Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2734–2738, July 1977
Biochemistry

Importance of 5′-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis*

(eukaryotic mRNA/confronting nucleotide structure/tobacco pyrophosphatase)

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Communicated by Motoo Kimura, April 19, 1977

ABSTRACT **The 7-methylguanylic acid residue confronting the 5′-terminal nucleotide of mRNA through two pyrophosphate linkages was completely removed by tobacco pyrophosphatase from mRNAs of cytoplasmic polyhedrosis virus, tobacco mosaic virus (viral RNA), and globin without any scission in the inner part of the RNA chain. Protein synthesis ability in a wheat germ cell-free system was lost after this treatment of all three kinds of mRNA. The initiation complexes for protein synthesis of these three RNAs were not obtained after using tobacco phosphodiesterase-treated mRNA. On incubation of mRNA in a wheat germ extract, the mRNA lacking m^7G was quickly degraded from the 5′ terminus in an exonucleolytic way, whereas the intact mRNA remained stable. These results show that one of the confronting nucleotide structure's functions is to stabilize the mRNA, to prevent its degradation.**

Since the discovery (1, 2) of the methylated blocked structure—the confronting nucleotide structure—at the 5′ terminus of the mRNA strand of cytoplasmic polyhedrosis virus (CPV), $m^7G^5′pppAm$-, the presence of a similar modified structure became known to be general for mRNAs in a eukaryotic system (3). Although there are some exceptional cases, the confronting structure is considered to function in protein synthesis. Some experiments have already suggested this possibility as follows. When pm^7G was added to an *in vitro* protein synthesizing system, translation of the mRNA carrying the methylated blocked structure was inhibited but the translation of the mRNA without the m^7G modified structure was not inhibited (4–6). Removal of the m^7G residue by β-elimination from the native mRNA resulted in a decrease in protein synthesizing ability as well as in ribosome binding ability (7–11). Similar results were obtained with viral mRNA lacking the methylation or the blocking nucleotide residue at the 5′ terminus (12). However, there were some incompatible results, causing confusion (13). These may be mainly due to incomplete elimination of the 7-methylguanylic acid residue in those experiments.

Recently it was shown that a pyrophosphatase purified from a tobacco cell culture specifically splits 7-methylguanylic acid and phosphate in the pyrophosphate linkages from the 5′ terminus of eukaryotic mRNA without any scission in the other parts of the RNA molecule (14, 15). Using this enzyme, we have investigated the function of the confronting nucleotide structure at the 5′ terminus of mRNA.

MATERIALS AND METHODS

Preparation of RNA. CPV mRNA was prepared *in vitro* as described (16, 17). Tobacco mosaic virus (TMV) RNA was prepared from infected leaves of *Nicotiana tabacum* (18). Hemoglobin mRNA was prepared from rabbit reticulocytes as described by Nienhuis *et al.* (19).

Hydrolysis of the 5′-Terminal Blocked Structure of mRNA with Tobacco Phosphodiesterase. Ten A_{260} units of mRNA or TMV RNA was incubated with 0.4 unit of tobacco phosphodiesterase (20) in the presence of 0.1 M sodium acetate (pH 5.5) and 5 mM EDTA in 0.4 ml of reaction mixture at 30° for 1.5 hr. The reaction was stopped by adding 0.2 ml of 80% phenol and shaking to inactivate the enzyme. Then the RNA was precipitated with ethanol after sufficient extraction of the phenol remaining in the aqueous phase with ether.

Protein Synthesizing System *In Vitro*. The protein synthesizing system was prepared from a wheat germ extract according to Roberts and Paterson (21). For one assay, 50 μl of the reaction mixture was used. It contained 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) (adjusted to pH 7.6 with KOH), 2.5 mM magnesium acetate, 100 mM KCl, 4 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 2 μg of creatine phosphate kinase, amino acid mixture (3 μM each, without leucine), 2 μCi of [^{3}H]leucine (50 Ci/mmol), 10 μg of RNA, and about 5 A_{260} units of wheat germ extract. After the reaction was over, 5% (wt/vol) ice-cold trichloroacetic acid was added and the mixture was heated at 90° for 20 min. The acid-precipitable fraction was collected on a glass fiber filter (Whatman GF/A, 24 mm in diameter) and its radioactivity was assayed in a liquid scintillation spectrometer.

Formation of the Initiation Complex for Protein Synthesis in Wheat Germ Extract. ^{3}H-Labeled CPV mRNA or ^{32}P-labeled TMV RNA was added to a cell-free protein synthesizing system, to which sparsomycin had been added (to 0.1 mM) to inhibit the elongation of peptide chains; the mixture was incubated at 30° for 15 min. Then the reaction mixture was put on a sucrose density gradient (10–30%) containing 10 mM Hepes (pH 7.6), 4 mM magnesium acetate, 100 mM KCl, and 2 mM dithiothreitol and centrifuged at 27,000 rpm for 4 hr in a Beckman-Spinco ultracentrifuge and an SW 40 swinging bucket rotor. From the bottom of the tube, 0.4-ml fractions were collected. Absorbancy at 260 nm and radioactivity of each fraction were measured.

Incubation of RNA with S-23 or S-160 Fraction in Wheat Germ Extract. Wheat germ (6 g) was ground with an equal weight of sea sand and extracted with 16 ml of a buffer containing 80 mM Hepes (adjusted to pH 7.6 with KOH), 0.1 M KCl, 1 mg of magnesium acetate, 2 mM $CaCl_2$, and 6 mM 2-mercaptoethanol. The extract obtained by centrifugation at 23,000 × *g* for 15 min was added to magnesium acetate (final concentration 3.5 mM)/ATP (1 mM)/GTP (20 μM)/dithiothreitol (2 mM)/creatine phosphate (8 mM)/creatine kinase (40 μg/ml). This mixture was incubated at 30° for 12 min. It was then applied to a Sephadex G-25 column to separate cellular enzymes from low-molecular-weight material. The proteins

Abbreviations: CPV, cytoplasmic polyhedrosis virus; TMV, tobacco mosaic virus; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

* Contribution no. 1150 from National Institute of Genetics.

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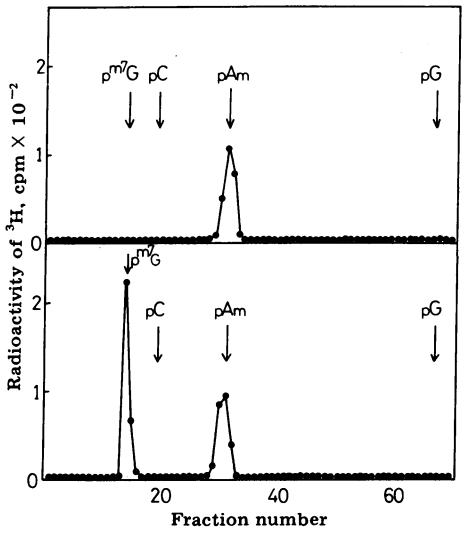

FIG. 1. Terminal nucleotide analysis of CPV mRNA by column chromatography (31). The *methyl*-^{3}H-labeled intact CPV mRNA (*Lower*) and tobacco pyrophosphatase-treated RNA (*Upper*) were digested with *Penicillium* nuclease P_1 and then with tobacco pyrophosphatase. The digests were chromatographed with nucleotide markers on a column of Bio-Rad AG 1 (0.6 × 20 cm). The nucleotides were eluted with a linear gradient of NaCl from 0 to 0.27 M in 0.01 M HCl (50 ml of each); 1-ml fractions were collected and absorbancy at 260 nm (indicated with arrows) and radioactivity of ^{3}H were measured.

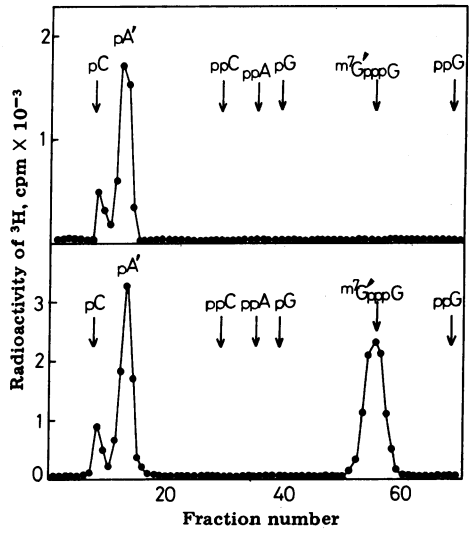

FIG. 2. Terminal nucleoside analysis of TMV RNA by column chromatography. TMV RNA, incubated with tobacco pyrophosphatase (*Upper*) and not incubated with it (*Lower*), was labeled at the terminal nucleosides by NaB^3H_4 after oxidation with sodium metaperiodate. After the RNA was digested with *Penicillium* nuclease P_1, the sample was chromatographed as described in Fig. 1.

were centrifuged at 23,000 × *g* for 15 min. This supernatant was used as the S-23 fraction. This fraction was then centrifuged at 160,000 × *g* for 2 hr in a no. 65 rotor in a Beckman-Spinco ultracentrifuge. The last supernatant was used as the S-160 fraction.

Labeled CPV mRNA (as C^3H_3 and ^{32}P) was incubated with the S-23 (or S-160) fraction from the wheat germ extract in 100 μl of a reaction mixture containing 10 mM Hepes (pH 7.6), 1 mM magnesium acetate, 64 mM KCl, and 2 mM dithiothreitol. The reaction mixture was incubated at 30° for various times. At each indicated time, 100 μl of water-saturated phenol was added and the mixture was shaken immediately. The aqueous phase was applied to a glycerol density gradient (10–30%) in 20 mM EDTA/50 mM sodium acetate (pH 5.5) and centrifuged at 38,000 rpm for 15 hr in an SW 40 swinging bucket rotor in a Beckman-Spinco ultracentrifuge. Fractions (4 drops) were collected from the bottom of the tube, and the radioactivity of ^{3}H and ^{32}P in each fraction was assayed in a dioxane-based scintillator.

Column Chromatography. Anion exchange resin Bio-Rad AG 1 was used for separation of the nucleotides including the 5′–5′ confronting nucleotides. The chromatographic procedure is in the legend to Fig. 1.

RESULTS

Removal of the 7-Methylguanylate at the 5′ Terminus of mRNA by Tobacco Phosphodiesterase. When CPV mRNA was treated with tobacco phosphodiesterase, pm^7G and inorganic phosphate were released (14, 15). Thus, the 5′-terminal structure of CPV mRNA was changed completely from $m^7G^5'pppAm$-G- to pAm-G-. This was confirmed again when the native CPV mRNA labeled with *methyl*-^{3}H and the tobacco pyrophosphatase-treated RNA were digested by *Penicillium* nuclease P_1 to yield terminal nucleotides as $m^7G^5'pppAm$ from the former and pAm from the latter; these nucleotides were then analyzed chromatographically after further digestion with tobacco phosphodiesterase to separate [^{3}H]pm^7G and [^{3}H]pAm. The native RNA gave both of them in nearly equal amounts, while the tobacco pyrophosphatase-treated RNA gave only [^{3}H]pAm (Fig. 1).

During the treatment of mRNA with tobacco pyrophosphatase, neither the cleavage of internucleotide bonds nor exonucleolytic attack from the 3′ terminal of mRNA was observed (14, 15; specific data will be reported elsewhere).

In order to confirm this characteristic splitting by tobacco phosphodiesterase for TMV RNA, the following experiment was carried out. Because it is difficult to label *in vivo* the terminal modified group of TMV RNA with radioisotopes sufficiently for analysis, the following method was used. RNA was oxidized by sodium metaperiodate and then reduced by sodium [^{3}H]borohydride. Because the *cis*-diol structure is labeled with ^{3}H by this treatment, the ^{3}H radioactivity should be introduced equally into the 3′-terminal nucleoside and the 7-methylguanosine residue confronting the 5′ terminus. This can be examined by digestion with *Penicillium* nuclease P_1, which produces the ^{3}H-labeled nucleoside dialcohol-5′-phosphate (pN′) from the 3′ terminus of RNA and the ^{3}H-labeled $m^7G'pppN$ (here m^7G' indicates 7-methylguanosine dialcohol) from the 5′-terminus. The products, pN′ and $m^7G'pppN$, were analyzed as shown in Fig. 2. With the labeled native TMV RNA, nearly equal amounts of [^{3}H]pA′ and [^{3}H]$m^7G'pppG$ [7618 cpm (51%) and 7273 cpm (49%), respectively] were detected. On the other hand, with the labeled tobacco phosphodiesterase-treated TMV RNA, only [^{3}H]pA′ and a small amount of [^{3}H]pC′ were detected, with no $m^7G'pppG$. This result indicates that the 7-methylguanosine blocking structure at the 5′ terminus of TMV RNA had been completely removed by tobacco phosphodiesterase.

Loss of mRNA Activity after Removal of the 5′-Terminal Modified Structure. CPV mRNA, globin mRNA, and TMV RNA were treated with tobacco phosphodiesterase to split pyrophosphate linkages at the 5′ terminus. The protein synthesizing ability of these mRNAs was compared with that of the native RNAs in the wheat germ extract. The results are presented in Fig. 3. For each RNA used, the protein synthesizing activity was decreased drastically by tobacco phosphodiesterase treatment. Because the removal of the modified structure was carried out almost completely by this enzyme, it was concluded

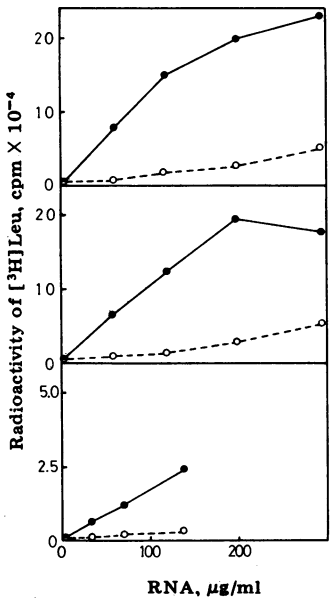

FIG. 3. Comparison of protein synthesizing abilities of intact mRNA (●) and mRNA without blocking structure (○). The reaction mixture for protein synthesis was as given in the *text*. The mixture was incubated at 30° for 30 min. After addition of cold trichloroacetic acid, radioactivity in the acid-precipitable fraction was measured. (*Top*) CPV mRNA. (*Middle*) TMV RNA. (*Bottom*) Globin mRNA.

that the confronting nucleotide structure at the 5′ terminus of mRNA is required for protein synthesis in all of the different types of eukaryotic mRNA. However, the translational ability of tobacco phosphodiesterase-treated RNA was not abolished completely. In an optimal translation condition, the tobacco phosphodiesterase-treated RNA showed 10–20% of the protein synthesizing ability of intact mRNA.

Effect of Removal of the Modified Structure of mRNA on Initiation Complex Formation. Because the 5′-terminal modification of mRNA may be concerned with the initial step of protein synthesis, the ability to form the initiation complex was tested with CPV mRNA after the confronting 7-methylguanylic acid at the 5′ terminus had been deleted by tobacco phosphodiesterase. ^{3}H-Labeled RNA was added to the *in vitro* protein synthesizing system from wheat germ as described in the preceding section, but sparsomycin was added to prevent peptide chain elongation and thus permit detection of the initiation complex of protein synthesis. Formation of the initiation complex was analyzed by sucrose density gradient centrifugation (Fig. 4). A significant proportion of the intact CPV mRNA was recovered as the 80S initiation complex, whereas with the tobacco phosphodiesterase-treated CPV mRNA only 15% of the amount of intact mRNA at the 80S position was recovered. A similar loss of initiation complex formation was obtained when 7-methylguanylic acid was added (to 1 mM) to a reaction mixture containing either intact mRNA or tobacco phosphodiesterase-treated mRNA. A remarkable decrease in initiation complex formation was also observed when tobacco phosphodiesterase-treated TMV RNA was used instead of mRNA (data will be published elsewhere).

Comparison of Stability of the Intact mRNA and mRNA without the Modification. One reason for the fact that initiation complex formation is decreased by removal of the 5′-terminal modified structure in mRNA may depend on the lability of the enzyme-treated RNA. This was tested investigating the stability of mRNAs in the wheat germ extract. Labeled CPV mRNA was incubated with the S-23 fraction (the supernatant

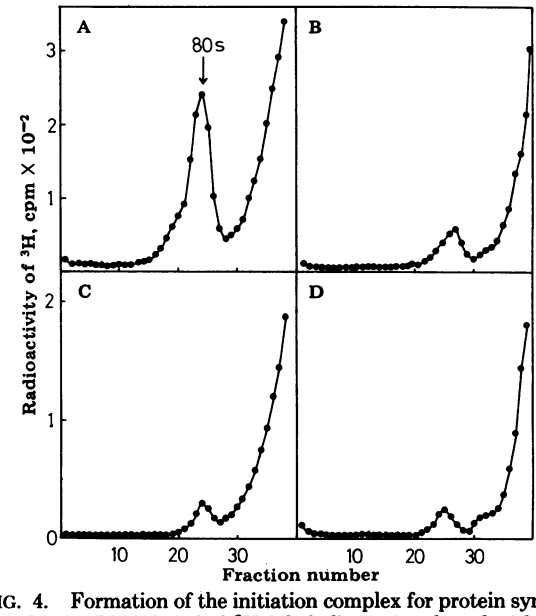

FIG. 4. Formation of the initiation complex for protein synthesis. Intact CPV mRNA (*methyl*-^{3}H-labeled) was incubated with wheat germ extract in the presence of sparsomycin as described in the *text*. The reaction mixture was placed on a sucrose density gradient, 10–30%, and centrifuged in a Beckman-Spinco SW 40 swinging bucket rotor at 27,000 rpm for 4 hr; 6-drop fractions were collected from the bottom of the centrifuge tube. ^{3}H radioactivity in each fraction was measured. (*A*) Intact CPV RNA. (*B*) Tobacco pyrophosphatase-treated CPV RNA. (*C*) Intact CPV RNA plus 1 mM pm^{7}G. (*D*) Tobacco pyrophosphatase-treated CPV RNA plus 1 mM pm^{7}G.

fraction after 23,000 × *g* centrifugation of the wheat germ extract) under the same conditions as for the *in vitro* protein synthesizing system. After 2–3 min of incubation, degradation of RNA was analyzed by glycerol density gradient centrifugation (Fig. 5). A large proportion of the intact CPV mRNA did not change in size. Although a small portion of ^{32}P radioactivity

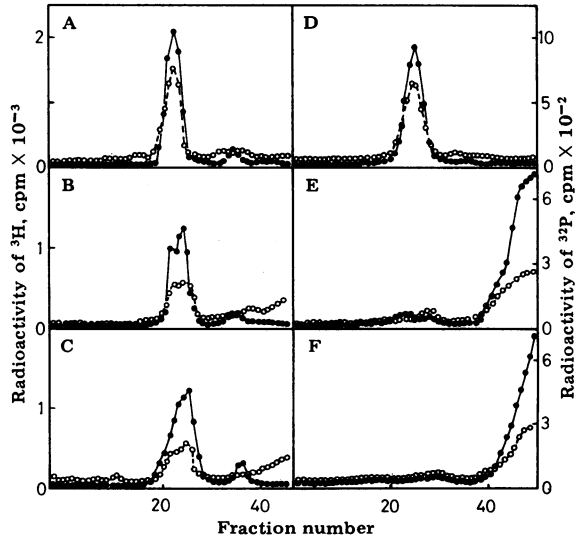

FIG. 5. Stability of CPV mRNA during incubation with the wheat germ cell-free fraction S-23. The labeled CPV mRNA (*A, B, C*) and the tobacco pyrophosphatase-treated RNA (*D, E, F*), which had lost the 5′-terminal modified structure, were incubated with the S-23 fraction in wheat germ extract at 30° as described in the *text* for 0 min (*A* and *D*), 2 min (*B* and *E*), and 3 min (*C* and *F*). After incubation, the reaction mixture was mixed with phenol, and the extracted RNA was analyzed on a glycerol density gradient as described in the *text*. ●, ^{3}H; ○, ^{32}P.

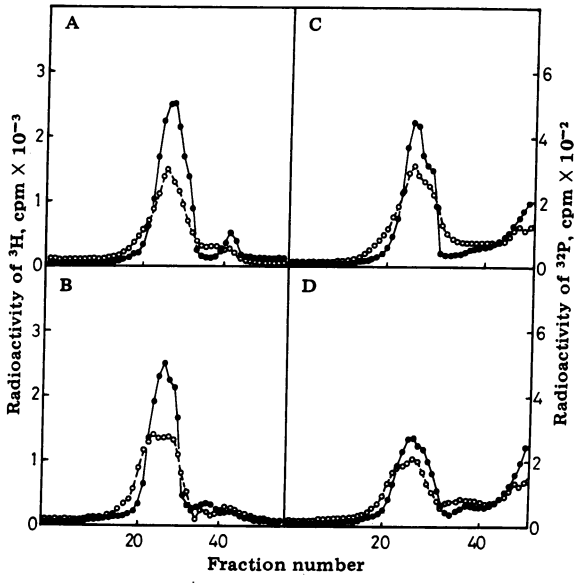

FIG. 6. Stability of CPV mRNA during incubation with the S-160 fraction in wheat germ cell-free extract. The experimental conditions were as in Fig. 4 with S-160 fraction in wheat germ extract instead of S-23. (*A* and *B*) Intact labeled CPV mRNA was incubated for 1 and 3 min, respectively. (*C* and *D*) Tobacco pyrophosphatase-treated CPV mRNA incubated for 1 and 3 min, respectively. ●, ^{3}H; ○, ^{32}P.

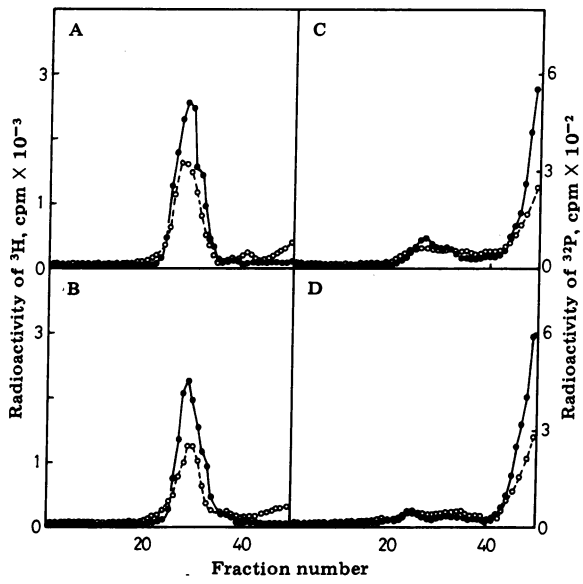

FIG. 7. Stability of CPV mRNA during incubation with wheat germ extract S-23 in the presence of pm^{7}G. The experimental conditions were the same as in Fig. 4 but 7-methylguanylic acid was added to the incubation mixture to a final concentration of 1 mM. (*A* and *B*) Intact CPV mRNA incubated for 1 and 3 min, respectively. (*C* and *D*) Tobacco pyrophosphatase-treated CPV mRNA incubated for 1 and 3 min, respectively. ●, ^{3}H; ○, ^{32}P.

of CPV mRNA was transferred to smaller RNA fractions, none of the *methyl*-^{3}H radioactivity located at the 5′ terminus of mRNA was detected in the top fraction of the density gradient. In contrast with the intact mRNA, the tobacco phosphodiesterase-treated CPV mRNA was immediately broken down by incubation with the S-23 fraction. Both the ^{3}H and the ^{32}P radioactivity moved to the top fraction of the density gradient. These results clearly indicate that the intact mRNA is resistant to the nuclease attack due to the presence of the 7-methylguanylic acid confronting 5′-terminal structure.

There is a possibility that initiation complex formation of mRNA with ribosomes or any other complex formation with some protein, which binds to the 5′-terminal modified structure specifically, may protect the mRNA from degradation. This was investigated in two ways. One experiment was incubated of mRNA with the S-160 fraction, which was the supernatant fraction after ribosomes had been centrifuged off. The other was incubation of mRNa with the S-23 fraction in the presence of pm^{7}G, by which protein-binding to the 5′-terminal modified structure seemed to be inhibited. Tobacco phosphodiesterase-treated CPV mRNA was degraded by incubation with S-160 and transferred to the top fraction of the density gradient (Fig. 6 *C* and *D*), whereas the intact mRNA was not converted into smaller molecules (Fig. 6 *A* and *B*). Similar results were obtained with incubation of mRNA with S-23 in the presence of pm^{7}G (Fig. 7). Thus, it was strongly suggested that the 5′-terminal modified structure itself in mRNA was necessary to stabilize the mRNA molecule.

Degradation products from the incubation of the modification-deleted mRNA in the wheat germ extract were analyzed by DEAE-cellulose/7 M urea chromatography to determine the molecular size or net charge (22). Because the *methyl*-^{3}H-labeled original CPV mRNA was treated with tobacco phosphodiesterase, only the 5′-terminal nucleotide contains 2′-*O*-*methyl*-^{3}H before incubation with the wheat germ extract. The radioactivity of ^{3}H in the degradation products was detected in the nucleoside fraction and the mononucleotide fraction but not in any oligonucleotide larger than dinucleotide. This means that the first nucleotide at the 5′ terminus of the tobacco phosphodiesterase-treated mRNA was released as a mononucleotide, and some of it was converted to nucleoside by phosphatase action, which may be contained in the S-23 fraction of the wheat germ extract. From these results, it is suggested that an exonucleolytic activity 5′ → 3′ in the wheat germ extract cannot cleave the confronting nucleotide structure at the 5′ terminus of mRNA, m^{7}G^5′ppp^5′N(m), and therefore cannot degrade the intact mRNA; it degrades RNA only after the 5′-terminal modification is removed. In other words, the confronting structure at the 5′ terminus of mRNA seems to protect the RNA molecule from exonucleolytic degradation; the degradation of mRNA would start by removal of the 5′-terminal modification.

DISCUSSION

In order to elucidate the function of the 5′-terminal modification in eukaryotic mRNA, protein synthesis activity should be compared for the native mRNA and the modification-deleted mRNA. Because the 7-methylguanosine residue confronting the 5′ terminus of mRNA carries a 2′,3′-*cis*-diol, it is deleted by oxidation with periodate followed by β-elimination with amine. However, this procedure is sometimes not perfect for all the residues in question (22). Moreover, it also splits a nucleoside residue at the 3′ terminus of RNA. In contrast to this, a phosphodiesterase purified from cultured tobacco cells (20) cleaves only pyrophosphate linkages in mRNA specifically, so that only 7-methylguanylic acid residues confronting the 5′ terminus of mRNA through a pyrophosphate linkage can be removed (14, 15). As shown here, removal of the 7-methylguanylic acid residue was complete under the given conditions, and no other splitting in the mRNA molecule was detected.

mRNAs of CPV and globin as well as TMV RNA can stimulate protein synthesis acting as a template in wheat germ extract. When the 5′-terminal blocking structure had been removed by tobacco phosphodiesterase, all the mRNAs lost the ability to synthesize protein. Thus, the confronting nucleotide

structure is necessary for protein synthesis. This was also shown by the inhibitory effect of added 7-methylguanylic acid (5′-phosphate) to the native mRNA (Y. Kodama, K. Shimotohno, and K. Miura, unpublished results; refs. 4 and 5).

Although a CPV mRNA preparation consisted of 10 different-sized RNA molecules (17), each RNA molecule seemed to be monocistronic, as also is globin mRNA. A TMV RNA molecule contains much information for proteins, being polycistronic, but it was recently reported that TMV RNA functions continuously as a long template molecule to translate the template into a large peptide molecule which is processed later into individual functional protein molecules (23). Therefore, the 7-methylguanylic acid-blocking structure at the 5′ terminus of mRNAs seems to be necessary for one read-through on the template mRNA from near the 5′ terminus, not for the start of the inner cistrons.

Because protein synthesis proceeds in the direction 5′ → 3′ of the template mRNA, the 5′-terminal modification may be related to the formation of an initiation complex in protein synthesis. Removal of the modified structure at the 5′ terminus of mRNA caused loss of the initiation complex formation capability of mRNA. Addition of 7-methylguanylic acid to the native mRNA caused a similar effect. Therefore, 7-methylguanylic acid at the 5′ terminus of mRNA is necessary at the step of formation of the initial complex in protein synthesis.

The 7-methylguanylic acid residue or the confronting nucleotide structure may be necessary for mRNA to bind specifically with ribosomes or some factors of the formation of the initiation complex in protein synthesis, to maintain the functional structure of mRNA, or to protect mRNA from degradation. The first possibility was suggested by Filipowicz *et al.* (24). The last possibility was clearly shown here by comparison of the stability of the modification-deleted mRNA with the native mRNA by incubation in a wheat germ extract (Fig. 6). If the 5′-terminal modification is removed, the RNA molecule seems to be degraded exonucleolytically from the 5′ terminus by an enzyme in the wheat germ extract.

In the experiments on protein synthesis and initiation complex formation, it was noticed that the tobacco phosphodiesterase-treated RNA did not lose its mRNA activity completely, 10–20% of that of native mRNA remaining (Figs. 3 and 4). Even if the modification had been removed, a small number of the mRNA molecules would be able to form an initiation complex to synthesize protein before degradation.

In any case, the 7-methylguanylic acid residue at the 5′ terminus protects the mRNA from exonucleolytic degradation in the cell. The presence of 7-methylguanylic acid confronting the 5′ terminus of mRNA is now known to be common for eukaryotic systems. Although there are some exceptions—for example, poliovirus mRNA and satelite tobacco necrosis virus RNA (25–27)—there may be a device to stabilize mRNA even in these cases as shown in the protein component attached to the 5′ terminus of the polio mRNA (28). Absence of the 5′-terminal modification in prokaryotic mRNA (K. Miura, M. Hiruta, M. Yamamoto, and F. Imamoto, unpublished data; refs. 29 and 30) may be one of the causes of short lifetime or rapid turnover of bacterial mRNA.

The authors are grateful to Dr. Masanao Miwa and Mr. Hideaki Shinshi for preparation of tobacco pyrophosphatase and to Drs. Yoshito Kaziro and Mitsuaki Yoshida for suggestions on the experiments of protein synthesis. This work was supported partly by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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1. Furuichi, Y. & Miura, K. (1975) *Nature* **253**, 374–375.
2. Miura, K., Furuichi, Y., Shimotohno, K., Urushibara, T. & Sugiura, M. (1975) *Fed. Eur. Biochem. Soc. Meet. 10th [Proc.]*, 95–108.
3. Griffin, B. (1975) *Nature* **255**, 9.
4. Hickey, E. D., Weber, L. A. & Baglioni, C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 19–23.
5. Hickey, E. D., Weber, L. A. & Baglioni, C. (1976) *Nature* **261**, 71–72.
6. Weber, L. A., Femen, E. R., Hickey, E. D., Williams, M. C. & Baglioni, C. (1976) *J. Biol. Chem.* **251**, 5657–5661.
7. Muthukrishnan, S., Both, G. W., Furuichi, Y. & Shatkin, A. J. (1975) *Nature* **255**, 33–37.
8. Leung, D. W., Gilbert, C. W., Smith, R. E., Sasavage, N. L. & Clark, J. M. (1976) *Biochemistry* **15**, 4943–4950.
9. Kozak, M. & Shatkin, A. J. (1976) *J. Biol. Chem.* **251**, 4259–4266.
10. Rose, J. K. & Lodish, H. F. (1976) *Nature* **262**, 32–37.
11. Roman, R., Brooker, J. D., Seal, S. N. & Marcus, A. (1976) *Nature* **260**, 359–360.
12. Both, G. W., Banerjee, A. K. & Shatkin, A. J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1189–1193.
13. Griffin, B. (1976) *Nature* **263**, 188–189.
14. Shinshi, H., Miwa, M., Sugimura, T., Shimotohno, K. & Miura, K. (1976) *FEBS Lett.* **65**, 254–257.
15. Ohno,T., Okada, Y., Shimotohno, K., Miura, K., Shinshi, H., Miwa, M. & Sugimura, T. (1976) *FEBS Lett.* **67**, 209–213.
16. Shimotohno, K. & Miura, K. (1973) *Virology* **53**, 283–286.
17. Shimotohno, K. & Miura, K. (1973) *J. Biochem.* **74**, 117–125.
18. Fraenkel-Conrat, H., Singer, B. & Tsugita, A. (1961) *Virology* **14**, 54–58.
19. Nienhuis, A. W., Falvey, A. K. & Anderson, W. F. (1974) in *Methods in Enzymology*, eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. 30, pp. 621–630.
20. Shinshi, H., Miwa, M., Kato, K., Noguchi, M., Matsushima, T. & Sugimura, T. (1976) *Biochemistry* **15**, 2185–2190.
21. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2330–2334.
22. Miura, K., Watanabe, K. & Sugiura, M. (1974) *J. Mol. Biol.* **86**, 31–48.
23. Roberts, B. E., Paterson, B. M. & Sperling, R. (1974) *Virology* **59**, 307–313.
24. Filipowicz, W., Furuichi, Y., Sierra, J. M., Muthukrishnan, S., Shatkin, A. J. & Ochoa, S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1559–1563.
25. Nomoto, A., Lee, Y. F. & Wimmer, E. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 375–380.
26. Hewlett, M. J., Rose, J. K. & Baltimore, D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 327–330.
27. Wimmer, E., Chang, A. Y., Clark, J. M. & Reichman, M. E. (1968) *J. Mol. Biol.* **38**, 59–73.
28. Lee, Y. E., Nomoto, A., Detjen, B. M. & Wimmer, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 59–63.
29. Wang, S., Marcu, K. B. & Inouye, M. (1976) *Biochem. Biophys. Res. Commun.* **68**, 1194–1200.
30. Squires, C., Lee, F., Bertrand, K., Squires, C., Bronson, M. & Yanofsky, C. (1976) *J. Mol. Biol.* **103**, 351–381.
31. Shimotohno, K. & Miura, K. (1976) *FEBS Lett.* **64**, 204–208.