

# Purification of mRNA Guanylyltransferase and mRNA(guanine-7-)methyltransferase from Vaccinia Virions

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The sequences m<sup>7</sup>G(5')pppG<sup>m</sup>- and m<sup>7</sup>G(5')pppA<sup>m</sup>- are located at the 5' termini of vaccinia mRNAs. Two novel enzymatic activities have been purified from vaccinia virus cores which modify the 5' terminus of unmethylated mRNA. One activity transfers GMP from GTP to mRNA and is designated a GTP:mRNA guanylyltransferase. The second activity transfers a methyl group from S-adenosylmethionine to position 7 of the added guanosine and is designated a S-adenosylmethionine:mRNA(guanine-7-)methyltransferase. Advantage was taken of the selective binding of these activities to homopolyribonucleotides relative to DNA to achieve a 200-fold increase in specific activity. The guanylyl- and methyltransferase remained inseparable during chromatography on DNA-agarose, poly(U)-Sephadex, poly(A)-Sephadex, and Sephadex G-200 and during sedimentation through sucrose density gradients suggesting they were associated. A Stokes radius of 5.0 nm, an  $s_{20,w}$  of 6.0, and a molecular weight of 127,000 were obtained by gel filtration on Sephadex G-200 and sedimentation in sucrose density gradients. Under denaturing conditions of sodium dodecyl sulfate-polyacrylamide gel electrophoresis two major polypeptides were detected in purified enzyme preparations. Their molecular weights of 95,000 and 31,400 suggested they were polypeptide components of the 127,000 molecular weight enzyme system.

Unique methylated structures have been identified at the 5' terminus of viral mRNAs (1-6), viral genome RNA (7), and mammalian cellular mRNAs (8-11). These structures consist of a terminal 7-methylguanosine that is attached to a penultimate ribosemethylated nucleoside via a triphosphate bond and may be represented as the sequence m<sup>7</sup>G(5')pppN<sup>m</sup>-.<sup>1</sup>

It is evident that such structures cannot be formed by the usual transcriptive mechanism. Vaccinia, a DNA virus that replicates within the cytoplasm of infected cells, is an ideal system for determining the enzymatic formation of these structures. Messenger RNA synthesized *in vitro* in the presence of S-adenosylmethionine by the vaccinia core-associated RNA nucleotidyltransferase has the 5'-terminal sequences m<sup>7</sup>G(5')pppG<sup>m</sup>- and m<sup>7</sup>G(5')pppA<sup>m</sup>- (1). Furthermore, disruption of the vaccinia core releases the activities which are capable of completely modifying the 5' termini of unmethylated vaccinia mRNA (12). Three activities have been identified: mRNA guanylyltransferase, mRNA(guanine-7-)methyltransferase, and mRNA(nucleoside-2'-)methyltransferase. In this paper we present the purification and physical characterization of the activities which catalyze the transfer of GMP to RNA and the methylation of the added terminal

guanosine residue. In the accompanying paper (13) we describe in detail the reactions catalyzed by these activities.

## 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 as previously described (14, 15). Radioactive virus was grown in the presence of [<sup>3</sup>H]leucine and was purified in a similar manner.

**Preparation of mRNA Substrate**—Vaccinia virus *in vitro* mRNA was prepared from 500-ml reaction mixtures which contained 500 A<sub>260</sub> units of vaccinia virus, 50 mM Tris-HCl (pH 8.4), 10 mM dithiothreitol, 0.05% Nonidet P-40, 7.5 mM MgCl<sub>2</sub>, 3.75 mM ATP, and 1.25 mM each GTP, CTP, and UTP. Following incubation for 2 hours at 37°, viral cores were removed by centrifugation at 25,000 × *g* in a Sorvall SS-34 rotor for 30 min at 4°. From the pooled supernatants the RNA was precipitated with cetyltrimethylammonium bromide according to the procedure of Sibatani (16), and was collected by centrifugation at 12,000 × *g* in a Sorvall HB-4 rotor for 20 min at 4°. The pooled RNA pellets were dried under an air stream, dissolved in absolute ethanol, and precipitated as the sodium salt from 0.1 M sodium acetate/70% ethanol overnight at -20°. Following ethanol precipitation the RNA was purified further by passage through a column (55 × 0.9 cm) of Sephadex G-50. The yield of RNA was 5 mg.

A procedure for the preparation of synthetic poly(A) that is a suitable acceptor for the guanylyltransferase is described in the accompanying paper (13).

**Assay for Guanylyltransferase**—The standard reaction mixture contained 50 mM Tris-HCl (pH 7.8), 2.5 mM MgCl<sub>2</sub>, 2.7 μM [<sup>3</sup>H]GTP (9.4 Ci/mmol), 1 mM dithiothreitol, and 10 μg of RNA in a final volume of 100 μl. After incubation with enzyme for 30 min at 37°, the incorporation of GMP into RNA was determined by measuring

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<sup>1</sup> The abbreviations used are: m<sup>7</sup>G, 7-methylguanosine; N<sup>m</sup>, 2'-O-methylribonucleoside; A<sup>m</sup>, 2'-O-methyladenosine; G<sup>m</sup>, 2'-O-methylguanosine; NP-40, Nonidet P-40.

radioactivity either bound to DEAE-cellulose (Whatman DE81) filters (17) or precipitated by cold trichloroacetic acid and collected onto nitrocellulose filters. All filters were counted in toluene-based scintillation fluid. By definition, 1 unit of guanylyltransferase will incorporate 1 pmol of GMP into RNA in 30 min.

**Assay for Methyltransferase**—The standard 100- $\mu$ l reaction mixture contained 50 mM Tris-HCl (pH 7.8), 2.5 mM each  $MgCl_2$  and GTP, 0.5  $\mu$ M S-adenosyl[methyl- $^3H$ ]methionine (12.6 Ci/mmol), 1 mM dithiothreitol, and 10  $\mu$ g of RNA. After incubation with enzyme for 30 min at 37°, the incorporation of methyl groups into RNA was determined by either of the filter methods described above. By definition, 1 unit of methyltransferase will incorporate 1 pmol of methyl groups into RNA in 30 min.

**Analysis of Nucleoside Products of Methyltransferase Reaction**—Vaccinia mRNA was incubated in the standard methyltransferase assay and was separated from S-adenosyl[methyl- $^3H$ ]methionine by passage through a column (25  $\times$  0.6 cm) of Sephadex G-50. The RNA was recovered in the excluded volume, lyophilized, and digested with ribonucleases A and  $T_1$ , followed by snake venom phosphodiesterase and alkaline phosphatase as previously described (1). This material was then applied directly to Eastman cellulose sheets (20  $\times$  20 cm). Ascending chromatography was performed in ethyl acetate/7.5 M  $NH_4OH$ /2-propanol/1-butanol (3/2/2/1). After the sheets were dried, the position of authentic standards was determined by examination under ultraviolet light. The [ $^3H$ ]methyl-labeled nucleosides were detected by counting 0.5-cm strips in toluene-based scintillation fluid containing 33% Triton X-100 and 10%  $H_2O$ .

**Preparation of Materials for Nucleic Acid-Affinity Chromatography**—Single-stranded calf thymus DNA was used to prepare DNA-agarose by the method of Schaller *et al.* (18) and DNA-cellulose according to Alberts and Herrick (19). Poly(A)-Sepharose was prepared by the procedure of Poonian *et al.* (20) with the modification that following the coupling reaction with poly(A) the Sepharose slurry was reacted an additional 4 hours with 1 M glycine to block any remaining CNBr-activated sites.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—For estimation of molecular weights of polypeptides and for monitoring the purification procedure cylindrical 7.5% polyacrylamide gels containing 5 M urea and 0.1% sodium dodecyl sulfate were employed. Samples for electrophoresis were concentrated by precipitation with 5% trichloroacetic acid, neutralized with  $NH_4OH$ , and solubilized in 5 M urea/2% sodium dodecyl sulfate/0.2% 2-mercaptoethanol for 2 min at 100°. Electrophoresis was performed at 3 mA/gel (2 volts/cm) for 22 hours. Following electrophoresis gels were fixed in 25% trichloroacetic acid, stained in 0.1% Coomassie brilliant blue R-250 in 25% trichloroacetic acid, and destained in 7% acetic acid. Gels containing [ $^3H$ ]leucine-labeled proteins were sliced into 1-mm sections, dissolved in  $H_2O_2$ , and counted as described previously (21).

**Sephadex G-200 Chromatography**—For the determination of Stokes radius, a 0.5-ml sample containing markers and the purified enzyme was applied to a column (51  $\times$  0.9 cm) of Sephadex G-200 equilibrated with 0.15 M Tris-HCl (pH 8.4), 0.1% Triton X-100, 0.25 M NaCl, 2 mM dithiothreitol, and 0.05% bovine serum albumin. The sample was eluted with the same buffer, and column fractions were assayed for guanylyl- and methyltransferase activities. Excluded and included column volumes were determined by measuring absorbance at 265 nm for blue dextran and by measuring radioactivity for [ $^{14}C$ ]glucose, respectively. Proteins of known Stokes radii present in the sample included catalase (0.07 mg), yeast alcohol dehydrogenase (0.36 mg), and bovine serum albumin (0.9 mg) and were assayed by the procedures of Beers and Sizor (22), Vallee and Hoch (23), and by absorbance at 235 nm, respectively.

**Sucrose Gradient Sedimentation**—The sedimentation coefficient of the purified enzyme was determined in linear 5 to 20% sucrose density gradients which contained buffer of the same composition as used for gel filtration with the omission of bovine serum albumin. Purified enzyme and sedimentation markers in a 0.2-ml volume were layered onto 10-ml gradients and centrifuged in a SW 41 rotor at 165,000  $\times g$  for 25 hours at 4°. Fractions were collected from the bottom of the tube and were assayed for guanylyl- and methyltransferase activities. Sedimentation markers included in the sample were catalase (0.03 mg), horse liver alcohol dehydrogenase (0.1 mg), and bovine serum albumin (1 mg) and were assayed as described above. In some experiments sperm whale myoglobin (0.2 mg) was added and assayed by measuring absorbance at 410 nm.

**Source of Materials**—Poly(U)-Sepharose and Sepharose 4B were

purchased from Pharmacia Fine Chemicals. DEAE-cellulose (Whatman DE52) was obtained from Reeve-Angel. Seakem agarose was from Bausch & Lomb. Munktell 410 cellulose was purchased from Bio-Rad Laboratories. Worthington Biochemical Corp. was the source of calf thymus DNA, bovine liver catalase, yeast and horse liver alcohol dehydrogenase, glyceraldehyde phosphate dehydrogenase,  $\beta$ -galactosidase, carbonic anhydrase, snake venom phosphodiesterase, *Escherichia coli* alkaline phosphatase, and ribonucleases A and  $T_1$ . Myoglobin and chymotrypsinogen were purchased from Schwarz/Mann. Ovalbumin and 7-methylguanosine were the products of Sigma. Phosphorylase A was from Boehringer-Mannheim. Nucleoside triphosphates and 2'-O-methyladenosine were obtained from P-L Biochemicals. New England Nuclear was the source of Triton X-100 and radioisotopes. Nonidet P-40 was a gift from the Shell Oil Co., and 2'-O-methylguanosine was kindly provided by M. Sporn, National Cancer Institute, N.I.H.

## RESULTS

**Purification of Guanylyl- and Methyltransferase**—The procedure for preparing soluble guanylyltransferase and methyltransferases from vaccinia virus cores has been reported previously (12) and is the initial step in the purification of all other enzymes which have been isolated from vaccinia virus, namely two nucleic acid-dependent nucleoside triphosphate phosphohydrolases (24), a deoxyribonuclease (25), a protein kinase (26), and a poly(A) polymerase (27). Briefly, this method involves first the removal of the viral envelope with dithiothreitol and the detergent Nonidet P-40 and isolation of the viral cores by centrifugation through a cushion of sucrose. Soluble proteins are extracted from the cores by treatment with sodium deoxycholate, dithiothreitol, and 0.25 M NaCl, followed by high speed centrifugation. The supernatant contains approximately 15% of the initial proteins and is passed through two successive DEAE-cellulose columns in 0.2 M and 0.05 M NaCl to remove viral DNA. Except during the preparation of viral cores, all steps were performed at 0–4°.

The flow-through of the second DEAE-cellulose column was applied to a column (4  $\times$  1.5 cm) of DNA-agarose containing 1.7 mg of DNA/ml bed volume and equilibrated with Buffer A (0.15 M Tris-HCl, pH 8.4/0.1% Triton X-100/2 mM dithiothreitol/1 mM  $Na_3EDTA$ /10% glycerol) containing 0.05 M NaCl, and the column was washed in the same buffer. About 50% of the applied protein including the guanylyl- and methyltransferase activities was recovered in the flow-through. DNA-cellulose was less satisfactory because the activities apparently adsorbed to the cellulose matrix.

After determining that the enzymes did not bind to DNA-agarose we decided to use RNA-affinity columns as selective steps for further purification. The flow-through of the DNA-agarose column was applied directly to a column (10  $\times$  0.6 cm) of poly(U)-Sepharose equilibrated with Buffer A containing 0.05 M NaCl. Following a wash with this same buffer, a linear gradient from 0.05 to 0.5 M NaCl in Buffer A was applied to the column. Fractions were collected and assayed for guanylyl- and methyltransferase activities, and in the experiments in which [ $^3H$ ]leucine-labeled virus was used, portions were counted for total protein. Fig. 1 illustrates a typical profile of the elution of total protein and guanylyl- and methyltransferase activities from poly(U)-Sepharose. Approximately 80% of the applied protein eluted in the flow-through. Ten per cent of the applied protein eluted at 0.36 M NaCl and was coincident with the major peak of both guanylyl- and methyltransferase activities. No further elution of either protein or enzyme activities was detected at salt concentrations greater than 0.5 M. Variable but always minor amounts of both guanylyl- and

methyltransferase activities eluted at 0.25 M NaCl. The properties of the activities eluting at this ionic strength do not differ from those in the major peak of activity, except for their lower specific activities. This is due to the presence of other proteins eluting in this region as demonstrated by gel electrophoretic analysis of the fractions (data not shown).

The entire major peak of activities from the poly(U)-Sephacose column was pooled, dialyzed against Buffer A containing 0.05 M NaCl, and applied to a column (5 × 0.6 cm) of poly(A)-Sephacose equilibrated with Buffer A containing 0.05 M NaCl. The column was washed with the same buffer, and a linear gradient from 0.05 to 0.4 M NaCl in Buffer A was applied to the column. Fractions were collected and assayed as

described for the poly(U)-Sephacose column. The elution of total protein and the enzyme activities from poly(A)-Sephacose is shown Fig. 2. Only two major peaks of protein are present. The peak eluting at 0.17 M NaCl is coincident with both guanylyl- and methyltransferase activities, whereas the second protein peak elutes in a region of diminishing enzyme activities. A 2-fold increase in specific activity is achieved in this step. Chromatography on either phosphocellulose, CM-Sephadex, or DEAE-cellulose resulted in no additional purification of the activities.

For experiments with purified enzyme, fractions around the peak of activity on poly(A)-Sephacose comprising approximately two-thirds of the total activity were pooled (e.g.

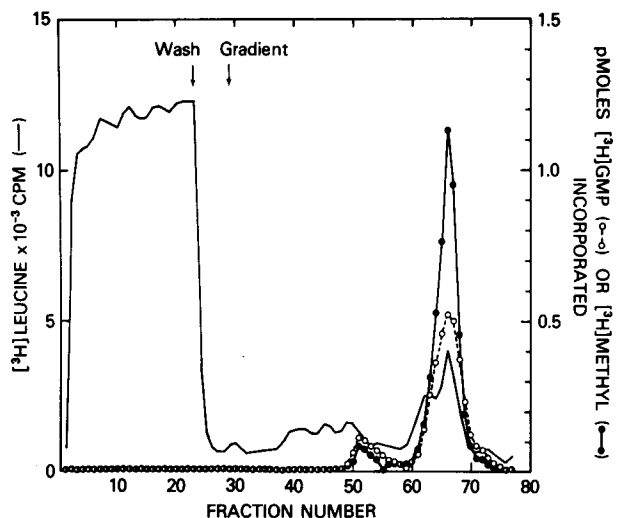


FIG. 1. Chromatography of guanylyl- and methyltransferase on poly(U)-Sephacose. Flow-through material from a DNA-agarose column was applied to a column of poly(U)-Sephacose, and the proteins were eluted with a 200-ml gradient from 0.05 to 0.5 M NaCl in Buffer A at a flow rate of 15 ml/hour. Fractions of 4.1 ml were collected and assayed for guanylyltransferase (O—O) and methyltransferase (●—●). For determination of [<sup>3</sup>H]leucine-labeled protein (—), 0.5 ml of each fraction was counted in toluene-based scintillation fluid containing 33% Triton X-100.

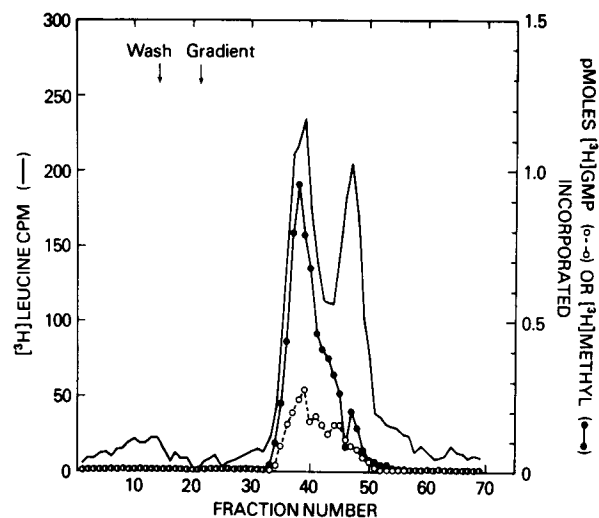


FIG. 2. Purification of guanylyl- and methyltransferase by poly(A)-Sephacose column-chromatography. The fractions of enzyme activity from the column of poly(U)-Sephacose were pooled and dialyzed against Buffer A containing 0.05 M NaCl and applied to a column of poly(A)-Sephacose. The proteins were eluted with a 100-ml gradient from 0.05 to 0.4 M NaCl in Buffer A at a flow rate of 15 ml/hour. Fractions (1.9 ml) were assayed for guanylyltransferase (O—O) and methyltransferase (●—●). [<sup>3</sup>H]Leucine-labeled protein (—) was determined by counting 0.1 ml of each fraction.

TABLE I  
Summary of purification of mRNA guanylyl- and mRNA (guanine-7-)methyltransferase activities

Fraction	Volume	Total Protein	Total guanylyl transferase activity	Specific guanylyl transferase activity	Total methyl transferase activity	Specific methyl transferase activity
	ml	mg*	units†	units/mg	units†	units/mg
I. NP-40 detergent treated virus	10.4	58.8	955	16.2	5,750	97.8
II. High speed supernatant	12.0	9.8	388	39.6	8,830	901
III. DEAE-cellulose II	65.0	6.9	410	59.3	9,500	1,380
IV. DNA-Agarose	97.0	4.3	1,180	275	6,500	1,510
V. Poly(U)-Sephacose	45.4	0.32	699	2,190	1,030	3,220
VI. a. Poly(A)-Sephacose	30.4	0.22	520	2,360	960	4,360
b. Poly(A)-Sephacose peak fraction	1.9	0.016	61	3,810	167	10,400

\* Determined from the relationship of 64 μg viral protein per absorbance at 260 nm using [<sup>3</sup>H]leucine-labeled virus with a specific activity of 8.0 × 10<sup>5</sup> cpm per mg.

† Expressed as pmoles of substrate incorporated in 30 minutes.

Fractions 35 to 41 in Fig. 2). The enzyme has been stored for several months at 4° or -20° without loss of activity.

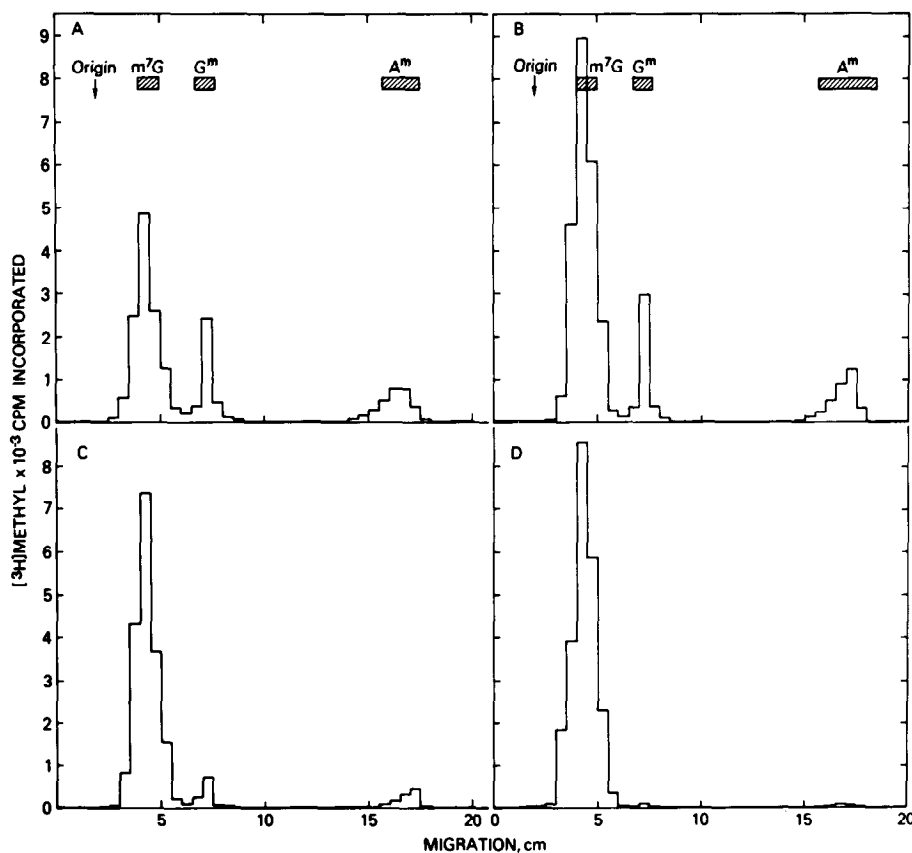
A summary of the purification of the guanylyl- and methyltransferase activities from vaccinia virus is presented in Table I. Following high speed centrifugation of the disrupted viral cores 17% of the initial viral proteins are recovered. In this step there is a 9-fold increase in specific activity and a 150% recovery of the methyltransferase which may result from an increased accessibility of the enzyme to the substrates as it is solubilized from the core particle. By contrast, a 2.4-fold increase in specific activity and an apparent 40% recovery of the guanylyltransferase is observed in this step. One explanation for this is that the vaccinia core-associated nucleoside triphosphatase, capable of hydrolyzing GTP to GDP in the presence of nucleic acid (28), is also released in this step. The hydrolysis of GTP would produce artificially low values for the recovery of the guanylyltransferase. Passage of the material through DNA-agarose, onto which the nucleoside triphosphatase adsorbs, results in full recovery of the guanylyltransferase activity and a 17-fold increase in its specific activity. At this stage only 70% of the methyltransferase is recovered; however, as is discussed in the following section this loss of total methyltransferase activity is due to removal of the mRNA(nucleoside-2'-)methyltransferase. Indeed the separation of the methyltransferases is accomplished most effectively in this step. Further increases in specific activities of both guanylyl- and methyltransferases are achieved by chromatography on poly(U)-Sepharose and poly(A)-Sepharose. The recovery of the guanylyl- and methyltransferase activities after these steps is 54 and 17%, respectively, of that observed in the detergent-treated virus. Following poly(A)-Sepharose chromatography, 0.4% of the initial viral protein remains, and there is an ap-

parent 150-fold purification of the guanylyltransferase and 50-fold purification of the methyltransferase. An additional increase in the specific activities of the guanylyl- and methyltransferase activities is observed in the peak fraction from poly(A)-Sepharose chromatography corresponding to an ultimate 235-fold and 106-fold purification of these activities, respectively. Since the virus contains two separate methyltransferase activities is observed in the peak fraction from mRNA(guanine-7-)methyltransferase, as shown below, the true purification achieved is nearly twice the indicated value.

**Identification of Purified Methyltransferase**—The nucleoside products formed by the methyltransferase at various stages of purification were analyzed by thin layer chromatography after the RNA was digested completely with ribonucleases A and T<sub>1</sub>, followed by snake venom phosphodiesterase and alkaline phosphatase. Viral cores exhibit mRNA(guanine-7-)methyltransferase, mRNA(adenosine-2'-)methyltransferase, and mRNA(guanosine-2'-)methyltransferase activities as indicated in Fig. 3A. Sixty-six per cent of the methyl groups are incorporated into 7-methylguanosine by the methyltransferases associated with the viral cores, and the remainder of the methyl groups are incorporated into the 2'-O-methylribonucleosides. The ratio of the mRNA(guanine-7-)methyltransferase activity to the mRNA(nucleoside-2'-)methyltransferase activity increases during the subsequent steps of purification as shown in Panels B through D in Fig. 3. After poly(U)-Sepharose chromatography 99% of the methyl groups are incorporated by the enzyme into 7-methylguanosine. Thus the purified enzyme exhibits, in addition to guanylyltransferase activity, mRNA(guanine-7-)methyltransferase activity but no significant mRNA(nucleoside-2'-)methyltransferase activity.

*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electro-*

FIG. 3. Thin layer chromatography of methylated nucleoside products of methyltransferase at representative stages of enzyme purification. Vaccinia virus mRNA synthesized *in vitro* was incubated with various enzyme fractions in the standard methyltransferase assay. The RNA was then digested, and the nucleosides were analyzed by thin layer chromatography as described under "Experimental Procedures." The origin and locations of markers, m<sup>7</sup>G, G<sup>m</sup>, and A<sup>m</sup> are indicated. The panels show the methylated nucleosides obtained by incubating mRNA with viral cores in A, with flow-through from the second DEAE-cellulose column in B, with flow-through from the DNA-agarose column in C, and with the pooled activity from the poly(U)-Sepharose column in D.



*phoresis*—Samples taken at various stages of the purification procedure were analyzed for their polypeptide composition on 7.5% polyacrylamide gels containing 5 M urea and 0.1% sodium dodecyl sulfate. Fig. 4 shows the polypeptide composition of whole virions, viral cores, flow-through from the second DEAE-cellulose column, flow-through from the DNA-agarose column, and the pooled enzyme fractions from the poly(U)-Sephadex column in *Gels A* through *E*, respectively. In *Gel E* only one major polypeptide is clearly seen in the photograph although additional minor bands were evident on visual inspection. Gel electrophoretic analysis of individual fractions from the poly(A)-Sephadex column suggested that the major band and one minor band were associated with enzyme activity. The stained gels in Fig. 5 correspond to Fractions 35 to 41 and 44 to 50 of the poly(A)-Sephadex column shown in Fig. 2. Both the major band and the minor band indicated by the arrows paralleled the enzyme activity while the other visible bands

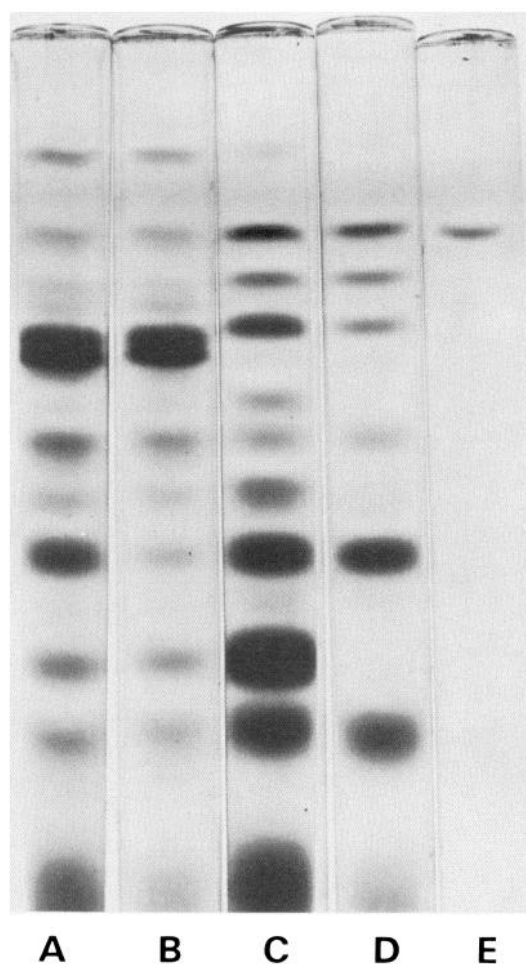


FIG. 4. Purification of guanylyl- and methyltransferase shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples of material at various stages of purification were analyzed by gel electrophoresis as described under "Experimental Procedures." The polypeptide composition of whole virions, viral cores, flow-through from the second DEAE-cellulose column, flow-through from the DNA-agarose column, and of the pooled active fractions from the poly(U)-Sephadex column is shown in *Gels A* through *E*, respectively. The approximate amount of viral protein applied to the gels was 40  $\mu$ g in *A*, 60  $\mu$ g in *B*, 120  $\mu$ g in *C*, 40  $\mu$ g in *D*, and 3  $\mu$ g in *E*. Faint bands were present in *Gel E* which cannot be observed in this photograph. Electrophoresis was from top to bottom.

did not. This was confirmed by gel electrophoretic analysis of the fractions from the poly(A)-Sephadex column during purification of the enzyme from [ $^3$ H]leucine-labeled virus. The distribution of radioactivity in one sliced gel is shown in Fig. 6. The ratio of the amounts of the radioactivity in the two more abundant polypeptides was constant across the peak of enzyme activity. By contrast, the ratio of the radioactivity in the very minor polypeptide with respect to either of the other two polypeptides decreased across the peak of enzyme activity suggesting it was a contaminant.

The molecular weights of the two enzyme-associated polypeptides were determined on 7.5% polyacrylamide gels containing 5 M urea and 0.1% sodium dodecyl sulfate. By comparison with the mobilities of polypeptides of known molecular weights (29, 30) values of 95,000 and 31,400 were derived for the two polypeptides. Using these molecular weights and assuming similar leucine compositions of both polypeptides, the molar ratio of the larger to the smaller polypeptide is 0.7. Alternately, when the molar ratio for these same polypeptides was determined from the area under curves of densitometric tracings of stained gels, the ratio 1.4 was derived. This range of 0.7 to 1.4 presumably reflects the invalid intrinsic assumptions of similar leucine composition and similar binding characteristics to Coomassie blue.

**Sephadex G-200 Chromatography**—Utilizing gel filtration with Sephadex G-200, Ackers has demonstrated the correlation of elution volume of a protein with its Stokes radius (31). For determination of this parameter of the guanylyl- and methyltransferase, purified enzyme was applied to a column of Sephadex G-200 as described under "Experimental Procedures." Internal standards of known Stokes radii (32, 33), catalase (5.22 nm), yeast alcohol dehydrogenase (4.55 nm), and bovine serum albumin (3.50 nm), were used to determine the effective gel pore radii. Values of 14.8, 14.6, and 15.8 nm were determined from the respective standards. The guanylyl- and methyltransferase eluted in the same fractions as shown in Fig. 7. Using the average gel pore radius, 15.1 nm, and the tables published by Ackers (31) the Stokes radius of both guanylyl- and methyltransferase was calculated to be 5.03 nm.

**Sucrose Gradient Sedimentation**—For determination of sedimentation coefficients of guanylyl- and methyltransferase, samples of purified enzyme were sedimented into 5 to 20% linear sucrose density gradients according to the method of Martin and Ames (33). Internal markers with established sedimentation coefficients included catalase (11.3 S, Ref. 34), horse liver alcohol dehydrogenase (4.88 S, Ref. 35), and bovine serum albumin (4.3 S, Ref. 36). The sedimentation profile of the guanylyl- and methyltransferase activities and the positions of the markers are shown in Fig. 8. Both activities co-sedimented; their  $s_{20,w}$  was determined to be 6.0.

**Determination of Molecular Weights**—Siegel and Monty have described the applicability of determining the molecular weights of proteins from their sedimentation coefficients and Stokes radii (32). From these parameters determined by sucrose gradient sedimentation and gel filtration, and assuming a partial specific volume of 0.73 cm<sup>3</sup>/g, a value of 127,000 was obtained for the molecular weight of the guanylyl- and methyltransferase. Additionally a frictional ratio of 1.51 was calculated.

#### DISCUSSION

Unique enzyme activities which specifically modify the 5' terminus of mRNA have been purified from vaccinia virus



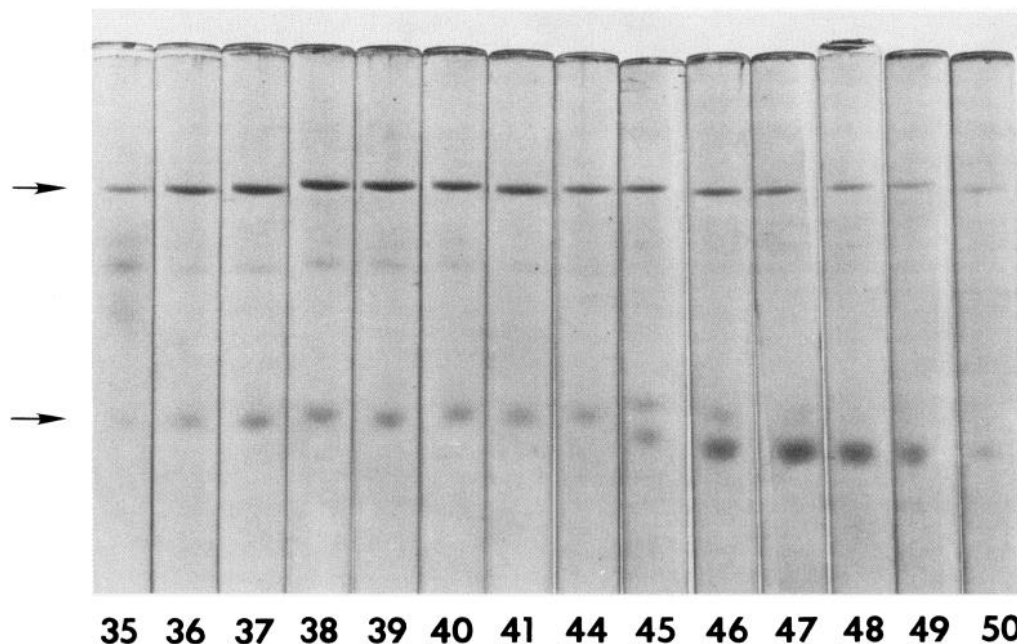


FIG. 5. Polypeptide composition of individual fractions from the poly(A)-Sephacel column. One-milliliter portions of fractions from the poly(A)-Sephacel column were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Experimental Procedures." From left to right the gels correspond to Fractions 35

through 41 and to Fractions 44 through 50 of the poly(A)-Sephacel column shown in Fig. 2. Electrophoresis was from top to bottom. The locations of the 95,000 and 31,400 molecular weight polypeptides are indicated by the arrows in the margin.

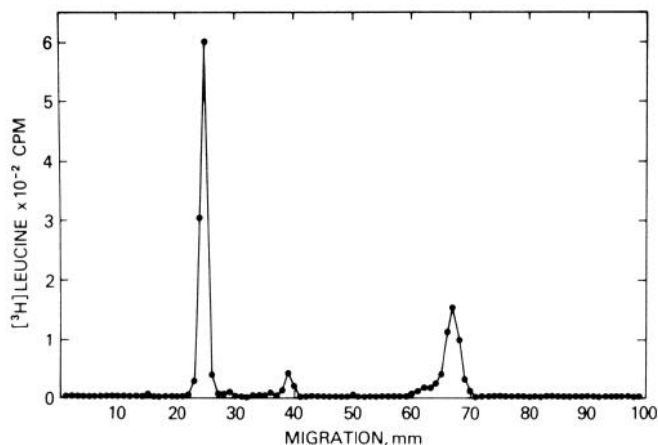


FIG. 6. Polypeptide composition of the peak fraction of enzyme activity on the poly(A)-Sephacel column. The peak fraction of purified enzyme isolated from [ $^3\text{H}$ ]leucine-labeled virus was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the gel was sliced into 1-mm sections which were solubilized and counted for radioactivity. The direction of migration was from left to right. In addition to the principal 95,000 and 31,400 molecular weight species, a minor amount of a 67,500 molecular weight polypeptide is present.

cores. These activities incorporate GMP into the 5' terminus of mRNA and incorporate methyl groups into the terminal 7-methylguanosine. These activities are inseparable by affinity or ion exchange chromatography, gel filtration, and sucrose density gradient sedimentation. The purified enzyme has a molecular weight of 127,000 and contains two polypeptides whose molecular weights are 95,000 and 31,400 and whose molar ratios are close to unity suggesting the mRNA guanylyltransferase and mRNA(guanine-7-)methyltransferase are components of an enzyme system.

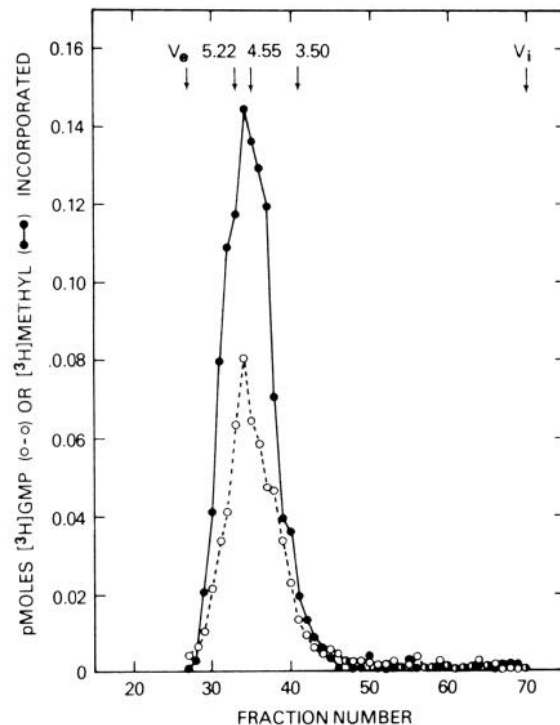


FIG. 7. Sephadex G-200 chromatography of purified guanylyl- and methyltransferase. Purified enzyme and standard proteins of known Stokes radii were applied to a column ( $51 \times 0.9$  cm) of Sephadex G-200. Fractions of 0.5 ml were collected and assayed for guanylyltransferase (O---O) and methyltransferase (●---●). Elution volumes of the standard proteins were determined as described under "Experimental Procedures." The excluded ( $V_e$ ) and included ( $V_i$ ) column volumes are shown, and the positions of elution of the standard proteins are designated by their Stokes radii: 5.22 nm for catalase, 4.55 nm for yeast alcohol dehydrogenase, and 3.50 nm for bovine serum albumin.

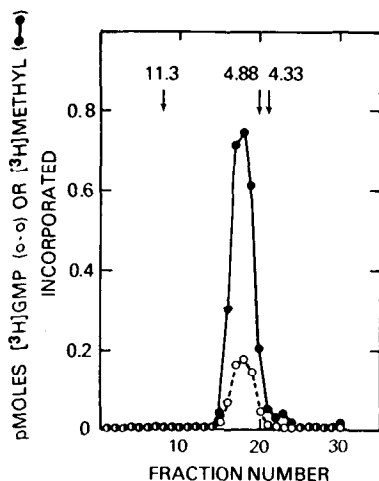


FIG. 8. Sucrose gradient sedimentation of purified guanylyl- and methyltransferase. Purified enzyme and proteins with known sedimentation coefficients were sedimented into 5 to 20% sucrose density gradients as described under "Experimental Procedures." Fractions (0.34 ml) were collected from the bottom of the tube and were assayed for guanylyltransferase (O--O) and methyltransferase (●--●). The locations of the protein markers are indicated by their sedimentation coefficients: 11.3 S for catalase, 4.88 S for horse liver alcohol dehydrogenase, and 4.33 S for bovine serum albumin. Sedimentation was from right to left.

By DNA- and RNA-affinity chromatography an approximately 200-fold purification of the enzyme has been achieved; the recovered enzyme comprises 0.4% of the total protein of vaccinia virus. Assuming the vaccinia virion has a particle weight of  $5.34 \times 10^{-16}$  g (37), of which 90% is protein, there would be approximately 90 molecules of enzyme per virion. This value is similar to those calculated for the nucleoside triphosphate phosphohydrolase I, the deoxyribonuclease, and the poly(A) polymerase.

Although the mRNA guanylyltransferase and mRNA(guanine-7-)methyltransferase isolated from vaccinia virus cores appear to be components of a single enzyme system, the mRNA(nucleoside-2'-)methyltransferase is a different enzyme. It is separated from the mRNA guanylyl- and (guanine-7-)methyltransferase during the purification by its affinity for DNA-agarose.

The modified 5'-terminal sequences of mRNAs from a variety of viruses and mammalian cells are similar to those found in vaccinia mRNA. Therefore, analogous enzymes must be present in cells and other viruses which perform this modification. Thus far, only the vaccinia enzyme has been purified, enabling us to study the process of modification of the 5' terminus of mRNA *in vitro*. The characterization of the reactions catalyzed by the mRNA guanylyltransferase and the mRNA(guanine-7-)methyltransferase is presented in the following paper (13).

Upon entry of vaccinia virus into the cytoplasm of the host cell the RNA nucleotidyltransferase (RNA polymerase) associated with the viral core (38, 39) is responsible for viral mRNA synthesis. Of the enzymes successfully isolated from vaccinia virus cores, the poly(A) polymerase (27) and the mRNA guanylyltransferase and mRNA(guanine-7-)methyltransferase are involved clearly in the modification of RNA. The biological roles of two nucleic acid-dependent nucleoside triphosphate phosphohydrolases (24, 28), a single strand-specific deoxyribonuclease (25, 40), and a protein kinase (26, 41) are obscure. It

seems reasonable, nevertheless, to consider that some of these enzymes may be involved in transcription. For example, Kates and Beeson have proposed that in vaccinia virus there may be an association between the adenosine triphosphatase and the ATP-dependent extrusion of mRNA from viral cores (42). In *Escherichia coli*  $\rho$  factor, which is responsible for termination of RNA synthesis, has been shown to possess RNA-dependent nucleoside triphosphate phosphohydrolase activity (43). Additionally, a bacteriophage T7-induced protein kinase which can phosphorylate the  $\beta'$  subunit of the host cell RNA nucleotidyltransferase appears to be important in regulation of transcription (44, 45).

Although proof that the enzymes within the vaccinia core are encoded by the viral genome is lacking, the RNA nucleotidyltransferase (46, 47), deoxyribonuclease (48), nucleoside triphosphate phosphohydrolases (49, 50), and poly(A) polymerase (51) are all induced after viral infection. Preliminary experiments<sup>2</sup> suggest that HeLa cell mRNA(guanine-7-)methyltransferase exhibits different chromatographic properties from the vaccinia virus core-associated enzyme indicating that the latter is also viral-induced.

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