Methylated Nucleotides Block 5'-Terminus of Vaccinia Virus Messenger RNA

(RNA methyl transferase/7-methylguanosine/2'-O-methylguanosine/2'-O-methyladenosine)

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ABSTRACT Studies on the nature and location of the methylated nucleotides in mRNA synthesized *in vitro* by vaccinia virus particles revealed an unusual 5'-terminal structure. Evidence that the pyrophosphate group is blocked by 7-methylguanosine and that both 2'-O-methyladenosine and 2'-O-methylguanosine occupy penultimate positions was presented. According to this model, the 5'-termini of vaccinia virus mRNAs are:

7MeG$^{5'}$ppp$^{5'}$GMepNp and 7MeG$^{5'}$ppp$^{5'}$AMepNp.

The presence of a low number of methylated nucleotides is a common, although recently discovered, characteristic of mRNAs synthesized *in vivo* by eukaryotic cells (1, 2) and *in vitro* by virion-associated enzymes of cytoplasmic polyhedrosis virus of insects (3), vaccinia virus (4), and reovirus (5). In the case of mRNA made by the double-stranded RNA viruses, cytoplasmic polyhedrosis virus (3) and reovirus (5), evidence for the location of a single 2'-O-methylribonucleotide at or near the 5'-terminus was presented. Other studies had suggested a terminal 2'-O-substituted ribonucleotide in the + strands of the genomes of cytoplasmic polyhedrosis virus (6) and reovirus (7). Experiments with mRNA synthesized by vaccinia, a double-stranded DNA virus, indicated the presence of either 5'-terminal methylated nucleotides or an internal sequence of adjacent methylated nucleotides (4). Further studies with the latter RNA now reveal an unusual structure consisting of a terminal base-methylated nucleotide linked to a ribosemethylated nucleotide through a 5'–5' pyrophosphate bond.

MATERIALS AND METHODS

Preparation of Methylated Vaccinia Virus mRNA. Procedures for the purification of vaccinia virus and synthesis *in vitro* of methylated mRNA by virus particles have been described (4).

Chemical and Enzymatic Treatment of RNA. Alkali digestion and DEAE–cellulose chromatography in 7 M urea at pH 7.6 were carried out as described (4). ^{3}H was measured by liquid scintillation counting, and ^{32}P by either the latter method or from Čerenkov radiation. Nucleotides were desalted by readsorption to and elution from a DEAE–cellulose column equilibrated with ammonium carbonate. Procedures for depurination and chromatography on Dowex 50(H$^+$) were adapted from Culp and Brown (8). Periodate oxidation followed by β-elimination with aniline was performed as described by Fraenkel-Conrat and Steinschneider (9). Digestion with alkaline phosphatase (0.1 mg/ml) and/or snake venom phosphodiesterase (0.2 mg/ml) was in 50 mM Tris·HCl (pH 8.5) and 5 mM MgCl$_2$ for 2 hr at 37°.

Thin-layer Electrophoresis and Chromatography. Cellulose-coated sheets (Eastman, 20 × 20 cm) were used for both electrophoresis and chromatography. Electrophoresis was performed under the following conditions: (A) 50 mM sodium formate (pH 3.5), 1000 V, 75 min; (B) 0.2 M sodium borate (pH 9), 800 V, 110 min; (C) 1 M formic acid, 800 V, 70 min. Chromatography solvents that were used included: (A) ethyl acetate–isopropanol–7.5 M NH$_4$OH–n-butanol (3:2:2:1); (B) isopropanol–concentrated HCl–H$_2$O (68:17.6:14.3); (C) isopropanol–H$_2$O–NH$_4$OH (7:2:1); (D) methanol–concentrated HCl–H$_2$O (7:2:1).

Enzymes, Chemicals, and Isotopes. Alkaline phosphatase and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp.; 2'-O-methylguanosine, synthesized by R. Robins, was a gift of M. Sporn; other 2'-O-methylribonucleosides were obtained from P-L Biochemicals and Ash Stevens, Inc.; base methylated compounds were from Sigma Chemical Co. [β,γ-^{32}P]GTP (5.5 Ci/mmol) and S-adenosyl [*methyl*-^{3}H]methionine (8.5 Ci/mmol) were products of ICN and New England Nuclear Corp., respectively.

RESULTS

Location of Methylated Nucleotides at the 5'-Terminus of Vaccinia mRNA. mRNA synthesized *in vitro* by vaccinia virus particles in the presence of S-adenosylmethionine contained about 2.3 methyl groups per 1000 nucleotides (4). After alkali digestion, the major methyl-labeled material chromatographed on DEAE-cellulose with a net charge of slightly more than −5. This charge suggested either a 5'-terminal methylated nucleotide containing a pyrophosphate group or an internal alkali-resistant sequence of ribose-methylated nucleotides (4). Attempts were made to label the pyrophosphate group with [β,γ-^{32}P]ribonucleoside triphosphates and to simultaneously label the nucleosides with S-adenosyl[*methyl*-^{3}H]methionine. When [β,γ-^{32}P]GTP was used and the RNA then subjected to alkali hydrolysis and DEAE–cellulose chromatography, the major peaks of ^{3}H and ^{32}P eluted together with a net charge of slightly more than −5 (Fig. 1B, peak II). A minor ^{3}H peak (Fig. 1B, peak I) had a net charge between −4 and −5 and was overlapped by a broader peak of ^{32}P-labeled material. By contrast, after alkali hydrolysis of RNA made in the absence of S-adenosylmethionine, very little ^{32}P-labeled material chromatographed with a net charge of −5 (Fig. 1A).

Pyrophosphate Group at the 5'-Terminus of Vaccinia mRNA Is Blocked. Experiments in the preceding section were consistent with a structure of the type ppNMepNp– at the 5'-
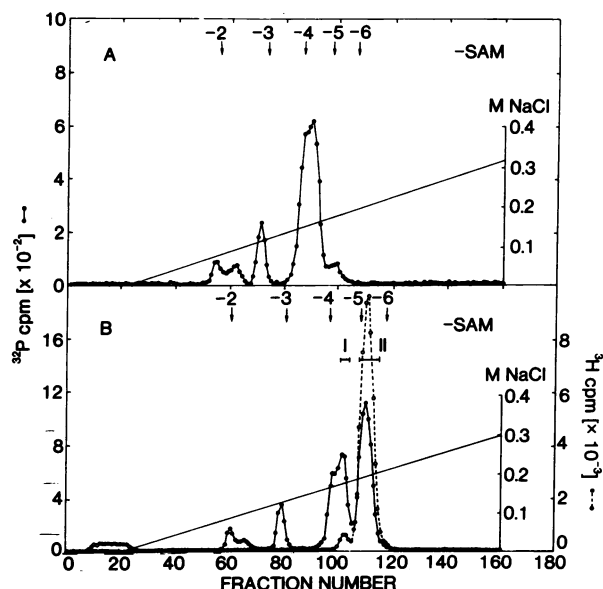
FIG. 1. DEAE–cellulose column chromatography of alkali hydrolysate of [β,γ-³²P]GTP-labeled vaccinia mRNA synthesized *in vitro* in the absence and presence of S-adenosyl[*methyl*-³H]methionine. The reaction mixture (3 ml) containing 50 mM Tris·HCl (pH 8.5), 10 mM dithiothreitol, 5 mM MgCl₂, 2.5 mM each of ATP, CTP, and UTP, 0.1 mM [β,γ-³²P]GTP (1 Ci/mmol), 0.05% Nonidet P-40 detergent, 0.52 mg of vaccinia virus in the absence or presence of 1.68 μM S-adenosyl[*methyl*-³H]methionine (8.5 Ci/mmol) was incubated at 37° for 30 min. Unincorporated radioactivity was removed by three cycles of trichloroacetic acid precipitation, and the RNA was digested with KOH and chromatographed on a DEAE–cellulose column (4). The arrows indicate the absorbance peaks of marker oligonucleotides, and the numbers above them represent their net charges.

terminus of vaccinia mRNA. However, the 5'-terminal phosphates were now found to be resistant to alkaline phosphatase, which suggested the presence of a blocking group. Treatment of intact [*methyl*-³H]RNA with this enzyme had no effect on the net charge of the labeled products obtained by alkali hydrolysis. Furthermore, when isolated material from peak II, which had a net charge of slightly more than −5, was treated with alkaline phosphatase and rechromatographed on DEAE–cellulose, most of the ³H and ³²P still eluted together but now with a net charge between −3 and −4 (Fig. 2B). This 2 charge reduction was consistent with the removal of only an unlabeled 3'-phosphate. Methyl-labeled material in peak I, which originally had a charge between −4 and −5, also lost 2 charges when treated with alkaline phosphatase (Fig. 2A). However, only a fraction of the ³²P co-chromatographed with the ³H and the remainder, presumably formed from unmethylated material that overlapped peak I, was converted to ³²Pi with a charge of −2. The −4 peak derived from RNA made in the absence of S-adenosylmethionine (Fig. 1A) was entirely converted to ³²Pi (not shown).

Isolation of Methylated Nucleotides. Peak II material that had been treated with alkaline phosphatase to remove the 3'-phosphate and purified by DEAE–cellulose chromatography as in Fig. 2B was completely resistant to spleen phosphodiesterase, a 5'-exonuclease. However, after venom phosphodiesterase treatment, three [³H]*methyl*-labeled nucleotides were detected on electrophoresis: one migrated with marker
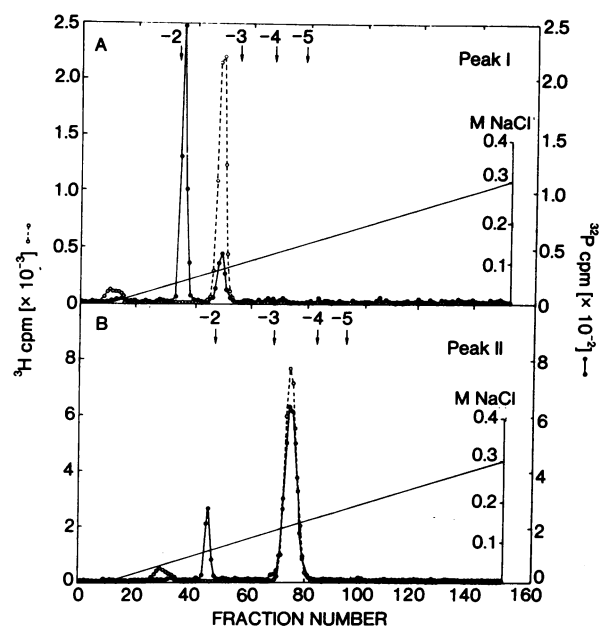

FIG. 2. DEAE–cellulose column chromatography of alkaline phosphatase-treated material in peaks I and II of Fig. IB. (A) Phosphatase-treated peak I material; (B) phosphatase-treated peak II material.

AMP; another with marker GMP; and the third, which was the only one also labeled with ³²P derived from [β,γ-³²P]GTP, migrated ahead of all the marker mononucleotides (Fig. 3). The latter nucleotide was eluted from the thin-layer sheet and shown to chromatograph on a DEAE–cellulose column as a nucleoside diphosphate (Fig. 4). A methylated nucleoside diphosphate and methylated derivatives of AMP and GMP could result from venom phosphodiesterase cleavage of MeN⁵'pp/p⁵'AMe/pN and MeN⁵'pp/p⁵'GMe/pN at the positions indicated by the slashes. The derivatives of AMP and GMP were thought to be methylated on the ribose because of previous evidence for 2'-O-methyl groups (4). The additional product of venom phosphodiesterase digestion, pN, could not be detected since it was unlabeled. An alternative model such as MeN⁵' ppp⁵' GMepAMepN seemed un-
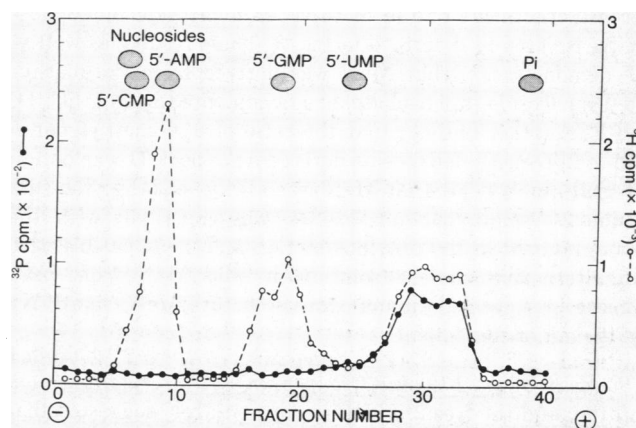

FIG. 3. Thin-layer electrophoresis of nucleotides produced by venom phosphodiesterase digestion of alkaline phosphatase-treated material in peak II. The −3 peak of Fig. 2B was isolated, desalted, treated with phosphodiesterase, and analyzed by electrophoresis in system A.
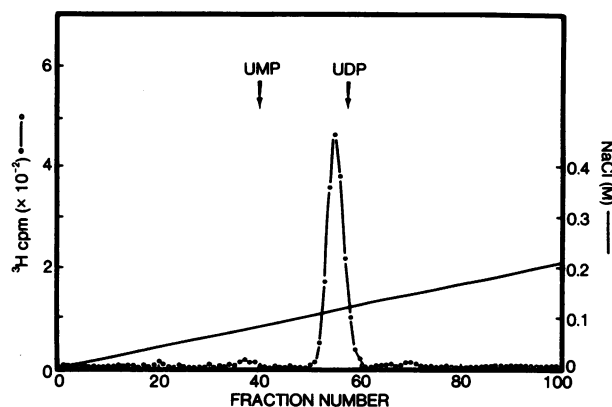
FIG. 4. DEAE–cellulose column chromatography of nucleoside diphosphate. The rapidly migrating nucleotide from Fig. 3 was chromatographed with UMP and UDP markers. ³²P was not indicated because of the lower levels of radioactivity.

likely because of its greater net charge and for additional reasons to be discussed.

Isolation of Methylated Nucleosides. Although venom phosphodiesterase digestion alone produced nucleotides, combined digestion with venom phosphodiesterase and alkaline phosphatase resulted in the formation of nucleosides. A single methylated nucleoside, designated MeN, was derived from peak I (Fig. 5A), whereas MeN plus two additional nucleosides were derived from peak II (Fig. 5B). The latter two nucleosides were identified as 2'-O-methyladenosine and 2'-O-methylguanosine by chromatography with authentic markers as indicated in Fig. 5B. MeN did not chromatograph with any of the 2'-O-methylribonucleoside markers, suggesting that it might be base-methylated. Since ribonucleosides with free cis-glycols complex with borate and migrate on electrophoresis toward the anode at pH 9 whereas 2'-O-methylribonucleosides migrate toward the cathode (10), this procedure provided a useful discriminatory test. As anticipated, MeN migrated toward the anode and the other two nucleosides migrated toward the cathode.

Additional experiments were done to correlate the results of this section with those of the previous one. The expected 2'-O-methylribonucleosides were produced by alkaline phosphatase treatment of the methylated nucleoside monophosphates isolated as described in Fig. 3. These results, as well as the demonstration that MeN was derived from the methylated nucleoside diphosphate, are documented in Fig. 5C, D, and E. Another finding, that the ratio of 2'-O-methylguanosine to 2'-O-methyladenosine derived from peak II was not an integral number but that MeN was always equal to the sum of the 2'-O-methylribonucleosides, was consistent with the model favored in the previous section and incompatible with the alternative model. Isolation of only MeN from peak I material suggested an analogous structure MeN⁵' ppp⁵' Np for the minor component.

Removal of the Blocking Group from mRNA. The terminal location and the free 2',3'-OH groups on MeN suggested that it should be possible to remove the latter nucleoside from vaccinia mRNA by periodate oxidation and β-elimination. As predicted, after such treatment the phosphates at the 5'-terminus of the RNA were susceptible to alkaline phosphatase and a major component with a net charge of slightly
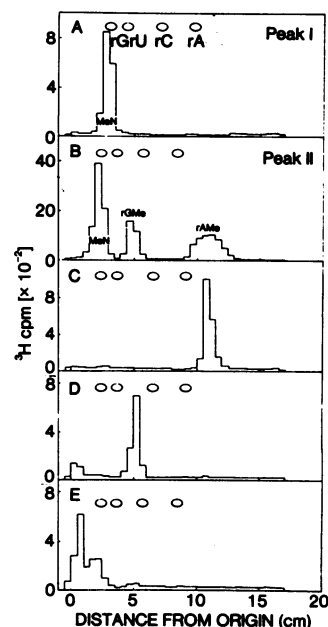

FIG. 5. Thin-layer chromatography of nucleosides produced by venom phosphodiesterase and alkaline phosphatase. Material of (A) peak I and (B) peak II of Fig. 1B was treated with phosphodiesterase and phosphatase. (C) Material in the peak corresponding to AMP, (D) in the peak corresponding to GMP, and (E) in the rapidly migrating peak of Fig. 3, was treated with phosphatase. Chromatography solvent A was used. The positions of the four internal ribonucleoside markers are indicated in each segment. The positions of 2'-O-methylguanosine (rGMe) and 2'-O-methyladenosine (rAMe) in relation to the radioactively labeled peaks were determined in a separate experiment but is indicated in (B).

more than −3, corresponding to GMepNp and AMepNp, was obtained by subsequent alkali hydrolysis and DEAE–cellulose chromatography (Fig. 6B). Control methylated RNA that had not undergone treatment to remove the blocking group was unaffected by alkaline phosphatase since following alkali hydrolysis the major product still had a charge of slightly more than −5 (Fig. 6A).

To prove that MeN was removed from the 5'-terminus of the RNA by periodate oxidation and β-elimination, the material with a net charge of −3 (Fig. 6B) was digested with a combination of alkaline phosphatase and venom phosphodiesterase and analyzed by thin-layer chromatography. 2'-O-Methyladenosine and 2'-O-methylguanosine were identified, whereas MeN was virtually absent (Fig. 7).

Identification of MeN. Our results thus far suggested that MeN was a base-methylated nucleoside. Accordingly, methylated vaccinia RNA was depurinated and analyzed by chromatography on a Dowex 50 (H⁺) column. Approximately half of the methyl-labeled material, derived from the 2'-O-methylribonucleosides, did not adsorb and the remainder eluted soon after the guanine marker, suggesting that it was a methylguanine derivative (Fig. 8A). By contrast, when the material from the −3 peak (Fig. 6B) was depurinated, a methylated purine was not detected (Fig. 8B).

The guanine derivative was identified as 7-methylguanine by chromatography in several systems (B, C, and D), one of which is shown in Fig. 9A. In addition, marker 7-methylguanosine migrated as MeN in the alkaline chromatography
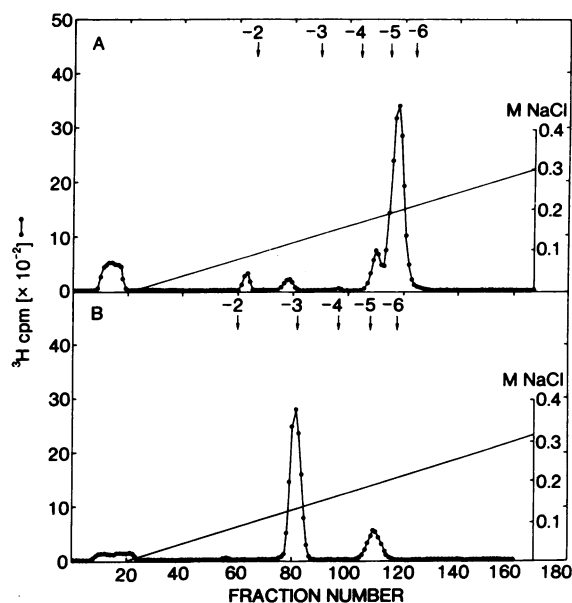
FIG. 6. DEAE–cellulose column chromatography of alkali hydrolysate of phosphatase-treated RNA. (A) Control, [³H]-methyl-labeled vaccinia mRNA was treated with phosphatase and then hydrolyzed with 0.3 M KOH. (B) Prior to phosphatase treatment and alkali hydrolysis, [³H]methyl-labeled RNA was subjected to periodate oxidation and aniline cleavage.

FIG. 8. Column chromatography of products of depurination reaction. [³H]Methyl-labeled RNA was mixed with 1 mg of yeast tRNA and heated in 1 M HCl at 100° for 30 min and chromatographed on a Dowex 50 (H⁺) column. (A) From intact RNA. (B) From −3 charge peak of Fig. 6B.

system used in Fig. 5. Actually 7-methylguanosine undergoes ring scission at neutral or alkaline pH values (11) and MeN was isolated predominantly in the ring-opened form as shown in Fig. 9B. This alteration may explain why, in a previous section, venom phosphodiesterase did not also cleave in the following manner: 7MeG⁵′p/pp⁵′MeG/pN.

DISCUSSION

We propose that the 5'-terminus of vaccinia mRNA, synthesized *in vitro* by purified vaccinia virus particles in the presence of S-adenosylmethionine, is blocked and has the unusual structure:

7MeG⁵′ppp⁵′GMepNp- and 7MeG⁵′ppp⁵′AMepNp-.
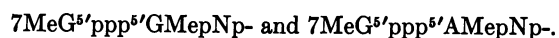
In addition, since vaccinia mRNA is on the order of 1000 nucleotides long (12) and there are 2.3 methyl groups per 1000 nucleotides (4), the majority of mRNA molecules must contain such a methylated terminus.
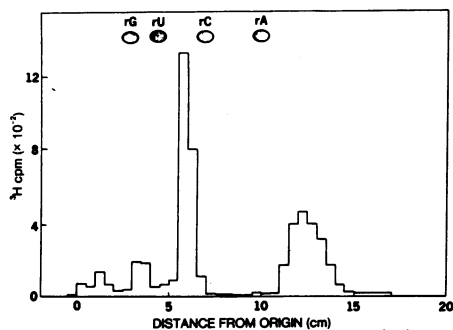
Most of our analyses were carried out on 7MeG⁵′ppp⁵′-GMepNp and 7MeG⁵′ppp⁵′AMepNp obtained by alkali hydrolysis of RNA labeled with [β,γ-³²P]GTP and S-adenosyl[*methyl-³H*]methionine. The 3'-terminal phosphate was removed with alkaline phosphatase and the remaining product was digested with venom phosphodiesterase to yield ³²P- and ³H-labeled pp7MeG, ³H-labeled pAMe and pGMe, and unlabeled pN. Formation of these products provided evidence for three phosphates in the pyrophosphate bridge. The number of phosphates had been a stumbling block since the

FIG. 7. Thin-layer chromatography of digest of material in −3 charge peak of Fig. 6B. The material in the −3 peak was isolated, desalted, and digested with a mixture of phosphatase and phosphodiesterase. Chromatography solvent A was used.
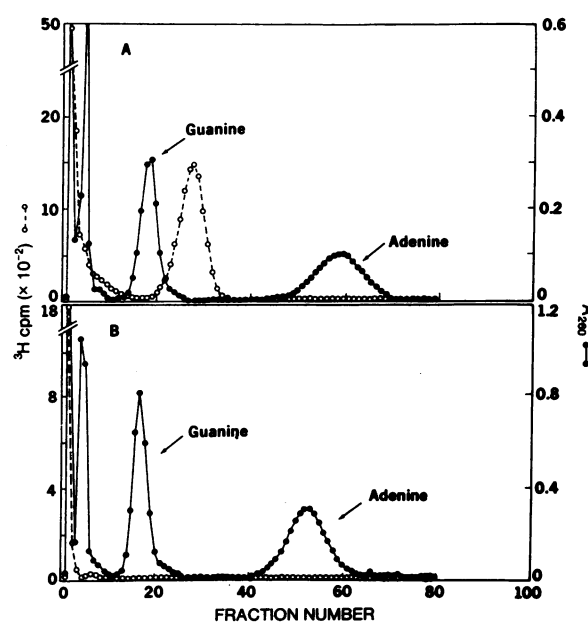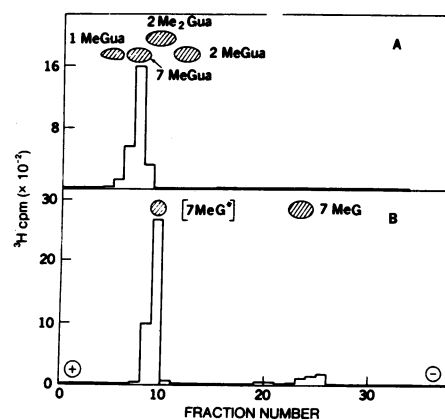

FIG. 9. Identification of 7-methylguanine and ring-open form of 7-methylguanosine. (A) Methylated vaccinia mRNA was depurinated and chromatographed in solvent B with methylated guanine standards. (B) Methylated RNA was digested with RNase A and T1 followed by a combination of alkaline phosphatase and venom phosphodiesterase, and MeN was then isolated by chromatography as in Fig. 5. Purified MeN, marker 7-methylguanosine (7MeG), and the ring-opened form [7MeG*], obtained by treatment of the latter with Tris·HCl (pH 8.5) or 0.3 M KOH, were analyzed by electrophoresis in system C.

net charge of the alkali hydrolysis fragments, as determined by DEAE–cellulose chromatography, was slightly more than −5 whereas a charge of −6 was predicted for this structure. Further digestion of the nucleotides with alkaline phosphatase produced 7-methylguanosine (ring-opened form), 2′-O-methyladenosine, and 2′-O-methylguanosine. Intact 7-methylguanine was isolated by depurination of RNA, indicating that the ring-opened form was an artifact of isolation. In another sequence of experiments, 7-methylguanosine was removed from RNA by periodate oxidation and β-elimination, leaving the unblocked terminal structures pppGMepNp- and pppAMepNp-, which were then susceptible to alkaline phosphatase digestion. A minor alkali hydrolysis product, 7MeG$^{5'}$ppp$^{5'}$Np, was derived from undermethylated RNA since it was not detected when saturating concentrations of S-adenosylmethionine were used for synthesis.

The terminal modification of vaccinia mRNA appears to occur post-transcriptionally since S-adenosylmethionine had no effect on the rate of mRNA synthesis *in vitro* (4) and, moreover, enzymes capable of this modification have been isolated and are being studied in our laboratory.

The possibility that 5′-termini of the double-stranded RNAs of cytoplasmic polyhedrosis virus and reovirus are blocked is under investigation (5, 7). Sequences quite similar to those of vaccinia mRNA have been proposed for the 5′-termini of certain low molecular weight, nonpolyadenylated RNA species of unknown function from nuclei of Novikoff hepatoma cells (13). One of these sequences is 2,2,7Me$_3$G$^{5'}$-pp$^{5'}$AMepUMep. Although Perry and Kelley (1) did not determine the location of the methylated nucleotides of L cell mRNA, a portion of the alkali digest eluted from DEAE–cellulose at 0.3 M NaCl, which suggested either an attached pyrophosphate group or an alkali-resistant internal sequence. More recently, the sequences 7MeG$^{5'}$ppp$^{5'}$NMep and 7-MeG$^{5'}$ppp$^{5'}$NMepNMep were found at the 5′-termini of HeLa cell mRNAs (C. M. Wei, A. Gershowitz, and B. Moss, submitted for publication). At present there is no evidence

for the function of modified 5′-termini, although the resistance of the blocked 5′-terminal oligonucleotide of vaccinia mRNA to spleen phosphodiesterase, a 5′-exonuclease, suggests one possibility.

Note Added in Proof. Additional evidence for the presence of three phosphates in the pyrophosphate bridge was obtained by digesting vaccinia mRNA with nuclease P$_1$ to yield 7Me-G$^{5'}$ppp$^{5'}$GMe and 7MeG$^{5'}$ppp$^{5'}$AMe. 7MeG was then removed by periodate oxidation and β-elimination, and pppGMe and pppAMe were identified by DEAE–cellulose chromatography.

1. Perry, R. P. & Kelley, D. E. (1974) *Cell* 1, 37–42.
2. Desrosiers, R., Frederici, K. & Rottman, F. (1974) *Fed. Proc.* 33, 1432.
3. Furuichi, Y. (1974) *Nucl. Acids Res.* 1, 809–822.
4. Wei, C. M. & Moss, B. (1974) *Proc. Nat. Acad. Sci., USA* 71, 3014–3018.
5. Shatkin, A. J. (1974) *Proc. Nat. Acad. Sci., USA* 71, 3204–3207.
6. Miura, K., Watanabe, K. & Sugiura, M. (1974) *J. Mol. Biol.* 86, 31–48.
7. Miura, K., Watanabe, K., Sugiura, M. & Shatkin, A. J. (1974) *Proc. Nat. Acad. Sci., USA* 71, 3979–3983.
8. Culp, L. & Brown, G. M. (1968), *Arch. Biochem. Biophys.* 124, 483–492.
9. Fraenkel-Conrat, H. & Steinschneider, A. (1967) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12, Sect. B, pp. 243–246.
10. Khym, J. X. (1967) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12, Sect. A, pp. 93–101.
11. Hall, R. (1971) *The Modified Nucleosides in Nucleic Acids* (Columbia University Press, New York).
12. Kates, J. & Beeson, J. (1970) *J. Mol. Biol.* 50, 1–18.
13. Ro-Choi, T. S., Reddy, R., Choi, Y. C., Raj, N. B. & Hennings, D. (1974) *Fed. Proc.* 33, 1832.