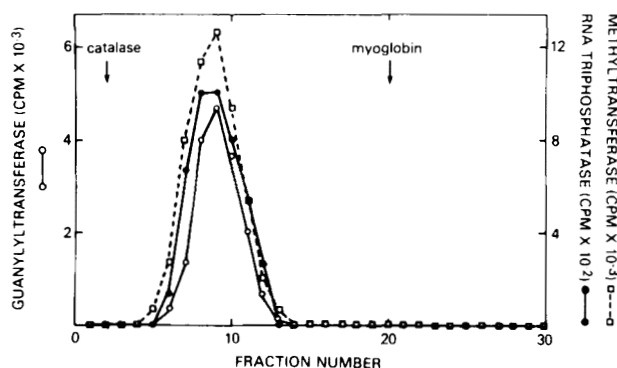


FIG. 7. Glycerol gradient centrifugation. The purified RNA triphosphatase-RNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex, obtained after successive chromatography on DEAE-cellulose, DEAE-Bio-Gel, CM-Sephadex, QAE-Sephadex A-50, heparin-agarose, and phosphocellulose, was adjusted to 1 M NaCl and stored at 0-4°C for 17 h. The enzyme (0.2 ml) was layered on a 3.6-ml linear 15 to 35% glycerol gradient in 0.05 M Tris-HCl (pH 8.4), 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100 and centrifuged in a SW 60 rotor at 50,000 rpm at 2°C for 39 h. Fractions (0.12 ml) were collected and assayed as described under "Experimental Procedures." The arrows indicate the sedimentation positions of protein markers analyzed on parallel gradients.

shown by glycerol gradient centrifugation of purified enzyme (Fig. 7). Previously, using both sucrose gradient centrifugation and gel filtration, a molecular weight of 127,000 was determined for the RNA guanylyltransferase (3).

**Functional Relationship between RNA Triphosphatase and RNA Guanylyltransferase**—The data in Table I indicate that on a molar basis the purified enzyme complex has much greater triphosphatase than guanylyltransferase activity. To examine this relationship more closely, the two activities were measured simultaneously in the same reaction mixture using  $\gamma$ - $^{32}$ P-labeled poly(A) both as substrate for the RNA triphosphatase and acceptor for the RNA guanylyltransferase. Quantitative release of  $P_i$  occurred within 2 min while cap formation, measured using [ $^3$ H]GTP, proceeded more slowly (Fig. 8). This difference was seen even more clearly by increasing the substrate/enzyme ratio (Fig. 9). RNA triphosphatase ac-

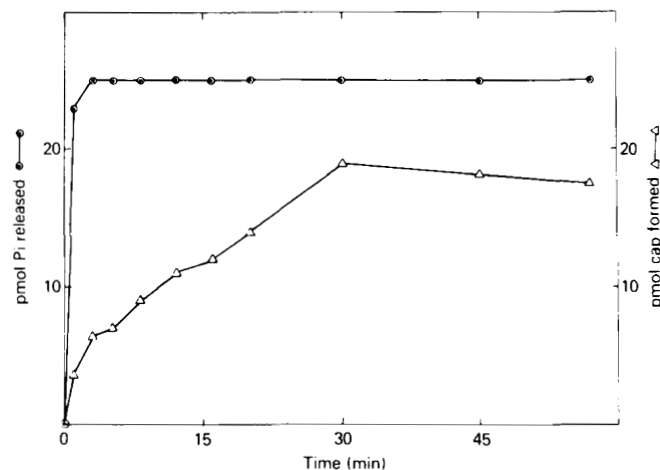


FIG. 8. Time course of RNA triphosphate hydrolysis and capping at low substrate/enzyme ratio. Approximately 25 pmol of  $\gamma$ - $^{32}$ P-labeled poly(A) (2,500 cpm/pmol) was incubated with 32 units of purified RNA guanylyltransferase in the presence of 37.5  $\mu$ M [ $^3$ H]GTP (10 Ci/mmol) for designated intervals and analyzed as described under "Experimental Procedures." The picomoles of  $^{32}P_i$  released and picomoles of incorporated [ $^3$ H]GMP were computed for each time point.

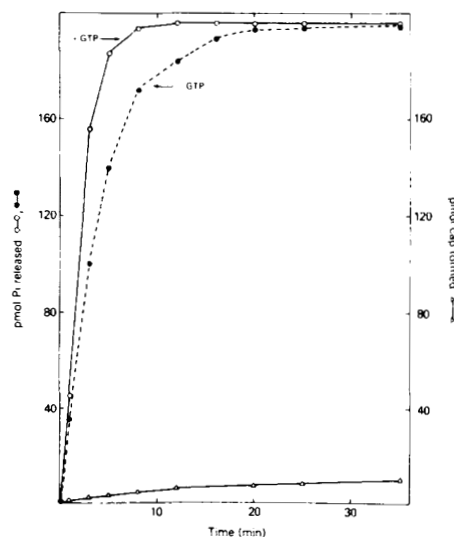


FIG. 9. Time course of RNA triphosphate hydrolysis and capping at high substrate/enzyme ratio. Approximately 200 pmol of  $\gamma$ - $^{32}$ P-labeled poly(A) (500 cpm/pmol) was incubated with 8 units of purified RNA guanylyltransferase in the presence ( $\Delta$ — $\Delta$ ,  $\circ$ — $\circ$ ) or absence ( $\bullet$ — $\bullet$ ) of 37.5  $\mu$ M [ $^3$ H]GTP (10 Ci/mmol) for designated intervals and analyzed as previously described.

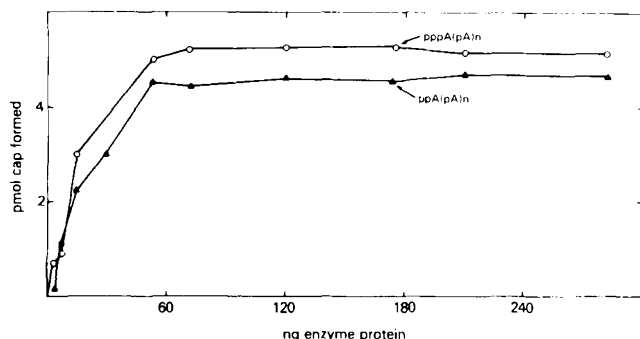


FIG. 10. Quantitative capping of triphosphate- and diphosphate-ended poly(A) by purified RNA guanylyltransferase.  $\beta$ - $^{32}$ P-labeled poly(A) with a 5'-triphosphate or diphosphate end (500 cpm/pmol) was prepared as described. Approximately 5 pmol of either poly(A) preparation was incubated with indicated amounts of enzyme, and incorporation of 25  $\mu$ M [ $8$ - $^3$ H]GTP (10 Ci/mmol) during a 30-min interval was measured.

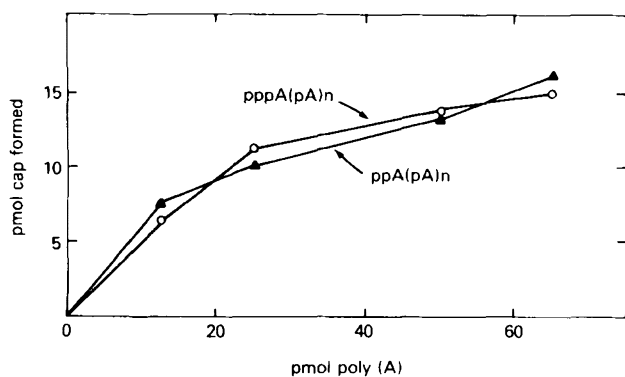


FIG. 11. Effect of concentration of triphosphate- and diphosphate-ended poly(A) on capping. Approximately 10 units of RNA guanylyltransferase were incubated with indicated amounts of poly(A) in the presence of 25  $\mu$ M [ $8$ - $^3$ H]GTP (10 Ci/mmol), and incorporation was measured after 30 min.

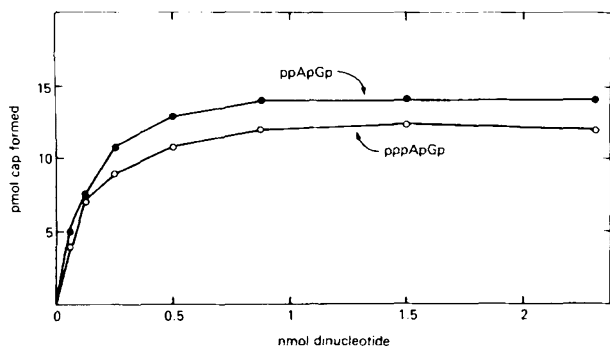


FIG. 12. Capping of diphosphate- and triphosphate-ended dinucleotides. Approximately 5 units of RNA guanylyltransferase were incubated with indicated amounts of pppApGp or ppApGp in the presence of 25  $\mu$ M [ $8$ - $^3$ H]GTP (10 Ci/mmol) in a total volume of 50  $\mu$ l for 30 min at 37°C. Residual GTP was destroyed by digestion with 2 units of calf intestinal alkaline phosphatase for 30 min at 37°C. The reaction mixture then was spotted on DE81 filters, which were washed and processed as indicated for the RNA guanine-7-methyltransferase assay under "Experimental Procedures."

tivity was nearly 100-fold greater than RNA guanylyltransferase under these conditions. Omission of GTP from the reaction mixture to prevent capping had a small effect on the rate of  $P_i$  release.

The high RNA triphosphatase activity of the enzyme complex suggested that there would be little difference in the

effectiveness of triphosphate- or diphosphate-ended poly(A) as acceptors for the RNA guanylyltransferase. To prepare suitable substrates, poly(A) was synthesized using [ $\beta$ - $^{32}$ P]-ATP. A portion of this material was then treated with RNA triphosphatase to remove the  $\gamma$ -phosphate and then repurified. The completeness of the digestion was monitored by alkaline hydrolysis and electrophoresis on DEAE-paper. With the untreated poly(A) preparation, 93% of the  $^{32}$ P was recovered as pppAp and 7% as ppAp, while with the triphosphatase-treated poly(A) this ratio was reversed (*i.e.* 7% was pppAp and 93% ppAp). The effectiveness of the polyribonucleotides as acceptors was measured under conditions of excess enzyme (Fig. 10) and excess substrate (Fig. 11). We concluded that diphosphate- and triphosphate-ended poly(A) were equally efficient acceptors and that both could be quantitatively capped. Additional experiments indicated that pppApGp as well as ppApGp were acceptors (Fig. 12) although, as previously indicated (5), the  $K_m$  is higher for dinucleotides than for polymers.

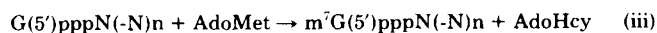
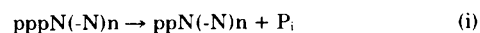
#### DISCUSSION

RNA guanylyltransferase and RNA(guanine-7-)methyltransferase from vaccinia virus were previously shown to exist as a  $M_r = 127,000$  complex containing two major polypeptides with  $M_r = 95,000$  and 31,400 (3). We now find that a third activity, an RNA triphosphatase, co-chromatographs with this complex on numerous ion exchange and affinity columns and also co-sediments with it on a glycerol gradient. With each of these purification procedures, only a single peak of RNA triphosphatase and a single co-eluting peak of RNA guanylyltransferase · RNA(guanine-7-)methyltransferase were detected, suggesting to us that all three activities are physically associated. Although Tutas and Paoletti (8) cited unpublished data regarding a separation of RNA triphosphatase from RNA guanylyltransferase by chromatography on ADP-agarose, we repeatedly found that the two activities co-eluted under conditions similar to those that they described (Fig. 13).

When a triphosphate-ended polyribonucleotide was used as a substrate, removal of the  $\gamma$ -phosphate preceded capping. Indeed, under conditions optimal for capping, the activity of the RNA triphosphatase was approximately 2 orders of magnitude greater than the activity of the RNA guanylyltransferase. These data explain the similar rates of capping diphosphate- and triphosphate-ended polyribonucleotides and indicate that cleavage of the  $\gamma$ -phosphate and capping of RNA are consecutive but not necessarily coupled reactions.

We found slight stimulation of RNA triphosphatase activity upon addition of GTP but not the obligatory coupling of the activities reported by Monroy and co-workers (16). Neither could we find the much greater activity with triphosphate-ended polyribonucleotides compared to diphosphate-ended polyribonucleotides, which they described (16). A dependence of the triphosphatase on GTP was not obtained by varying either the salt concentration or pH of the reaction mixture.<sup>3</sup>

The physical association of the vaccinia virus RNA triphosphatase, RNA guanylyltransferase, and RNA(guanine-7-)methyltransferase in a single complex would seem to be an efficient design for capping and methylating nascent RNA in the following sequence:



As previously discussed (4, 5, 16), methylation occurring in

<sup>3</sup> E. Barbosa, personal communication.

Step (iii) greatly inhibits reversal of Step (ii).

If the analogous eukaryotic enzymes exist in a similar complex, it must be less stable since RNA (guanine-7-)methyltransferase (17) and RNA guanylyltransferase<sup>4</sup> have been purified as separate enzymes from HeLa cells. In addition, the relative ability of the HeLa cell RNA guanylyltransferase to cap triphosphate- compared to diphosphate-ended poly(A) decreased markedly during purification, suggesting separation from RNA triphosphatase.

*Acknowledgments*—We thank E. Barbosa for carrying out preliminary experiments and N. Cooper for purifying vaccinia virus.

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<sup>4</sup> S. Venkatesan, and B. Moss (1979) in *From Gene to Protein: Information Transfer in Normal and Abnormal Cells*, Miami Winter Symposium, p. 135, Department of Biochemistry, University of Miami School of Medicine, Miami.

## SUPPLEMENTAL MATERIAL

TO

Modification of the 5' End of mRNA: Association of RNA Triphosphatase with the RNA Guanylyltransferase-RNA (Guanine-7-Methyltransferase Complex from Vaccinia Virus

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## RESULTS

Co-chromatography of RNA triphosphatase and RNA guanylyltransferase was found using columns of denatured DNA cellulose (Fig. 1, accompanying manuscript), CM-Sephadex (Fig. 2, accompanying manuscript), QAE-Sephadex (Fig. 3), heparin agarose (Fig. 4), phosphocellulose (Fig. 5), and ADP-agarose and GTP-agarose (Fig. 6). In addition, the enzymes co-eluted from ADP-agarose using a protocol similar to that described by Tutas and Paoletti (8) as shown in Fig. 13 (see "Discussion").

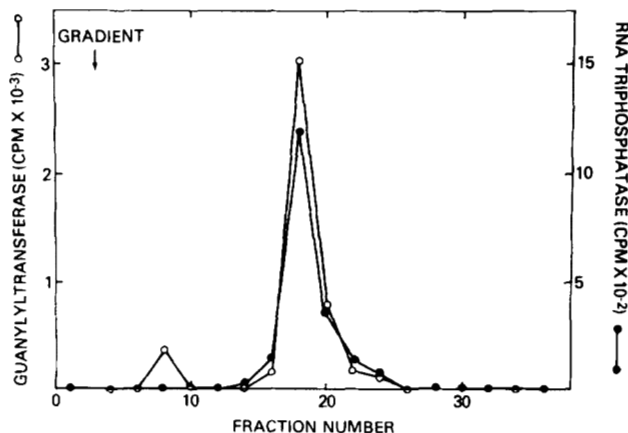


FIG. 3. QAE-A50 Sephadex column chromatography. The fractions from the CM-Sephadex column (Fig. 2, accompanying manuscript) that contained RNA triphosphatase and RNA guanylyltransferase were pooled, dialyzed against 250 volumes of 25 mM Tris-HCl (pH 9.0), 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol and then applied to a 1 X 5-cm column of QAE-A50 Sephadex equilibrated with the above buffer. After washing, a linear gradient of 0.1 M to 0.6 M NaCl in the above buffer was applied at 10 ml/h. Enzyme activities were assayed as described under "Experimental Procedures."

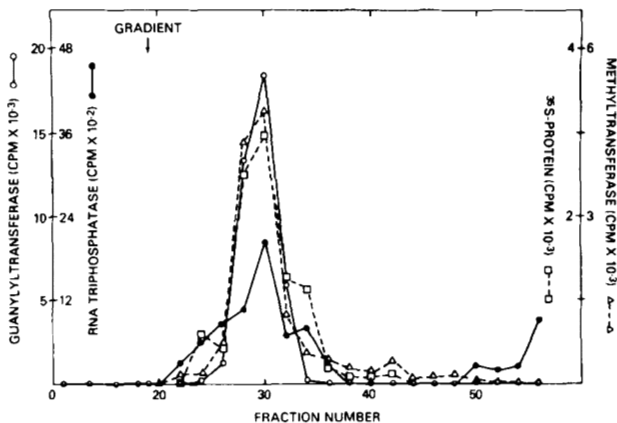


FIG. 4. Heparin-agarose column chromatography. The fractions from the QAE-A50 Sephadex column that contained RNA triphosphatase and RNA guanylyltransferase were pooled, dialyzed with 2.5 volumes of 25 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol (buffer A) to achieve a conductivity of 4 mS and applied at 20 ml/h to a 0.7 X 3.5-cm column of heparin agarose equilibrated with buffer A containing 0.05 M NaCl. The column was washed with the latter buffer, and a 40-ml linear gradient from 0.05 M NaCl to 0.75 M NaCl in buffer A was applied at 10 ml/h. Enzyme activities were assayed as described under "Experimental Procedures."

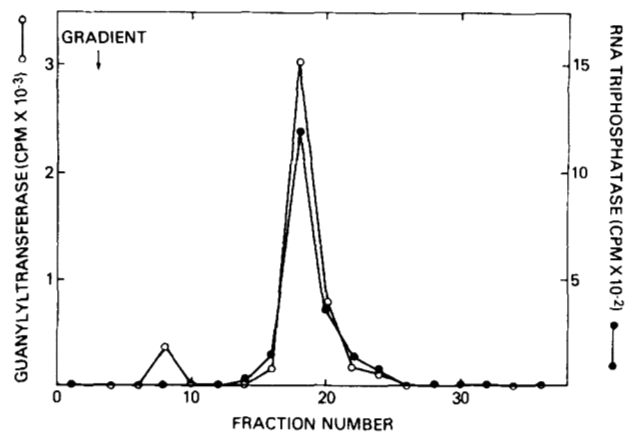


FIG. 5. Phosphocellulose column chromatography. Approximately 20 units of RNA guanylyltransferase obtained after chromatography on CM-Sephadex was diluted with buffer A to a conductivity of 4 mS and applied to a 0.7 X 2-cm column of phosphocellulose. The column was developed with a 40-ml linear gradient of NaCl from 0.05 M to 0.75 M in buffer A. Enzymes were assayed as described under "Experimental Procedures."

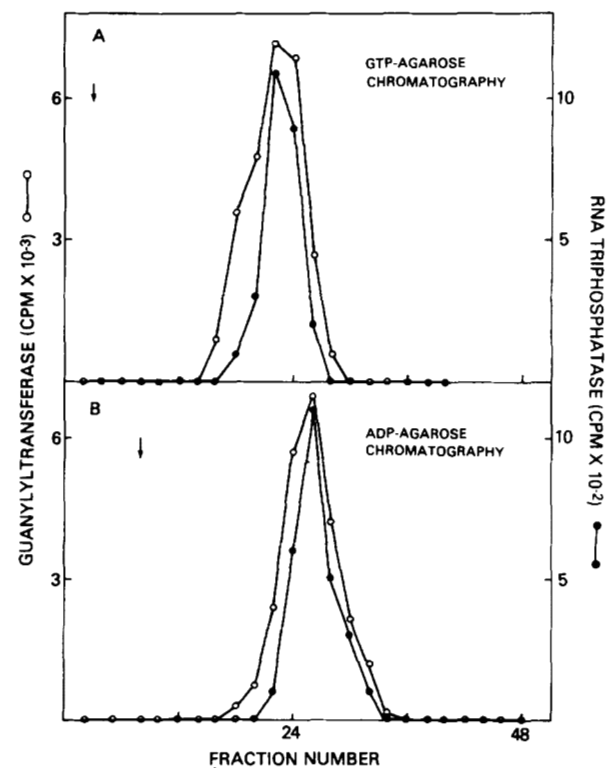


FIG. 6. GTP-agarose and ADP-agarose column chromatography. Approximately 20 units of RNA guanylyltransferase obtained by CM-Sephadex column chromatography were applied to 0.7 X 2-cm columns of GTP-agarose (A) and ADP-agarose (B). The columns were developed with 40-ml linear gradients of NaCl from 0.05 M to 0.75 M in buffer A. The arrows indicate the starting points of the gradients. Enzymes were assayed as described under "Experimental Procedures."

FIG. 13. ADP-agarose chromatography according to the procedure of Tutas and Paoletti (8). Approximately 500 units of RNA guanylyltransferase obtained as the effluent from DEAE-BioGel chromatography were dialyzed overnight against 300 volumes of buffer A containing 0.05 M NaCl and applied to a 1 X 5-cm column of ADP-agarose. The column was then washed successively with 10 ml of buffer A containing 0.05 M NaCl, with 25 ml of buffer A containing 0.05 M NaCl and 1 mg of denatured calf thymus DNA per ml, again with 10 ml of buffer A containing 0.05 M NaCl and finally with 40 ml of 0.5 M NaCl in buffer A. Fractions (2.5 ml) were collected. Enzyme assays were performed as described under "Experimental Procedures."

