Inhibition of initiation of protein synthesis by 7-methylguanosine-5'-monophosphate

(protein synthesis/methylated nucleotides/modified 5'-termini)

EILEEN D. HICKEY, LEE A. WEBER, AND CORRADO BAGLIONI*

Department of Biological Sciences, State University of New York, Albany, N.Y. 12222

ABSTRACT   Translation of rabbit globin mRNA in a wheat germ protein-synthesizing system is inhibited by the nucleotide   7-methylguanosine-5'-monophosphate   (m^{7}G^{5}p) but not by other guanosine nucleotides without the 7-methyl group or with the phosphate in a different position. Translation of RNA of tobacco mosaic virus and poly(A) + HeLa RNA is also inhibited by m^{7}G^{5}p. We show that m^{7}G^{5}p prevents the association of mRNA with ribosomal subunits to form an initiation complex. We propose that m^{7}G^{5}p interacts with a site on initiation factor(s) or ribosomes which is involved in mRNA recognition, presumably by binding to the 5'-terminal sequence m^{7}G^{5}ppp. m^{7}G^{5}p does not inhibit translation of poly(U) and RNA of satellite tobacco necrosis virus, which do not have the 5'-terminal sequence m^{7}G^{5}ppp. In the case of RNA of satellite tobacco necrosis virus, some stimulation of its translation is consistently observed in the presence of m^{7}G^{5}p; possible interpretations of this finding are discussed.

Rottman et al. (1) have described a dinucleotide at the 5'-terminus of a variety of mRNAs that consists of 7-methylguanosine linked through its 5'-hydroxyl group via a triphosphate to the 5'-hydroxyl group of a ribosemethylated nucleotide. The 5'-terminal sequence m^{7}G^{5}ppp . . . has been reported for reovirus (2), vesicular stomatitis (3), vaccinia (4), and silkworm polyhedrosis virus RNAs (5) and for rabbit globin (6), HeLa (7), myeloma (8), and L cell mRNAs (6). These reports suggest that methylated blocked 5'-termini may be a rather general feature of eukaryotic mRNA.

Both et al. (9) have demonstrated a specific role for the 7-methyl group of the 5'-terminal guanosine of reovirus RNA. Viral RNA can be synthesized in vitro by virion-associated RNA polymerase. This RNA is methylated when S-adenosylmethionine is added to the incubation mixture, but is unmethylated when S-adenosylhomocysteine, an inhibitor of methylation, is added. The methylated RNA is translated effectively and with fidelity by a wheat germ protein-synthesizing system; whereas the unmethylated RNA is translated only after being methylated during the incubation in the wheat germ extract (9). Addition of S-adenosylhomocysteine to the wheat germ extract prevents methylation and abolishes translation of unmethylated reovirus RNA. Similarly, the removal of the 5'-terminal m^{7}G^{5}p from rabbit globin mRNA causes a loss of the template activity of this mRNA (6). The presence of the methylated 5'-terminal nucleotide seems to be specifically required for the interaction

of reovirus RNA with ribosomal subunits in the formation of initiation complexes (10). A functional role for the m^{7}G^{5}p . . . nucleotide sequence in mRNA translation has not yet been directly shown. It seems possible, however, that this group acts as a recognition signal for mRNA. We report here that this is the most likely function of the 5'-end of mRNA by showing that the nucleotide m^{7}G^{5}p specifically inhibits translation of mRNAs with the sequence m^{7}G^{5}ppp. . ., but not of other RNAs that do not have this group at the 5'-terminus. Other analogs of guanosine do not show inhibitory activity.

MATERIALS AND METHODS

7-Methylguanosine and 7-methylguanosine monophosphates were purchased from Terra-Marine Bioresearch (La Jolla, Calif.). Globin mRNA was isolated by repeated sucrose gradient centrifugation as described (11). Tobacco mosaic virus (TMV) was given to us by Dr. Bryan E. Roberts, Massachusetts Institute of Technology. TMV RNA was prepared by phenol extraction (12). Poly(A) + RNA was prepared from HeLa cell polysomes as described (13). Reovirus RNA synthesized by virions and labeled with S-adenosyl[CH$_3$-^{3}H]methionine (10) was a gift of Gerald W. Both and Aaron J. Shatkin of the Roche Institute of Molecular Biology, Nutley, N.J. Satellite tobacco necrosis virus RNA was given to us by Dr. Abraham Marcus, Institute for Cancer Research, Fox Chase, Philadelphia. Sparsomycin was a gift of Upjohn Co., Kalamazoo, Mich. Radioactive amino acids were purchased from New England Nuclear.

Cell-Free Protein Synthesis. Previously published methods were used to prepare the wheat germ extract (14). The wheat germ protein-synthesis assay contains in 50 μl: 15 μl of wheat germ extract, 1.2 mM ATP, 0.25 mM GTP, 15 mM creatine phosphate, 0.67 unit of creatine phosphokinase (Sigma), 22 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (Hepes)/KOH pH 7.1, 3.0 mM Mg-acetate, 0.1 M KCl, 2.6 mM dithiothreitol, 1.8 mM mercaptoethanol, 50 μM unlabeled amino acids minus lysine or phenylalanine, and 5 μCi of [^{3}H]lysine (38 Ci/mmol) or [^{3}H]phenylalanine (59 Ci/mmol). RNA, guanosine nucleotides, and inhibitors were added at the concentrations indicated in the figure legends. The incubations were carried out at 24°, and 5-μl samples were taken in duplicate before starting the incubation and at the specified time intervals. The samples were processed for determination of radioactivity as described (15).

Binding of Reovirus [^{3}H]RNA to Initiation Complexes. The binding reactions were carried out in 50 μl of the wheat germ cell-free system containing 50 μM unlabeled amino acids, 0.1 mM sparsomycin, 2500 cpm of reovirus
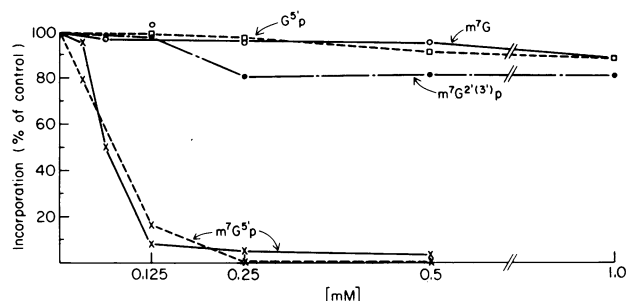
FIG. 1. Inhibition of translation of rabbit globin mRNA by m⁷G⁵′p. The incubations were carried out in the wheat germ cell-free system as described in *Materials and Methods*. Aliquots (5 μl) were sampled in duplicate after 60 min at 24°. Globin mRNA was added at 13 μg/ml in all incubations except those measuring endogenous incorporation (X- - -X). The concentration of the guanosine nucleotides added is indicated on the abscissa. Translation in the presence of the nucleotides is expressed as % of the untreated control; the endogenous incorporation of the control was 1160 cpm. The control incorporation with globin mRNA added was 37,000 cpm.

[³H]mRNA (specific activity 8000 cpm/μg), and the indicated concentration of m⁷G⁵′p. After 10 min at 24° the incubations were stopped by addition of 0.45 ml of 1 mM cycloheximide in 0.1 M KCl, 20 mM Tris·HCl (pH 7.5), and 5 mM Mg-acetate. The samples were applied to 15–30% sucrose gradients in the same buffer and centrifuged at 22,000 rpm in a Beckman SW-27 rotor. The A_{260} of the gradients was monitored in a Gilford recording spectrophotometer, and the radioactivity of the fractions collected was determined after addition of 12 volumes of 1 part Triton X-100, 2 parts 0.8% butyl-PBD (C.I.B.A.) in toluene, and 10% water.

RESULTS

The presence of a 5′-terminal m⁷G⁵′p in rabbit globin mRNA affects its translation by wheat germ extracts (6). When the 5′-terminal m⁷G⁵′p is removed by periodate oxidation and β-elimination, the mRNA loses most of its ability to stimulate protein synthesis (6). In experiments directed at establishing a specific role for m⁷G⁵′p in protein synthesis, we have translated rabbit globin mRNA in the presence of mononucleotide analogs of the 5′-terminal end of this mRNA. Only m⁷G⁵′p inhibits protein synthesis, whereas the other compounds tested (m⁷G, m⁷G²⁽³⁾′p, and G⁵′p) show negligible inhibition and only at very high concentrations (Fig. 1). This indicates that the chemical groups essential for
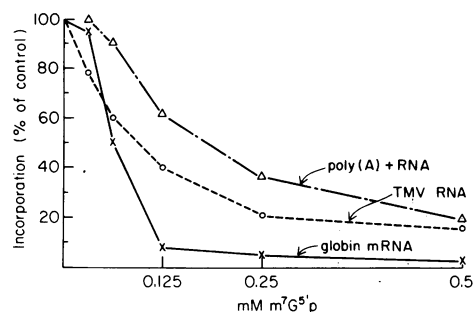
FIG. 2. Inhibition of translation of TMV RNA and poly(A) + HeLa cell RNA by m⁷G⁵′p. The incubations are carried out as described in Fig. 1. TMV RNA is added at 80 μg/ml; poly(A) + RNA is added at 36 μg/ml. Translation in the presence of m⁷G⁵′p is expressed as % of the untreated control; the control incorporation for TMV RNA is 41,143 cpm, and for poly(A) + RNA 11,960 cpm. The curve for globin mRNA translation is the same shown in Fig. 1.

inhibition of globin mRNA translation by m⁷G⁵′p are the methyl group in position 7 and the phosphate in 5′. Compounds with the phosphate group in a different position or without the methyl group show no inhibition. Endogenous protein synthesis of the wheat germ extract is also inhibited by m⁷G⁵′p (Fig. 1).

We have observed inhibition of translation of other mRNAs by m⁷G⁵′p in wheat germ extracts and also in a different cell free system. Translation of TMV RNA and of HeLa cell poly(A) + RNA in the wheat germ extract is inhibited, though incompletely or at slightly higher concentrations of the inhibitor (Fig. 2). Poly(A) + RNA of HeLa cells has been shown to have 5′-terminal m⁷G⁵′p (7). Protein synthesis programmed by endogenous mRNA in a HeLa cell extract is also inhibited by m⁷G⁵′p (to be published elsewhere).

Mechanism of inhibition of protein synthesis by m⁷G⁵′p

A direct demonstration that m⁷G⁵′p acts at the level of initiation of protein synthesis has been obtained by studying the formation of initiation complexes between reovirus RNA labeled in the methyl group of the 5′-terminal guanosine (2) and wheat germ ribosomes (Fig. 3). The binding reaction is carried out in the presence of sparsomycin, an inhibitor of elongation, as suggested by Darnbrough *et al.* (16). In the control experiment (Fig. 3A) 80% of the labeled RNA becomes associated with ribosomes. However, the association of reovirus RNA with ribosomes is partially inhibited by the presence of 0.125 mM m⁷G⁵′p (Fig. 3B) and is inhibited by
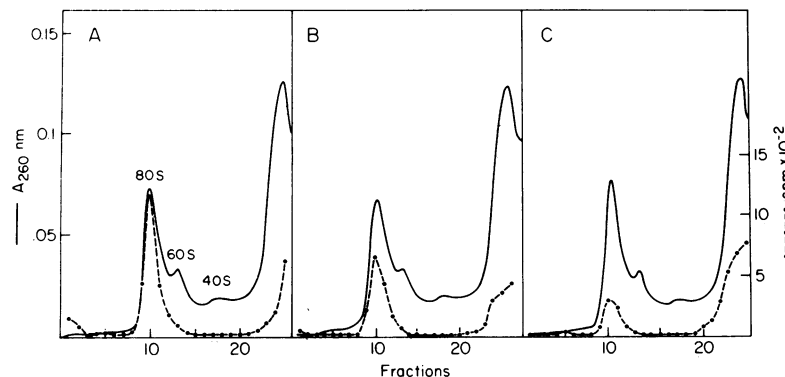

FIG. 3. Binding of reovirus [³H]RNA to the 80S initiation complex. The binding assay is carried out as indicated under *Materials and Methods*. The incubation mixtures are analyzed by sucrose gradient centrifugation. (A) Control; (B) 0.125 mM; and (C) 0.5 mM m⁷G⁵′p.

Biochemistry: Hickey et al.
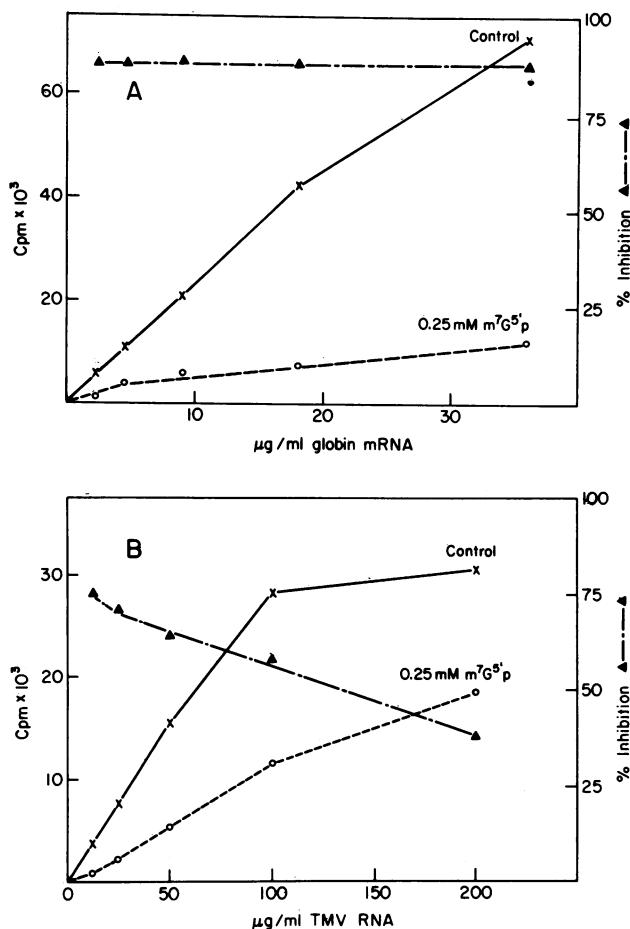
Proc. Nat. Acad. Sci. USA 73 (1976)   21

FIG. 4. The effect of globin mRNA (A) and TMV RNA (B) concentration on the inhibition of translation by $m^7G^{5'}p$. The assays are carried out in the wheat germ cell-free system as described in the legend of Fig. 1.

FIG. 5. Translation of STNV RNA and of poly(U) in the presence of $m^7G^{5'}p$. The incubations are carried out as described in the legend of Fig. 1. STNV RNA is added at 20 μg/ml and, in a separate experiment, at 50 μg/ml. The values reported as % of control (4186 and 6417 cpm, respectively) are the average of the two experiments; the endogenous incorporation in the presence of each concentration of $m^7G^{5'}p$ has been subtracted. Poly(U) is added at 20 μg/ml; the control incorporation with poly(U) is 4529 cpm of [³H]phenylalanine incorporated and the endogenous incorporation is 366 cpm. The globin mRNA translation curve is the same shown in Fig. 1 and is presented here for the sake of comparison.

over 70% by 0.5 mM $m^7G^{5'}p$ (Fig. 3C). No binding of reovirus RNA to 80S initiation complexes occurs in an incubation mixture kept at 0° (not shown). The RNA that does not bind to the initiation complexes sediments at the top of the gradients because of degradation during the incubation. Several other labeled mRNAs have been shown to be degraded in a 10-min incubation (our unpublished observations). This degradation does not affect our results since the label in the reovirus RNA that we have used is in the 5'-terminal nucleotide, which is protected once the RNA is bound to ribosomes (10).

$m^7G^{5'}p$ has no effect on the binding of initiator Met-tRNAf to the 40S ribosomal subunit (to be published elsewhere). It seems possible that $m^7G^{5'}p$ interferes with the binding of mRNA in the formation of the 80S initiation complex by occupying one site with which the 5'-terminal nucleotide of mRNA interacts. This implies that the 5'-terminal nucleotide acts as a specific recognition signal during initiation. This suggestion is supported by the experiments of Both et al. (9), who have shown that unmethylated reovirus RNA is not translated by a wheat germ extract, whereas methylated reovirus RNA is efficiently translated. Messenger RNA and $m^7G^{5'}p$ may thus compete for binding to the same site with different affinity. We have calculated that the concentration of $m^7G^{5'}p$ at which we observe 50% inhibition of globin mRNA translation (0.063 mM; Fig. 1) is at least 1000-fold higher than the molar concentration of
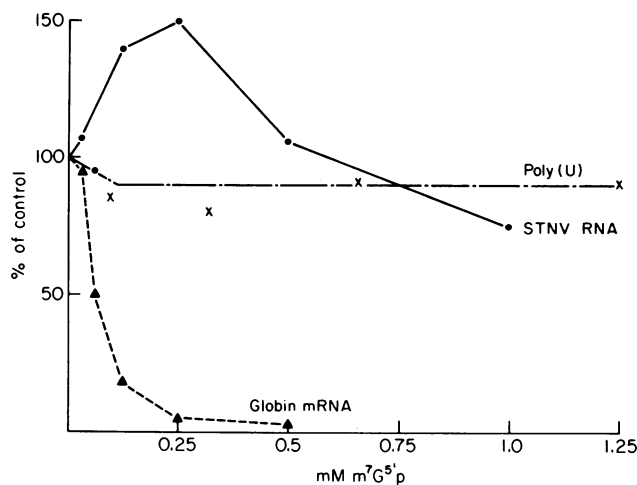
mRNA in the assay. We have used 220,000 as the molecular weight for globin mRNA (17) and have made the assumption that all the RNA added to the wheat germ extract is pure and active globin mRNA. In so doing, we have presumably overestimated the concentration of mRNA.

We postulate that $m^7G^{5'}p$ inhibits mRNA translation by binding to a site that interacts with the 5'-terminus of mRNA. We have attempted to learn more about this interaction by translating increasing concentrations of mRNA in the presence of a constant inhibitory concentration of $m^7G^{5'}p$ (Fig. 4). In the case of globin mRNA we have observed that the inhibition is constant over a 10-fold range of mRNA concentration (Fig. 4A). This can be explained by assuming that $m^7G^{5'}p$ inhibits an initiation factor in a dose-dependent fashion. Even in the presence of relatively high concentrations of the inhibitor there is some active factor that can participate in the initiation process. Translation of globin mRNA is linearly related to the amount of mRNA added under these conditions (Fig. 4A).

In the case of TMV RNA we find that the inhibition by $m^7G^{5'}p$ is overcome by increasing the RNA concentration (Fig. 4B). We propose a possible explanation for the discrepancy between the result obtained with globin mRNA and that with TMV RNA. We have seen previously that $m^7G^{5'}p$ does not completely inhibit translation of TMV RNA even at the highest concentrations tested (Fig. 2). This raises the possibility that TMV RNA is heterogeneous with respect to inhibition of translation by $m^7G^{5'}p$. The result of the experiment shown in Fig. 4B may be explained by the addition of increasing amounts of RNA moieties that are not inhibited by $m^7G^{5'}p$. Since these molecules may not have this methylated nucleotide at the 5'-terminus (see Discussion), it becomes extremely relevant to establish whether the translation of this type of RNA is inhibited by $m^7G^{5'}p$.

Satellite tobacco necrosis virus (STNV) RNA is unique among a number of mRNAs in that it has a 5'-terminal se-

quence of either ppApGpUp... (18, 19) or pppApGpUp... (20). Furthermore, accurate translation of STNV RNA occurs without the attachment of a 5'-terminal $m^7G^{5'}p$ moiety (J. Brooker, S. N. Seal, and A. Marcus, in preparation). It is therefore of interest to ascertain whether or not $m^7G^{5'}p$ has any effect on the translation of this mRNA. As shown in Fig. 5, in contrast to the results obtained with other mRNAs, STNV RNA translation is not inhibited, but is instead stimulated about 50% by relatively low concentrations of $m^7G^{5'}p$.

Both *et al.* (9) have shown that unmethylated reovirus RNA can be methylated by an endogenous methylating enzyme of the wheat germ and is then translated as well as methylated reovirus RNA. In some incubations we have added 0.32 mM S-adenosylhomocysteine, a competitive inhibitor of this enzyme (9), to the wheat germ extract. At this concentration S-adenosylhomocysteine prevents translation of unmethylated reovirus RNA (9). We have observed no effect on the translation of STNV RNA, TMV RNA, globin mRNA, and sea urchin egg mRNA in the presence of S-adenosylhomocysteine (R. Enos, L. Weber, and C. Baglioni, unpublished observations).

The translation of poly(U) is not inhibited by $m^7G^{5'}p$ (Fig. 5). We have translated poly(U) under the same ionic conditions used for translation of natural mRNAs. At the relatively low Mg^{2+} concentration used, stimulation of protein synthesis by poly(U) is relatively modest, about 12-fold over the endogenous incorporation at 20 μg/ml of poly(U). This result supports our hypothesis that $m^7G^{5'}p$ inhibits translation of only those RNAs having this nucleotide at the 5'-terminus.

DISCUSSION

The experiments described above show that $m^7G^{5'}p$ can block the translation of some mRNAs, whereas it does not inhibit the translation of RNAs that lack the $m^7G^{5'}ppp$... 5'-terminal sequence. Therefore, $m^7G^{5'}p$ does not inhibit protein chain elongation. We have shown that $m^7G^{5'}p$ inhibits the binding of labeled reovirus mRNA to 80S initiation complexes. The inhibitory activity of guanosine nucleotides requires both the presence of the methyl group in the 7 position and the 5' phosphate. We have not yet tested the activity of $m^7G^{5'}pp$ or $m^7G^{5'}ppp$. These compounds may prove to be even more effective inhibitors, since they more closely resemble the 5'-terminus of mRNA than $m^7G^{5'}p$.

Translation of rabbit globin and HeLa poly(A) + RNA is inhibited by $m^7G^{5'}p$. These RNAs are known to have 5'-terminal $m^7G^{5'}ppp$ (6, 7). Translation of TMV RNA is also inhibited by $m^7G^{5'}p$. While this manuscript was being prepared for publication, we learned that Keith and Frankel-Conrat (21) and Zimmern (22) have examined the 5'-terminal sequence of TMV RNA by the methods introduced for the study of other methylated viral and mammalian RNAs, and have determined that the majority of the molecules have $m^7G^{5'}p$ at the 5'-terminus. Translation of TMV RNA is inhibited by $m^7G^{5'}p$ to a less degree than globin mRNA (Fig. 2). We believe that TMV RNA is a homogeneous template of molecular weight about 2×10^6 (12), containing enough nucleotides to code for 10 proteins the size of rabbit globin. We find that about 10–15% of the translational activity of TMV RNA is resistant to $m^7G^{5'}p$ inhibition. The RNA translated in the presence of $m^7G^{5'}p$ may correspond to molecules that do not have this nucleotide at the 5'-terminus; hidden breaks in TMV RNA may result in the formation of these molecules (22). Another explanation for the translation

of TMV RNA being resistant to $m^7G^{5'}p$ is worth mentioning. TMV RNA is a polycistronic mRNA, and it is possible that initiation of polypeptide chains can take place at internal sites, though the preferred initiation site involves the $m^7G^{5'}ppp$... 5'-terminal sequence.

The observation that the translation of STNV RNA is not inhibited by $m^7G^{5'}p$ demonstrates the specificity of this inhibitor and also raises the possibility of the existence of separate mechanisms for the initiation of protein synthesis for classes of mRNAs that contain different 5'-termini. This has intriguing possibilities as a potential translational control mechanism. The stimulation of STNV RNA translation by $m^7G^{5'}p$ is rather surprising. A possible explanation for this observation is that $m^7G^{5'}p$ inhibits translation of endogenous mRNA or mRNA fragments present in the wheat germ extract, and thus eliminates competition between this RNA and STNV RNA.

Translation of HeLa poly(A) + RNA in the wheat germ extract does not appear to be completely inhibited at high $m^7G^{5'}p$ concentrations. This result is open to different interpretations. One possibility is that, in the heterogeneous populations of mRNAs present in the poly(A) + RNA, there are mRNAs that do not possess the $m^7G^{5'}ppp$... terminal sequence and are not inhibited by $m^7G^{5'}p$. Whether the fraction of HeLa cell mRNA resistant to $m^7G^{5'}p$ inhibition codes for proteins different from those coded for by the HeLa mRNA that is inhibited is a matter of speculation.

We believe that $m^7G^{5'}p$ will be a very useful tool in elucidating the role of methylated 5'-termini of mRNA in protein synthesis. Our preliminary results suggest that this compound might be used to determine whether a template for protein synthesis has the 5'-terminal sequence $m^7G^{5'}ppp$.... It seems possible, moreover, that labeled $m^7G^{5'}p$ can be used to establish whether a specific initiation factor or a ribosomal protein interacts with the 5'-terminus of mRNA.

1. Rottman, F., Shatkin, A. J. & Perry, R. P. (1974) *Cell* 3, 197–199.
2. Furuichi, Y., Muthukrishnan, S. & Shatkin, A. J. (1975) *Proc. Nat. Acad. Sci. USA* 72, 742–745.
3. Abraham, G., Rhodes, D. P. & Banerjee, A. K. (1975) *Nature* 255, 37–40.
4. Wei, C. M. & Moss, B. (1975) *Proc. Nat. Acad. Sci. USA* 72, 318–322.
5. Furuichi, Y. & Miura, K. I. (1975) *Nature* 253, 374–375.
6. Muthukrishnan, S., Both, G. W., Furuichi, Y. & Shatkin, A. J. (1975) *Nature* 255, 33–37.
7. Furuichi, Y., Morgan, M., Shatkin, A. J., Jelinek, W., Dalditt-Georgiev, M. & Darnell, J. E. (1975) *Proc. Nat. Acad. Sci. USA* 72, 1904–1908.
8. Adams, J. & Cory, S. (1975) *Nature* 255, 28–33.
9. Both, G. W., Banerjee, A. K. & Shatkin, A. J. (1975) *Proc. Nat. Acad. Sci. USA* 72, 1189–1193.
10. Both, G. W., Furuichi, Y., Muthukrishnan, S. & Shatkin, A. J. (1975) *Cell* 6, 185–195.
11. Jacobs-Lorena, M. & Baglioni, C. (1972) *Proc. Nat. Acad. Sci. USA* 69, 1425–1428.
12. Roberts, B. E., Mathews, M. B. & Bruton, C. J. (1973) *J. Mol. Biol.* 80, 733–742.
13. Pemberton, R. E., Liberti, P. & Baglioni, C. (1975) *Anal. Biochem.* 66, 18–28.

14. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2330–2334.
15. Jacobs-Lorena, M. & Baglioni, C. (1973) *Eur. J. Biochem.* **35**, 559–565.
16. Darnbrough, C. H., Legon, S., Hunt, T. & Jackson, R. J. (1973) *J. Mol. Biol.* **76**, 379–403.
17. Gaskill, P. & Kabat, D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 72–75.
18. Wimmer, E. & Reichmann, M. (1968) *Science* **160**, 1452–1454.
19. Lesnaw, J. & Reichmann, M. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 140–145.
20. Horst, J., Frankel-Conrat, H. & Mendeles, S. (1971) *Biochemistry* **10**, 4748–4752.
21. Keith, L. & Frankel-Conrat, H. (1975) *FEBS Lett.* **57**, 31–33.
22. Zimmern, D. (1975) *Nucleic Acid Res.* **2**, 1189–1201.