Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 742–745, February 1975

5′-Terminal m^{7}G(5′)ppp(5′)G^{m}p *In Vivo*: Identification in Reovirus Genome RNA

(7-methylguanosine/double-stranded RNA/methylation)

Y. FURUICHI, S. MUTHUKRISHNAN, AND A. J. SHATKIN

Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Communicated by Sidney Udenfriend, December 9, 1974

ABSTRACT **Methylated reovirus mRNA was synthesized *in vitro* in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]-methionine. Viral genome double-stranded RNA that was uniformly labeled with ^{32}P was isolated from purified virions. The RNAs were mixed and their 5′-terminal structures compared by electrophoretic and chromatographic analyses after enzymatic digestion. Both the mRNA and the corresponding strand in the genome RNA contain m^{7}G(5′)ppp(5′)G^{m}pCp, indicating that infected cells synthesize viral RNA with blocked, methylated 5′ termini.**

Blocked, methylated 5′-terminal structures were recently observed in the mRNAs synthesized *in vitro* in the presence of *S*-adenosylmethionine by the virion-associated polymerase of cytoplasmic polyhedrosis virus (CPV, refs. 1 and 2), reovirus (3, 4), and vaccinia virus (5–7). The blocking group was identified as 7-methylguanosine in 5′-5′-pyrophosphate linkage to a 2′-*O*-methylnucleotide, i.e., m^{7}G(5′)ppp(5′)A^{m}p and m^{7}G(5′)-ppp(5′)G^{m}p in CPV (2) and reovirus mRNA (4), respectively, and a mixture of the same structures with 2 (6) or 3 phosphates (7) in pyrophosphate linkage in vaccinia mRNAs. These unusual 5′-terminal structures were obtained in viral mRNAs produced *in vitro*. It was therefore of interest to determine if similar structures are present in RNA synthesized by infected cells.

MATERIALS AND METHODS

Reovirus type 3 Dearing strain was purified from infected mouse L cells as described previously (8). The double-stranded RNA was isolated by phenol extraction and gel filtration in Sephadex G-100 (9). Reovirus mRNA was synthesized *in vitro* with chymotrypsin-treated virions under standard conditions (10). For RNase T_2 digestion, genome RNA (about 200 μg) was denatured by heating for 30 min at 45° in 90% dimethyl sulfoxide, alcohol precipitated, and incubated with 50 units of enzyme in 0.1 ml of 0.01 M sodium acetate, pH 4.5, for 4 hr at 37°. To ensure complete hydrolysis, the digest was then heated for 30 sec at 90°, another 25 units (50 μl) of RNase T_2 was added, and the incubation continued for 1 hr at 37°. The digest was analyzed by chromatography on DEAE-cellulose in 7 M urea (4, 9). Conditions for enzymatic digestion of RNA and sources of Penicillium nuclease, alkaline phosphatase, and nucleotide pyrophosphatase were as described (4). Hydrolyzed samples were analyzed by high voltage paper electrophoresis in 0.66% morpholine/5% acetic acid (v/v), pH 3.5 at 2600 V for 40 min and by descending paper chromatography in isobutyric acid/0.5 N NH_4OH (10:6 v/v). β-Elimination by periodate oxidation and aniline treatment of [*methyl*-^{3}H]mRNA and oxidation followed by reduction with [^{3}H]borohydride of denatured genome RNA have been described (11–13). *S*-Adenosyl-L-[*methyl*-^{3}H]-methionine (specific activity = 8.5 Ci/mmol) and carrier-free [^{32}P]phosphoric acid were purchased from New England Nuclear and ^{3}H-labeled potassium borohydride (specific activity = 9.3 Ci/mmol) from Amersham/Searle. RNase T_2 (Sankyo) was purchased from Calbiochem.

Abbreviation: CPV, cytoplasmic polyhedrosis virus.

RESULTS

Isolation of the 5′-Terminal Structures from Genome and mRNA. Previously it was demonstrated that reovirus mRNA, synthesized *in vitro* by the virion-associated polymerase in the presence of *S*-adenosylmethionine, contains 5′-terminal m^{7}G(5′)ppp(5′)G^{m}pCp (4). The double-stranded genome RNA isolated from purified reovirions also was found to contain blocked 5′ termini in the plus strand, i.e., the chain corresponding to viral mRNA, but the identity of the blocking group was not determined (14, 15). To determine if the blocking moiety in the double-stranded RNA is the same as that in viral mRNA, genome RNA uniformly labeled with ^{32}P was mixed with ^{3}H-labeled mRNA synthesized *in vitro* in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The double-labeled mixture was digested with RNase T_2 and analyzed by column chromatography on DEAE-cellulose in 7 M urea. As shown in Fig. 1, the ^{3}H-labeled material eluted as a single symmetrical peak with a net negative charge close to −5 as observed previously for m^{7}GpppG^{m}pCp (4). In contrast, most of the [^{32}P]RNA was hydrolyzed to mononucleotides (−2 charge). No ^{32}P eluted with a −3 or −4 net charge, suggesting the absence of internal 2′-*O*-methylnucleotides. However, a fraction (0.2%) of the total ^{32}P eluted in the position of the 5′-terminal structures. The ^{32}P-labeled material appeared to be heterogeneous and also did not elute coincidentally with the ^{3}H-labeled 5′ termini.

Although the plus strand of reovirus genome RNA contains a blocked 5′-terminal structure, the 5′ end of the complementary (minus) strand has an unblocked sequence, ppGp-ApUp (14, 15). Treatment of this sequence with T_2 RNase yields ppGp which also elutes from DEAE-cellulose close to the position of net charge −5. The asymmetry of the ^{32}P peak in fractions 62–72 (Fig. 1) is consistent with the presence of two types of 5′-terminal structures of similar net charge: ppGp from the minus strand and m^{7}GpppG^{m}pCp from the plus strand. The two types of ^{32}P-labeled 5′-termini were separated by rechromatography of the indicated peak on DEAE-cellulose at pH 5.5 (Fig. 2). One peak of radioactivity, comprising about 40% of the ^{32}P associated with the 5′-termini eluted in 0.16 M sodium acetate. It was shown to be ppGp on the basis of electrophoretic mobility and release of

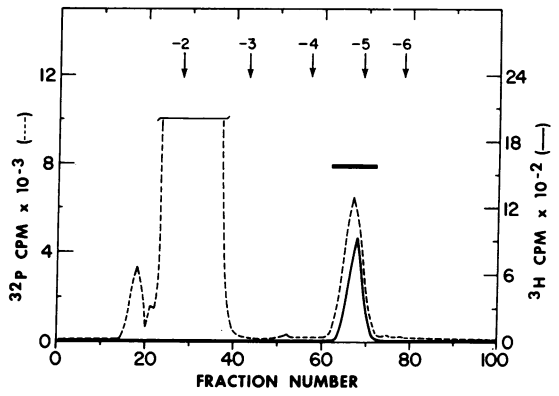

FIG. 1. Column chromatography of T_2 RNase digest of a mixture of [*methyl*-^{3}H]mRNA and double-stranded [^{32}P]RNA. Reovirus mRNA was synthesized *in vitro* with *S*-adenosyl-L-[*methyl*-^{3}H]methionine as labeled precursor and mixed with double-stranded RNA uniformly labeled with ^{32}P isolated from purified virions. After digestion with RNase T_2, the sample was applied to a column (1 × 20 cm) of DEAE-cellulose in 0.05 M Tris·HCl buffer, pH 8.0 that contained 7 M urea. Samples of 2 ml were eluted with a NaCl gradient of 0.05–0.3 M and aliquots of 0.03 ml were counted in 10 ml of Aquasol (New England Nuclear).

$^{32}P_i$ by alkaline phosphatase treatment (refs. 4 and 15, and data not shown). A second peak containing 50% of the ^{32}P co-eluted at 0.22 M NaCl with the single peak of ^{3}H-labeled m⁷GpppG^mpCp which was quantitatively recovered.

Identity of the 5'-Termini in the mRNA and Genome Plus Strand. The ^{3}H-labeled and ^{32}P-containing structures that co-eluted from DEAE-cellulose also migrated together when analyzed by high voltage paper electrophoresis (Fig. 3A). This material, presumptive 5'-terminal m⁷GpppGp^mCp, was digested with *Penicillium* nuclease, an enzyme which converts the terminal sequence in viral mRNA to m⁷GpppG^m, pC, and P_i (4). *Penicillium* nuclease hydrolyzed the ^{32}P-labeled termini to the same three constituents (Fig. 3B). Sixty percent of the ^{32}P migrated with the ^{3}H-labeled m⁷GpppG^m, 20% with authentic pC, and 20% with P_i. After incubation with *Penicillium* nuclease followed by alkaline phosphatase, the ^{32}pC was converted to $^{32}P_i$ (Fig. 3C). Again, 60% of the ^{32}P was resistant to hydrolysis and migrated with

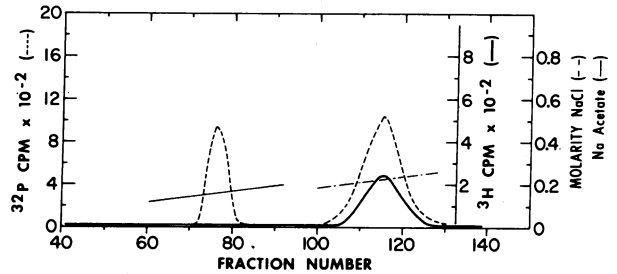

FIG. 2. Separation of 5'-terminal structures by DEAE-cellulose chromatography. Fractions 62–72 of Fig. 1 were pooled, diluted 5-fold with water, and adsorbed onto a 1 × 20-cm column of DEAE-cellulose. A gradient of 0.05–0.25 M Na acetate buffer at pH 5.5 containing 7 M urea (100 ml of each) was applied and samples of 2 ml were collected. The column was then eluted with a second gradient of 0.05–0.25 M NaCl in 0.05 M Na acetate buffer at pH 5.5 that contained 7 M urea, and an aliquot of each fraction was counted as in Fig. 1.

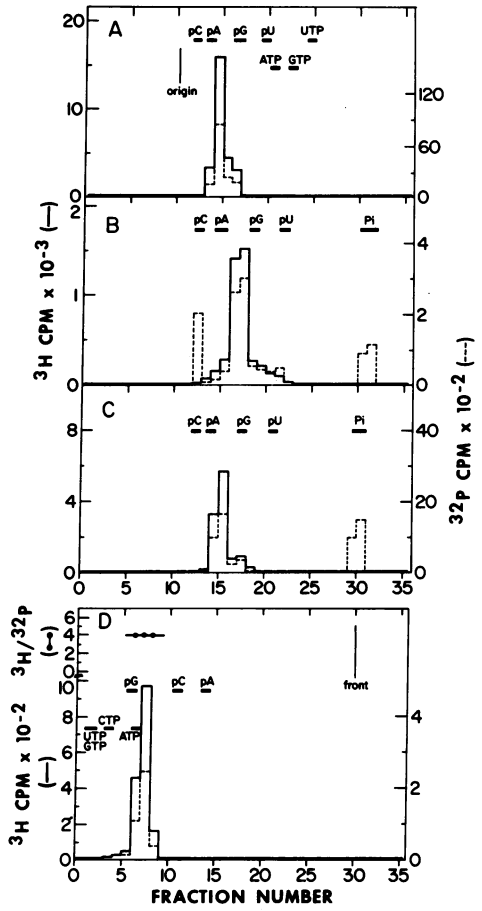

FIG. 3. Characterization of 5'-terminal structures by paper electrophoresis and chromatography. The peak of ^{3}H- and ^{32}P-labeled material eluting at 0.18–0.22 M NaCl (fractions 65–85) in Fig. 2 was desalted by adsorption to a DEAE-cellulose column (0.6 × 3 cm), which was washed with 0.1 M triethylammonium bicarbonate (5 column volumes) before elution in 1 M triethylammonium bicarbonate. The sample was lyophilized, dissolved in sterile water, and a portion analyzed by paper electrophoresis (A). Portions were also digested with *Penicillium* nuclease (22) (B) or *Penicillium* nuclease plus alkaline phosphatase (C) before electrophoresis. For panel (D), the nuclease- and phosphatase-resistant material in fractions 15 and 16 in (C) were eluted and analyzed by paper chromatography.

the ^{3}H-labeled m⁷GpppG^m. The mixture of ^{3}H- and ^{32}P-labeled 5'-termini in Fig. 3C was eluted and subjected to paper chromatography. The two isotopes moved as a single component, and the ratio of ^{3}H to ^{32}P was constant across the peak (Fig. 3D).

To confirm that the 5'-termini from ^{32}P-labeled genome RNA and ^{3}H-labeled mRNA are identical, the peak of m⁷GpppG^m in Fig. 3C was eluted, hydrolyzed with nucleotide pyrophosphatase, and re-analyzed by paper electrophoresis (Fig. 4a). Two ^{32}P-, ^{3}H-labeled peaks were obtained in the positions of authentic pm⁷G and pG^m. A third peak of ^{33}P migrating as P_i comprised about half of the ^{32}P, somewhat more than the expected one-third, presumably due to contaminating phosphatase activity. The two methylated, radioactive nucleotides were eluted and reanalyzed by descending chromatography. Both the ^{3}H-, ^{32}P-labeled pm⁷G (Fig. 4b) and pG^m (Fig. 4c) migrated with authentic samples.

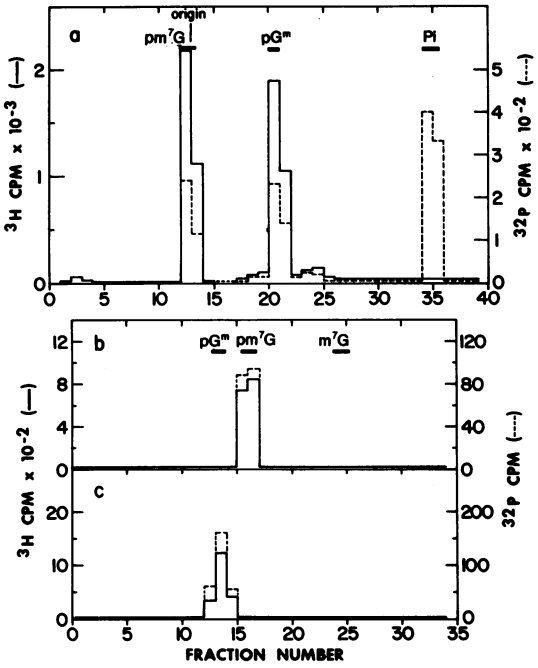

FIG. 4. Identification of pm^{7}G and pG^m in mRNA and double-stranded RNA 5′ termini. Another portion of the eluted ^{3}H-, ^{32}P-labeled 5′-terminal material (Fig. 3C) was digested with nucleotide pyrophosphatase and reanalyzed by paper electrophoresis (a). Peaks corresponding in position to pm^{7}G and pG^m were eluted and analyzed by paper chromatography in (b) and (c), respectively.

Estimation of the Number of Blocked 5′-Terminal Phosphates. The characteristics of the 5′-terminal m^{7}GpppG^m from reovirus mRNA are consistent with the presence of three blocked, phosphatase-resistant phosphates (4). However, 5′-terminal methylated sequences in Novikoff hepatoma cell nuclear RNA (16) and in vaccinia mRNA (6) have been reported to contain only two blocked phosphates. A direct test of the number of blocked, terminal phosphates in reovirus mRNA was possible because the m^{7}G which is in 5′-linkage to the pyrophosphate group contains free 2′,3′-hydroxyl groups and can be removed by β-elimination (4, 11). Viral mRNA was synthesized in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]-

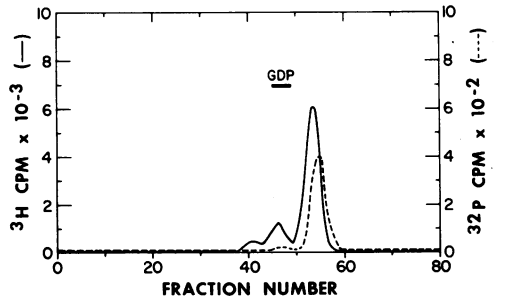

FIG. 5. Column chromatography of pppG^m derived by *Penicillium* nuclease digestion of β-eliminated [*methyl*-^{3}H]mRNA. Reovirus mRNA was synthesized *in vitro* in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine and purified by gel filtration. The RNA was subjected to periodate oxidation and β-elimination by aniline treatment. The β-eliminated mRNA was digested with *Penicillium* nuclease, mixed with [α ^{32}P]GTP and unlabeled GDP, and chromatographed on DEAE-cellulose in 7 M urea.

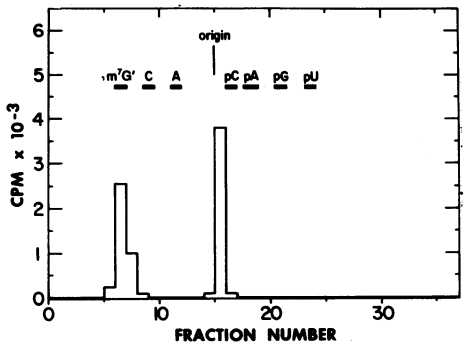

FIG. 6. Electrophoresis of ^{3}H-labeled m^{7}G trialcohol (m^{7}G′) from double-stranded RNA that had been reduced with ^{3}H-labeled borohydride. Purified virion double-stranded RNA was denatured with dimethyl sulfoxide, oxidized with periodate, and reduced with 20 mCi of ^{3}H-labeled potassium borohydride. The ^{3}H-labeled RNA was digested with *Penicillium* nuclease and alkaline phosphatase and the enzyme-resistant 5′-terminal structure was purified by paper electrophoresis as in Fig. 3. The 5′-terminal material was eluted, digested with nucleotide pyrophosphatase and alkaline phosphatase, and reanalyzed by paper electrophoresis with authentic marker compounds.

methionine, oxidized with periodate, and treated with aniline to remove the 5′-terminal blocking group. The treated RNA was digested with *Penicillium* nuclease and mixed with GDP and [α-^{32}P]GTP before analysis by chromatography on DEAE-cellulose in 7 M urea (Fig. 5). The ^{3}H-labeled methylated nucleotide eluted after GDP with the ^{32}P-labeled authentic GTP, although the elution positions of the peak fractions were slightly different, presumably due to 2′-*O*-methylation. Small amounts of ^{3}H-labeled 2′-*O*-Me-GDP and 2′-*O*-Me-GMP present in the sample were also resolved. Alkaline phosphatase digestion converted the ^{3}H-labeled material to pG^m and G^m (data not shown). The results demonstrate that there are three blocked phosphates at the 5′ end of reovirus mRNA and in the identical 5′ terminus of the plus strand of viral genome RNA.

5′ Linkage of m^{7}G in Genome RNA. The m^{7}G in reovirus mRNA is removed by β-elimination, indicating that it contains unblocked 2′,3′-hydroxyls, i.e., it is linked at the 5′ position to the terminal phosphates. The same type of linkage occurs in genome RNA. Double-stranded RNA was oxidized with periodate, reduced with ^{3}H-labeled borohydride and hydrolyzed with *Penicillium* nuclease and alkaline phosphatase. The enzyme-resistant, modified 5′-terminal m^{7}G′pppG^m was purified by high voltage paper electrophoresis as in Fig. 3C. It was eluted, digested with nucleotide pyrophosphatase and alkaline phosphatase, and reanalyzed by electrophoresis (Fig. 6). Half of the ^{3}H-labeled material migrated in the position of authentic m^{7}G trialcohol (m^{7}G′), and the remainder was of neutral charge at the origin. The latter presumably represents the modified, ring-opened structure of m^{7}G that forms 2-amino-4-hydroxy-5-(*N*-methyl)carboxamide-6-ribosylamino-pyrimidine at neutral and alkaline pH (4, 17).

DISCUSSION

Recent studies of a variety of animal cells and viruses indicate that methylation of mRNA is a general phenomenon. In mouse L fibroblast (18), monkey BSC-1 kidney cell (Lavi and Shatkin, unpublished results), Novikoff hepatoma cell (19)

and HeLa cell mRNA (Furuichi, Morgan, Shatkin, and Darnell, unpublished results), methylation occurs on both the base moieties and the 2′-hydroxyl position of ribose. Simian virus 40-specific mRNA from infected BSC-1 cells is also methylated, and the pattern of methylation is different in nuclear and cytoplasmic RNA (Lavi and Shatkin, unpublished results). Viral mRNAs synthesized *in vitro* in the presence of *S*-adenosylmethionine by the virion-associated transcriptases of CPV (1, 2), reovirus (3, 4), vaccinia virus (5–7) and vesicular stomatitis virus (20) are also methylated on both components of the constituent ribonucleotides to the extent of about two methyl groups per molecule. The methylated residues are exclusively at the 5′ ends of the viral mRNAs, and transcription *in vitro* by the CPV polymerase is coupled to methylation of the initiated RNA chains (1). The 5′ structures of the *in vitro* CPV mRNA (2) and reovirus mRNA (4) are m^{7}G(5′)-ppp(5′)A^{m}p and m^{7}G(5′)ppp(5′)G^{m}p, respectively. Vaccinia mRNAs contain m^{7}G(5′)p(p)p(5′)G^{m}p and m^{7}G(5′)p(p)p(5′)-A^{m}p (6, 7). Vesicular stomatitis virus mRNAs are also blocked at the 5′ termini by methylated nucleosides (20).

A blocked, methylated 5′ sequence has been found in the plus strand of the double-stranded genome RNA from purified reovirus (14, 15). Its structure is identical to that of the viral mRNA synthesized *in vitro* in an incubation mixture containing a methyl donor. Viral mRNA isolated from infected cells has the same 5′-terminal structure (Furuichi, La Fiandra, and Shatkin, unpublished results). Thus, reovirus-infected L cells are capable of producing RNA with the blocked 5′ sequence, m^{7}G(5′)ppp(5′)G^{m}p. This sequence is very similar to the 5′ structure, m$_3^{2,2,7}$G(5′)pp(5′)A^{m}p identified in the low-molecular-weight nuclear RNA isolated from Novikoff hepatoma cells (16). A single, internal 2′-*O*-methyladenosine is also present in the hepatoma nuclear RNA.

The functional importance of the 5′-terminal methylation of viral and cellular mRNAs may be at the level of transcription (1), translation (4), and/or processing of precursor molecules to mRNA (21). The contiguous location of m^{7}G and a 2′-*O*-methylnucleotide suggests that for cellular mRNA, the heterogeneous nuclear RNA may be 2′-*O*-methylated at a specific sequence, cleaved by an endonuclease at the site of methylation, and modified further by a capping reaction at the 5′ terminus in the resulting 3′ portion of the molecule (21). Messenger RNA with blocked 5′ sequences may be more stable to nucleolytic digestion. In addition, recognition of different mRNA molecules, i.e., viral as compared to cellular or viral early as compared to late mRNA, for protein synthesis may be at least partially determined by the nature of the 5′ sequence. It will be of interest to study the effect of methylation on the capacity of mRNA to bind to ribosomes and initiate polypeptide synthesis.

Note Added in Proof. Similar results were obtained for reovirus genome RNA by K.-I. Miura.

We thank Alba LaFiandra and Carol Carter for the preparations of purified ^{32}P-labeled reovirus.

1. Furuichi, Y. (1974) *Nucleic Acids Res.* **1**, 809–822.
2. Furuichi, Y. & Miura, K.-I. (1975) *Nature*, in press.
3. Shatkin, A. J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3204–3207.
4. Furuichi, Y., Morgan, M., Muthukrishnan, S. & Shatkin, A. J. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 362–365.
5. Wei, C. M. & Moss, B. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3014–3018.
6. Urushibara, T., Furuichi, Y., Nishimura, C. & Miura, K.-I. (1975) *FEBS Lett.*, in press.
7. Wei, C. M. & Moss, B. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 318–322.
8. Shatkin, A. J. & LaFiandra, A. J. (1972) *J. Virol.* **10**, 698–706.
9. Banerjee, A. K. & Shatkin, A. J. (1971) *J. Mol. Biol.* **61**, 643–653.
10. Both, G. W., Lavi, S. & Shatkin, A. J. (1975) *Cell*, in press.
11. Steinschneider, A. & Fraenkel-Conrat, H. (1966) *Biochemistry* **5**, 2735–2743.
12. Muthukrishnan, S. & Shatkin, A. J. (1975) *Virology*, in press.
13. Furuichi, Y. & Miura, K.-I. (1973) *Virology* **55**, 418–425.
14. Miura, K.-I., Watanabe, K., Sugiura, M. & Shatkin, A. J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3979–3983.
15. Chow, N.-L. & Shatkin, A. J. (1975) *J. Virol.*, in press.
16. Reddy, R., Ro-Choi, T. S., Henning, D. & Busch, H. (1974) *J. Biol. Chem.* **249**, 6486–6494.
17. Haines, J. A., Reese, C. B. & Todd, A. R. (1962) *J. Chem. Soc.*, 5281.
18. Perry, R. R. & Kelley, D. E. (1974) *Cell* **1**, 37–42.
19. Desrosiers, R., Friderici, K. & Rottman, F. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3971–3975.
20. Rhodes, D. P., Moyer, S. A. & Banerjee, A. K. (1974) *Cell* **3**, 327–333.
21. Rottman, F., Shatkin, A. J. & Perry, R. R. (1974) *Cell* **3**, 197–199.
22. Fujimoto, M., Kuminaka, A. & Yoshino, H. (1974) *Agr. Biol. Chem.* **38**, 1555–1561.