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mRNA(nucleoside-2′-)-methyltransferase from Vaccinia Virus

CHARACTERISTICS AND SUBSTRATE SPECIFICITY*

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An mRNA(nucleoside-2′-)-methyltransferase purified from vaccinia virus was shown to methylate the penultimate nucleoside of RNA ending in m^{7}G(5′)pppN-. By contrast, RNAs ending in pN-, ppN-, or even G(5′)pppN- are not methyl acceptors. This specificity indicates that 2′-*O*-methylation is the final step in the formation of the m^{7}G(5′)pppN^m- cap structure. Both adenosine and guanosine are methylated, in accordance with the presence of these nucleosides in the penultimate position of vaccinia virus mRNAs. Studies with homopolyribonucleotides containing m^{7}G(5′)pppN ends indicated that poly(A) and poly(I) were the best methyl acceptors while significant but much less activity was obtained with poly(G), poly(U), and poly(C). Simple dinucleotides of the type m^{7}G(5′)pppN, however, are poor substrates and do not compete with capped RNA. Additional studies indicate that the methyltransferase has a pH optimum of 7.5, does not require divalent cations, is inhibited by *S*-adenosylhomocysteine, has a K_m of 2.0 μM for *S*-adenosylmethionine, a K_m of approximately 5 nM for brome mosaic virus RNA, and kinetics consistent with a random bireactant mechanism.

Relatively little is known regarding the sequence of steps involved in the synthesis and processing of eukaryotic mRNAs. As one approach to this problem, we are attempting to reconstruct some of the events *in vitro* by isolating and then characterizing viral and cellular enzymes that form the 5′-terminal m^{7}G(5′)pppN^m- cap[1] structure (1–6). Previous studies with a guanylyltransferase·guanine-7-methyltransferase complex purified from vaccinia virus particles indicated that a GMP residue is transferred from GTP to a nascent RNA chain containing at least two 5′-terminal phosphates (2). The resulting unmethylated G(5′)pppN- structure can serve as a substrate for the guanine-7-methyltransferase leading to the formation of m^{7}G(5′)pppN-. For this and other (7) reasons, we proposed that the third step in cap formation is the conversion of m^{7}G(5′)pppN- to m^{7}G(5′)pppN^m- by a specific nucleoside-2′-methyltransferase. Nevertheless, an alternative sequence was possible since RNA ending in pppN^m-, obtained by chemical removal of the m^{7}G residue, could serve as a substrate for the guanylyltransferase (8). Moreover, the G(5′)pppN^m- structure formed could be further methylated by the guanine-7-methyltransferase. That our original proposal is correct, however, and that the steps in the biosynthesis of the cap must occur in a unique sequence are indicated by the present studies of the substrate specificity of the purified (6) mRNA(nucleoside-2′-)-methyltransferase.

EXPERIMENTAL PROCEDURES

Enzyme Assays—mRNA(nucleoside-2′-)-methyltransferase was purified from vaccinia virus and assayed in a 0.1-ml reaction as described in the preceding communication (6). At appropriate times, 75-μl samples were chilled and sodium dodecyl sulfate was added to a concentration of 0.1% before applying to DE81 filters. When alternative substrates, temperatures, or incubation times were used, details are provided in the appropriate figure legends.

Preparation of RNA Substrates—Brome mosaic virus RNA was obtained and purified as described in the accompanying report (6). Unmethylated vaccinia virus mRNA containing predominantly ppA- and ppG- ends were synthesized in reaction mixtures containing AdoHcy and pyrophosphate (7). Conversion of the ends to G(5′)pppA- and G(5′)pppG- was carried out in 50 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.1 mM GTP, 10 μCi/ml of [α-^{32}P]GTP (250 Ci/mmol) 100 to 150 μg/ml of RNA, 50 μg/ml of bovine serum albumin, and 10 units/ml of mRNA guanylyltransferase guanine-7-methyltransferase purified from vaccinia virus (1). After incubation at 37°C for 2½ h, the RNA was passed through a Sephadex G-75 column (1.5 × 25 cm) that had been equilibrated with 10 mM ammonium acetate (pH 6.8). RNA eluting in the excluded volume of the column was extracted three times with buffer saturated phenol:chloroform (1:1) and then precipitated from the aqueous layer with 3 volumes of 95% ethanol at −20°C overnight. The RNA was recovered by centrifugation, washed three times with 95% ethanol, dried, and resuspended in H_2O at 1 mg/ml. Similarly, RNA ending in m^{7}G(5′)pppA- and m^{7}G(5′)pppG- was prepared from unmethylated vaccinia virus mRNA in reaction mixtures containing 0.1 mM AdoMet. With both preparations of RNA, the extent of capping was monitored by incorporation of [α^{32}P]GTP. Unmethylated RNA, containing G(5′)pppN- ends, was shown to be a substrate for the guanine-7-methyltransferase while methylated RNA, containing m^{7}G(5′)pppN- ends was not. The latter result indicated that methylation was complete. For some purposes, vaccina virus mRNA containing m^{7}G(5′)pppN- ends was prepared using 5 μM Ado[*methyl*-^{3}H]Met (6.9 Ci/mmol).

Synthetic homopolyribonucleotides with diphosphates at the 5′ ends (2) were further modified to contain either G(5′)pppN- or m^{7}G(5′)pppN- ends as described above for vaccinia virus mRNA. The extent of capping was also monitored using [α-^{32}P]GTP and by analysis of products by electrophoresis after digestion with nuclease P_1 alone and with snake venom phosphodiesterase.

Paper Electrophoresis and Chromatography—High voltage electrophoresis was performed on 100-cm-long sheets of Whatman No. 3MM paper in 0.05% pyridine, 0.5% acetic acid, and 1 mM EDTA (pH 3.5). After drying, relevant markers were located under ultraviolet illumination and the paper was cut into 1-cm strips. Usually, the strips were placed in vials and 0.5 ml of H_2O was added to elute the materials prior to counting in Aquasol scintillation fluid. In experiments where further analysis was required, the 1-cm strips were counted directly in a nonaqueous toluene-based scintillation fluid; the strips were then washed five times with 5 ml of toluene and five times with ethyl ether and allowed to dry at room temperature. Following

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[1] The abbreviations used are: cap, m^{7}G(5′)pppN or m^{7}G(5′)pppN^m; m^{7}G, 7-methylguanosine; N^m, any 2′-*O*-methylribonucleoside; BMV, brome mosaic virus; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

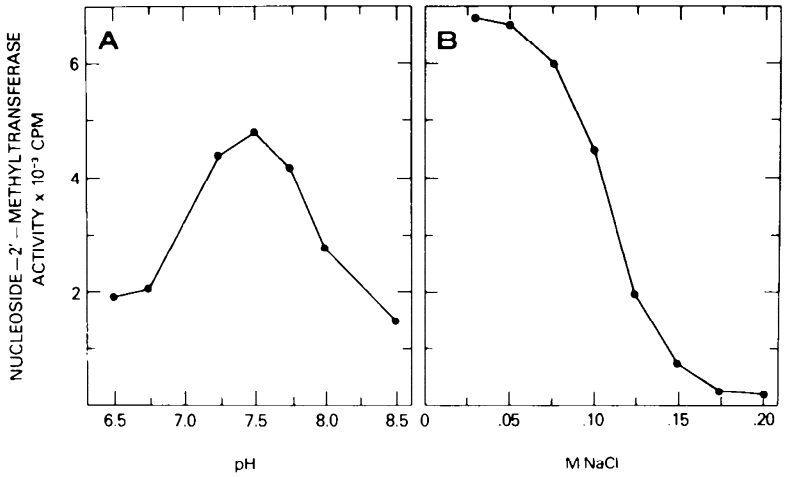

FIG. 1 (*left*). Effect of pH and salt concentration on mRNA-(nucleoside-2′-)-methyltransferase activity. *A*, activity was measured in the standard reactions containing 0.05 M Hepes/NaOH buffers. The pH values of the Hepes buffers were determined at 25°C and at a concentration of 0.1 M. *B*, activity was measured under standard conditions at the indicated concentrations of NaCl.

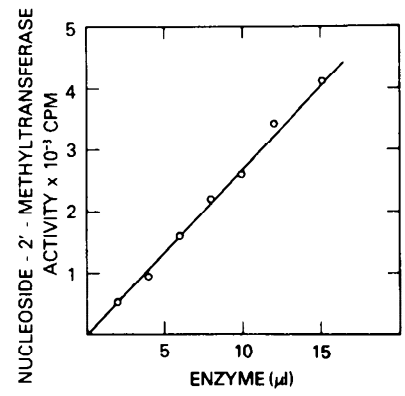

FIG. 2 (*right*). Effects of varying amounts of enzyme. mRNA-(nucleoside-2′-)-methyltransferase activity was measured in standard 0.1-ml reaction mixtures containing 3.3 μM *S*-adenosyl[*methyl*-^{3}H]-methionine (6.9 Ci/mol) and 5 μg (6.6 pmol) of BMV RNA. Varying amounts of enzyme (4 μg/ml) were assayed for 6 min at 37°C.

this, the labeled materials were eluted from the paper with water, dried, and then applied to Whatman No. 1 paper for descending chromatography.

Sources of Materials—Radioisotopes were obtained from New England Nuclear and synthetic polyribonucleotides and dinucleoside triphosphates were purchased from P-L Biochemicals. AdoMet and AdoHcy came from Boehringer Mannheim. Nucleotides and nucleosides were either from P-L Biochemicals or from Sigma.

RESULTS

Conditions for Optimal mRNA(nucleoside-2′-)-methyltransferase Activity—Brome mosaic virus RNA, ending in m^{7}G(5′)pppG-, was used as the methyl acceptor for all optimization studies. Control experiments indicated that methyl groups are transferred exclusively into position 2′ of the penultimate guanosine residue. Enzyme activity was maximal at pH 7.5 (Fig. 1*A*) but was inhibited by concentrations of NaCl greater than 0.05 M (Fig. 1*B*). No requirements for divalent cations could be demonstrated; neither EDTA at 2 mM nor Mg^{2+}, Mn^{2+}, or Ca^{2+} at 5 mM had a notable affect on methyltransferase activity. By contrast, Zn^{2+} at 5 mM reduced activity by 88%.

Under standard conditions, methyl incorporation was proportional to enzyme concentration over at least a 5-fold range (Fig. 2) and was linear with time for at least 20 min at 25°, 30°, and 37°C (Fig. 3). Greatest activity was obtained at the latter temperature. In the presence of 10% glycerol and 250 μg/ml of bovine serum albumin at pH 7.5, the enzyme was relatively stable at temperatures up to 50°C but rapidly lost activity at 59°C (Fig. 4).

Enzyme Kinetics—Initial rates of methyl incorportion were measured while holding one substrate at a fixed concentration and varying the concentration of the other. Results obtained with variable AdoMet at three concentrations of brome mosaic virus RNA are shown in Fig. 5. When plotted according to Lineweaver-Burk, a family of straight lines intersecting the *x* axis to the left of the *y* axis was obtained. An apparent K_m value of approximately 2.0 μM AdoMet was calculated. A similar family of curves was obtained in another series of experiments in which RNA concentration was varied at three fixed AdoMet concentrations (Fig. 6). Both sets of results (Figs. 5 and 6) are consistent with a random bireactant system in which both substrates bind to the enzyme before any

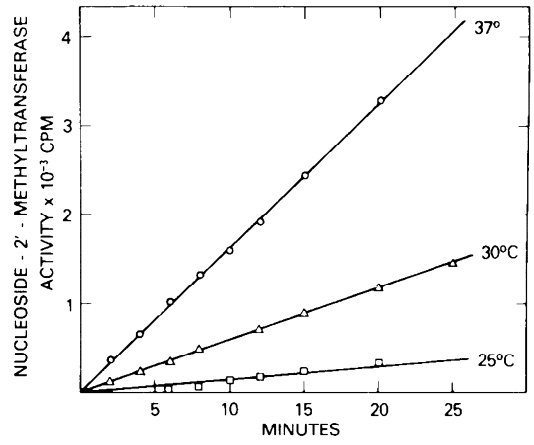

FIG. 3. Time course of methyl incorporation into BMV RNA. Reaction mixtures (700 μl) containing 3 μM *S*-adenosyl[*methyl*-^{3}H]methionine (6.9 Ci/mmol) and 35 μg of BMV RNA were preincubated for 10 min at the indicated temperatures. Following the addition of 40 μl of purified enzyme (4 μg/ml), 75-μl samples were withdrawn at various times and assayed.

products are released (9). By plotting the apparent reciprocal V_{max} (*i.e.* y intercept) values from Fig. 5 *versus* reciprocal RNA concentrations, a K_m of 0.38 mg of brome mosaic virus RNA per liter was deduced. Since this amount of RNA contains approximately 5 nmol of m^{7}G(5′)pppG- ends, the K_m is 5 nM. The number of ends was estimated by using excess enzyme to methylate a measured quantity of brome mosaic virus RNA with Ado[*methyl*-^{3}H]Met of known specific radioactivity.

Methylation of the m^{7}G(5′)pppG- and m^{7}G(5′)pppA- Ends of Vaccinia Virus mRNA—During purification of the mRNA(nucleoside-2′-)-methyltransferase, the enzyme was assayed exclusively with brome mosaic virus RNA, which contains only m^{7}G(5′)pppG- ends. Vaccinia virus mRNAs, however, contain both m^{7}G(5′)pppG^m- and m^{7}G(5′)pppA^m-. To check whether the purified enzyme can methylate adenosine as well as guanosine, appropriate substrates were prepared. Unmethylated vaccinia virus mRNA, which contains predominantly ppA- and ppG- ends as well as some G(5′)pppA- and G(5′)pppG- ends (7), was synthesized *in vitro* by vaccina virus

particles in the presence of AdoHcy and pyrophosphate. The isolated RNA was then capped and methylated with guanylyltransferase·guanine-7-methyltransferase complex purified from vaccinia virus. The RNA, now containing $m^7G(5')pppA$- and $m^7G(5')pppG$- ends, was offered as a substrate to the purified nucleoside-2'-methyltransferase. After gel filtration to remove unreacted Ado[*methyl*-^{3}H]Met, the RNA was digested with nuclease P_1 and bacterial alkaline phosphatase. The labeled material co-chromatographed in an

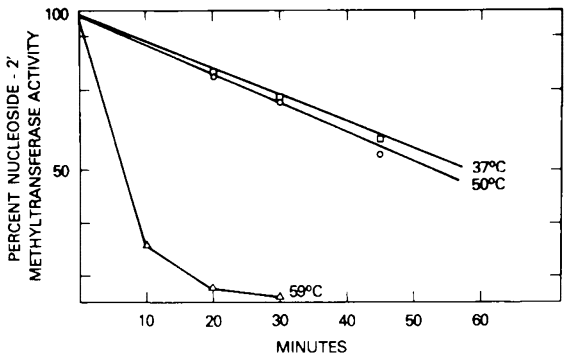

FIG. 4. Heat stability of mRNA(nucleoside-2'-)-methyltransferase. Fifty microliters of purified enzyme (4 μg/ml) in the presence of 10% glycerol, 250 μg/ml of bovine serum albumin at pH 7.5 was incubated at 37°C (□), 50°C (○), or 59°C (△) for the indicated times. Residual activity was measured by adding 5 μl of enzyme to a 95-μl standard reaction mixture for 30 min at 37°C.

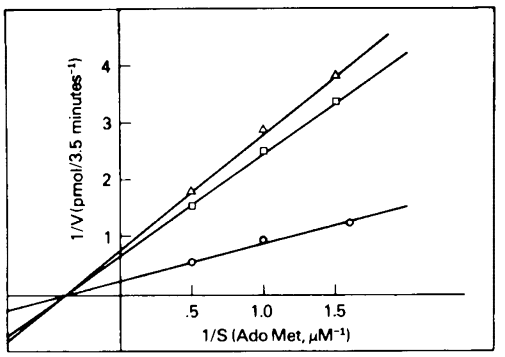

FIG. 5. Effect of AdoMet concentration. Standard 0.1-ml reaction mixtures containing varying concentrations of Ado[*methyl*-^{3}H]Met and fixed amounts of BMV RNA were incubated for 3½ min at 30°C following the addition of 4 μl of purified enzymes. The reaction was terminated and methyl incorporation was determined from the specific activity of the isotope. The quantity of BMV RNA was 5 μg (△), 10 μg (□), or 15 μg (○).

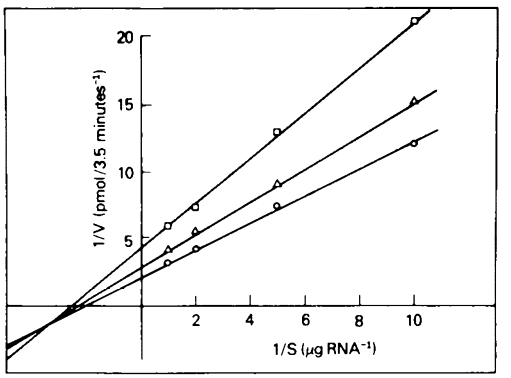

FIG. 6. Effect of RNA concentration. Standard reaction mixtures containing varying amounts of BMV RNA and fixed concentrations of Ado[*methyl*-^{3}H]Met were incubated for 3½ min at 30°C. AdoMet concentrations were 1 μM (□), 2 μM (△), and 3 μM (○).

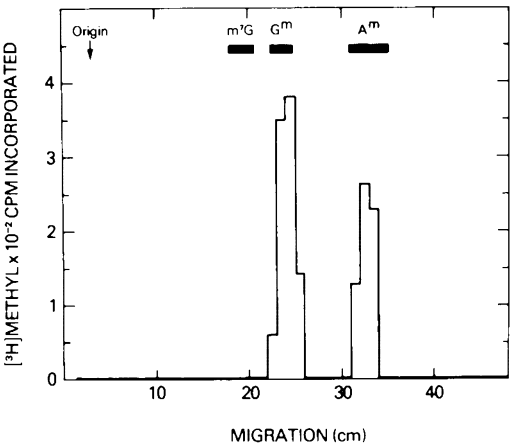

FIG. 7. Thin layer chromatography of methylated nucleosides. Vaccinia mRNA synthesized *in vitro* and modified to have $m^7G(5')pppG$-, and $m^7G(5')pppA$- caps as described in the text was used as substrate in a 250-μl standard reaction mix containing 4 μM Ado[*methyl*-^{3}H]Met. After gel filtration and lyophilization, the material was digested successively with nuclease P_1, snake venom phosphodiesterase, and bacterial alkaline phosphatase as described under "Experimental Procedures." The products were analyzed by chromatography on cellulose thin layer plates in ethyl acetate/isopropyl alcohol/7.5 M NH_4OH/1-butanol (3:2:2:1). The origin and locations of markers m^7G, G^m, and A^m are indicated.

isobutyric acid/NH_3/H_2O system with $m^7G(5')pppG^m$ and $m^7G(5')pppA^m$ standards (not shown), indicating that both adenosine and guanosine were labeled. This was confirmed by direct identification of A^m and G^m after further digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase (Fig. 7).

To compare the rate and extent of methylation of adenosine and guanosine, vaccina virus mRNAs containing methyl-labeled $m^7G(5')pppA$- and $m^7G(5')pppG$- ends were prepared, again using purified guanylyltransferase·guanosine-7-methyltransferase complex. The labeled RNA was then incubated with purified nucleoside-2'-methyltransferase and unlabeled AdoMet. Samples were withdrawn at intervals and the isolated RNA was digested with nuclease P_1 and bacterial alkaline phosphatase. Separation of the products was obtained by the following two-step procedure. First, co-migrating $m^7G(5')pppA$ and $m^7G(5')pppA^m$ were separated from comigrating $m^7G(5')pppG$ and $m^7G(5')pppG^m$ by paper electrophoresis at pH 3.5. Next, the oligonucleotide pairs were eluted and resolved into individual components by paper chromatography and the amount of radioactivity associated with each was determined. The conversions of $m^7G(5')pppA$ to $m^7G(5')pppA^m$ and of $m^7G(5')pppG$ to $m^7G(5')pppG^m$ are indicated in Table I. Methylation of adenosine occurred more rapidly than that of guanosine although both reactions went virtually to completion. The latter result indicated that the purified nucleoside-2'-methyltransferase is capable of methylating the 5'-terminal sequences of virtually all vaccinia virus mRNAs synthesized *in vitro*.

Specificity for mRNAs Containing Terminal m^7G—The absence of methylation of internal sites within the RNA chain indicates that the nucleoside-2'-methyltransferase is specific for the 5' end. Although we have shown that $m^7G(5')pppN$- ends of mRNAs are substrates, the possibility that ends lacking the m^7G residue or containing an unmethylated guanosine are equal or even better substrates has not yet been considered. Accordingly, vaccinia virus mRNA containing (i) ppA- and ppG- ends, (ii) G(5')pppA- and G(5')pppG- ends, and (iii) $m^7G(5')pppA$- and $m^7G(5')pppG$- ends were prepared. When similar amounts of RNA were incubated with purified

TABLE I

Methylation of $m^7G(5')pppA$- and $m^7G(5')pppG$- ends of RNA

Vaccinia virus mRNAs containing methyl-labeled $m^7G(5')pppA$- and $m^7G(5')pppG$- were prepared and used as substrate for the purified mRNA (nucleoside-2'-)methyltransferase. At varying times the RNA was isolated and $m^7G(5')pppA$, $m^7G(5')pppA^m$, $m^7G(5')pppG$, and $m^7G(5')pppG^m$ were separated as described in the text.

Time	% $m^7G(5')pppA^m$	% $m^7G(5')pppG^m$
min		
1	0.64	0.28
2½	0.84	0.47
5	0.92	0.64
8	0.94	0.71
12		0.82
20		0.87
40		0.95

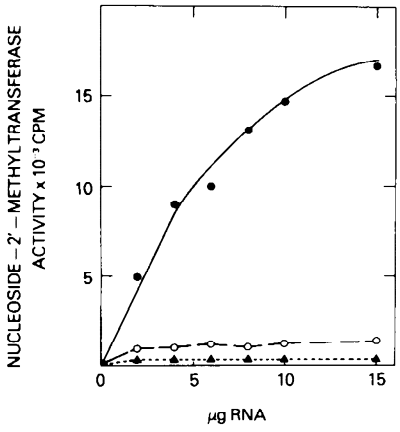

FIG. 8. Substrate specificity. Vaccinia virus mRNA containing 5'-terminal ppA- and ppG- (▲-----▲), G(5')pppA- and G(5')pppG- (○- - -○), or $m^7G(5')pppA$- and $m^7(5')pppG$- (●——●) were tested as substrates for the nucleoside-2'-methyltransferase under standard conditions.

TABLE II

Relative activity with polyribonucleotides containing m^7GpppN- ends

Polyribonucleotides were capped by purified guanylyltransferase · guanine-7-methyltransferase using [α^{32}P]GTP and unlabeled AdoMet as described under "Experimental Procedures." A similar concentration of labeled $m^7G(5')pppN$- ends was added in each case and incorporation of [^{3}H]AdoMet was measured. No activity was detected with uncapped polymers.

Capped polyribonucleotide	cpm
Poly(A)	3764
Poly(I)	3570
Poly(G)	568
Poly(U)	725
Poly(C)	730

mRNA(nucleoside-2'-)-methyltransferase and Ado[*methyl*-^{3}H]Met, only those containing $m^7G(5')pppA$- and $m^7G(5')pppG$- were suitable methyl acceptors (Fig. 8). From the specificity of this enzyme, we deduced that methylation at position 2' must follow capping and guanine-7-methylation.

Activity with Synthetic Substrates—To further study the substrate specificity of the mRNA(nucleoside-2'-)-methyltransferase, a series of capped homopolyribonucleotides were prepared. As anticipated from the previous section, each of the diphosphate-terminated homopolyribonucleotides, prior to capping, were completely unreactive. Of the capped homopolyribonucleotides tested, poly(A) and poly(I) were the best methyl acceptors (Table II). Significant but less activity was obtained with capped poly(G), poly(C), and poly(U). Since guanosine is a natural substrate, we attribute the low reactivity of poly(G) to the high degree of secondary structure of this polymer.

Very low or no activity was obtained with the dinucleotides $m^7G(5')pppG$ and $m^7G(5')pppA$, indicating a requirement for additional nucleotides beyond the cap structure (data not shown).

Inhibitors of Methylation—AdoHcy, one of the presumptive reaction products of methylation was a potent inhibitor of the mRNA(nucleoside-2'-)-methyltransferase. In the presence of 2.5 μM AdoMet, 1 μM AdoHcy was sufficient to inhibit the enzyme completely.

We checked a number of compounds including Gp, pG, ppG, m^7G, pm^7G, $m^7G(5')pppA$, $m^7G(5')pppA^m$, $m^7G(5')pppG$, and $m^7G(5')pppG^m$ that contain part or all of the $m^7G(5')pppN$ structure as possible inhibitors of the methyltransferase. At concentrations up to 6 mM, there was little or no effect on the methylation of brome mosaic virus RNA. That the $m^7G(5')pppN$ dinucleotides were not good competitive inhibitors was consistent with their inability to serve as efficient substrates. In addition, commercial poly(A), poly(G), poly(C), and poly(U) at concentrations up to 10 μg/ml were not inhibitory.

DISCUSSION

An mRNA(nucleoside-2'-)-methyltransferase purified from vaccinia virus cores (6) has been shown to specifically methylate the penultimate nucleoside of RNA ending in $m^7G(5')pppN$-. By contrast, RNAs ending in pN-, ppN-, or G(5')pppN- were ineffective. This enzyme specificity indicates that 2'-*O*-methylation must be the final step in the formation of the $m^7G(5')pppN^m$- structure. Therefore, the sequence of reactions is:

$$\text{GTP} + \text{ppN-} \rightleftharpoons \text{G(5')pppN-} + \text{PP}_i \quad (1)$$

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

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

Reactions 1 and 2 are carried out by an mRNA guanylyltransferase · mRNA(guanine-7-)-methyltransferase complex (1-3) and Reaction 3 by the mRNA(nucleoside-2'-)-methyltransferase described here.

These results are consistent with transcriptional studies carried out with intact vaccinia virus cores; it was found that at reduced AdoMet concentrations, RNA ending in $m^7G(5')pppN$- was formed (7).

Since vaccina virus mRNA contains $m^7G(5')pppA^m$- and $m^7G(5')pppG^m$- ends, it was essential to determine whether one enzyme could methylate position 2'-*O* of both adenosine and guanosine. This was found to be the case, although adenosine appeared to be methylated more rapidly by the purified enzyme. In accord with the above result, we have repeatedly found that when RNA made by vaccinia virus cores is incompletely methylated, the ratio of $m^7G(5')pppG$/$m^7G(5')pppG^m$ ends is greater than the ratio of $m^7G(5')pppA$/$m^7G(5')pppA^m$. Experiments with homopolyribonucleotides ending in $m^7G(5')pppN$ indicated that poly(A) and poly(I) were excellent substrates for the 2'-methyltransferase. In contrast, poly(G), poly(U), and poly(C) were poor substrates. The low efficiency of poly(G) may result from its high degree of secondary structure. Additional substrates are needed to further analyze the methylation of pyrimidines, which are not found in the penultimate position of vaccinia virus mRNAs.

From the substrate specificities of the purified mRNA guanylyltransferase and mRNA(guanine-7-)-methyltransferase, we concluded that capping and methylation occurred post-

transcriptionally (3). The low ability of mRNA(nucleoside-2'-)-methyltransferase to utilize dinucleotides such as $m^7G(5')pppG$ suggests that a longer polyribonucleotide chain is required. However, since the enzyme can methylate all vaccinia virus mRNAs, and adenosine, guanosine, cytidine, or uridine may be in the penultimate position,[2] there seems to be no specific sequence requirement.

The mRNA(nucleoside-2'-)-methyltransferase is extremely sensitive to AdoHcy. With 1 μM AdoHcy, in the presence of 2.5 μM AdoMet, activity was virtually undetectable. Since the 2'-methyltransferase is more sensitive to AdoHcy than is the guanine-7-methyltransferase (2), we suspect that at low concentrations of AdoHcy, 2'-*O*-methylation would be preferentially inhibited during transcription by vaccinia virus cores.

While mRNA(nucleoside-2'-)-methyltransferase has not yet been isolated from other sources, it appears likely that 2'-*O*-methylation also occurs after capping and guanine-7-methylation occurs in intact reovirus cores (10). However, a different sequence, in which 2'-*O*-methylation precedes guanine-7-methylation, has been proposed for vesicular stomatitis virus based on the formation of $G(5')pppA^m$-ended RNA at low AdoMet concentrations (11). No information is currently available concerning the step at which 2'-*O*-methylation of cellular mRNA occurs.

[2] J. Keith, A. Gershowitz, and B. Moss, unpublished observation.

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