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mRNA Guanylyltransferase and mRNA (Guanine-7-)-methyltransferase from Vaccinia Virions

DONOR AND ACCEPTOR SUBSTRATE SPECIFICITES

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Characterization of the donor and acceptor specificities of mRNA guanylyltransferase and mRNA (guanine-7-)-methyltransferase isolated from vaccinia virus cores has enabled us to discriminate between alternative reaction sequences leading to the formation of the 5'-terminal $m^7G(5')pppN$- structure. The mRNA guanylyltransferase catalyzes the transfer of a residue of GMP from GTP to acceptors which possess a 5'-terminal diphosphate. A diphosphate-terminated polyribonucleotide is preferred to a mononucleoside diphosphate as an acceptor suggesting that the guanylyltransferase reaction occurs after initiation of RNA synthesis. Although all of the homopolyribonucleotides tested ($pp(A)_n$, $pp(G)_n$, $pp(I)_n$, $pp(U)_n$, and $pp(C)_n$) are acceptors for the mRNA guanylyltransferase indicating lack of strict sequence specificity, those containing purines are preferred. Only GTP and dGTP are donors in the reaction; 7-methylguanosine (m^7G) triphosphate specifically is not a donor indicating that guanylylation must precede guanine-7-methylation. The preferred acceptor of the mRNA (guanine-7-)-methyltransferase is the product of the guanylyltransferase reaction, a polyribonucleotide with the 5'-terminal sequence G(5')pppN-. The enzyme can also catalyze, but less efficiently, methylation of the following: dinucleoside triphosphates with the structure G(5')pppN, GTP, dGTP, ITP, GDP, GMP, and guanosine. The enzyme will not catalyze the transfer of methyl groups to ATP, XTP, CTP, UTP, or to guanosine-containing compounds with phosphate groups in either positions 2' or 3' or in 3'-5' phosphodiester linkages. The latter specificity provides an explanation for the absence of internal 7-methylguanosine in mRNA. In the presence of PP_i, the mRNA guanylyltransferase catalyzes the pyrophosphorolysis of the dinucleoside triphosphate G(5')pppA, but not of $m^7G(5')pppA$. Since PP_i is generated in the process of RNA chain elongation, stabilization of the 5'-terminal sequences of mRNA is afforded by guanine-7-methylation.

Two distinguishing features of eukaryotic mRNAs are their polyadenylylated 3'-terminals and their guanylylated and methylated 5'-terminals. Vaccinia, a member of the poxvirus group, contains enzymes which are responsible for the synthesis and modification of mRNA and thus provides an excellent system for study of these processes. When purified vaccinia virions are incubated *in vitro* in the presence of a nonionic detergent, dithiothreitol, the four ribonucleoside triphosphates, Mg^{2+}, and *S*-adenosylmethionine, mRNA is synthesized which contains approximately 100 adenylate residues at the 3'-terminals (1) and $m^7G(5')pppA^m$- and $m^7G(5')pppG^m$-sequences[1] at the 5'-terminals (2, 3). Incubation of the vaccinia virus cores with sodium deoxycholate results in their disruption and release of soluble proteins including a poly(A) polymerase (4) and all of the 5'-terminal modification enzymes (5). The purified poly(A) polymerase exhibits primer dependency, has a molecular weight of 80,000, and contains two polypeptides with molecular weights of 51,000 and 30,000 (6). Two of the 5'-terminal modification enzymes, an mRNA guanylyltransferase and an mRNA (guanine-7-)-methyltransferase, have been purified as a complex with a molecular weight of 127,000 which contains polypeptides with molecular weights of 95,000 and 31,400 (7). A third activity involved in the 5'-terminal modification, an mRNA (nucleoside-2'-)-methyltransferase, appears to be a separate enzyme. Based upon the reactions shown to be catalyzed by the soluble enzymes (5), we have proposed that the 5'-terminal modification of mRNA occurs by a post-transcriptional mechanism in the following steps:

$$\overset{\gamma\beta\alpha}{pppG} + \overset{\beta\alpha}{ppN}\text{-} \rightleftarrows G(5')\overset{\alpha\beta\alpha}{ppN}\text{-} + \overset{\beta\gamma}{PP_i} \quad (1)$$

$$G(5')pppN\text{-} + AdoMet \rightarrow m^7G(5')pppN\text{-} + AdoHcy \quad (2)$$

$$m^7G(5')pppN\text{-} + AdoMet \rightarrow m^7G(5')pppN^m\text{-} + AdoHcy \quad (3)$$

[1] The abbreviations used are: m^7G, 7-methylguanosine; m^7Gua, 7-methylguanine; m^7Hyp, 7-methylhypoxanthine; A, G, C, U, I, X, adenosine, guanosine, cytidine, uridine, inosine, and xanthosine, respectively; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; PEI-cellulose, polyethyleneimine-substituted cellulose.

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This sequence of reactions, in which ppN- represents the 5′-terminal of the mRNA chain, is also supported by results from experiments with intact vaccinia virus cores in which the incorporation of phosphate groups from specifically labeled precursors into the modified 5′-terminals was examined under conditions of mRNA synthesis in the absence or presence of either limiting or saturating concentrations of *S*-adenosylmethionine (8). Reaction 1, catalyzed by the mRNA guanylyltransferase, involves the transfer of GMP from GTP to an acceptor mRNA containing a 5′-terminal diphosphate. A divalent cation, preferably Mg^{2+}, is required. The reverse reaction, pyrophosphorolysis of guanylylated 5′-terminal sequences, readily occurs in the presence of PP_i, a potent inhibitor of the net forward reaction (9). The mRNA (guanine-7-)-methyltransferase, which catalyzes reaction 2, requires neither GTP nor Mg^{2+} and is inhibited by the putative product *S*-adenosylhomocysteine (9). The observation that *S*-adenosylmethionine decreases the inhibitory effect of PP_i on the net guanylyltransferase reaction suggested to us that methylation of the terminal guanosine might prevent the pyrophosphorolysis reaction.

Although evidence obtained thus far was consistent with the sequence of reactions 1 to 3 above in the formation of modified 5′-terminals of mRNA, several alternative mechanisms were not ruled out. We considered that instead of a post-transcriptional mechanism the formation of the G(5′)pppN sequence might precede or be coupled with initiation of mRNA synthesis. This would require that the mRNA guanylyltransferase be capable of catalyzing the transfer of GMP from GTP to a mononucleoside diphosphate rather than to a diphosphate-terminated polyribonucleotide. The resulting dinucleoside triphosphate might then serve as a primer for transcription. A second alternative was that the methyltransferase reaction precedes the guanylyltransferase reaction instead of following it as outlined in reactions 1 and 2. This mechanism would require both the efficient methylation of GTP and the utilization of 7-methylguanosine triphosphate as a donor by the mRNA guanylyltransferase. Such a reaction order might ensure stabilization of these 5′-terminal sequences if, in fact, methylation does confer upon these sequences resistance to the PP_i-mediated pyrophosphorolysis described above. In order to evaluate the above possibilities and to characterize further the reactions catalyzed by these novel enzymes we have examined in detail the donor and acceptor specificities of the mRNA guanylyl- and mRNA (guanine-7-)-methyltransferase.

EXPERIMENTAL PROCEDURES

Purification of Viral Enzymes—Vaccinia virus (strain WR) was purified from infected HeLa cells according to previously published methods (10, 11). The procedure for the purification of the mRNA guanylyltransferase and mRNA (guanine-7-)-methyltransferase complex from vaccinia virus cores is described in detail (7).

Preparation of Synthetic Homopolyribonucleotides Containing 5′-Terminal Diphosphate—The chemical addition of a second phosphate to the 5′-terminals of poly(A), poly(G), poly(I), poly(U), and poly(C) was performed essentially according to the procedure which we have previously described in detail (9). Briefly, to 5 mg each of the polyribonucleotides, as their cetyltrimethylammonium salts, was added 0.25 mmol of diimidazolyl[^{32}P]phosphonate (3.25 mCi/mmol) which was prepared from [^{32}P]orthophosphoric acid. Following the reaction, the polyribonucleotides were recovered as their sodium salts and separated from unreacted radioisotope by sequential dialysis against two changes of 1 M NaCl, three changes of 0.01 M Na_2HPO_4 (pH 7.4), and exhaustively against 0.1 M NaCl/0.01 M Tris/HCl (pH 7.4) until no radioactivity was detectable in the dialysate. Subsequently, the polyribonucleotides were precipitated overnight at −20° after the addition of 2.5 volumes of ethanol, were collected by centrifugation at 3000 × *g* in a Sorvall SS-34 rotor for 10 min at −10°, and were dissolved in H_2O.

Preparation of 7-Methylguanosine Triphosphate and 7-Methylguanosine Diphosphate—The conversion of GTP and GDP into their respective 7-methyl derivatives was carried out with dimethyl sulfate as the alkylating agent as described by Hendler *et al.* (12). Purification of the methylated nucleotides was effected by ion exchange chromatography on a column of DEAE-cellulose equilibrated with 7 M urea/0.01 M Tris/HCl (pH 7.6). With linear gradients of NaCl from 0 to 0.2 M containing 7 M urea/0.01 M Tris/HCl (pH 7.6), the nucleotides were eluted from the column and were detected by continuous ultraviolet absorption measurements. The peaks containing methylated nucleotides were identified by characteristic blue fluorescence of dried samples under ultraviolet light (13). Fractions of the column eluate containing the methylated nucleotide were pooled, diluted 5-fold, and desalted by readsorption to a column of DEAE-cellulose equilibrated with 5 mM ammonium acetate, after which the column was washed to remove NaCl and urea. The methylated nucleotide was then eluted with 0.5 M ammonium acetate, repeatedly lyophilized, dissolved in H_2O, and stored at −20°.

Radioactive 7-methylguanosine triphosphate was prepared similarly. To 30 nmol of [α-^{32}P]GTP was added 30 μl (0.32 μmol) of dimethyl sulfate. After 3 h, the addition of dimethyl sulfate was repeated and the reaction was continued for 3 h more. The product was purified by preparative thin layer ascending chromatography on PEI-cellulose in 4 M sodium formate (pH 3.4). The reaction products were located by autoradiography of the thin layer sheet, and the PEI-cellulose containing the radioactive 7-methylguanosine triphosphate was scraped into a column and washed with H_2O. The isotope was eluted from the adsorbent with 2 M ammonium acetate, repeatedly lyophilized, dissolved in H_2O, and stored at −20°.

Guanylyltransferase Reactions—The standard reaction mixtures contained 50 mM Tris/HCl (pH 7.8), 2.5 mM $MgCl_2$, 5.3 μM [^{3}H]GTP (9.4 Ci/mmol), 1 mM dithiothreitol, 10 units/ml of mRNA guanylyltransferase, and defined concentrations of polyribonucleotides or nucleoside diphosphates. Incubation was at 37° for 30 min unless stated otherwise. Incorporation of GMP into polyribonucleotides was measured as radioactivity precipitated by cold 10% trichloroacetic acid and collected onto nitrocellulose filters. With nucleoside diphosphate acceptors in the mixture, the guanylyltransferase reaction was terminated by heating the reaction mixtures for 5 min at 100°. Then, the pH of the reaction mixture was adjusted to 8.5 with Tris/HCl; *Escherichia coli* alkaline phosphates was added to a final concentration of 1 unit/ml. After a 1-h incubated at 37° the reaction mixture was diluted 5-fold, and the addition of alkaline phosphate was repeated. The reaction mixture was incubated again for 1 h at 37° and then diluted with 7 M urea. The incorporation of GMP into alkaline phosphatase-resistant nucleotides with the general structure G(5′)pppN was determined by measuring radioactivity which could be adsorbed to columns of DEAE-cellulose in the presence of 7 M urea and eluted with 0.5 M ammonium acetate.

Methyltransferase Reactions—The standard reaction mixture contained 50 mM Tris/HCl (pH 7.8), 2.5 mM $MgCl_2$, 2 μM *S*-adenosyl-[*methyl*-^{3}H]methionine (11.6 Ci/mmol), 1 mM dithiothreitol, 30 units/ml of mRNA (guanine-7-)-methyltransferase, and defined concentrations of substrates. After incubation at 37° for 30 min, the incorporation of methyl groups into the substrate was measured by one of the following methods unless stated otherwise. In experiments containing polyribonucleotides, methyl incorporation was measured as radioactivity precipitated by cold 10% trichloroacetic acid and collected onto nitrocellulose filters. Methyl incorporation into simple nucleotides was determined by measuring radioactivity which was adsorbed to and eluted from DEAE-cellulose as indicated for the guanylyltransferase assay. For comparative quantitative analysis of the incorporation of methyl groups with guanosine and guanosine-containing nucleotides as substrates the reaction mixtures were heated for 30 min at 100° in the presence of 1 M HCl (14). The mixtures were then applied to Eastman thin layer cellulose sheets (20 × 20 cm) and ascending chromatography was performed in 5% aqueous NaH_2PO_4 saturated at room temperature with isoamyl alcohol. The sheets were subsequently cut into 1-cm strips and the amount of 7-methylguanine present in each sample was determined by liquid scintillation spectrometry.

Identification of Products of Methyltransferase Reactions—Chromatographic techniques were employed to establish the identity of products formed in the methyltransferase reactions. The

reaction mixture containing guanosine was analyzed by applying the sample to Whatman No. 1 paper and performing ascending chromatography in 2-propanol/H_2O/concentrated HCl (650/184/166). The paper was cut into 1-cm strips and one-half of each strip was analyzed by liquid scintillation spectrometry. From the remaining one-half of those strips containing radioactivity corresponding to the position of migration of authentic 7-methylguanosine the radioactivity was eluted with H_2O, lyophilized, and then hydrolyzed with 1 M HCl at 100° for 30 min. The hydrolysis product was identified as 7-methylguanine as described above.

The reaction products in mixtures with guanosine-containing nucleotides as substrates were identified first by chromatography of the reaction products on columns (12.5 × 0.9 cm) of DEAE-cellulose in the presence of 7 M urea. Fractions of the column eluate comprising the peak of radioactivity were pooled, diluted 4-fold, and desalted as described above. This material was then analyzed directly and after treatment with alkaline phosphatase by descending paper chromatography in isobutyric acid/0.5 M NH_4OH (5/3).

In all experiments in which chromatographic or electrophoretic methods were used to identify enzymatic reaction products, authentic compounds were applied with the sample and the positions of migration of these substances were ascertained by examining the chromatogram or electrophoretogram under ultraviolet illumination.

Source of Materials—DEAE-cellulose (Whatman DE52) was obtained from Whatman, Inc., and PEI-cellulose thin layer sheets were the products of J. T. Baker. Unless stated otherwise, P-L Biochemicals was the source of all nucleosides, nucleotides, and polyribonucleotides; GTP was purified further by ion exchange chromatography on columns of DEAE-cellulose before use. *E. coli* alkaline phosphatase was from Boehringer Mannheim. Inosine, 1,1′-carbonyldiimidazole, and *E. coli* K-12 RNA nucleotidyltransferase were obtained from Sigma Chemical Co. *Penicillium citrinum* nuclease P_1 was from Yamasa Shoyu Co., Japan. Amersham/Searle was the source of [^{3}H]GTP; other radioisotopes were the product of New England nuclear. We are grateful to Drs. E. Hamel and M. Cashel, National Institutes of Child Health and Human Development, National Institutes of Health, who kindly provided guanosine 2′,5′-diphosphate and guanosine 3′,5′-diphosphate.

RESULTS

Acceptor Substrate Specificity of mRNA Guanylyltransferase—The purified vaccinia mRNA guanylyltransferase transfers GMP from GTP to the 5′-terminals of added vaccinia mRNA to form the sequences G(5′)pppG- and G(5′)pppA-. Using poly(adenylic acid) we demonstrated that RNA must possess a 5′-terminal diphosphate to serve as an acceptor in the reaction; furthermore, that a homopolyribonucleotide is an efficient acceptor in the reaction indicated that the mRNA guanylyltransferase exhibits no strict sequence specificity (9). To probe further the limits of the acceptor specificity of the mRNA guanylyltransferase, a variety of diphosphate-terminated homopolyribonucleotides were prepared from the commercial monophosphate-terminated polymers by the method described under "Experimental Procedures." Quantitation of the ppN-terminated structures formed was achieved by using [^{32}P]orthophosphate for their syntheses; this was important since neither was the chemical reaction complete nor were the polymers equally reactive with the diimidazolyphosphonate.[2] When the polyribonucleotides were incubated with [^{3}H]GTP and enzyme under the conditions described under "Experimental Procedures," [^{3}H]GMP was incorporated in each case into cold trichloroacetic acid-precipitable material. These results are summarized in Table I. More GMP was incorporated with $pp(A)_n$ than with any of the other polymers as the acceptor. As a group, the polyribonucleotides containing purines were more efficient than those containing pyrimidines as acceptors in the guanylyltransferase reaction. This specificity is in accord with the presence of a purine base in the nucleoside adjacent to the terminal guanosine residue in vaccinia mRNA.

[2] S. Martin, unpublished results.

TABLE I

Incorporation of GMP into homopolyribonucleotides by vaccinia mRNA guanylyltransferase

Polyribonucleotide	Termini of Polymer Present in Reaction	GMP Incorporated
	pmol	pmol
$pp(A)_n$	620	3.1
$pp(G)_n$	590	1.2
$pp(I)_n$	690	1.6
$pp(C)_n$	870	0.74
$pp(U)_n$	470	0.74

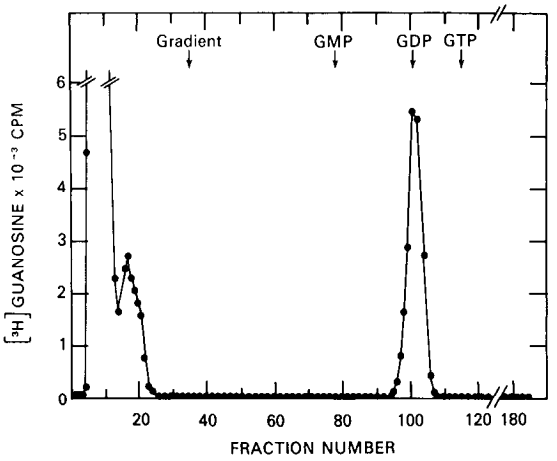

FIG. 1. DEAE-cellulose chromatography of alkaline phosphatase-resistant material produced by incubation of mRNA guanylyltransferase, [^{3}H]GTP, and ADP. The standard guanylyltransferase reaction mixture containing 1 mM ADP was incubated and treated with alkaline phosphatase as described under "Experimental Procedures." This mixture was then diluted with 7 M urea and adsorbed to a column (12.5 × 0.9 cm) of DEAE-cellulose equilibrated with 7 M urea/0.01 M Tris/HCl (pH 7.6). GMP, GDP, and GTP absorbance markers were then applied to the column. After the column was washed, a 300-ml linear gradient from 0 to 0.2 M NaCl containing 7 M urea/0.01 M Tris/HCl (pH 7.6) was applied. Fractions of 2 ml were collected at a rate of 15 ml/h, and 1 ml of each fraction was counted in toluene-based scintillation fluid containing 33% Triton X-100.

Although the transfer of GMP to a preformed polyribonucleotide by the mRNA guanylyltransferase would account for post-transcriptional modification of the 5′-terminal of mRNA, one may alternatively consider a mechanism in which the formation of the 5′-terminal sequence is coupled with the initiation of mRNA synthesis. Such a mechanism would require the initial condensation of GMP with a mononucleoside diphosphate resulting in the formation of a dinucleoside triphosphate with the structure G(5′)pppN. We incubated [^{3}H]GTP and enzyme with nucleoside diphosphates, treated the reaction mixtures subsequently with alkaline phosphatase, and analyzed the reaction products by DEAE-cellulose chromatography. Fig. 1 shows the elution profile of the radioactivity in one experiment in which 1 mM ADP and 5 μM [^{3}H]GTP were incubated with the mRNA guanylyltransferase. A single peak of alkaline phosphatase-resistant radioactivity was detected which eluted coincidentally with the absorbance marker GDP with a net charge of approximately −2.9. Identical

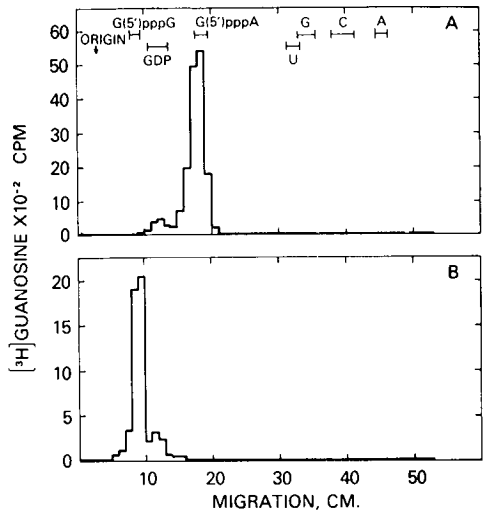

FIG. 2. Characterization of alkaline phosphatase-resistant material formed by incubation of mRNA guanylyltransferase, [^{3}H]GTP, and nucleoside diphosphates. Standard guanylyltransferase reaction mixtures each containing 1 mM nucleoside diphosphate were first analyzed by DEAE-cellulose chromatography as outlined in the legend to Fig. 1. Fractions comprising the peak of radioactivity with a net charge of −2.9 were pooled, diluted 4-fold, and desalted, and then applied directly to Whatman No. 1 paper, and descending chromatography was performed in isobutyric acid/0.5 M NH_4OH (5/3). The paper was cut into 1-cm strips, and the radioactivity was eluted with H_2O and counted in toluene-based scintillation fluid containing 33%. Triton X-100. The reaction mixtures contained ADP in *Panel A* and GDP in *Panel B*. The location of marker compounds are shown in *Panel A*. Direction of migration was from *left* to *right*.

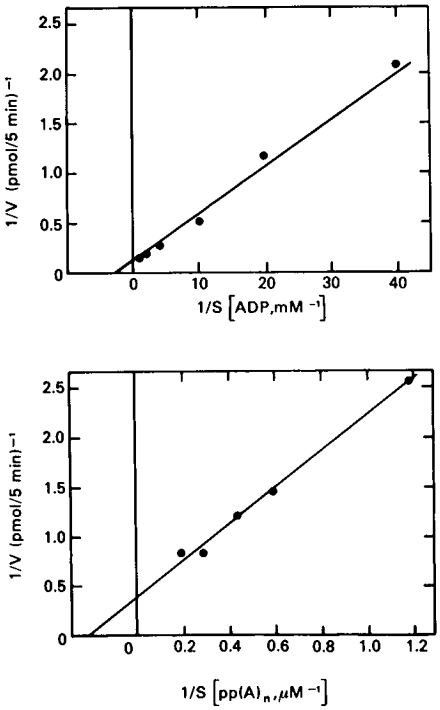

FIG. 3. Effect of concentrations of acceptor substrates ADP and pp(A)$_n$ on the guanylyltransferase reaction. Conditions for the guanylyltransferase reaction and the methods for analyzing the products were as described under "Experimental Procedures" except that the concentration of [^{3}H]GTP (17 Ci/mmol) was 5.9 μM and incubations were for 5 min at 37°. The reaction mixtures contained different concentrations of either ADP (*upper plot*) or pp(A)$_n$ (*lower plot*).

radioactive profiles also were observed when reaction mixtures containing either GDP, UDP, or CDP were analyzed similarly, whereas no alkaline phosphatase-resistant material was detected when a nucleoside diphosphate was omitted from the reaction mixture. The [^{3}H]guanosine-containing, alkaline phosphatase-resistant material isolated by DEAE-cellulose chromatography was characterized further by paper chromatography. The results of such an analysis on the material formed by incubating enzyme, [^{3}H]GTP, and ADP are shown in *Panel A* of Fig. 2. The product co-migrates with authentic G(5′)pppA. Similarly, in *Panel B* migration of the material resulting from the incubation of enzyme, [^{3}H]GTP, and GDP is identical to that of authentic G(5′)pppG. When [^{3}H]GTP was incubated with UDP, the resulting alkaline phosphatase-resistant product, the putative dinucleoside triphosphate G(5′)pppU, migrated slightly slower than authentic G(5′)pppG. This is what would be expected since in this system the rate of migration of uridine is sightly less than that of guanosine as shown in *Panel A*. Likewise, the alkaline phosphatase-resistant material, the putative product G(5′)pppC, formed during the incubation of enzyme, [^{3}H]GTP, and CDP migrated on paper chromatography faster than G(5′)pppG and slower than G(5′)pppA, just as the rate of migration of cytidine is intermediate between those of guanosine and adenosine. This experiment establishes that the vaccinia mRNA guanylyltransferase can, in fact, utilize a nucleoside diphosphate as an acceptor in the reaction.

The relative acceptor activity of the nucleoside diphosphates in the guanylyltransferase reaction was determined from measurements of radioactivity incorporated into alkaline phosphatase-resistant nucleotides. In 0.1-ml reactions containing 1 mM nucleoside diphosphate and 5 μM [^{3}H]GTP the amount of GMP incorporated was 3.7, 1.5, 0.46, and 0.14 pmol with ADP, GDP, UDP, and CDP, respectively. Just as with the polyribonucleotides, the nucleoside diphosphates containing purine bases were more efficient acceptors than those containing pyrimidine bases.

The preceding experiments demonstrate that the vaccinia mRNA guanylyltransferase will incorporate GMP into structures with the sequence G(5′)ppp(N)$_n$ with a variety of polyribonucleotide acceptors or into structures with the sequence G(5′)pppN with simple nucleoside diphosphate acceptors. In an attempt to answer the question whether the occurrence of the guanylyltransferase reaction during synthesis of RNA by viral cores is post-transcriptional or is coupled with initiation of mRNA synthesis we examined the effect of the concentration of the acceptors, ADP and pp(A)$_n$, on the initial velocity of the reaction with the purified enzyme. Fig. 3 is a Lineweaver-Burk double reciprocal plot of these data showing the reaction with ADP in the upper plot and the reaction with pp(A)$_n$ in the lower plot. From these data we calculated the apparent dissociation constants for the acceptor species, ADP and pp(A)$_n$, in the guanylyltransferase reaction. These values are 360 μM for ADP and 5.2 μM for pp(A)$_n$, suggesting that the reaction occurs much more efficiently with the diphosphate-terminated polyribonucleotide than with the mononucleoside diphosphate as an acceptor.

Donor Substrate Specificity of mRNA Guanylyltransferase—Previously, we found that of the four ribonucleoside triphosphates the mRNA guanylyltransferase will utilize only GTP as a donor. The corresponding deoxyribonucleotide, dGTP, will also serve as a donor in the reaction. In separate

reaction mixtures with either [^{3}H]GTP or [^{3}H]dGTP at comparable substrate concentrations (5 μM), the incorporation of the deoxyribonucleotide was 30% of that of the ribonucleotide.

Since, as will be shown below, the mRNA (guanine-7-)-methyltransferase can utilize GTP as a substrate and thus form 7-methylguanosine triphosphate, it was important to determine whether this methylated nucleotide could serve as a donor in the guanylyltransferase reaction. Standard 0.1-ml guanylyltransferase reaction mixtures containing either [α-^{32}P]GTP (4.9 Ci/mmol) or the 7-methyl derivative of the isotope, prepared from [α-^{32}P]GTP as described under "Experimental Procedures," at concentrations of 4 μM were incubated with 40 μg of pp(A)$_n$ acceptor. Whereas under these conditions, 13 pmol (1.4×10^5 cpm) of [^{32}P]GMP was incorporated into the polymer with GTP as the donor, there was no detectable incorporation of ^{32}P into polymer when 7-methylguanosine [α-^{32}P] triphosphate was the donor. That the 7-methylguanosine triphosphate which had been synthesized was biologically active material was verified by its incorporation into poly(G, m^{7}G) by *Escherichia coli* RNA nucleotidyltransferase in the presence of poly(C) template (12). Similarly, the vaccinia core-associated RNA nucleotidyltransferase was demonstrated to possess the ability to incorporate radioactive label from 7-methylguanosine [α-^{32}P]triphosphate in the presence of all four naturally occurring ribonucleoside triphosphates into mRNA. This mRNA was purified, digested with *Penicillium* nuclease P$_1$ (15), and analyzed by chromatography on a column of DEAE-cellulose in the presence of 7 M urea. Only a single peak of radioactivity was eluted from the column with a net charge of approximately -1.5 which is that of pm^{7}G.[3] This indicated that the detectable incorporation of 7-methylguanosine phosphate was into internal sequences and not preferentially into 5′-terminals. Such 5′-terminal sequences would have been eluted from the DEAE-cellulose column after digestion with P$_1$ nuclease as the structures m^{7}G(5′)pppA and m^{7}G(5′)pppG with net charges of -2.5. Therefore, the lack of incorporation of radioactivity from 7-methylguanosine [α-^{32}P]-triphosphate into added RNA by the purified enzyme or into the 5′-terminals of mRNA synthesized in the vaccinia virus cores indicated the donor specificity of the mRNA guanylyltransferase for GTP and not the 7-methyl derivative. Furthermore, this is evidence that the sequence for the guanylyltransferase and methyltransferase reactions is that order which we had proposed, namely, first the formation of the guanylylated 5′-terminal sequence followed by the methylation of the terminal guanosine residue.

Effect of Methylation on Reversibility of Guanylyltransferase Reaction—In a previous report we presented evidence that the guanylyltransferase reaction is reversible (9). This was demonstrated by incubating enzyme with ^{32}PP$_i$ and unmethylated vaccinia mRNA containing [^{3}H]guanosine-labeled 5′-terminals and identifying [^{3}H, ^{32}P]GTP in the reaction products. In addition, PP$_i$ was found to be an inhibitor of the guanylyltransferase reaction in the forward direction. However, in the presence of *S*-adenosylmethionine, the inhibitory effect of PP$_i$ on the forward guanylyltransferase reaction is reduced suggesting that the methylated 5′-terminal sequences of the mRNA might not participate in the pyrophosphorolysis (reverse) reaction. To test this possibility directly we incubated the mRNA guanylyltransferase and ^{32}PP$_i$ with m^{7}G(5′)pppA and with G(5′)pppA as described in the legend to Fig. 4. The reaction products were analyzed by paper electrophoresis and were detected by autoradiography as shown in Fig. 4. In a similar experiment, the paper electrophoretogram was cut into strips which were analyzed by liquid scintillation spectrometry; this procedure provided greater sensitivity and gave identical results. The incubation of ^{32}PP$_i$ with either m^{7}G(5′)pppA or G(5′)pppA in the absence of enzyme revealed that only P$_i$ and PP$_i$ were detectable in the reaction mixture as shown in *Lanes 1* and *2* in Fig. 4. The reaction mixture containing ^{32}PP$_i$, m^{7}G(5′)pppA, and enzyme (Fig. 4, *Lane 3*) likewise exhibited radioactivity associated only with P$_i$ and PP$_i$. By contrast, in the reaction mixture containing ^{32}PP$_i$, G(5′)pppA, and enzyme (Fig. 4, *Lane 4*) an additional spot was identified on the autoradiogram which co-migrated with GTP. The radioactivity in the region of the electrophoretogram which corresponded to this spot was eluted from the paper and was analyzed further by thin layer chromatography on PEI-cellulose. With this technique, the ^{32}P was again identified as GTP as shown in Fig. 5. This experiment established that methylation of the terminal guanosine residue prevents reversal of the guanylyltransferase reaction. This is in accord with data in the previous section which showed that 7-methylguanosine triphosphate was not a substrate for the forward reaction catalyzed by the mRNA guanylyltransferase. Consequently, the corresponding 7-methylguanosine residue in the 5′-terminal sequence should not be a substrate in the reverse reaction catalyzed by the mRNA guanylyltransferase.

Acceptor Substrate Specificity of mRNA (Guanine-7-)-methyltransferase—The vaccinia mRNA (guanine-7-)-methyltransferase transfers a methyl group from *S*-adenosylmethionine to position 7 of the terminal guanosine residue of either unmethylated vaccinia mRNA or a synthetic polyribonucleotide with the structure G(5′)ppp(A)$_n$; neither Mg^{2+} nor GTP is required (9). These findings established that mRNA guanylylation and mRNA (guanine-7-)-methylation are not requisitely coupled processes and suggested that guanylylation precedes methylation. Nevertheless, an alternative sequence of reactions, namely first the methylation of GTP followed by a guanylyltransferase reaction in which a residue of 7-methylguanosine phosphate would be transferred to a polyribonucleotide was considered. Although this reaction sequence has been eliminated as a possibility since the data presented above indicate that 7-methylguanosine triphosphate is not a donor in the guanylyltransferase reaction, additional studies revealed that the mRNA (guanine-7-)-methyltransferase could utilize GTP as an acceptor substrate. The enzyme was incubated with GTP and *S*-adenosyl [*methyl*-^{3}H]methionine, and the products of the reaction were analyzed by DEAE-cellulose chromatography as shown in Fig. 6. A Single peak of radioactivity was eluted from the column with a net charge of approximately -3.1. This position of elution from DEAE-cellulose is identical to that of chemically synthesized 7-methylguanosine triphosphate. The 7-methyl derivative of GTP does not co-elute with GTP because the methylation at position 7 of the guanine ring introduces a positive charge which partially neutralizes the net negative charge of the nucleotide. This peak of radioactive material from the DEAE-cellulose column was characterized further, and its identity as 7-methylguanosine triphosphate was established by (*a*) its co-chromatography with chemically synthesized 7-methylguanosine triphosphate on PEI-cellulose thin layers, (*b*) its conversion to 7-methylguanosine by treatment with *E. coli* alkaline phosphatase, and (*c*) its conversion

[3] S. Martin, unpublished results.

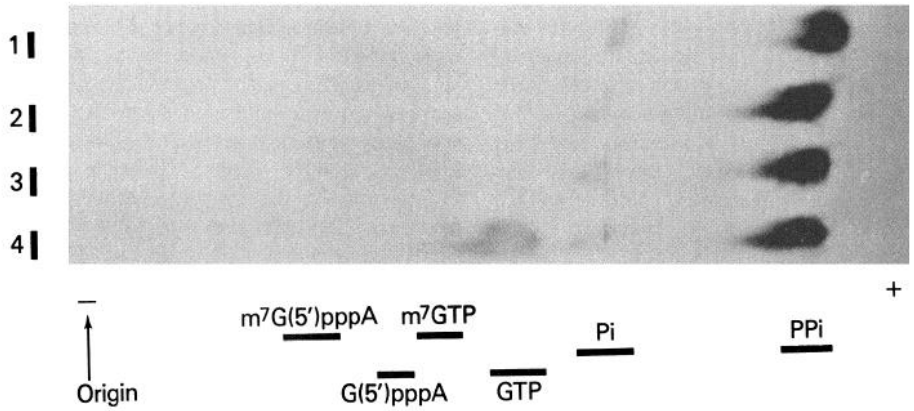

FIG. 4. Formation of GTP by reversal of the guanylyltransferase reaction. Each 100-μl reaction mixture contained 50 mM Tris/HCl (pH 7.8), 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.5 μM $^{32}PP_i$ (1.4 Ci/mmol), 5 A_{260} units/ml of the dinucleoside triphosphate, and 20 units/ml of mRNA guanylyltransferase in those reactions containing the enzyme. Reaction mixtures were incubated for 30 min at 37° in the presence of: *1*, $m^7G(5')pppA$; *2*, G(5′)pppA; *3*, $m^7G(5')pppA$ and enzyme; and *4*, G(5′)pppA and enzyme, indicated in *Lanes 1*, *2*, *3*, and *4*, respectively, in the autoradiogram. The reactions were terminated by the addition of 1 μmol of EDTA and 75-μl portions of each mixture were applied to Whatman No. 3MM paper. Electrophoresis was performed at 30 V/cm for 3.25 h in 50 mM sodium citrate buffer, pH 3.5. The electrophoretogram was exposed for 72 h to Kodak RP/R2 x-ray film. Electrophoresis was from *left* to *right*.

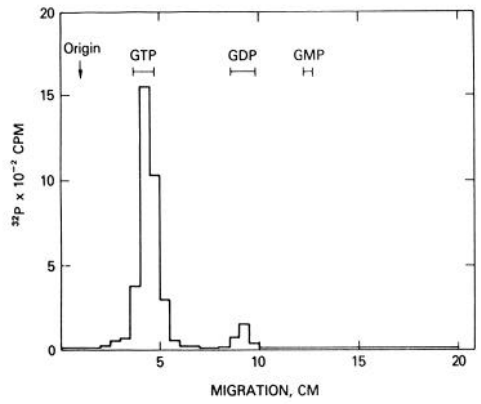

FIG. 5. Identification of reaction product obtained by incubation of $^{32}PP_i$, G(5′)pppA, and mRNA guanylyltransferase. The radioactivity in the region of the electrophoretogram corresponding to a unique reaction product, shown in Fig. 4, *Lane 4*, was eluted from the paper with H_2O, lyophilized, redissolved in H_2O, and applied to a thin layer sheet of PEI-cellulose. Ascending chromatography was performed in 2 M LiCl/2 M HCOOH (1/1). The sheet was cut into 0.5-cm strips which were counted in toluene-based scintillation fluid.

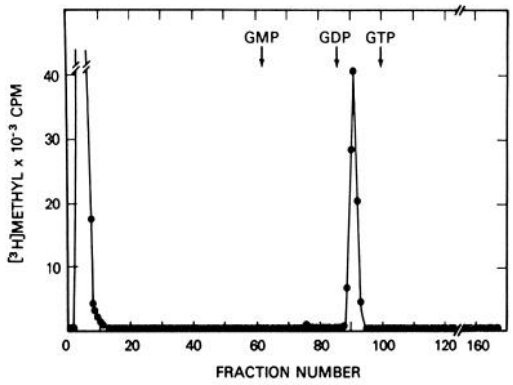

FIG. 6. Formation of 7-methylguanosine triphosphate by mRNA (guanine-7-)-methyltransferase. A standard methyltransferase reaction containing 2.5 mM GTP was incubated at 37° for 30 min, was diluted 4-fold with 7 M urea, and was subsequently analyzed by DEAE-cellulose chromatography as described in the legend to Fig. 1.

to 7-methylguanine by treatment with 1 M HCl at 100° for 30 min (data not shown). Despite the fact that GTP can be methylated by the enzyme this reaction is relatively inefficient. At identical concentrations of *S*-adenosylmethionine, the rate of incorporation of methyl groups was measured with GTP, G(5′)pppG, and $G(5')ppp(A)_n$ as substrates. Fig. 7 shows the double reciprocal plots of these data in experiments with GTP and $G(5')ppp(A)_n$ substrates. From such plots we were able to calculate the apparent dissociation constants of the acceptor substrates in these reactions. These values were 530 μM for GTP, 120 μM for G(5′)pppG, and 0.21 μM for $G(5')ppp(A)_n$. Undoubtedly, the terminal residue in the polymer $G(5')ppp(A)_n$ is methylated preferentially in comparison to the other substrates tested. This is consistent with both the preferential guanylylation of a preformed polyribonucleotide, suggesting that these two reactions occur as post-transcriptional modifications, and the sequence of the guanyly- and methyltransferase reactions established above.

Since GTP was utilized as a substrate by the mRNA (guanine-7-)-methyltransferase additional studies were carried out to define more precisely the specific substrate requirements of the enzyme. The ability of a number of other compounds to act as acceptors of methyl groups was determined, and the results of these experiments are presented in Table II. Just as with GTP, the incorporation of methyl groups was observed with guanosine, GMP, and GDP as substrates. At identical substrate concentrations, the transfer of methyl groups to guanosine was only 11% of that transferred to GTP. Relative to the incorporation of methyl groups with guanosine as a substrate, the incorporation of methyl groups with the simple guanosine-containing 5′-nucleotides exhibits a direct correlation with the number of phosphate groups present. By contrast, compounds containing a phosphate group located in either position 2′ or 3′ do not accept methyl groups; no methyl incorporation was detected with guanosine 2′-phosphate, gua-

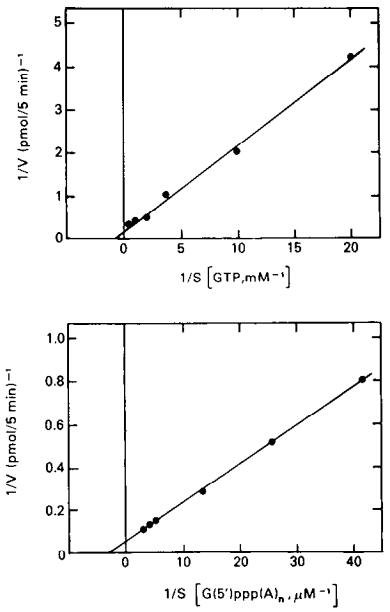

FIG. 7. Effect of concentration of acceptor substrates GTP and G(5')ppp(A)$_n$ on the methyltransferase reaction. Standard methyltransferase reaction mixtures as described under "Experimental Procedures" containing different concentrations of GTP or G(5')ppp(A)$_n$ were incubated 5 min at 37°. Reactions were terminated by the addition of 0.2 ml of 7 M urea, and then 0.1-ml portions of each reaction were applied to DEAE-cellulose (Whatman DE81) filters. Unincorporated *S*-adenosyl[*methyl*-^{3}H]methionine was removed by washing the filters in five changes of 25 mM ammonium bicarbonate (pH 8.0). The filters were dried and counted in toluene-based scintillation fluid. Methyl incorporation with GTP as the substrate is shown in the *upper panel* and with G(5')ppp(A)$_n$ as the substrate is shown in the *lower panel*.

TABLE II

Acceptor substrate specificity of vaccinia mRNA (guanine-7-)-methyltransferase

Substrate	Methyl Groups Incorporated[a]
G	0.11
pG	0.18
Gp	<0.01
G(2')p	<0.01
GpG	<0.01
pG + Gp	0.17
pGp	<0.01
pG(2')p	<0.01
ppG	0.39
pppG	1.0
ppppG	1.9
pppA	<0.01
ppp I	0.11
pppX	<0.01
pppU	<0.01
pppC	<0.01
G(5')pppG	4.6
m^{7}G(5')pppG	0.30

[a] Numbers are expressed as relative to the incorporation of methyl groups observed with pppG at identical substrate concentrations. Except with dinucleoside triphosphates which were 1 mM, all substrates were 2.5 mM.

nosine 3'-phosphate, guanosine 2',5'-diphosphate, or guanosine 3',5'-diphosphate as substrates. Compounds containing a phosphate group existing in a conventional 3'-5' phosphodiester bond, as in guanylyl-(3'-5')-guanosine, also are not methyl acceptors. The mere presence of a nucleoside 3'-phosphate does not inhibit the enzyme since the transfer of methyl groups to guanosine 5'-phosphate is not significantly affected by the addition of equimolar guanosine 3'-phosphate to the reaction mixture. These data demonstrate the effect of the number and position of phosphates in the substrate on the transfer of methyl groups to nucleotides and provide an explanation for the observation that only the 5'-terminals, not internal guanosine residues, are the site of methylation by mRNA (guanine-7-)-methyltransferase. With dGTP as a substrate, almost twice the incorporation of methyl groups is observed as compared with GTP as a substrate. The steric fit of the substrate at the active site of the enzyme may be influenced by the size of the moiety located at position 2' or 3' as suggested by the marked effect of the location of the phosphate groups in the substrate on its acceptor activity in the methyltransferase reaction.

We next examined the effect of the base moiety of the substrate on the transfer of methyl groups to nucleoside triphosphates. As shown in Table II, neither of the pyrimidine-containing nucleotides, UTP and CTP, were acceptors of methyl groups. With the purine-containing nucleotides, no incorporation of methyl groups was observed with either ATP or XTP as substrates. With ITP, the incorporation of methyl groups was 11% of that observed when GTP was the substrate in the reaction. The methyl-labeled product resulting from the incubation of *S*-adenosyl[*methyl*-^{3}H]methionine and enzyme with ITP was purified by chromatography on DEAE-cellulose, was hydrolyzed with 1 M HCl at 100°, and was then analyzed by paper chromatography as shown in Fig. 8. The methyl-labeled hydrolysis product was identified as 7-methylhypoxanthine indicating that the enzyme reaction product was 7-methylinosine triphosphate. It is evident from these data that a common feature of the substrates for the mRNA (guanine-7-)-methyltransferase, GTP, and ITP, is an oxygen atom at position 6 of the purine ring; ATP, which is not substrate, contains an amino group in position 6. Since XTP which contains the base 2,6-dioxopurine (xanthine) is not a substrate for the enzyme, we can infer that the moiety at position 2 of the purine ring also exerts an effect on the acceptor substrate activity, possibly by its influence on the distribution of electrons in the purine ring and, consequently, on the ionization constants of the reactive sites on the ring (16, 17).

Table II also shows that the dinucleoside triphosphates G(5')pppG and m^{7}G(5')pppG are substrates for the mRNA (guanine-7-)-methyltransferase. With these substrates, G(5')pppG and m^{7}G(5')pppG, the reaction products were identified by paper chromatography as a monomethylated diguanosine triphosphate in the case of the former and as a dimethylated diguanosine triphosphate in the case of the latter. Compared with GTP at 1 mM, there is an almost 5-fold increase in the incorporation of methyl groups with G(5')pppG as a substrate. When m^{7}G(5')pppG instead of G(5')pppG was incubated as a substrate in the methyltransferase reaction the methyl incorporation was reduced approximately 15-fold. Whereas in the symmetric molecule, G(5')pppG, either guanosine residue may be a site for methyl incorporation, only 1 guanosine residue in m^{7}G(5')pppG is available for methylation. Moreover, one would expect that binding the enzyme to the alternate end of the molecule, the 7-methylguanosine moiety, would inhibit the incorporation of methyl groups by a mechanism analogous to product inhibition.

DISCUSSION

The modified 5'-terminals of vaccinia mRNA synthesized by viral cores *in vitro* in the presence of *S*-adenosylmethionine are m^{7}G(5')pppG^m- and m^{7}G(5')pppA^m- (3). The addition and

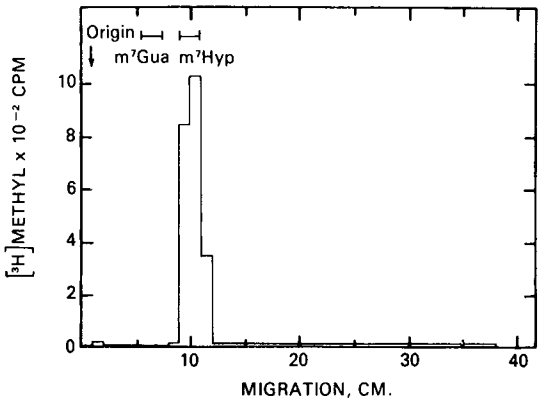

FIG. 8. Identification of the methyl-labeled product of a methyltransferase reaction with ITP as the substrate. A standard methyltransferase reaction mixture as described under "Experimental Procedures" containing 2.5 mM ITP was incubated for 30 min at 37°, diluted with 7 M urea, and adsorbed to a column (2 × 0.6 cm) of DEAE-cellulose. The column was washed with 7 M urea/0.01 M Tris/HCl (pH 7.6) and then with 5 mM ammonium acetate. The methyl-labeled product was eluted from the column with 0.5 M ammonium acetate, lyophilized, and then dissolved in 1 N HCl. This was then heated at 100° for 30 min. As controls, 100 μg each of authentic 7-methylinosine and 7-methylguanosine were similarly hydrolyzed with 1 N HCl. The sample was then applied to Whatman No. 1 paper, and portions of the acid-hydrolytic products, 7-methylhypoxanthine and 7-methylguanine, were applied as markers. Ascending chromatography was performed in ethyl acetate/HCOOH/H_2O (7/2/1). The paper was cut into 1-cm strips which were counted in toluene-based scintillation fluid to locate the methyl-labeled reaction product.

methylation of the terminal guanosine residue are catalyzed by specific mRNA guanylyl- and mRNA (guanine-7-)-methyltransferases which have been extensively purified as a 127,000-dalton complex in our laboratory (7). Through the use of appropriate acceptors it has been possible to study the mRNA guanylyltransferase and mRNA (guanine-7-)-methyltransferase independently.

The properties of the mRNA guanylyltransferase are as follows: a divalent cation is required; Mg^{2+} is more effective than Mn^{2+}. Of the four ribonucleoside triphosphates only GTP is a suitable donor; dGTP can also be utilized, but less efficiently than GTP. The derivative 7-methylguanosine triphosphate is completely inactive as a donor. In contrast to the restricted donor specificity of the enzyme, a variety of polyribonucleotides are active acceptors. The only stringent requirement of an acceptor appears to be that it possess a 5′-terminal diphosphate. Polyribonucleotides containing a 5′-terminal monophosphate are not acceptors; polyribonucleotides with a 5′-terminal triphosphate can, however, be utilized as acceptors. This is explained by the detection in our mRNA guanylyltransferase preparations of an additional enzyme, which has been purified from vaccinia virions by D. J. Tutas and E. Paoletti,[4] that removes the γ-phosphate from the 5′-terminal of RNA. Chain length of the acceptor is important; diphosphate-terminated polyribonucleotides are far more efficient acceptors than mononucleoside diphosphates. Polyribonucleotide acceptors need no specific sequence. Homopolyribonucleotides containing purines are better acceptors than those containing pyrimidines, consistent with the presence of adenosine and guanosine in the 5′-terminals of the naturally occurring vaccinia mRNA. In the presence of PP_i, the mRNA guanylyltransferase can readily catalyze the pyrophosphorolysis of the 5′-terminals of polyribonucleotides with the sequences G(5′)pppN- or of dinucleoside triphosphates such as G(5′)pppA. By contrast, m⁷G(5′)pppA is not a substrate for the mRNA guanylyltransferase-catalyzed pyrophosphorolysis reaction.

The mRNA (guanine-7-)-methyltransferase catalyzes efficiently the methylation of polyribonucleotides containing the 5′-terminal sequence G(5′)pppN-. Also, methylated but with considerably less efficiency than polyribonucleotides are the following: G(5′)pppN, GTP, dGTP, ITP, GDP, GMP, and guanosine. No methylation of ATP, XTP, CTP, UTP, guanosine 2′- or 3′-phosphate, guanosine 2′ or 3′,5′-diphosphate, or guanylyl-(3-5′)guanosine was detectable. These results indicate that the specificity of the enzyme for the acceptor is determined by the number and position of phosphate groups on the ribose moiety and by the chemical nature and location of groups on the purine base.

From this detailed analysis of the reactions catalyzed by the mRNA guanylyltransferase and mRNA (guanine-7-)-methyltransferase several inferences can be made regarding the modification of vaccinia mRNA. Since polyribonucleotides are the preferred acceptors for both the mRNA guanylyltransferase and mRNA (guanine-7-)-methyltransferase it appears likely that the modifications occur after the initiation of the RNA chain. Furthermore, methylation does not appear to occur at the level of simple nucleotides since 7-methylguanosine triphosphate is neither formed efficiently from GTP by the mRNA (guanine-7-)-methyltransferase nor utilized as a donor by the mRNA guanylyltransferase. The observation that methylation does not occur with guanosine residues containing 3′-5′ phosphodiester linkages provides an explanation for the absence of internal 7-methylguanosine in vaccinia mRNA. We have postulated that one function of 7-methylation of the 5′-terminal guanosine in mRNA is to prevent reversal of the guanylyltransferase reaction by the PP_i generated during RNA elongation (9). This concept originated with the finding that *S*-adenosylmethionine diminishes the inhibitory effect of PP_i on the net mRNA guanylyltransferase reaction and is now directly supported by the demonstration that while G(5′)pppA is a substrate for the mRNA guanylyltransferase-catalyzed pyrophosphorolysis, the methylated analog, m⁷G(5′)pppA, is not a substrate.

Attempts are currently in progress to separate the mRNA (nucleoside-2′-)-methyltransferase of vaccinia virus from residual mRNA guanylyl- and mRNA (guanine-7-)-methyltransferase. The mRNA (nucleoside-2′-)-methyltransferase is the remaining enzyme involved in the formation of the 5′-terminal sequence of vaccinia mRNA synthesized *in vitro*. We have proposed that this enzyme requires a substrate with the 5′-terminal sequence m⁷G(5′)pppN-, and although data supporting this concept has been obtained, definite studies must await final purification of the enzyme. Thus far, none of the 5′-terminal modification enzymes of other viruses have been obtained in a soluble form thus precluding any detailed characterization. Activities which methylate the 5′-terminals of exogenous mRNA have been detected in extracts of mouse L-cells (18), wheat germ (18, 19), and *Artemia salina* embryos (20); one cellular enzyme, an mRNA (guanine-7-)-methyltransferase, has been purified and characterized from HeLa cells (21). The HeLa cell enzyme catalyzes the methylation of polyribonucleotides with G(5′)pppN- sequences and of the dinucleoside triphosphate G(5′)pppG but not of GTP. This specificity suggests that the cellular mechanism for the forma-

[4] Personal communication.

tion of modified 5′-terminals of mRNA also involves G(5′)pppN- intermediates. The characterization of other enzymes needed to modify the 5′-terminal of cellular mRNA should contribute to our understanding of mRNA synthesis and processing.

Note Added in Proof—We have now shown that the purified guanylyltransferase utilizes ppGpC as an acceptor more efficiently than ppG thereby supporting our argument that guanylylation occurs after initiation of RNA synthesis. At concentrations of 10 μM the rate of guanylylation of ppGpC was 12 times higher than that of ppG. The ppGpC used for these experiments was synthesized by J. Tomasz and kindly provided by A. J. Shatkin.

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