

Influence of 5'-Terminal Cap Structure on the Initiation of Translation of Vaccinia Virus mRNA*

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The ability of methylated vaccinia virus mRNA to bind to ribosomes derived from wheat germ or rabbit reticulocyte lysates has been studied after β elimination, to remove the 5'-terminal m⁷G, and after "recapping" of β -eliminated mRNA molecules using guanylyltransferase-guanine-7-methyltransferase complex from vaccinia virions. Removal of m⁷G from the mRNA results in significant loss of ability to bind to ribosomes and to stimulate protein synthesis *in vitro*. Readdition of m⁷G, but not of unmethylated guanosine to the 5' end results in recovery of both of these functions. To evaluate the role of 2'-O-methylation of the penultimate ribonucleoside, mRNAs containing m⁷G-(5')pppA- and m⁷G(5')pppG- as well as m⁷G(5')pppA^m- and m⁷G(5')pppG^m- ends were synthesized *in vitro* at limiting S-adenosylmethionine concentrations by vaccinia virus cores. By comparing the cap sequences of ribosome-bound and unbound mRNAs, we concluded that 2'-O-methylation has at most a minor effect compared to that of m⁷G upon ribosome binding under *in vitro* conditions. Only at high input mRNA concentrations, at which competition might occur, was there some ribosomal enrichment of mRNAs containing a specific terminal structure, namely m⁷G(5')pppA^m-.

Messenger RNA synthesized *in vitro* by purified vaccinia virus particles, in the presence of the methyl donor, S-adenosylmethionine (AdoMet) has been shown to contain a mixture of molecules with two types of 5' termini, m⁷G(5')pppG^m and m⁷G(5')pppA^m (1, 2). Virus-specific mRNA isolated from vaccinia-infected HeLa cells has similar as well as more highly modified termini that carry extra methyl groups on the N⁶ of the penultimate adenosine and on the 2'-OH of the third nucleoside (3). The presence of such methylated 5' ends (caps) appears to be a feature of eukaryotic mRNA (4-7), heterogeneous nuclear RNA (8-9), and low molecular weight nuclear RNAs (10, 11). Notable exceptions are viral RNAs such as

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[†] The abbreviations used are: m⁷G, 7-methylguanosine; G^m, 2'-O-methylguanosine; A^m, 2'-O-methyladenosine; N, nucleoside; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

polio (12-14), encephalomyocarditis (15), tobacco necrosis and satellite tobacco necrosis virus (16-18), and yeast killer particle RNA (19). A significant amount of information has been gathered concerning the possible role of such cap structures in mRNA translation (20-22). However, some controversy exists as to whether 1) the requirement for m⁷G depends on the particular mRNA and *in vitro* translation system (23), 2) chemical methods used to remove m⁷G may produce unrelated changes in the RNA that lowers its ability to be translated (24), and 3) unmethylated viral mRNAs formed in the absence of S-adenosylmethionine have other structural anomalies that affect translation (24). In addition, little information is available regarding the influence of the state of methylation of the penultimate nucleoside on translation.

Vaccinia virus offers a useful system for addressing some of the questions raised regarding the influence of mRNA 5'-terminal structures on the initiation of protein synthesis. The *in vitro* transcription product of vaccinia cores consists of a large population of RNAs and by varying the concentration of AdoMet both uncapped and partially methylated termini are formed (25). Furthermore, enzymes involved in the synthesis of cap structures have been isolated from this virus and can be used to add m⁷G to unmethylated mRNA or restore m⁷G following chemical removal (18, 26, 27). Here, we report the result of studies on the influence of the m⁷G residue and of the penultimate 2'-O-methylated nucleoside on vaccinia virus mRNA binding to ribosomes and translation.

MATERIALS AND METHODS

Synthesis and Purification of Vaccinia Virus mRNA —The WR strain of vaccinia virus was grown and purified as previously described (28). The synthesis of mRNA was carried out *in vitro* in reaction mixtures containing 1.3 A_{260 nm} vaccinia virus/ml, 50 mM Tris/HCl (pH 8.4), 5 mM dithiothreitol, 0.05% Nonidet P-40, 2.5 mM each of ATP, GTP, CTP, and UTP, 10 mM MgCl₂, 2 μ M Ado[methyl-³H]Met (7.5 to 9.7 Ci/mmol), or 0.1 mM AdoHcy. After 30 min at 37°, the viral cores were removed by centrifugation, EDTA added to 10 mM, and the RNA was purified by sodium dodecyl sulfate-phenol-chloroform extraction, alcohol precipitation, and passage through a Sephadex G-75 column.

Periodate Oxidation, β Elimination, and Recapping of mRNA —Periodate oxidation and β elimination were carried out as previously described (29), except that the β -eliminated mRNA was passed through a Sephadex G-75 column. The guanylyltransferase-guanine-7-methyltransferase complex obtained after DEAE-cellulose column chromatography (27) was used for recapping β -eliminated or unmethylated mRNA. The reaction mixtures contained 25 mM Tris/HCl (pH 7.5), 5 mM dithiothreitol, 2 mM GTP, 2 mM MgCl₂, 0.1 mM AdoMet, or 0.1 mM AdoHcy, 5 μ g of RNA and 10 μ l of enzyme in a

total volume of 0.2 ml. After incubation at 37° for 30 min, the RNA was purified by sodium dodecyl sulfate-phenol-chloroform extraction and ethanol precipitation.

Ribosome Binding Studies — The binding reactions were usually carried out in a 50- μ l volume using mRNA at a concentration of 20 pmol/ml, unless otherwise specified. RNA concentration was determined from optical density measurements assuming an average chain length of 1000 nucleotides. The wheat germ incubation mixture contained 25 μ l of wheat germ S₂₀ (approximately 70 A/ml), 10 mM Hepes/NaOH (pH 7.6), 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 40 μ g/ml creatine phosphokinase, 70 mM KCl, 3.5 mM magnesium acetate, 20 μ M unlabeled amino acids, and 200 μ M sparsomycin. These conditions were found to be optimal for mRNA bindings and saturation was reached at around 120 pmol of mRNA/ml. The incubation was at 25° for 5 min.

The reticulocyte lysate incubation mixture contained in a 50- μ l assay, the following: 30 μ l of rabbit reticulocyte lysate, 10 mM Hepes/NaOH (pH 7.6), 70 mM KCl, 3.0 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 25 μ g/ml of creatine phosphokinase, 20 μ g/ml of hemin, 20 μ M unlabeled amino acids, and 200 μ M sparsomycin. Incubation was for 5 min at 30°.

Reactions were stopped by chilling and diluting to 0.2 ml with 10 mM Tris/HCl (pH 7.6), 70 mM KCl, and 3 mM magnesium acetate and loaded onto 5 ml of 10 to 30% (v/v) glycerol gradients in the above buffer. Centrifugation was for 90 min at 48,000 rpm at 4° in an SW 50.1 rotor. Fractions of 0.2 ml were collected and counted in aqueous scintillant (Amersham/Searle). Fractions of the reticulocyte lysate incubation mixture were decolorized with H₂O₂ before addition of scintillation fluid.

Translation Assays — The incubation mixtures were the same as in ribosome-binding experiments with the following changes: for the wheat germ extract KCl was used at 90 mM, sparsomycin was omitted and ³H-labeled leucine, proline, and valine were included (2 μ Ci each). For the reticulocyte lysate incubation, a micrococcal nuclease-treated lysate (30) having low endogenous protein-synthesizing activity was used in the absence of sparsomycin. KCl was replaced with 200 mM potassium acetate, conditions that are optimal for translation according to Weber *et al.* (31). [³⁵S]Methionine was used as the radioactive amino acid. Five-microliter samples of the reaction mixtures were removed at different times to determine hot trichloroacetic acid-precipitable radioactivity.

Polypeptides formed during a 60-min incubation with [³⁵S]methionine were analyzed by electrophoresis on a discontinuous polyacrylamide gel (32). To each 5- μ l sample from the translation

reaction, ribonuclease A was added to 100 μ g/ml and EDTA to 10 mM. After a 10-min incubation at 30°, 25 μ l of 80 mM Tris/HCl (pH 6.8), 0.1 M dithiothreitol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.002% bromophenol blue were added. The samples were heated at 100° for 2 min before electrophoresis on a 15% polyacrylamide, 0.58% bisacrylamide gel.

Analysis of 5' Ends of Ribosome-bound and Free RNA — The RNAs were dissolved in 100 μ l of sodium acetate buffer (pH 6.0, 5 mM), 25 μ g of nuclease P₁ was added, and the mixture was incubated at 37° for 1 h. This was followed by addition of 0.2 unit of bacterial alkaline phosphatase for 1 h at 37°. The digest was then analyzed by paper electrophoresis on Whatman No. 3MM paper at pH 3.5 at 3000 V for 2 h. After electrophoresis the paper was dried, cut into 1-cm strips, and counted using toluene-based scintillation fluid. Radioactivity was recovered in two peaks corresponding to adenosine caps (m^7GpppA plus m^7GpppA') and guanosine caps (m^7GpppG plus m^7GpppG'). After washing in toluene, the strips were eluted with water and the radioactive material resolved into individual components by descending paper chromatography using isobutyric acid, 0.5 M NH₄OH (10:6 v/v) solvent system.

Sources of Radioactive Chemicals — L-[2,3,5-³H]Leucine, L-[2,3,4,5-³H]proline, and L-[2,3,³H]valine were obtained from New England Nuclear. The specific activities were 80 Ci/mmol, 60 Ci/mmol, and 14.5 Ci/mmol, respectively. L-[³⁵S]Methionine (600 Ci/mmol) was from Amersham/Searle.

RESULTS

Influence of 5'-Terminal m^7G on Formation of Initiation Complexes — To determine whether the m^7G residue plays an important role in mRNA selection by ribosomes during initiation of protein synthesis, we studied initiation complex formation using vaccinia mRNA labeled within the 5'-terminal methyl groups. [³H]Methyl-labeled mRNA was synthesized by vaccinia virus particles *in vitro* and a portion of the mRNA was subjected to periodate oxidation and β elimination procedure to remove the m^7G residue. The treated mRNA containing labeled $pppN^m$ ends was then "recapped" using guanylyltransferase-methyltransferase complex in the presence of unlabeled AdoMet or AdoHcy. The different mRNA preparations were tested for their ability to bind to wheat germ or reticulocyte ribosomes, in the presence of sparsomycin to

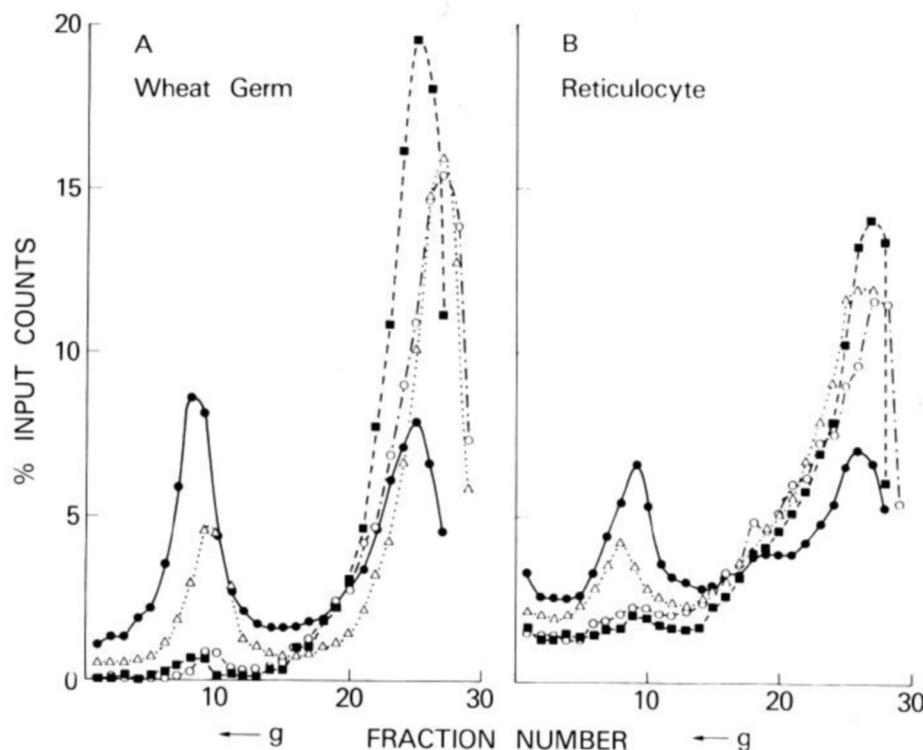


FIG. 1. Effect of β elimination and recapping on binding of methylated vaccinia mRNA to ribosomes. [³H]-Methyl-labeled vaccinia mRNA synthesized *in vitro* was periodate-oxidized and β -eliminated. A portion of the β -eliminated RNA was then recapped using the guanylyltransferase-methyltransferase complex in the presence of AdoMet or AdoHcy as described under "Materials and Methods." 1 pmol of each RNA was used in a 50- μ l ribosome binding assay (see "Materials and Methods") and analyzed by glycerol gradient centrifugation. Values are expressed as per cent of input counts per min in each fraction since the specific radioactivity of the mock-treated RNA was twice (7500 cpm/pmol) that of all the other treated RNAs (3750 cpm/pmol). ●—●, mock-treated RNA; ○—○, β -eliminated RNA; ■—■, GpppNm-ended recapped RNA; △—△, m,GpppNm-ended recapped RNA.

inhibit polypeptide chain elongation (33). The abilities of the control and treated RNAs to form 80 S initiation complexes are compared in Fig. 1. The values are expressed as per cent input counts per min because the removal of the labeled m⁷G lowers the specific activity of the treated RNAs. Following β elimination, the binding of vaccinia mRNA is decreased from 46 to 4% in the wheat germ extract and from 43 to 12% in the reticulocyte lysate. This loss of binding is not irreversible, however, since recapping of the treated RNA to restore m⁷G residues results in recovery of about half the ability to bind to ribosomes (22% and 23% of input in the two extracts). Recapping in the presence of AdoHcy to form GpppN^m-ended RNA lacking the N⁷ methylation does not improve the binding efficiency of the β -eliminated RNA. This suggests that the presence of blocked ends *per se* is not responsible for the recovery of ribosome binding and stresses the importance of the methyl group at position 7 of the 5'-terminal guanosine.

The failure to recover all of the original ribosome binding activity of β -eliminated vaccinia virus mRNA was partially due to incomplete recapping. The results of an analysis of the 5' termini of mRNA preparations used for ribosome binding are shown in Table I. Although β elimination results in the removal of 96% of the m⁷G residues, recapping in the presence of AdoMet in this experiment was incomplete, 5% of the RNA molecules ended in pppA^m and 21% in pppG^m. The different efficiencies of capping adenosine and guanosine ends appears to be a property of the enzyme (34). Since only 74% of the mRNA was recapped, we calculated that ribosome binding was restored to 68% of the theoretical value.

When AdoHcy was present during recapping, only a third of the mRNA 5' ends were blocked (Table I). These results are in agreement with the previous findings of Martin and Moss (34, 35) that AdoMet promotes capping possibly by preventing reversal of the reaction by pyrophosphate. To overcome this problem and directly compare the binding efficiencies of two RNA preparations having the same amounts of GpppN- and m⁷GpppN- ends, vaccinia virus mRNA containing predominantly 5'-diphosphate ends was

TABLE I
5' Termini of treated vaccinia mRNAs

Vaccinia virus mRNA was synthesized *in vitro* using Ado[methyl-³H]Met. Aliquots of this RNA were subjected to β elimination and recapping procedures as described under "Materials and Methods." Recapping was done in the presence of unlabeled GTP and AdoMet or AdoHcy. One picomole of each type of RNA was dissolved in 50 μ l of sodium acetate buffer (pH 6.0, 5 mM) digested with nuclease P₁ followed by bacterial alkaline phosphatase and the digest was analyzed by paper electrophoresis at pH 3.5 along with appropriate markers as described under "Materials and Methods."

RNA	5' ends ^a					
	m ⁷ G- pppA ^m	m ⁷ G- pppG ^m	GpppA ^m	GpppG ^m	pppA ^m ^b	pppG ^m ^b
Control	43	57				
β -eliminated RNA	2	2			43	53
mRNA recapped with AdoMet present	39	35			5	21
mRNA recapped with AdoHcy present	2.5	3	16.0	16.5	25	37

^a All values are expressed as per cent of total.

^b Determined as the corresponding methylated nucleoside after alkaline phosphatase treatment.

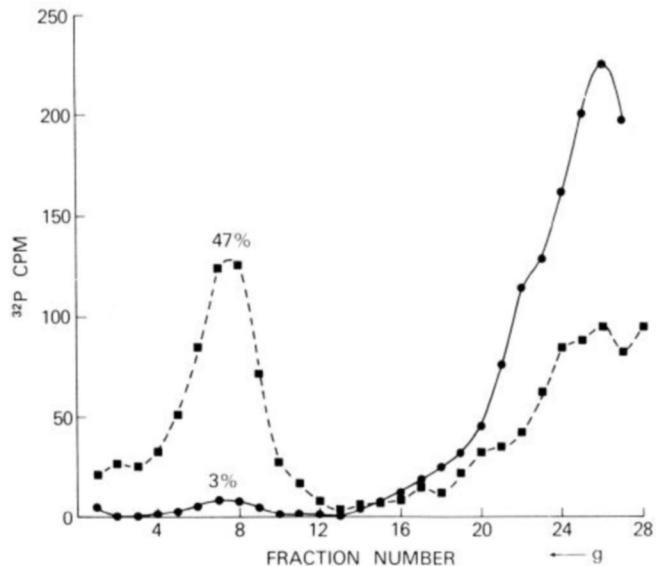


FIG. 2. Effect of methylation on the binding of recapped vaccinia mRNA to wheat germ ribosomes. 1950 cpm of ³²P-labeled GpppN-ended RNA or m⁷GpppN-ended RNA (approximately 0.7 pmol) was incubated for 5 min at 25° in 100 μ l of standard ribosome-binding assay mixture in the presence of sparsomycin and analyzed by glycerol gradient centrifugation as described in legend for Fig. 1.

●—●, GpppN-ended RNA; ■—■, m⁷GpppN-ended RNA.

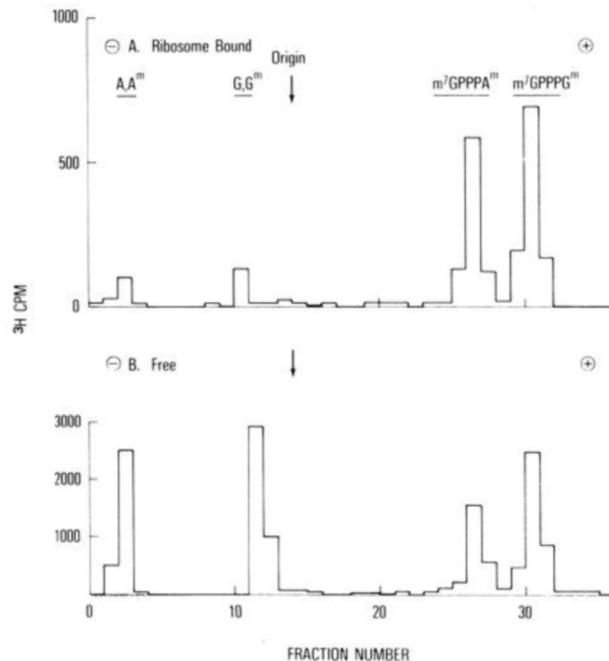


FIG. 3. Analysis of 5' ends of ribosome-bound and free mRNA. 4 pmol of [³H]methyl-labeled vaccinia mRNA and 8 pmol of β -eliminated methylated RNA were incubated in 100 μ l of a reticulocyte ribosome-binding assay mixture for 5 min at 30°. The reaction mixture was analyzed by glycerol gradient centrifugation as in Fig. 1 and aliquots of the fractions were counted. Fractions from the 80 S and the free mRNA regions were pooled, phenol-extracted, and RNA was precipitated by addition of ethanol. The RNA pellet was digested with nuclease P₁ followed by bacterial alkaline phosphatase and analyzed by paper electrophoresis at pH 3.5 as described under "Materials and Methods." The positions of the marker compounds visualized under ultraviolet light are indicated. One-centimeter strips were cut and counted in toluene-based scintillation fluid.

synthesized *in vitro* by replacing AdoMet with AdoHcy in the transcription reaction. This RNA was then enzymatically capped with [α -³²P]GTP, in the presence of AdoMet or AdoHcy, to form m⁷G⁺pppN- or G⁺pppN-ended RNA. Under these conditions the amount of radioactivity in each preparation is a measure of the extent of capping. Using equal amounts of radioactively labeled material, the binding efficiencies of the two mRNA preparations were compared in the wheat germ extract (Fig. 2). The RNA ending in m⁷GpppN- binds with much greater efficiency (47%) than the GpppN-ended RNA (binding efficiency 3%).

Competition between m⁷GpppN^m-ended versus pppN^m-ended mRNA Molecules for Ribosome Binding—It was of further interest to determine how mRNA molecules lacking m⁷G would compete with other molecules containing m⁷G for ribosome binding. A mixture of β -eliminated and untreated methylated vaccinia mRNAs was constituted to give a 2-fold molar excess of molecules lacking m⁷G. This mixture was then added to a reticulocyte protein-synthesizing system using an mRNA concentration that was slightly above saturation. Fig. 3 shows the results of the analysis of 5' termini of ribosome-bound and free mRNA by paper electrophoresis after nuclease P₁ and alkaline phosphatase digestion. The pppN^m ends are converted to A^m and G^m whereas the capped ends are resistant to digestion. Ribosome-bound RNA was found to be

predominantly m⁷G-capped, whereas free mRNA contained more pppN^m-ended RNA than capped mRNA. The results of this experiment are summarized in Table II. It appears that in a competitive situation RNA containing m⁷G is preferentially bound to the ribosomes.

Influence of 5'-Terminal m⁷G on Translation—Recapping of β -eliminated vaccinia virus mRNA also restored its ability to be translated in a protein synthesizing system. Fig. 4A shows that both unmethylated and β -eliminated methylated vaccinia mRNAs are poorly translated in the wheat germ extract. Following recapping, however, the β -eliminated RNA is translated nearly as well as control methylated RNA. Similar experiments could not be carried out with the reticulocyte lysate because of high levels of endogenous globin synthesis. In order to decrease this background, the reticulocyte lysate was treated with micrococcal nuclease according to the procedure of Pelham and Jackson (30). In such an extract, recapping of the β -eliminated RNA also restored translation (Fig. 4B). These results agree with the data of Weber *et al.* (31) that translation of methylated vaccinia mRNA is more efficient than unmethylated RNA, the difference being maximal at high potassium ion concentrations. The latter workers also reported that unmethylated vaccinia virus mRNA made in the presence of AdoHcy does not have abnormal length poly(A) as has been described for vesicular stomatitis virus (24).

The *in vitro* translation products of vaccinia virus mRNAs have only been characterized in a preliminary fashion (36–38). Nevertheless, it was considered important to determine whether mRNAs that had been β -eliminated and recapped, directed synthesis of the same polypeptides as unmodified mRNAs. In the wheat germ cell-free system programmed with unmodified and recapped mRNAs, an identical set of at least 22 [³⁵S]methionine-labeled polypeptides were detected on radioautographs of polyacrylamide gels (Fig. 5). Radioautographs of products synthesized in the reticulocyte cell-free system in response to unmodified and recapped mRNAs were also indistinguishable (data not shown). The wheat germ and reticulocyte cell-free systems, however, appeared to synthesize individual polypeptides with different efficiencies. Authentication by peptide mapping of vaccinia virus-specific polypeptides synthesized *in vivo* and *in vitro* is in progress.

TABLE II

Competition for binding of m⁷G-capped and β -eliminated vaccinia mRNA to reticulocyte ribosomes

The details of this experiment are described in the legend for Fig. 3.

mRNA 5' termini	80 S bound	Free
cpm		
pppA ^m ^a	121	3053
pppG ^m ^a	144	4017
m ⁷ GpppA ^m	837	2396
m ⁷ GpppG ^m	1059	3885
Capped/pppN ^m -ended ratio	7.1	0.9

^a Determined as the corresponding methylated nucleoside after bacterial alkaline phosphatase digestion.

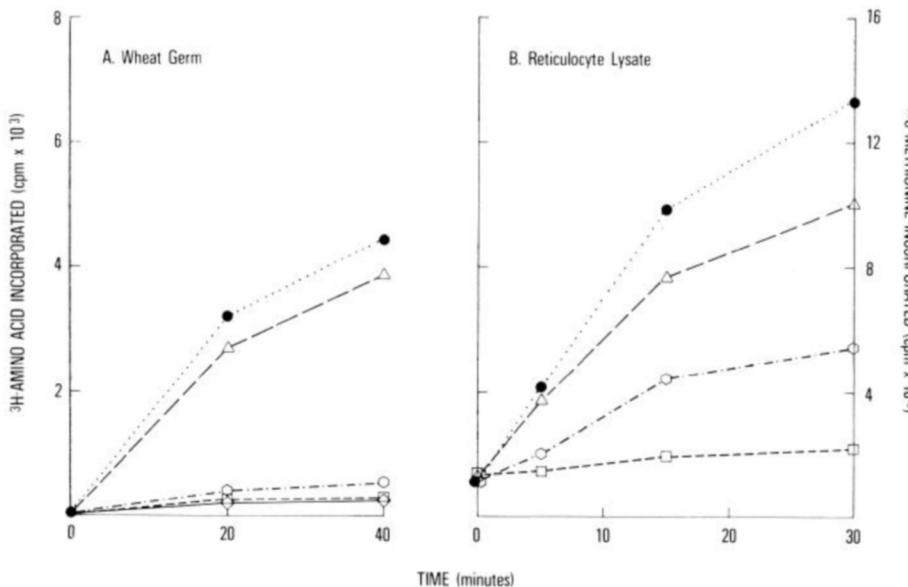


FIG. 4. Translation of vaccinia mRNA after β elimination and recapping. Translation assays were carried out in a final volume of 50 μ l using 1.5 pmol of each type of RNA per assay. At the indicated times 5- μ l aliquots were removed to determine incorporation into proteins. The wheat germ incubation was at 25° using 2 μ Ci each of ³H-labeled, leucine, valine, and proline as the labeled amino acids. Reticulocyte lysate was incubated at 30° and [³⁵S]methionine was used to follow protein synthesis. \square — \square , minus RNA; ●—●, mock-treated methylated RNA; ○—○, β -eliminated, methylated RNA; △—△, recapped, methylated RNA; ◇—◇, unmethylated RNA.

Influence of Methylation of Penultimate 5'-Nucleoside on Initiation Complex Formation—So far we have only considered the influence of the 5'-terminal m⁷G on mRNA binding and translation. Using capped synthetic ribopolymers, it has been suggested that the 2'-O-methyl group has a positive influence on mRNA binding to ribosomes (39). It is possible to vary the extent of 2'-O-methylation in vaccinia mRNA by

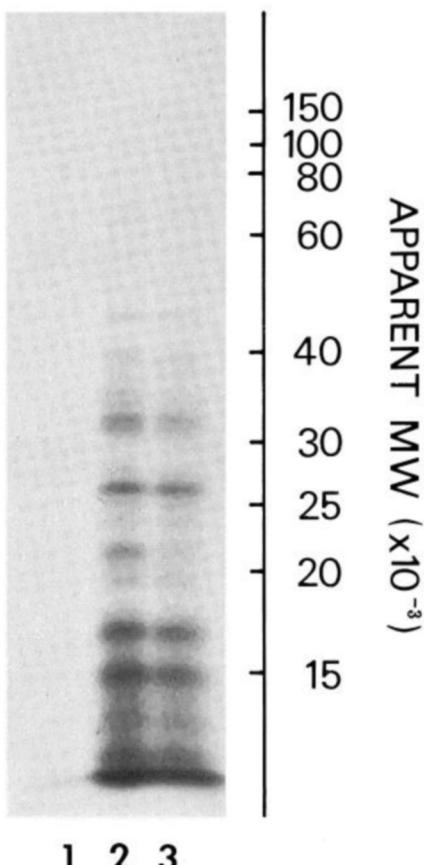


FIG. 5. Analysis of polypeptides synthesized in cell-free systems programmed with unmodified and with β -eliminated and recapped mRNAs. [³⁵S]Methionine-labeled polypeptides synthesized in wheat germ extract were analyzed on a 15% polyacrylamide gel (see "Materials and Methods"). 50- μ l incubations contained no added RNA (Track 1), 1.5 pmol of mock-treated methylated RNA (Track 2), or 1.5 pmol of recapped, β -eliminated RNA (Track 3). Five microliters of each incubation was analyzed. The gel was dried and autoradiographed for 7 days. Apparent molecular weights were determined from markers electrophoresed on the same gel.

altering the AdoMet concentration in the transcription reaction (25). RNA made using suboptimal amounts of AdoMet has the four different types of methylated 5' termini: m⁷GpppA^m, m⁷GpppA, m⁷GpppG^m, and m⁷GpppG.

To evaluate the influence of the 2'-O-methyl group on ribosome binding, we analyzed the 5' termini of ribosome-bound and unbound mRNAs. At 40 pmol/ml, an mRNA concentration below saturation, the proportion of each type of terminus in the wheat germ ribosome-bound mRNA was similar to that in the free mRNA as well as the input mRNA (Table III), suggesting that there is no selection. However, at 200 and 400 pmol/ml, RNA concentrations above saturation at which competition might occur, some differences were noted. In the ribosome-bound fraction, mRNA containing m⁷GpppA^m was enriched up to 2-fold. A small effect of this nature was also detected in binding reactions carried out in a reticulocyte lysate treated with micrococcal nuclease to minimize competition with endogenous globin mRNA (not shown). Nevertheless, mRNAs with m⁷GpppG^m were not selected by ribosomes over mRNAs ending in m⁷GpppG in either the wheat germ (Table III) or reticulocyte (not shown) lysates.

DISCUSSION

The present studies extend the evidence for the involvement of 5'-terminal m⁷G in protein synthesis to another system, namely translation of vaccinia virus mRNAs in wheat germ extract and rabbit reticulocyte lysate. Methylated mRNA synthesized *in vitro* by vaccinia virus particles in the presence of AdoMet are translated more efficiently than unmethylated mRNA made in the presence of AdoHcy. Furthermore, the translatability of methylated vaccinia mRNA is severely reduced by removal of the m⁷G residue by periodate oxidation and β elimination. Previous studies using a similar approach to show the function of m⁷G have been subjected to criticism that chemical treatment during oxidation and β elimination damaged the mRNA in other ways besides removal of m⁷G. A novel feature of the present study is the demonstration that the translational activity of the β -eliminated RNA could be largely restored by the specific readdition of the m⁷G residue with the guanylyltransferase-guanine-7-methyltransferase complex. Moreover, the original and recapped mRNAs directed the synthesis of similar polypeptides. In addition, the loss of ribosome-binding ability caused by the removal of m⁷G can also be reversed by restoration of the methylated cap structure. Similarly, mRNA made in the presence of AdoHcy binds to ribosomes with high efficiency after capping. The importance of the N⁷ methyl group is apparent from the finding that capping to form GpppN- or GpppN^m-ended RNA

TABLE III
Analysis of 5' termini of ribosome-bound and free mRNA in wheat germ S₂₀

Methylated vaccinia virus mRNA was synthesized *in vitro* using suboptimal amounts of Ado[methyl-³H]Met as described under "Materials and Methods." At the indicated concentration, ³H-labeled mRNA was incubated in 100 μ l of wheat germ extract in the presence of 200 μ M sparsomycin at 25° for 5 min. Fractions from the 80 S and free mRNA regions were pooled, phenol-extracted, and

RNA was precipitated with ethanol. The RNA was digested with nuclease P₁ and bacterial alkaline phosphatase and analyzed as described under "Materials and Methods." For calculation of per cent of total radioactivity recovered in each cap only half the counts per min associated with dimethylated caps was used.

5' Termini	40 pmol/ml				200 pmol/ml				400 pmol/ml				Input	
	80 S		Free		80 S		Free		80 S		Free		cpm	%
	cpm	%	cpm	%		cpm	%		cpm	%	cpm	%	cpm	%
m ⁷ GpppA	76	14	80	14	353	15	766	11	660	16	9953	13	894	13
m ⁷ GpppA ^m	212	20	330	20	1792	38	2548	19	3452	41	38916	25	3648	26
m ⁷ GpppG	179	34	206	35	504	22	2316	34	949	22	24170	31	2045	29
m ⁷ GpppG ^m	344	32	372	32	1188	25	4792	35	1808	21	49602	32	4382	32

does not improve the binding efficiency of unmethylated or of β -eliminated RNA. Competition experiments also emphasize the role of m⁷G since capped mRNA binds preferentially to ribosomes when mixed with uncapped mRNA. We wish to stress that all ribosome-binding experiments involved an incubation time of only 5 min and that the recoveries of pppN^m-, GpppN^m-, and m⁷GpppN^m-ended molecules after incubation in the wheat germ extract and the reticulocyte lysate were similar, thereby eliminating the possibility that the results are due to the 5'-exonuclease activity recently reported (40).

Previous studies had indicated that at limiting concentrations of AdoMet, vaccinia virus cores synthesize mRNA containing partially methylated m⁷GpppA- and m⁷GpppG- ends not found *in vivo* as well as fully methylated m⁷GpppA^m- and m⁷GpppG^m- ends (25). By comparing the cap sequences of ribosome-bound and unbound mRNAs, we conclude that 2'-O-methylation has at most a minor effect compared to that of m⁷G upon the binding of vaccinia virus mRNAs under the *in vitro* conditions used. Only at high input mRNA concentrations, at which competition might occur, was there some ribosomal enrichment of mRNAs containing a specific terminal structure, namely m⁷GpppA^m. Whether an apparent selection of mRNAs with m⁷GpppA^m over mRNAs with m⁷G(5')pppG^m represents a specific affinity of ribosomes for the m⁷GpppA^m- structure or is merely a coincidental association of this cap structure with mRNAs that have other structural features that account for their ribosome binding cannot be ascertained at this time.

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