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Biochemistry

Modification of the 5′-terminus of mRNA by soluble guanylyl and methyl transferases from vaccinia virus

[7-methylguanosine/2′-*O*-methyladenosine/2′-*O*-methylguanosine/poly(A)]

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ABSTRACT **RNA guanylyl and methyl transferases have been solubilized from vaccinia virus cores. The guanylyl transferase specifically adds a GMP residue to the 5′-terminus of unmethylated vaccinia virus mRNA to form the structures G(5′)ppp(5′)Gp- and G(5′)ppp(5′)Ap-. Studies with [α-^{32}P]GTP and [β,γ-^{32}P]GTP indicated that only the α-phosphate is transferred. In the presence of *S*-adenosylmethionine, the methyl transferases convert the blocked 5′-termini to m^{7}G(5′)ppp(5′)G^{m}p- and m^{7}G(5′)ppp(5′)A^{m}p-. Similarly, the enzymes can modify synthetic poly(A) to form the structure m^{7}G(5′)ppp(5′)A^{m}p-.**

Although modified 5′-terminal sequences of the type m^{7}G(5′)ppp(5′)N^{m}p- have been found in viral (1–6) and cellular (7–9) RNAs, little is presently known concerning their mode of formation. This paper describes the isolation from vaccinia virus cores of guanylyl and methyl transferases that catalyze the specific post-transcriptional modification of the 5′-terminus of mRNA as well as synthetic poly(A).

MATERIALS AND METHODS

Extraction of Guanylyl and Methyl Transferases. Cores, prepared from purified vaccinia virus by treatment with Nonidet P-40 (Shell) and dithiothreitol, were isolated and disrupted with sodium deoxycholate and dithiothreitol (10–12). Insoluble structural proteins were removed by high-speed centrifugation and DNA by DEAE-cellulose column chromatography. The enzyme extract contained approximately 1.8 mg of protein per ml (13).

Preparation of Unmethylated Vaccinia mRNA. mRNA was synthesized by vaccinia virus cores in the absence of *S*-adenosylmethionine (*S*AdoMet). The reaction mixture contained 0.6 A_{260} vaccinia virus per ml, 50 mM Tris·HCl (pH 8.4), 5 mM dithiothreitol, 0.05% Nonidet P-40, 3 mM ATP, 1 mM each CTP, GTP, and UTP, and 6 mM $MgCl_2$. After 90 min at 37°, the cores were removed by centrifugation and the RNA was precipitated with cetyltrimethylammonium bromide (14), redissolved, and passed through a Sephadex G-50 column equilibrated in 50 mM ammonium acetate.

Guanylyl and Methyl Transferase Assays. The standard reaction mixture (0.1 ml) for assaying the incorporation of *S*AdoMet into added RNA contained 50 mM Tris·HCl (pH 7.6), 1 mM dithiothreitol, 10 μg of unmethylated vaccinia mRNA, 0.67 μM *S*-adenosyl[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]SAdoMet, 8.5 Ci/mmol), 1 mM GTP, 1 mM $MgCl_2$, and 5 μl of enzyme extract. The incorporation of GTP was assayed in the same reaction mixture containing either 10 μM [*U*-^{14}C]GTP (545 Ci/mol) or 5 μM [^{3}H]GTP (9.4 Ci/mmol) instead of the labeled *S*AdoMet. After 30 min of incubation at 37°, 75 μl was applied to DEAE-cellulose filter discs (Whatman DE81) and the unincorporated precursor was removed by washing (15).

Thin-Layer Chromatography. Cellulose sheets (Eastman, 20 × 20 cm) were used for chromatography in (*A*) ethyl acetate/isopropanol/7.5 M NH_4OH/*n*-butanol (3:2:2:1) or (*B*) 5% (w/v) aqueous NaH_2PO_4 saturated with isoamyl alcohol.

Enzymes, Chemicals, and Isotopes. Alkaline phosphatase, snake venom phosphodiesterase, and RNase A were purchased from Worthington Biochemical Corp., and RNase T_1 from Schwarz/Mann. [^{3}H]GTP and [*methyl*-^{3}H]SAdoMet were obtained from New England Nuclear Corp., [^{14}C]GTP from Amersham/Searle, and [α-^{32}P]GTP and [β,γ-^{32}P]GTP from International Chemical and Nuclear Corp. m^{7}G and β,γ-methylene GTP were purchased from Sigma and poly(A) lot no. 341101 from P-L Biochemicals.

RESULTS

Demonstration of Guanylyl and Methyl Transferases. Determination of the 5′-terminal sequences, m^{7}G(5′)ppp(5′)G^{m}pNp- and m^{7}G(5′)ppp(5′)A^{m}pNp-, of mRNAs synthesized by vaccinia virus cores (1) led us to consider the existence of specific guanylyl and methyl transferases. Using unmethylated vaccinia mRNA as a substrate, we found that a soluble extract prepared from cores incorporated radioactivity from both GTP and *S*AdoMet into trichloroacetic-acid-precipitable material. Neither CTP nor UTP were incorporated. Incorporation of ATP was observed presumably because of the viral poly(A) polymerase in the extract (12).

The characteristics of the guanylyl transferase reaction are listed in Table 1. Activity was dependent on RNA, either unmethylated vaccinia mRNA or synthetic poly(A). The addition of equimolar quantities of the other three nucleoside triphosphates failed to stimulate the reaction, indicating the absence of RNA polymerase activity. Other nucleoside triphosphates, including β,γ-methylene GTP, at concentrations much higher than the labeled GTP inhibited the reaction (not shown). *S*AdoMet, at concentrations as low as 5 μM, stimulated the incorporation of GTP approximately 1.6-fold. Guanylyl transferase activity was completely dependent on divalent cations; of those tested, only Mg^{++} and Mn^{++} were effective, with maximum incorporation achieved in the presence of Mg^{++}.

Abbreviations: m^{7}G, 7-methylguanosine; G^m, 2′-*O*-methylguanosine; A^m, 2′-*O*-methyladenosine; *S*AdoMet, *S*-adenosylmethionine; *S*AdoHcy, *S*-adenosylhomocysteine.

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Table 1. Effect of various reagents on GTP incorporation

	Reaction mixture*	GTP incorporation (cpm)	% of standard
Exp. 1	Complete	6562	100
	− RNA, + poly(A)	5666	86
	− RNA	306	4.7
Exp. 2	Complete	2555	100
	+ 10 μM each ATP,CTP,UTP	2174	85
	+ *S*AdoMet 5 μM	4219	165
	10 μM	4483	163
Exp. 3	Complete	3031	100
	− Mg^{++}, + Mn^{++}	1155	38
	− Mg^{++}, + Ca^{++}	43	1.4
	− Mg^{++}	0	0

* The incorporation of [^{3}H]GTP (Exps. 1 and 3) and [^{14}C]GTP (Exp. 2) was measured in the standard reaction mixture (*Materials and Methods*).

A similar analysis of the methylation reaction is presented in Table 2. The methyl transferase was completely dependent on added RNA. The omission of GTP reduced methylation of vaccinia mRNA by 60% and of poly(A) by 100%. The other three nucleoside triphosphates as well as β,γ-methylene GTP, GDP, GMP, and dGTP could not substitute for GTP (not shown). Methylation in the presence of unlabeled GTP was stimulated by the divalent cations Mg^{++} and Mn^{++}, with Mg^{++} being the more effective. Ca^{++} was inactive. EDTA did not inhibit methylation of vaccinia mRNA in the absence of GTP but it did prevent the GTP stimulation of methyl incorporation (not shown). This result demonstrated that the small amount of methylation of vaccinia mRNA in the absence of GTP was not caused by low levels of GTP contaminating either the RNA or the extract, since the incorporation of GTP was dependent on divalent cations.

Characterization of the Enzymatic Product. Evidence that both the [^{3}H]GTP and [*methyl*-^{3}H]SAdoMet labels were incorporated into RNA was obtained. Both products were resistant to DNase and were rendered acid-soluble by hot trichloroacetic acid, 0.4 N KOH, RNase T_1, and RNase A in high or low salt. The latter result ruled out addition to the 3′-poly(A) terminus of vaccinia mRNA.

Identification of Labeled Nucleosides. Both unmethylated vaccinia mRNA and synthetic poly(A) were labeled by the enzyme extract with [*methyl*-^{3}H]SAdoMet and [^{14}C]GTP. After digestion with RNase A and T_1 followed by alkaline phosphatase and venom phosphodiesterase, the resulting nucleosides were separated by thin-layer chromotography (Fig. 1). Three [^{3}H]methyl-labeled nucleosides identified as m^7G, G^m, and A^m were obtained from the mRNA (Fig. 1A). m^7G was also labeled with [^{14}C]GTP, indicating the transfer of guanosine to the RNA substrate. The absence of ^{14}C label in G^m demonstrated that a guanosine residue in the RNA substrate was methylated. When poly(A) was used as substrate, [^{3}H]methyl was incorporated exclusively into m^7G and A^m; no labeled G^m was formed (Fig. 1B). Again m^7G was also labeled with [^{14}C]GTP, indicating the transfer of guanosine to the poly(A). Because guanosine and m^7G comigrate in this chromatography system, samples of the doubly labeled RNA were depurinated and chromatographed in system B (Fig. 2). The methyl-labeled ribose phosphate from

Table 2. Effect of various reagents on *S*-adenosyl[*methyl*-^{3}H]methionine incorporation*

	Reaction mixture	[^{3}H]Methyl incorporation	% of standard
Exp. 1	Complete	32,507	100
	− RNA	0	0
	− GTP	13,091	40
	− RNA, + poly(A)	18,907	58
	− RNA, − GTP, + poly(A)	49	0.15
Exp. 2	Complete	27,908	100
	− Mg^{++}, + Mn^{++}	19,747	71
	− Mg^{++}, + Ca^{++}	11,047	40
	− Mg^{++}	10,475	39

* [^{3}H]Methyl incorporation was measured in the standard reaction mixture.

G^m and A^m as well as the ^{14}C label in the ribose moiety of the [*U*-^{14}C]GTP migrated with the solvent front. There were two peaks of ^{14}C-label in addition to the [^{14}C]ribose.

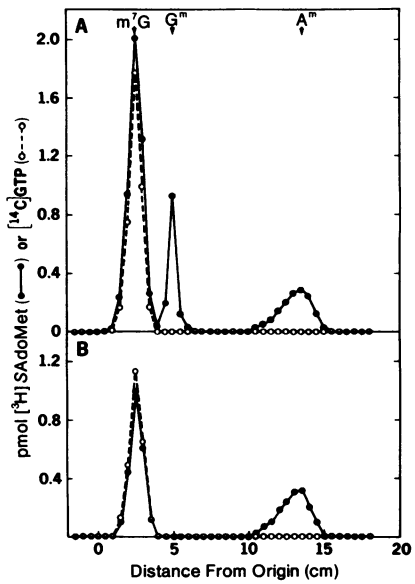

FIG. 1. Identification of labeled nucleosides by thin-layer chromatography. The enzymatic modification of unmethylated vaccinia mRNA and synthetic poly(A) was carried out in the standard reaction mixture containing [^{14}C]GTP (15 μM, 545 Ci/mol) and *S*-adenosyl[*methyl*-^{3}H]methionine (1.34 μM, 8.5 Ci/mmol). After 45 min at 37°, the reaction was terminated by heating at 100° for 2 min and the unincorporated precursors were removed by passage through a Sephadex G-50 column. A sample of each RNA was then digested with RNase A (0.1 mg/ml) and T_1 (0.05 mg/ml) in 10 mM Tris·HCl (pH 7.5) for 1 hr followed by alkaline phosphatase (0.1 mg/ml) and venom phosphodiesterase (0.2 mg/ml) in 50 mM Tris·HCl (pH 8.5), 5 mM $MgCl_2$ for 2 hr, and the resulting nucleosides were chromatographed in solvent A. (A) vaccinia mRNA: the total number of pmol recovered from the area of the chromatogram corresponding to guanosine and 7-methylguanosine was 3.97 based on the ^{14}C incorporation and 4.85 based on [^{3}H]methyl incorporation; 1.32 pmol of 2′-*O*-methylguanosine and 1.40 pmol of 2′-*O*-methyladenosine were recovered. (B) poly(A): the number of pmol recovered from the guanosine and 7-methylguanosine area was 2.52 based on the ^{14}C label and 2.27 based on the [^{3}H]methyl label; 1.50 pmol of 2′-*O*-methyladenosine were recovered.

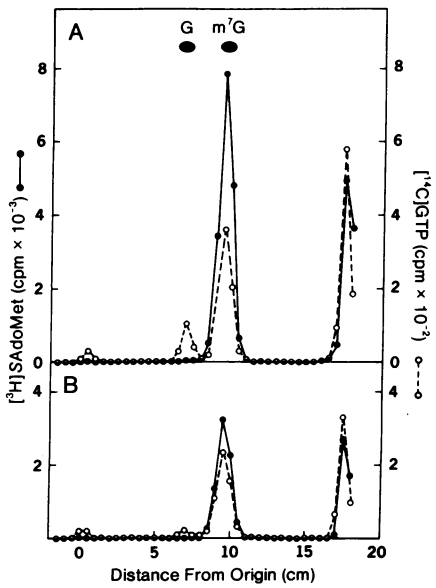

FIG. 2. Thin-layer chromatography of guanine and 7-methylguanine. A sample of the RNA labeled with [^{14}C]GTP and *S*-adenosyl[*methyl*-^{3}H]methionine described in Fig. 1 was depurinated (16) and chromatographed in solvent B. (A) vaccinia mRNA; (B) poly(A).

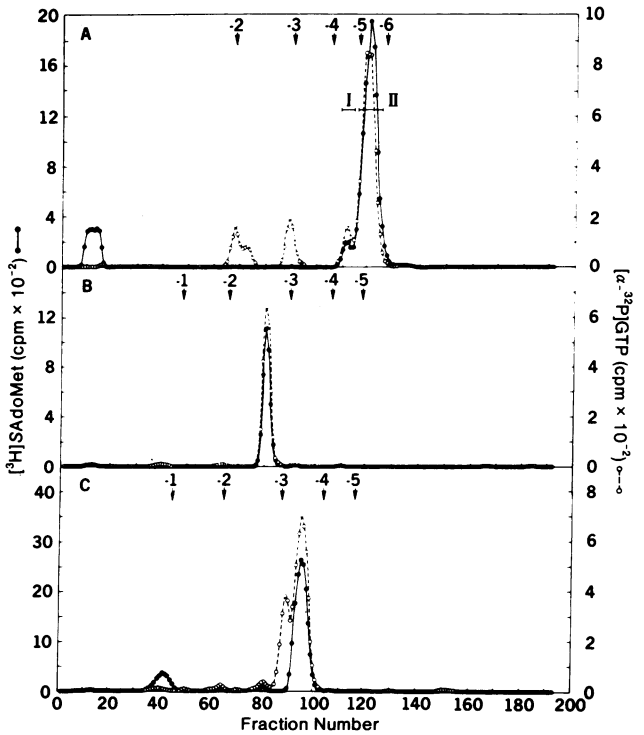

FIG. 3. DEAE-cellulose chromatography of alkali-digested RNA doubly labeled with [α-^{32}P]GTP and *S*-adenosyl[*methyl*-^{3}H]methionine. Enzymatic modification of unmethylated vaccinia mRNA was carried out in the standard reaction mixture containing [α-^{32}P]GTP (10 μM, 9.0 Ci/mmol) and *S*-adenosyl[*methyl*-^{3}H]methionine (1.34 μM, 8.5 Ci/mmol) and purified as described in Fig. 1. The RNA product was digested with 0.4 N KOH for 24 hr at 37°, applied to a DEAE-cellulose column in 7 M urea, and eluted with a 0–0.3 M NaCl gradient (13). The arrows indicate the position of oligonucleotide markers and the numbers above the arrows indicate their net charges. (A) alkali-digested RNA; (B) peak I material as indicated by the bar in Fig. 3A was pooled, treated with alkaline phosphatase, and rechromatographed; (C) alkaline-phosphatase-treated peak II.

The major ^{14}C-labeled peak derived from both vaccinia mRNA and poly(A) was coincident with a ^{3}H-labeled peak and the 7-methylguanine marker; the minor ^{14}C-labeled peak corresponded to guanine. With vaccinia mRNA as the substrate (Fig. 2A), 79% of the added guanine was methylated at the 7-position while 92% of that added to poly(A) was methylated (Fig. 2B). If only the added guanosine residues were methylated at the 7 position, then the number of pmol of m^{7}G in the RNA should be the same based on the incorporation of label from either [^{14}C]GTP or [*methyl*-^{3}H]S-AdoMet. From Figs. 1B and 2B, the poly(A) product contained 92% of 2.52 pmol or 2.30 pmol of [^{14}C]m^{7}G; this number is in close agreement with 2.27 pmol of methyl-labeled m^{7}G in the poly(A). Enzymatic digestion of the doubly labeled vaccinia mRNA yielded 3.14 pmol of m^{7}G based on the incorporation of ^{14}C compared with 4.85 pmol of methyl-labeled m^{7}G. This inequality suggested that some of the unmethylated vaccinia mRNA used as substrate contained G(5′)ppp(5′)Gp- or G(5′)ppp(5′)Ap- at the 5′-terminus. This is indeed the case, since all three methylated nucleosides, G^{m}, A^{m}, and m^{7}G, are found in the product obtained in a reaction without added GTP (not shown).

Table 3. Removal of the blocking group by periodate oxidation and β-elimination*

	Radioactive material released from RNA			
	^{3}H		^{14}C	
RNA	cpm	%	cpm	%
Vaccinia	82,390	71	7,560	93
Poly(A)	29,760	66	3,730	90

* Vaccinia mRNA and poly(A) doubly labeled with *S*-adenosyl-[*methyl*-^{3}H]methionine and [^{14}C]GTP as in Fig. 1 were treated with periodate followed by aniline (17). The RNA was separated from the eliminated base and ribose by ethanol precipitation.

Both vaccinia mRNA and poly(A) products contained more m^{7}G than the ribose-methylated nucleosides on a molar basis; 64% and 60% of the methyl groups incorporated into vaccinia mRNA and poly(A), respectively, were in m^{7}G (Fig. 1), suggesting the presence of small amounts of incompletely methylated molecules having the structures m^{7}G(5′)ppp(5′)Gp- and m^{7}G(5′)ppp(5′)Ap-.

Evidence That m^{7}G Is Located at the Terminus. Previous experiments with mRNA synthesized by vaccinia virus cores demonstrated that the 5′-terminal m^{7}G residue contained 2′,3′-hydroxyls and, therefore, could be removed from the RNA by periodate oxidation and β-elimination (1). Similarly more than 90% of the [^{14}C]guanosine attached by the soluble enzymes to the vaccinia mRNA and poly(A) was removed by this treatment (Table 3). Theoretically 50% of the [^{3}H]methyl label from m^{7}G(5′)ppp(5′)G^{m}pNp- and m^{7}G(5′)ppp(5′)A^{m}pNp- should be removed by periodate oxidation and β-elimination. The loss of 71% and 66% of the methyl groups from the enzymatically labeled vaccinia mRNA and poly(A) again indicated that ribose methylation of the RNA was incomplete

Incorporation of [α-^{32}P]GTP into the Blocked 5′-terminus. Studies with ^{32}P-labeled GTP were carried out to determine the number of phosphates transferred to the RNA and

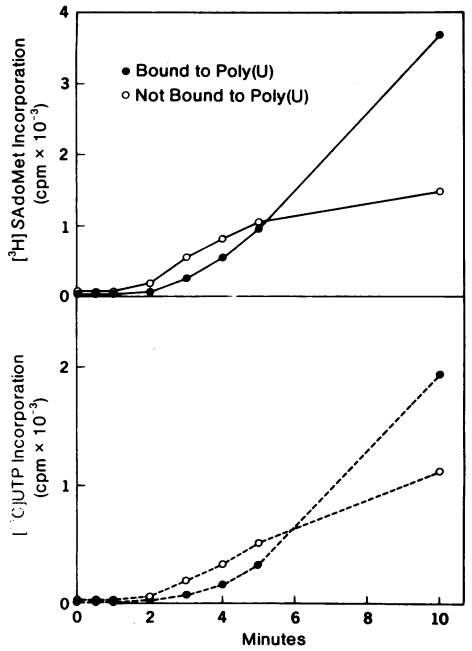

FIG. 4. Time course of incorporation of [^{3}H]methyl and [^{14}C]UMP into nascent and polyadenylylated RNA by vaccinia virus cores. RNA was synthesized in a standard reaction mixture containing vaccinia virus cores equivalent to 280 μg of viral protein per ml, 50 mM Tris·HCl (pH 8.4), 5 mM dithiothreitol, 5 mM $MgCl_2$, 2.5 mM ATP, 1.25 mM CTP and GTP, 0.05 mM [^{14}C]UTP (5 Ci/mol), and 2.9 μM *S*-adenosyl[*methyl*-^{3}H]methionine (8.5 Ci/mmol). Samples were withdrawn at various times into 2 mM EDTA and 0.5% sodium dodecyl sulfate to stop the reaction. The RNA was phenol extracted and the amount of labeled RNA bound and not bound to poly(U) filters was determined (19).

to demonstrate their presence in a phosphatase-resistant structure. Preliminary experiments revealed that the soluble enzyme extract incorporated [α-^{32}P]GTP but not [β,γ-^{32}P]GTP into RNA. Vaccinia mRNA was then labeled by the enzyme extract with [*methyl*-^{3}H]*S*AdoMet and [α-^{32}P]GTP; the product was digested with alkali and chromatographed on a DEAE-cellulose column in 7 M urea (Fig. 3A). The two peaks containing both methyl and ^{32}P label corresponded to peaks I and II, identified previously as m^{7}G(5′)ppp(5′)Gp and m^{7}G(5′)ppp(5′)Ap and as m^{7}G(5′)ppp(5′)G^{m}pNp and m^{7}G(5′)ppp(5′)A^{m}pNp, respectively, from vaccinia mRNA synthesized by cores (1). The materials corresponding to peak I and peak II were each pooled, digested with alkaline phosphatase, and rechromatographed on DEAE-cellulose. Little or no $^{32}P_i$ was removed from peaks I and II, although the loss of a −2 charge indicated the removal of the 3′-terminal phosphate (Fig. 3B and C). An additional peak of unmethylated ^{32}P-labeled material that was derived from peak II eluted with 0.4 less negative charge than the major doubly labeled peak. This material was probably G(5′)ppp(5′)G and G(5′)ppp(5′)A from RNA molecules that had been blocked but remained unmethylated. Alkali-digested RNA that was labeled by the enzyme extract with [^{3}H]GTP alone in the absence of *S*AdoMet yielded a labeled oligonucleotide that eluted from DEAE-cellulose with exactly a −5 charge (not shown). The presence of unmethylated blocked molecules in the double labeled RNA in Fig. 3A would account for the slight skewing of the ^{32}P-label in peak II toward a less negative charge than the methyl label.

Time of Methylation of Vaccinia mRNA. The following experiment with intact virus cores was designed to determine whether nascent or completed RNA chains are methylated. RNA was synthesized by cores in the presence of [*methyl*-^{3}H]*S*AdoMet and [^{14}C]UTP and samples were withdrawn at short-time intervals. The appearance of methyl label in completed RNA molecules containing poly(A) was measured by the amount bound to poly(U) filters, while label passing through the filters represented primarily nascent chains. The incorporation of methyl groups followed the same time course as UMP incorporation, appearing first in the RNA not bound to the filters, and, after a lag, in the poly(A)-containing RNA bound to the filters (Fig. 4). Thus, methylation of RNA synthesized by cores appears to occur prior to polyadenylylation.

DISCUSSION

Studies with intact vaccinia virus cores indicated that methylation occurs on the nascent RNA chain prior to addition of the poly(A) sequence. The identification of guanylyl and methyl transferases in a soluble extract of vaccinia virus cores provides a mechanism for the post-transcriptional modification of the nascent RNA. The first step appears to be addition by the guanylyl transferase of a single GMP residue to the 5′-terminus of the RNA to form the sequences G(5′)ppp(5′)Gp- and G(5′)ppp(5′)Ap-. *S*AdoMet is not required for the guanylyl transferase reaction although it does stimulate activity. Studies with [α-^{32}P]GTP and [β,γ-^{32}P]GTP indicated that only the α-phosphate is transferred. The other two phosphates are derived from the 5′-terminus of vaccinia mRNA (unpublished). Although unmethylated vaccinia mRNA has been reported to contain the 5′-terminal sequence pppAp- (20), the γ-phosphate appears to be removed under our conditions of synthesis. Accordingly, the proposed guanylyl transferase reaction is:

$$\text{GTP} + \text{pp}N_1\text{p}N_2\text{p}- \longrightarrow \text{G(5′)ppp(5′)}N_1\text{p}N_2\text{p}- + \text{pp}_i \quad [1]$$

where N_1 is guanosine or adenosine. Although we have obtained poly(A) synthesized by polynucleotide phosphorylase which is a good acceptor, usually it is inactive because of the presence of a single phosphate at the 5′-terminus (21). However, the latter has been activated (unpublished) by chemical addition of another phosphate (22, 23).

In the presence of *S*AdoMet the soluble methyl transferases add a methyl group to the 7-position of the added GMP and to the 2′-*O*-position of the now penultimate guanylate or adenylate residue to form the structures m^{7}G(5′)ppp(5′)G^{m}p- and m^{7}G(5′)ppp(5′)A^{m}p-. Since poly(A) is an ineffective substrate in the absence of GTP, guanylylation must precede methylation. Unmethylated vaccinia mRNA synthesized by virus cores contains some blocked sequences G(5′)ppp(5′)Gp- and G(5′)ppp(5′)Ap-, accounting for its limited role as a methyl acceptor in the absence of GTP. Evidence for partially methylated structures m^{7}G(5′)ppp(5′)Gp- and m^{7}G(5′)ppp(5′)Ap- suggests that they are possible intermediates in the formation of the completed structures. Thus the final two steps in modification may be

$$\text{G(5′)ppp(5′)}N_1\text{p}N_2\text{p}- + \textit{S}\text{AdoMet} \longrightarrow \text{m}^7\text{G(5′)ppp(5′)}N_1\text{p}N_2- + \textit{S}\text{AdoHcy} \quad [2]$$

$$m^7G(5')ppp(5')N_1pN_2p- + SAdoMet \longrightarrow m^7G(5')ppp(5')N_1{}^mpN_2- + SAdoHcy \quad [3]$$

The finding that poly(A) can function as a substrate for all the modification reactions indicates that the guanylyl transferase has little or no sequence specificity except perhaps for a terminal purine. The methyl transferases require the structures G(5')ppp(5')Gp- or G(5')ppp(5')Ap- since there is no methylation of internal nucleotides or of poly(A) in the absence of GTP.

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