Purification and Characterization of the Messenger Ribonucleic Acid Capping Enzyme GTP:RNA Guanylyltransferase from Wheat Germ†

Jerry M. Keith,* Sundararajan Venkatesan, Alan Gershowitz, and Bernard Moss

ABSTRACT: A GTP:RNA guanylyltransferase or capping enzyme has been purified approximately 2000-fold from wheat germ. The enzyme catalyzes the transfer of a GMP residue from GTP to the 5′ end of RNA or synthetic polyribonucleotides. Diphosphate-ended polymers were capped more efficiently than molecules with triphosphate ends, and molecules with monophosphate ends were not capped at all. There appears to be little sequence specificity since RNAs with purine or pyrimidine ends served as acceptors. Other features of the wheat germ RNA guanylyltransferase include relatively low K_m values for GTP (2.7 μM) and ppA(pA)$_n$ (14.2 nM), a divalent cation requirement satisfied by low (0.5 mM) concentrations of $MnCl_2$ or higher (5 mM) concentrations of $MgCl_2$, and a pH optimum around neutrality.

Formation of eukaryotic mRNA involves a complex series of reactions including capping, methylation, polyadenylylation, and splicing. While considerable information related to the biosynthesis of active mRNA molecules has been obtained from structural analysis and radioisotopic labeling studies, isolation and characterization of the individual enzymes involved are required for a more complete understanding of mRNA processing events. During the past several years, we have been particularly interested in the modification of the 5′ ends of mRNAs which result in cap structures. In its simplest form, the cap consists of a 7-methylguanosine residue connected from its 5′ position through a triphosphate bridge to the 5′ position of the adjacent nucleoside. In more complex cap structures, there are additional methylated sites [for a review, see Banerjee (1980)]. The key enzyme in cap formation catalyzes the transfer of a GMP residue from GTP to the 5′ end of RNA. Such an RNA guanylyltransferase has been purified to near homogeneity from vaccinia virus and has been extensively characterized (Martin & Moss, 1975, 1976; Martin et al., 1975; Monroy et al., 1978a,b; Venkatesan et al., 1980a; Shuman et al., 1980). This vaccinia capping enzyme has been isolated as an M_r 127 000 complex containing associated RNA triphosphatase and RNA (guanine-7-)methyltransferase activities. The ability to use the vaccinia virus guanylyltransferase to specifically label the 5′ ends of RNA has led to considerable interest in this enzyme as an analytical tool (Moss, 1977).

An analogous eukaryotic RNA guanylyltransferase has been partially purified from Hela cell nuclei (Wei & Moss, 1977; Venkatesan et al., 1980b; Venkatesan & Moss, 1980), rat liver (Mizumoto & Lipmann, 1979), and calf thymus (Laycock, 1976). The cellular RNA guanylyltransferase appears to be smaller than the capping enzyme isolated from vaccinia virus; furthermore, it is separable from both RNA triphosphatase and RNA (guanine-7-)methyltransferase activities. Indeed, RNA (guanine-7-)methyltransferase has been purified as a separate enzyme from HeLa cells (Ensinger & Moss, 1976). Additional enzymes involved in cap formation including an RNA (2′-*O*-methyladenosine-N^6-)methyltransferase (Keith et al., 1978) and two separate RNA (nucleoside-2′-)methyltransferases (Langberg & Moss, 1981) have also been purified from this source.

Until now, RNA guanylyltransferase had not been purified from plants. However, the presence of capping and methylating enzymes in wheat germ was suggested by the ability of extracts to enhance the translatability of viral (Muthukrishnan et al., 1975) and prokaryotic (Paterson & Rosenberg, 1979) mRNAs. The advantages of wheat germ for large-scale preparative work led us to undertake the isolation of RNA guanylyltransferase from this source. In the present paper, we describe the initial purification and characterization of the wheat germ RNA guanylyltransferase.

Experimental Procedures

Materials. Raw wheat germ was obtained from VioBin Corp., Monticello, IL, and was stored at −20 °C. Nucleotides, 5′-terminal cap derivatives, synthetic polynucleotides, and ADP–agarose (type 4) were obtained from P-L Biochemicals. Nuclease P_1 was produced by Yamasa Shoyu Co., Japan, and purchased from Accurate Chemical Co., NY. Calf intestinal alkaline phosphatase and snake venom phosphodiesterase were purchased from Boehringer-Mannheim and Worthington Biochemicals, respectively. Sephadex was from Pharmacia Fine Chemicals, and DEAE-cellulose (DE-52), phosphocellulose (P-11), and 3MM and DEAE-cellulose (DE81) paper were from Whatman. Cibacron blue–agarose (Affi-gel blue) and gel electrophoresis reagents were purchased from Bio-Rad. Tributylamine was obtained from Eastman Organic Chemicals, and 1,1′-carbonyldiimidazole was purchased from Sigma. The phosphorylation reagents (silylation grade) were purchased from Pierce Chemicals. Amersham/Searle and New England Nuclear were the suppliers of [α-^{32}P]GTP (300–350 Ci/mmol) and [γ-^{32}P]ATP (>2000 Ci/mmol). [β-^{32}P]ATP (>2000 Ci/mmol) was prepared as described by Furuichi & Shatkin (1977). *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes), dithiothreitol, and Miracloth were purchased from Calbiochem. Amido black (10B) was from Hartman-Leddon Co., Philadelphia, PA. Ethylene glycol was from Aldrich Chemical Co.

Buffers. Column chromatography buffers were prepared from Millipore membrane (0.22 μm) filtered stock solutions of 2.0 M Tris-HCl (pH 7.9, 25 °C) and 0.2 M Na_2EDTA (pH

† From the Department of Biochemistry, New York University Dental Center, New York, New York 10010 (J.M.K.), and the Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205 (S.V., A.G., and B.M.). *Received March 10, 1981; revised manuscript received August 4, 1981.* This project was supported in part by BRSG Grant RR07062, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by Research Grant GM 29124 from the National Institute of General Medical Sciences.

0006-2960/82/0421-0327$01.25/0

7.9). Buffer A, used in the purification procedure, contained 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM dithiothreitol, 25% (v/v) ethylene glycol, and 0.1% (v/v) Triton X-100.

Preparation of Chromatography Columns. Whatman DE-52 DEAE-cellulose was suspended in 10 volumes of buffer A containing 50 mM NaCl and adjusted to pH 7.9 with 1 M HCl. Columns were packed at room temperature and then equilibrated overnight at 0–4 °C with 500 mL of buffer A containing 50 mM NaCl.

Phosphocellulose was prepared by washing Whatman P-11 cellulose at room temperature for 30 min with 0.5 M NaOH containing 10 mM EDTA (15 mL/g of cellulose), rinsing with water, then washing with 0.5 M HCl, rinsing with water, washing with 0.5 M NaOH, and finally rinsing with water until the filtrate reached a neutral pH. Washed phosphocellulose was suspended in 10 volumes of buffer A containing 0.1 M NaCl and adjusted to pH 7.9 with NaOH. Columns were packed at room temperature and then equilibrated overnight at 0–4 °C with 200 mL of buffer A containing 0.1 M NaCl.

Cibacron blue–agarose (Bio-Rad Affi-gel blue) columns were packed at room temperature and then washed at 0–4 °C with buffer A containing 1 M NaCl and equilibrated with 100 mL of buffer A containing 50 mM NaCl.

ADP–agarose (P-L Biochemicals type 4) was packed at 0–4 °C. The column was washed with 20 mL of buffer A containing 2 M NaCl and then equilibrated with buffer A containing 75 mM NaCl.

Protein Determinations. Protein concentration was determined by either a modified Lowry method (Peterson, 1977) or a sensitive procedure utilizing amido black staining described by Schaffner & Weissmann (1973). Bovine serum albumin was used to determine the protein standard curve.

Preparation of Polynucleotide Acceptors. The 5′ terminus of synthetic poly(adenylic acid) [poly(A)] molecules 40–80 and 15–40 nucleotides long was modified by the chemical addition of phosphate groups to form (p)ppA ends exactly as previously described (Venkatesan et al., 1980b). Preparation of defined diphosphate- and triphosphate-ended poly(A), using *Escherichia coli* RNA polymerase and vaccinia virus RNA triphosphatase, was as previously described (Venkatesan & Moss, 1980).

RNA Guanylyltransferase Assay. The standard reaction mixture (0.1 mL) for assaying column fractions contained 50 mM Hepes (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1.65 μM [α-^{32}P]GTP (12–24 Ci/mmol), and 1.6 μg of chemically phosphorylated poly(A) as the guanosine monophosphate acceptor. Tes buffer, pH 7.5 (25 mM), was sometimes used in the standard reaction mixture in place of Hepes. For determination of enzyme units, diphosphate-ended poly(A) synthesized in vitro with *E. coli* RNA polymerase was used. After incubation for 30 min at 37 °C, 0.5 mL of stop buffer containing 25 mM Tris-HCl (pH 7.9), 0.1 mM GTP, 10 mM EDTA, and 50 μg of poly(A) was added to the reaction mixture. Acid-insoluble material was precipitated by adding 100% trichloroacetic acid to a final concentration of 7.5%. The precipitate was collected by centrifugation and the pellet washed with 70% ethanol containing 0.15 M sodium acetate. After the pellet was dried, the residue was treated with 25 μg of nuclease P_1 in 25 μL of 10 mM sodium acetate (pH 6.0) for 90 min at 37 °C. A 10-μL sample of a mixture containing 50 mM Tris–acetate (pH 9.0), 5 mM $MgCl_2$, and 2.5 μg of calf intestinal alkaline phosphatase was then added, and the incubation was continued for 60 min at 37 °C. Characterization of the nuclease P_1 and alkaline phosphatase resistant digestion products was by paper electrophoresis (Whatman 3MM) at pH 3.5 in pyridine–acetate (5%/0.5% v/v) buffer containing 1 mM EDTA.

Autoradiographs and fluorographs utilizing Du Pont Cronex intensifying screens and Kodak XR-2 X-ray film were used to locate ^{32}P-labeled material, and radioactivity was quantitated by liquid scintillation counting. In this assay, the nuclease P_1 and phosphatase-resistant ^{32}P-labeled material was identified as the cap product G(5′)pppA (see Characterization of Enzyme Product). By definition, 1 unit of guanylyltransferase incorporated 1 pmol of GMP into polymer cap structure G(5′)pppA in 30 min.

Characterization of Enzyme Product. Purified guanylyltransferase (10 μL of fraction 6) was used in the standard reaction mixture to label the poly(A) acceptor. After three successive trichloroacetic acid precipitations, the pellet was washed with 70% ethanol containing 0.15 M sodium acetate, and the remaining residue was treated with nuclease P_1 and alkaline phosphatase and then analyzed by pH 3.5 electrophoresis as described under RNA Guanylyltransferase Assay. The nuclease P_1 and phosphatase-resistant ^{32}P-labeled material was eluted from the electrophoretogram with water and lyophilized. A portion of the eluate was treated with 12.5 μg of snake venom phosphodiesterase in a 25-μL digestion mixture containing 50 mM Tris-HCl (pH 8.5) and 5 mM $MgCl_2$. The products of the digestion were analyzed by pH 3.5 electrophoresis on DEAE paper. Electrophoretograms were cut into 1-cm strips, and the radioactivity was determined by liquid scintillation counting.

Purification of the Guanylyltransferase. All operations were carried out at 0–4 °C.

Preparation of the Crude Extract. Wheat germ (250 g) was ground for 1 min at full speed in a Waring blender with 1 L of 50 mM Tris-HCl containing 0.1 mM EDTA, 1 mM dithiothreitol, and 75 mM ammonium sulfate. The homogenate was then diluted with 250 mL of the same buffer and centrifuged at 10400*g* for 15 min. The supernatant was filtered through one layer of Miracloth, resulting in approximately 1 L of crude extract (fraction 1) (Jendrisak & Burgess, 1975). Fraction 1 was then centrifuged at 143000*g* for 60 min and the supernatant filtered through one layer of Miracloth (fraction 2). Fraction 2 was adjusted to a final concentration of 25% (v/v) ethylene glycol and 0.1% Triton X-100 by adding 100% ethylene glycol (0.338 times the volume of fraction 2) and 10% Triton X-100 (v/v) (0.013 times the volume of fraction 2). This mixture (approximately 1.35 L) was further diluted by adding 1.1 volumes of buffer A containing 50 mM NaCl (fraction 2A).

DEAE-cellulose Column Chromatography. Fraction 2A was applied at a flow rate of 60–100 mL/h to a DEAE-cellulose column (4 × 39 cm) equilibrated with buffer A containing 50 mM NaCl. During the loading of the column, it was necessary to occasionally stir the top of the DEAE-cellulose to maintain an adequate flow rate. The column was washed with 500 mL of buffer A containing 50 mM NaCl and then eluted at 60 mL/h with about 800 mL of buffer A containing 0.3 M NaCl. Fractions (13 mL) of the 0.3 M NaCl eluate were collected, and a sample of each was extracted with an equal volume of chloroform/methanol (3:1 v/v) and tested for protein as described by Schaffner & Weissman (1973). Fractions containing protein were combined, resulting in approximately 220 mL of the 0.3 M NaCl eluate (fraction 3).

Phosphocellulose Column Chromatography. Fraction 3 was adjusted to a final concentration of 0.125 M NaCl by

Table I: Summary of Purification of Wheat Germ RNA Guanylyltransferase[a]

fraction no.	fraction description	volume (mL)	protein concn (mg)	activity[b] (units)	sp act. (units/mg)	x-fold purification	yield (%)
1	crude extract	1045	30430	3172	0.104	1	100
2	high-speed supernatant	965	20458	3949	0.193	1.9	125
3	DEAE-cellulose	220	4840	4791	0.99	9.5	151
4	phosphocellulose	73	36	1307	36	346	41
5	Cibacron blue-agarose	24	13.6	720	53	510	23
6	ADP-agarose	9	2.4	548	228	2192	17

[a] From 250 g of wheat germ. [b] One unit is defined as 1 pmol of GMP incorporated into G(5')pppA cap structure in 30 min.

adding buffer A and then applied at a flow rate of 100 mL/h to a phosphocellulose column (1 × 8 cm) equilibrated with buffer A containing 0.1 M NaCl. The column was washed with 200 mL of buffer A containing 0.1 M NaCl and then eluted at 20 mL/h with a 200-mL linear gradient of 0.1–0.5 M NaCl in buffer A. Fractions (7.5 mL) of the gradient eluate were collected and assayed for guanylyltransferase activity and protein by using a modified Lowry procedure (Peterson, 1977). Fractions containing the majority of the enzyme activity were combined, resulting in approximately 66 mL of eluate (fraction 4).

Cibacron Blue–Agarose Column Chromatography. Fraction 4 was diluted with 4 volumes of buffer A and then applied at a flow rate of 60–70 mL/h to an equilibrated Affi-gel blue column (1.5 × 6 cm). The column was washed with 50 mL of buffer A containing 50 mM NaCl and the bound material eluted at 20 mL/h with a 80-mL linear gradient of 50 mM to 0.75 M NaCl in buffer A. The flow-through and wash eluate were collected in 15-mL fractions, and the salt gradient eluate was collected in 3-mL fractions. Fractions containing guanylyltransferase activity were combined in approximately 21 mL as fraction 5.

ADP–Agarose Column Chromatography. Fraction 5 was diluted with an equal volume of both buffer A and water and then applied at a flow rate of 14 mL/h to a washed and equilibrated ADP–agarose column (0.7 × 5 cm). The flow-through material was collected in 4-mL fractions. After a brief wash with buffer A containing 75 mM NaCl, the bound material was eluted with a 40-mL linear gradient of 75 mM to 1 M NaCl in buffer A. The salt gradient eluate was collected in 1-mL fractions at 4 mL/h. Those fractions containing the majority of the purified guanylyltransferase activity were combined to give fraction 6 (approximately 9 mL) and stored at 0 or −70 °C until further use.

Sucrose-Gradient Sedimentation. RNA guanylyltransferase (0.25 mL) was dialyzed for 3 h at 4 °C against 0.2 M NaCl, 25 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100 and applied to a 5–20% (w/v) linear sucrose gradient in the same buffer containing 0.05 M NaCl. Centrifugation was in an SW 60 Ti rotor at 189560*g* for 16 h at 3 °C. In some experiments, 75 μg of bovine serum albumin and 75 μg of myoglobin were added with the RNA guanylyltransferase; in other experiments, the markers were added to a parallel tube. In one experiment, 0.2 mg of lysozyme per mL was added to the enzyme and to the sucrose solutions prior to formation of the gradient in an attempt to stabilize enzyme activity. The tubes were tapped from the bottom, and approximately 25 fractions were collected. RNA guanylyltransferase assays were performed on samples from each fraction. The positions of markers were determined by measuring the protein concentration at A_{410nm}.

Preparation of Labeled RNA To Test for RNase Contamination. Internally labeled RNA was synthesized by using *E. coli* RNA polymerase and p53 plasmid DNA (Neiman et al., 1981) as a template. A 250-μL reaction mixture contained 30 mM Tris-HCl (pH 7.9), 0.1 mM dithiothreitol, 0.1 mM EDTA, 150 mM KCl, 10 mM $MgCl_2$, 0.15 mM each of ATP, CTP, GTP, and UTP, 0.4 mM potassium phosphate buffer, 0.4 mg/mL BSA, 10 μg of DNA template, and 23 units of *E. coli* RNA polymerase. In addition, the reaction mixture contained either 20 μCi of [α-^{32}P]GTP at 410 Ci/mmol or 40 μCi of [2,8-$^{3}H_2$]ATP at 26 Ci/mmol. The reactions were incubated for 20 min at 37 °C.

End-labeled RNA was prepared by capping STNV RNA in vitro by utilizing [α-^{32}P]GTP and purified vaccinia virus guanylyltransferase (Moss, 1977). The reaction mixture (100 μL) contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, 40 μCi of [α-^{32}P]GTP (total of 2 mM concentration), 2 μg of STNV RNA, and 10 μL of purified guanylyltransferase from vaccinia virus. The reaction was incubated for 30 min at 37 °C.

The labeled RNAs prepared by both the RNA polymerase reaction and the capping reaction were purified from the reaction mixture by phenol extraction and Sephadex G-50 gel filtration in 50 mM ammonium acetate.

Results

Purification of mRNA Guanylyltransferase. Crude wheat germ extracts, fraction 1, were prepared following the procedure described by Jendrisak & Burgess (1975) for the isolation of wheat germ RNA polymerase II. From preliminary studies with a variety of liquid chromatography column materials, a purification scheme involving step elution from DEAE-cellulose and gradient elution from successive columns of phosphocellulose, Cibacron blue–agarose, and ADP–agarose was devised (Figure 1). A summary of one purification starting with 250 g of wheat germ is presented in Table I. The apparent increase in enzyme units during the initial steps presumably resulted from removal of inhibitors. A final purification of 2000-fold relative to that for the crude extract was obtained with an overall recovery of approximately 17%. However, analysis of the final purified fraction by polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed more than 12 major polypeptides, indicating that the enzyme was not yet homogeneous.

The final purified enzyme fraction was tested for ribonuclease contamination under standard capping assay conditions, except for the omission of [α-^{32}P]GTP. No decrease in trichloroacetic acid insoluble material was detected when 10 μL of RNA guanylyltransferase was incubated with uniformly [^{3}H]adenosine-labeled RNA or 5'-end ^{32}P-cap-labeled STNV RNA at 37 °C for periods up to 45 min. Under similar conditions, 10 units of RNase A converted 75% of the uniformly labeled RNA and 85% of the end-labeled RNA to acid-soluble products in 30 min. However, when uniformly ^{32}P-labeled RNA incubated with RNA guanylyltransferase was analyzed by electrophoresis on a 4% polyacrylamide gel, a slow but definite shift in the migration pattern was observed

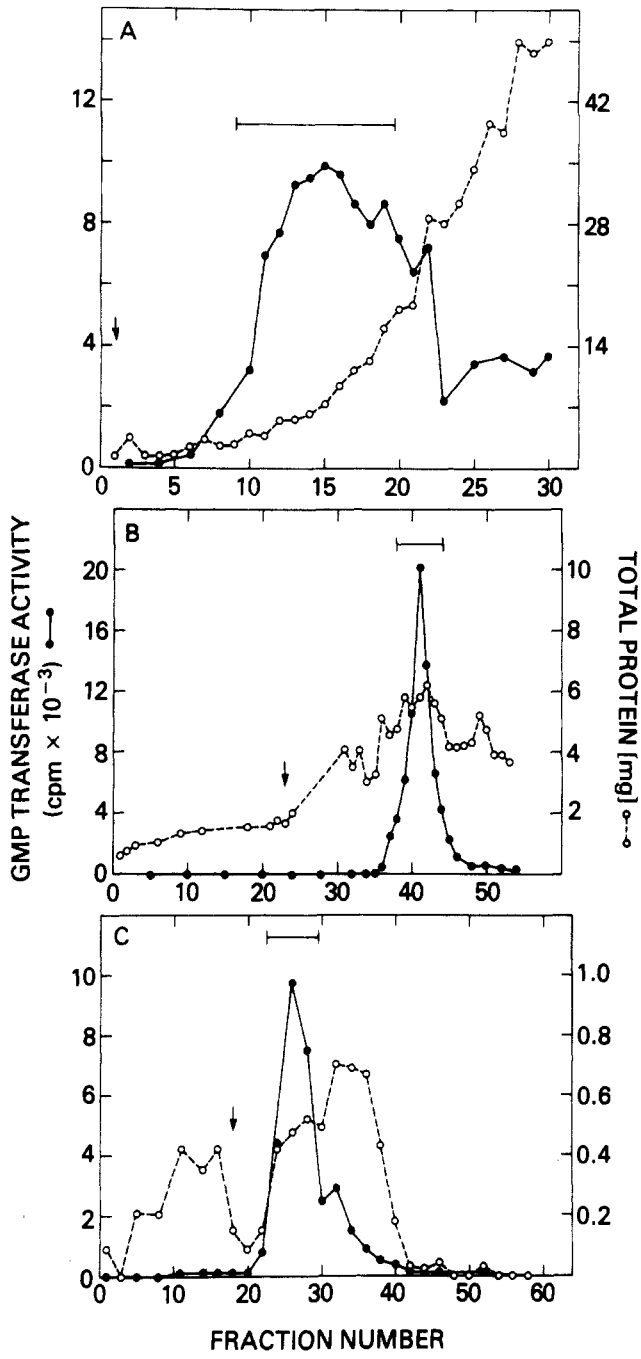

FIGURE 1: Purification of RNA guanylyltransferase by chromatography on (A) phosphocellulose, (B) Cibacron blue–agarose, and (C) ADP–agarose. Linear gradients were started at the positions indicated by arrows. Individual fractions were assayed for enzyme activity and protein content. Pooled fractions are indicated by bars.

over 30 min, indicating some residual RNase contamination.

The pooled enzyme fractions from the final ADP–agarose column (fraction 6) were stable for over 6 months at −20 °C and considerably longer at −70 °C.

Sucrose-Gradient Sedimentation. The purified guanylyltransferase was centrifuged with marker proteins through 5–20% sucrose gradients to estimate its molecular weight (Martin & Ames, 1961). The enzyme sedimented as a broad peak with and slightly ahead of bovine serum albumin which has a molecular weight of approximately 65 000.

Characterization of the RNA Guanylyltransferase Product. For evaluation of the specificity of the purified enzyme, poly(A) acceptor was used in a standard reaction mixture containing [α-^{32}P]GTP and the purified enzyme fraction from the ADP–agarose column. After trichloroacetic acid precipitation, the polymer was treated with nuclease P_1 and alkaline phosphatase. This method of analysis is based on the resistance of the cap or cap derivatives, i.e., G(5′)pppA, to phosphatase following nuclease P_1 treatment, which cleaves all phosphodiester bonds regardless of base or ribose methylation. After this digestion, all of the labeled material derived from [α-^{32}P]GTP migrated between Ap and Gp standards upon electrophoresis on Whatman 3MM paper as expected for a cap structure (Figure 2B), whereas all labeled material in an untreated sample remained at the origin (Figure 2A). Further identification was accomplished by eluting the nuclease P_1 and phosphatase-resistant material indicated by the bar in Figure 2B and then subjecting this material to analysis by DEAE paper electrophoresis both before and after digestion with snake venom phosphodiesterase. As shown in Figure 2C, prior to phosphodiesterase treatment all of the ^{32}P-labeled material comigrated upon electrophoresis with authentic marker G(5′)pppA and was well separated from pG. After phosphodiesterase treatment, the majority of the ^{32}P-labeled material comigrated with guanosine 5′-monophosphate, the expected product (Figure 2D). Some inorganic phosphate was also produced by the venom phosphodiesterase treatment, probably due to a slight contamination of monoesterase activity present in most preparations of this enzyme (Figure 2D). The results

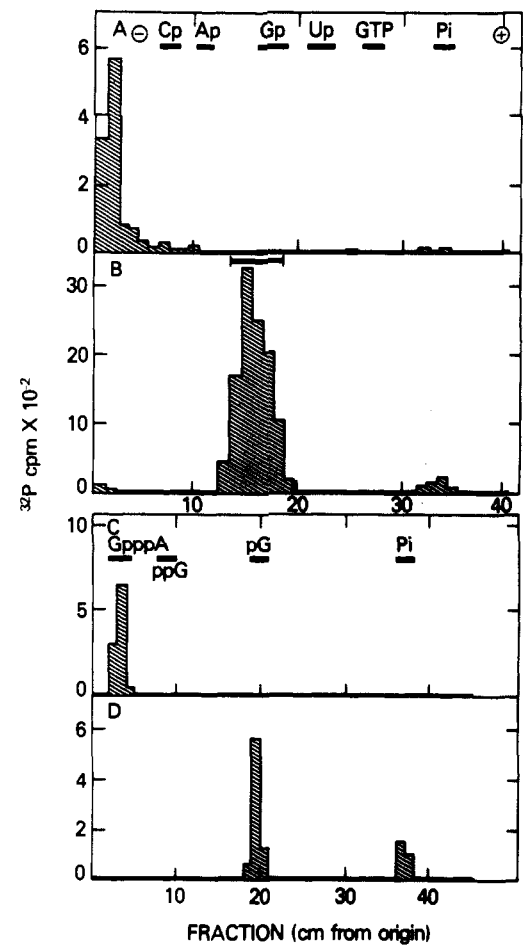

FIGURE 2: Characterization of the guanylyltransferase product. Purified enzyme, diphosphate-ended poly(A) acceptor, and [α-^{32}P]GTP were incubated under standard reaction conditions. A portion of the product was treated with nuclease P_1 and alkaline phosphatase and then analyzed by electrophoresis on Whatman 3MM paper at pH 3.5 as described under Experimental Procedures. (A) No nuclease P_1 or phosphatase treatment. (B) After nuclease P_1 and phosphatase treatment. The radioactive material indicated by the bar in (B) was eluted, and samples were reanalyzed by electrophoresis on DEAE-81 paper at pH 3.5. (C) Without further enzyme treatment. (D) After treatment with snake venom phosphodiesterase.

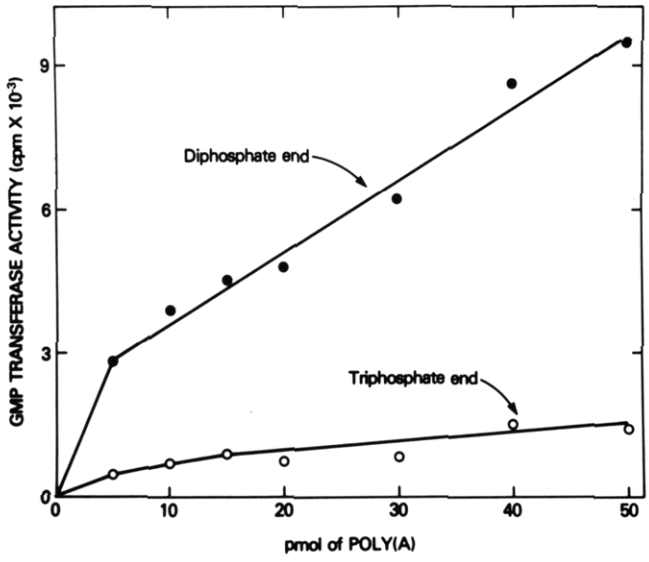

FIGURE 3: Polynucleotide acceptor specificity of guanylyltransferase. Reaction mixtures (50 μL) contained 5 μM [α-^{32}P]GTP (20 000 cpm/pmol), 1 unit of enzyme, and the indicated amounts of 5′-diphosphate- or 5′-triphosphate-ended poly(A). The reactions were at 37 °C for 30 min, and the products were analyzed and quantitated as described under Experimental Procedures.

of these analyses indicate that essentially all of the radioactivity derived from [α-^{32}P]GTP is incorporated into the cap structure, G(5′)pppA, at the 5′ end of the polymer.

Structural Requirements of the Polyribonucleotide Acceptor: Di- and Triphosphate Ends. For determination of the specificity of the guanylyltransferase for polyribonucleotides with di- or triphosphate ends, poly(A) was synthesized with *E. coli* RNA polymerase by using low specific activity [γ-^{32}P]ATP to label the 5′ end. A portion of the poly(A) was then treated with an extract of vaccinia virus containing RNA triphosphatase activity (Tutas & Paoletti, 1977) to form diphosphate-ended polymer. The efficiency of this step was determined by measuring the release of $^{32}P_i$. In other experiments, β-^{32}P-labeled poly(A) was used to analyze directly the diphosphate end. Similar amounts of the di- and triphosphate-ended polymers were then added to standard reaction mixtures containing [α-^{32}P]GTP and guanylyltransferase, and the amount of radioactively labeled cap formed was measured. Under these conditions, molecules with diphosphate ends were better acceptors than molecules with triphosphate ends (Figure 3). Omission of the di- or triphosphate-terminated poly(A) acceptor or the use of poly(A) with only a monophosphate 5′ end resulted in the complete loss of activity.

Pyrimidine-Terminated Polyribonucleotide as Acceptor for RNA Guanylyltransferase. To determine if an mRNA with a pyrimidine residue at the 5′ terminal could be capped by the purified guanylyltransferase, it was first necessary to synthesize the appropriate molecule in vitro. This was accomplished enzymatically by using a unique DNA template and *E. coli* RNA polymerase. The template was derived from a λ phage C17 mutant fragment which had been inserted into a pBR322 cloning vector (Rosenberg et al., 1978). After amplification of the recombinant plasmid in *E. coli*, the λ-mutant insert with a small portion of the plasmid DNA was cleaved from the vector by restriction endonuclease digestion. The desired DNA fragment was purified by gel electrophoresis and then used as template for transcription (Venkatesan & Moss, 1980). The predominantly triphosphate cytidine terminated RNA molecules were converted to the diphosphate configuration by treatment with RNA triphosphatase from vaccinia virus and

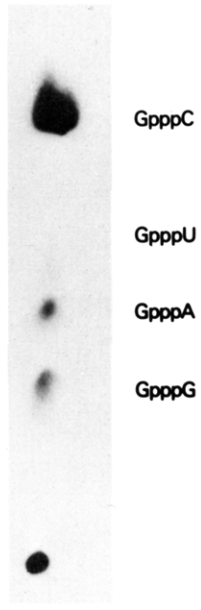

FIGURE 4: Capping of pyrimidine-initiated RNA by guanylyltransferase. Reaction mixture (0.1 mL) containing 10 μM [α-^{32}P]GTP (25 000 cpm/pmol) and 2 units of guanylyltransferase was incubated with 5 pmol of λ C17 RNA at 37 °C for 30 min. The capped products were processed as described under Experimental Procedures and resolved by high-voltage electrophoresis on DEAE-cellulose paper at pH 3.5 with authentic markers. A fluorograph is shown.

then used in a standard reaction mixture containing [α-^{32}P]-GTP and the purified guanylyltransferase. The products of the reaction were treated with nuclease P_1 and alkaline phosphatase and analyzed by DEAE paper electrophoresis. G(5′)pppC and smaller amounts of G(5′)pppA and G(5′)pppG were identified. These experiments clearly demonstrated that the enzyme can cap RNA molecules initiating with CTP (Figure 4).

Characteristics of the Guanylyltransferase Reaction. Under the standard reaction conditions, linear incorporation of GMP into the cap structure G(5′)pppA continued for over 45 min. When Tes buffer at various pH values was used, the enzyme exhibited a rather wide activity range with a neutral pH optimum. The enzyme required divalent cations for activity. Optimal cap formation was obtained with $MgCl_2$ concentrations of 5 mM or more or with 0.5 mM $MnCl_2$ (Figure 5). Lower or higher concentrations of $MnCl_2$ were less effective.

Inorganic pyrophosphate, which is a putative product of the reaction catalyzed by the guanylyltransferase, inhibited the enzyme by approximately 50% at 5 μM, and almost total inhibition was observed at 50 μM. At similar concentrations, inorganic phosphate had much less effect on enzyme activity.

The influence of substrate concentration on guanylyltransferase activity was studied by varying the concentration of GTP and diphosphate-terminated poly(A) acceptor. Data obtained with six different GTP concentrations in quadruplicate and 5 ppA$(pA)_n$ concentrations in triplicate were analyzed on a Hewlett-Packard 97 calculator using a Lineweaver–Burk program written by R. M. Bartholow. A Michaelis constant (K_m) of 2.7 μM was determined for GTP when saturating amounts of poly(A) acceptor were used and 14.2 nM for the polymer acceptor molecule when 16 μM GTP was used.

Discussion

Purification of RNA guanylyltransferase from wheat germ depended on the use of a highly specific and reliable assay: [α-^{32}P]GMP from GTP was incorporated into the 5′ end of

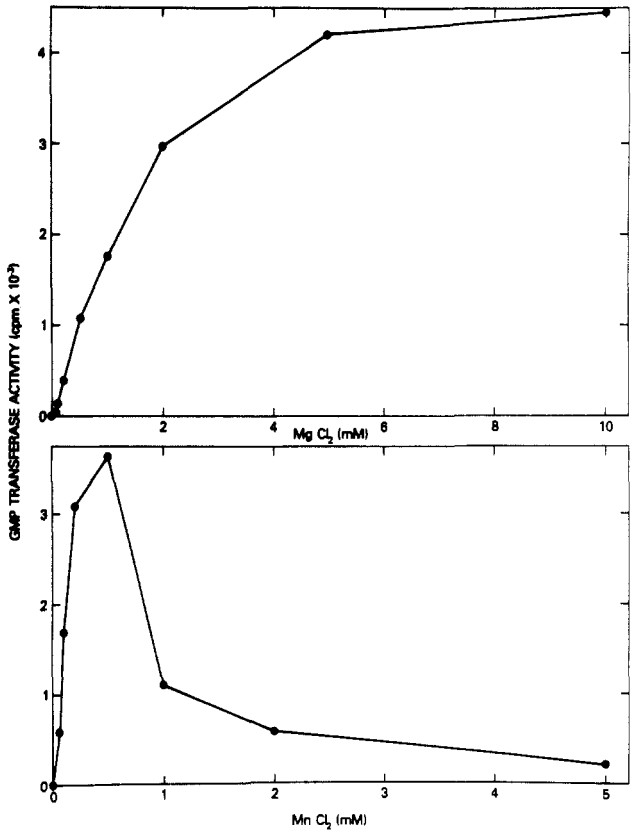

FIGURE 5: Effect of divalent cation concentration on guanylyltransferase activity. Enzyme activity was measured as described under Experimental Procedures with the exception that only $MgCl_2$ (top panel) or $MnCl_2$ (bottom panel) was used as the divalent cation.

poly(A) which was then digested with nuclease P_1 and alkaline phosphatase and subjected to paper electrophoresis. The enzyme-resistant cap structures were located by fluorography and quantitated by scintillation counting. Simpler procedures which measured the incorporation of [^{3}H]GTP into poly(A) by trichloroacetic acid precipitation or binding to DEAE filters did not provide reliable results due to relatively low amounts of RNA guanylyltransferase activity and high backgrounds.

The RNA guanylyltransferase purified from wheat germ resembles the analogous enzymes from HeLa cells (Venkatesan & Moss, 1980) and rat liver (Mizumoto & Lipmann, 1979). All three enzymes catalyze the transfer of a GMP residue from GTP to the 5′-diphosphate end of RNA or synthetic polyribonucleotides. Thus, cap formation appears to occur by a similar mechanism in plants and animals. The RNA guanylyltransferase from vaccinia virus also catalyzes a similar reaction; however, that enzyme has been shown to contain an associated RNA triphosphatase and consequently capped di- and triphosphate-ended RNAs at similar rates (Venkatesan et al., 1980a). Since the wheat germ and the HeLa cell (Venkatesan & Moss, 1980) and rat liver (Mizumoto & Lipmann, 1979) RNA guanylyltransferases cap diphosphate-ended RNAs most efficiently, the cellular triphosphatase may be dissociated or partially inactivated during purification. Our preliminary results indicate that the wheat germ RNA triphosphatase activity is removed during chromatography on Cibacron blue–agarose.

The greater complexity of the viral enzyme is also indicated by the presence of an associated RNA (guanine-7-)methyltransferase activity (Martin et al., 1975; Martin & Moss, 1975). By contrast, the HeLa cell (Ensinger & Moss, 1976) and the rat liver (Mizumoto & Lipmann, 1979) RNA (guanine-7-)methyltransferases have been purified as separate enzymes. Bajszar and co-workers (Bajszar et al., 1978), however, reported that 30S ribonucleoprotein particles from rat liver contain RNA guanylyltransferase as well as RNA (guanine-7-)methyltransferase and RNA (nucleoside-2′-)-methyltransferase, suggesting that all three exist as a complex in vivo. We have not yet attempted to purify the RNA (guanine-7-)methyltransferase from wheat germ; however, the enzyme has been partially purified from *Neurospora crassa* (Germershausen et al., 1978).

Wheat embryo mRNA has been shown to contain cap structures that lack a 2′-*O*-methyl group on the penultimate nucleoside, e.g., $m^7G(5')pppN$- (Haffner et al., 1978). By using the vaccinia virus RNA (nucleoside-2′-)methyltransferase to label wheat germ RNA with Ado[*methyl*-^{3}H]Met, S. Muthukrishnan has shown that the penultimate nucleoside may be cytidine as well as guanosine and adenosine (personal communication). For this reason, we wish to determine whether pyrimidine-ended as well as purine-ended RNAs could be capped by the same wheat germ RNA guanylyltransferase. A CTP-ended RNA is formed by transcription of phage λ C17 DNA with *E. coli* RNA polymerase (Rosenberg et al., 1978). Using triphosphatase-treated RNA transcribed from this cloned DNA segment, we previously reported that it could be capped by the HeLa cell RNA guanylyltransferase (Venkatesan & Moss, 1980). Similarly, we found that the CDP-ended RNA was capped by the wheat germ enzyme.

The amount of RNA guanylyltransferase extractable from eukaryotic cells appears to be quite low. The major advantage of wheat germ as an enzyme source is the ability to obtain large amounts of starting material conveniently and inexpensively. Thus far, a purification of approximately 2000-fold has been achieved. However, the enzyme has been only partially purified, and efforts are currently being made to scale up the procedure and explore additional chromatographic steps to remove residual traces of RNase.

References

Bajszar, G., Szabó, G., Simoncsits, A., & Molnár, J. (1978) *Mol. Biol. Rep. 4*, 93.

Banerjee, A. K. (1980) *Microbiol. Rev. 44*, 175.

Ensinger, M. J., & Moss, B. (1976) *J. Biol. Chem. 251*, 5283.

Furuichi, Y., & Shatkin, A. J. (1977) *Nucleic Acids Res. 4*, 3341.

Germershausen, J., Goodman, D., & Somberg, E. W. (1978) *Biochem. Biophys. Res. Commun. 82*, 871.

Haffner, M. H., Chin, M. B., & Lane, B. G. (1978) *Can. J. Biochem. 56*, 729.

Jendrisak, J. J., & Burgess, R. R. (1975) *Biochemistry 14*, 4639.

Keith, J. M., Ensinger, M. J., & Moss, B. (1978) *J. Biol. Chem. 253*, 5033.

Langberg, S. R., & Moss, B. (1981) *J. Biol. Chem. 256*, 10054.

Laycock, D. G. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 770.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.

Martin, S. A., & Moss, B. (1975) *J. Biol. Chem. 250*, 9330.

Martin, S. A., & Moss, B. (1976) *J. Biol. Chem. 251*, 7313.

Martin, S. A., Paoletti, E., & Moss, B. (1975) *J. Biol. Chem. 250*, 9322.

Mizumoto, K., & Lipmann, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4961.

Monroy, G., Spencer, E., & Hurwitz, J. (1978a) *J. Biol. Chem. 253*, 4481.

Monroy, G., Spencer, E., & Hurwitz, J. (1978b) *J. Biol. Chem. 253*, 4490.
Moss, B. (1977) *Biochem. Biophys. Res. Commun. 74*, 374.
Muthukrishnan, S., Both, G. W., Furuichi, Y., & Shatkin, A. J. (1975) *Nature (London) 255*, 33.
Neiman, P., Beemon, K., & Luce, J. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1896.
Paterson, B. M., & Rosenberg, M. (1979) *Nature (London) 279*, 692.
Peterson, G. (1977) *Anal. Biochem. 83*, 346.
Rosenberg, M., Court, D., Shimatake, H., Brady, C., & Wolff, D. L. (1978) *Nature (London) 272*, 414.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502.
Shuman, S., Surks, M., Furneaux, H., & Hurwitz, J. (1980) *J. Biol. Chem. 255*, 11588.
Tutas, D. G., & Paoletti, E. (1977) *J. Biol. Chem. 252*, 3092.
Venkatesan, S., & Moss, B. (1980) *J. Biol. Chem. 255*, 2835.
Venkatesan, S., Gershowitz, A., & Moss, B. (1980a) *J. Biol. Chem. 255*, 903.
Venkatesan, S., Gershowitz, A., & Moss, B. (1980b) *J. Biol. Chem. 255*, 2829.
Wei, C.-M., & Moss, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3758.