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7-Methyl-Guanosine and Efficiency of RNA Translation

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Brome mosaic virus RNAs 3 and 4 were chemically modified to remove the terminal 7-methyl-guanosine (m^7G) structure, and the modified RNAs were tested for their messenger activity in a cell-free system derived from wheat embryo. Amino acid incorporation and ribosome-binding data show that removal of m^7G results in reduction, but not complete abolition, of the messenger activity of the RNA. This suggests that the function of m^7G may be related to efficient translation of messenger RNA. Possible involvement of other structural factors in RNA translation is discussed.

A unique methylated structure, a 7-methyl-guanosine (m^7G) connected to its adjacent nucleotide through a 5′ to 5′ triphosphate linkage, has been found to occur at the 5′ termini of many cellular and viral RNAs (see reference 3 for a comprehensive list). Translation studies with reovirus, vesicular stomatitis virus, globin, and brine shrimp messengers, all of which contain the methylated structure, indicated that m^7G is important for RNA translation (2, 9, 10).

We have examined the involvement of m^7G in RNA translation by studying the messenger activities of brome mosaic virus (BMV) RNAs 3 and 4 in a cell-free system derived from wheat embryo. BMV is a small multicomponent virus infectious to wheat. Its genetic information is divided among four RNAs with molecular weights of 1.09×10^6, 0.99×10^6, 0.75×10^6, and 0.28×10^6, designated 1 to 4 in order of decreasing size (8). All the four BMV RNAs contain a sequence of $m^7G\ ^5'ppp^5'Gp$ at their 5′ termini (5). The four RNAs are active messengers in wheat embryo extracts, inducing the synthesis of four proteins. RNA 4 is a monocistronic messenger for the synthesis of BMV coat protein (13). RNA 3 also contains the coat protein cistron (8), but the product synthesized from this RNA is a protein (designated protein 3a) unrelated to the coat protein (13). RNAs 1 and 2 each direct the synthesis of a large protein of molecular weight over 100,000 (7; D. Shih and P. Kaesberg, J. Mol. Biol., in press). The nucleotide sequence of the first 53 bases of RNA 4 is known (4). The sequence includes the ribosome-binding site for wheat embryo ribosomes.

In the present study, BMV RNAs 3 and 4 were chosen in preference to RNAs 1 and 2 because they can be more readily obtained in large quantities. RNA 3 and RNA 4 were chemically modified to remove the terminal m^7G, and the modified RNAs were tested for their messenger activities. We have found that removal of m^7G results in the reduction of the amino acid incorporation and ribosome-binding activities of the two BMV RNAs. However, there is always some activity remaining. At saturation concentrations, modified RNAs can stimulate amino acid incorporation and can bind with ribosomes at about 40% of the efficiency of unmodified BMV RNAs.

MATERIALS AND METHODS

Preparation of BMV RNAs. BMV RNA was prepared from purified virus by phenol extraction. RNA 3 and RNA 4 were obtained by fractionation of whole BMV RNA on sucrose density gradients. The fractionation procedure used was the same as previously described (13), except that before the second sucrose density gradient centrifugation, the RNA samples were heated at 60°C for 10 min in a buffer containing 0.005 M Tris, pH 8.3, 0.02 M NaCl, and 6 M urea to prevent occurrence of any association between the two RNA components. ^{32}P-labeled BMV RNA was prepared as previously described (1).

Chemical modification reactions. To obtain better completion of chemical modification reactions, BMV RNAs 3 and 4 were treated wtih a large excess of modifying agents. To a solution of 0.5 mg of BMV RNA in 0.2 ml of 0.1 M Na acetate buffer, pH 5.3, sodium periodate was added to a 300-fold molar excess. The solution was incubated for 1 h at 21°C in the dark. The RNA was precipitated with ethanol and washed twice by dissolving in 0.2 ml of the Na acetate buffer and reprecipitated with ethanol. The periodate-oxidized RNA was then dissolved in a solution containing 0.1 ml of the Na acetate buffer and 0.5 ml of 0.33 N redistilled aniline (adjusted to pH 5 with HCl). This solution was incubated for 3 h at 21°C in the dark. The RNA was precipitated with ethanol and washed twice to remove all the contaminating aniline. For preparing control RNA samples (unmodified RNA), all manipulations of the chemi-

cal treatment were carried out except that the modifying agents (periodate and aniline) were omitted. ^{32}P-labeled BMV RNAs were treated in parallel with the nonradioactive BMV RNAs. The RNAs were intact after the chemical treatment as judged by polyacrylamide gel electrophoresis analyses.

Analysis of the T_1 RNase digestion products. ^{32}P-labeled BMV RNA was digested with T_1 RNase (at an enzyme-to-RNA ratio of 1 to 10) for 30 min at 37°C. The resulting oligonucleotides were fractionated by two-dimensional electrophoresis according to a procedure described by Sanger et al. (12).

Amino acid incorporation assay. Wheat embryo cell-free extracts were prepared as previously described (6). A standard reaction mixture (100 μl) contained 50 μl of S23, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.6), 2.5 mM ATP, 0.38 mM GTP, 10 mM creatine phosphate, 10 μg of creatine kinase per ml, 0.75 μCi of [^{14}C]leucine (274 μCi/μmol), and 0.04 mM each of the appropriate unlabeled amino acids. The reaction mixture was made 4 mM in magnesium acetate and 95 mM in potassium acetate. *S*-adenosylhomocysteine (0.32 mM) was added to prevent methylation. Incubation was at 30°C. Radioactive proteins were precipitated and counted as previously described (13).

Ribosome-binding assay. Unfractionated S23 extracts were used for ribosome-binding experiments. The reaction mixtures were the same as the amino acid incorporation assay mixture except that the antibiotic anisomycin (125 μg/ml) was added to prevent peptide chain elongation. Incubation was at 30°C for 20 min. The binding reaction mixtures were quickly cooled and applied to 10 to 40% sucrose density gradients prepared in 50 mM Tris (pH 7.6), 50 mM KCl, and 4 mM Mg acetate. The gradients were centrifuged at 4°C in a Spinco SW27 rotor for 4 h at 27,000 rpm.

RESULTS

Fingerprint analyses of modified BMV RNAs. For precise comparison of the activities of modified and unmodified BMV RNAs, it was necessary at first to establish the degree of completion of the chemical reactions. For this purpose, ^{32}P-labeled BMV RNAs were digested with RNase T_1, and the resulting oligonucleotides were fingerprinted. Figure 1a shows the upper portion of the fingerprint of unmodified RNA 4. The spot indicated by an arrow contains the terminal m^7GpppGp. (RNase T_1 does not cleave the phosphate linkage after the m^7G.) This spot is distinctive and is well isolated. The corresponding portion of the fingerprint of RNA 4 modified as described in Materials and Methods is shown in Fig. 1b. The m^7GpppGp spot is clearly absent. In addition, no radioactivity above background was detected when the area corresponding to the position of this dinucleotide was cut out and counted. Similarly, repeated analyses with several different RNA 4 samples and with modified RNA 3 samples gave the same results, indicating that removal of m^7G from both RNA 3 and RNA 4 is complete. Modified RNA has an additional spot that moves with authentic pppGp (beyond the leftmost spot of Fig. 1b).

Translation of modified RNA 4. The abilities of modified and unmodified RNA 4 to promote leucine incorporation were compared. At

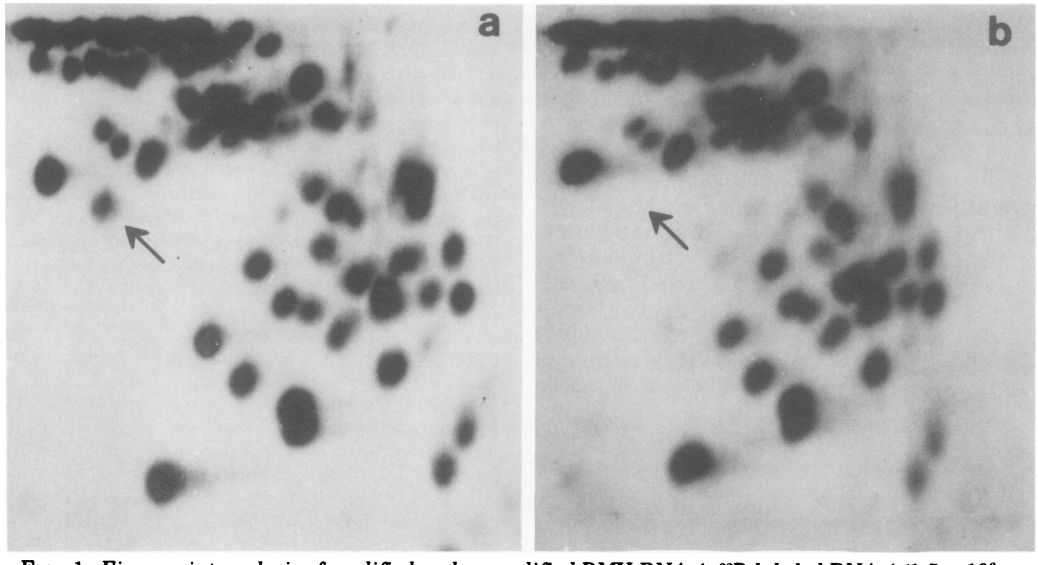

FIG. 1. *Fingerprint analysis of modified and unmodified BMV RNA 4. ^{32}P-labeled RNA 4 (1.5×10^6 cpm) was digested and analyzed as described in Materials and Methods. (a) Electropherogram of unmodified RNA 4; (b) electropherogram of modified RNA 4.*

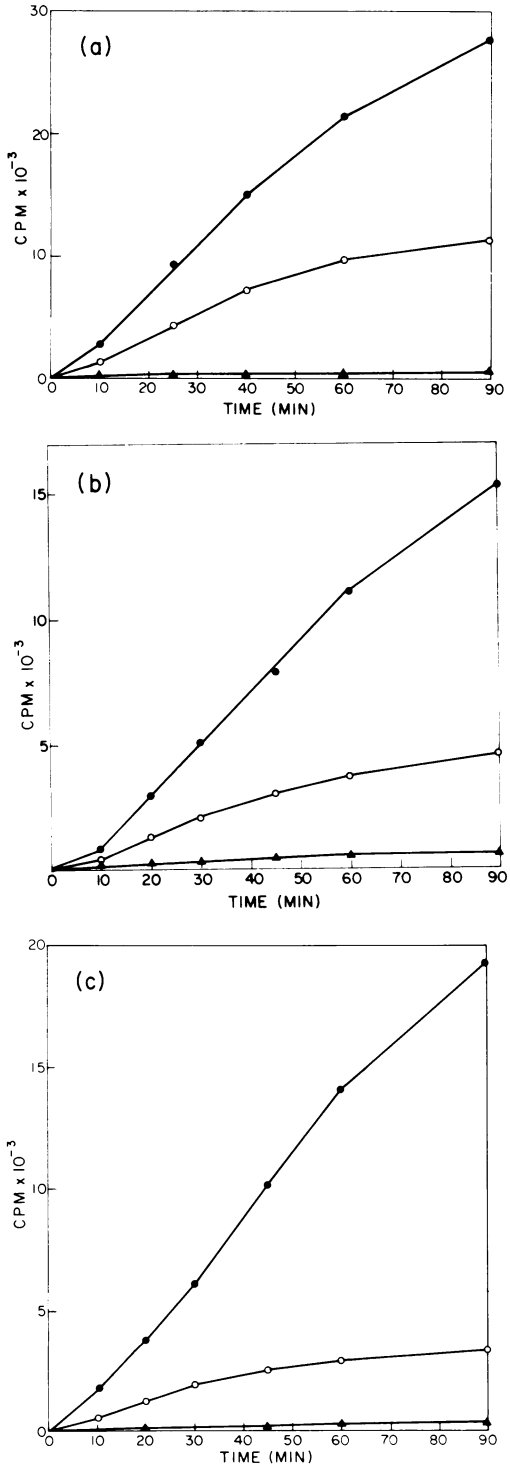

FIG. 2. *Comparison of the incorporation activities of modified and unmodified RNA 4. Amino acid incorporation assays were performed as described in Materials and Methods, except that the magnesium ion concentration was 3 mM in (b) and 5.5 mM in (c). The reaction mixtures contained 50 μg of RNA per ml. Ten-microliter samples were withdrawn from the reaction mixtures at the times indicated. Symbols: (▲) no RNA added; (○) modified RNA 4; (●) unmodified RNA 4.*

a concentration of 10 μg/ml, modified RNA 4 promoted an incorporation about 12% of that promoted by unmodified RNA 4. When used at higher RNA concentrations, modified RNA 4 can promote substantial incorporation. For example, at a concentration of 50 μg/ml, which is near the saturation concentration of normal RNA 4, modified RNA 4 promoted an incorporation about 40% of the amount promoted by unmodified RNA 4 (Fig. 2a). Incorporation activities of the two RNA 4 samples at different RNA concentrations are plotted in Fig. 3. The figure shows that incorporations increased with increasing concentration for both the modified and unmodified RNA, but the incorporation from modified RNA 4 was lower in all cases. We interpret these data to mean that removal of m7G results in a decrease of the translation efficiency of RNA 4. Although m7G is important for efficient RNA translation, it is not essential.

Incorporation with modified RNA has an increased sensitivity to magnesium ion concen-

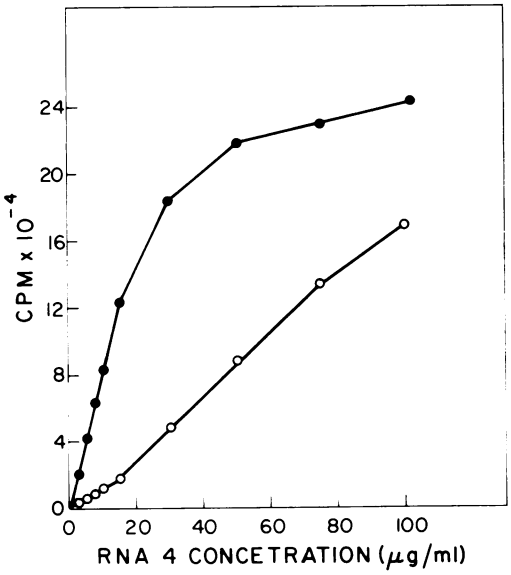

FIG. 3. *Comparison of the activities of modified and unmodified RNA 4 at different RNA concentrations. Amino acid incorporation assays were performed as described in Materials and Methods. The reaction mixtures were incubated for 90 min and radioactive proteins were precipitated with trichloroacetic acid and counted. Symbols: (○) modified RNA 4; (●) unmodified RNA 4.*

tration. Modified and unmodified RNA 4 (50 μg/ml) were translated at magnesium ion concentrations of 3 and 5.5 mM instead of at the optimal concentration of 4 mM. Under these conditions, the incorporations from both forms of RNA 4 were lower than the incorporations obtained at 4 mM magnesium (Fig. 2b and c). However, the decrease in incorporation was greater in the case of modified RNA 4. The incorporation from modified RNA 4 was only about 25% of that from unmodified RNA 4 when the RNAs were translated at 3 mM magnesium (Fig. 2b) and 16% when translated at 5.5 mM (Fig. 2c), compared with about 40% when translated at the optimal concentration of 4 mM (Fig. 2a).

The above data, however, do not distinguish between two possibilities. The observed lower efficiency may be due directly to the absence of m^7G or due to the increased lability of the triphosphate group. That is, the terminal triphosphate is involved in RNA translation and m^7G protects it from degradation. However, pppGp was still present in fingerprints of modified RNA that had been exposed to the translation conditions. Furthermore, when such modified RNA 4 was treated with alkaline phosphatase and the activity of this RNA was tested at different RNA concentrations, we found that the incorporations were identical to those shown in Fig. 3. Thus, removal of the triphosphate did not result in an additional loss in efficiency. We conclude, therefore, that it is not the triphosphate, but m^7G, which directly affects the efficiency of RNA translation.

The products synthesized from modified RNA 4 are normal BMV coat proteins. Figure 4 shows the polyacrylamide gel electrophoretic patterns of the products synthesized from the two RNA 4 samples. The major band of radioactivity represents the coat protein.

Binding of modified RNA 4 to wheat embryo ribosomes. The efficiency of binding to wheat embryo ribosomes was compared for modified and unmodified RNA 4. Figure 5a shows the sucrose density gradient centrifugation patterns of the binding complexes formed in reaction mixtures containing 60 μg of RNA per ml. From incorporation data at this RNA concentration, one would expect the binding efficiency of modified RNA 4 to be about half that of unmodified RNA 4. This is what is observed, as shown in Fig. 5a. The amount of radioactivity associated with ribosomes was 29.8% of the total added RNA for unmodified RNA 4 and 15% for the modified RNA 4 samples. (The reason for the appearance of a diribosome as well as a monoribosome band is probably due to the low anisomycin concentration used, which allows some peptide chain elongation to occur.) The ratios of the binding efficiencies of the two forms of RNA at other RNA concentrations correspond very well with the amino acid incorporation data. Figure 5b shows that with modified RNA 4 as a messenger, polyribosomes are formed if anisomycin is omitted from the binding reaction mixture. This indicates that translation can take place with modified RNA.

To prove that the observed binding and incorporation activities of modified RNA 4 are not due to any methylation or "repairing" of some RNA molecules, bound RNA from the monoribosome and diribosome regions of Fig. 5a was isolated and subjected to fingerprint analyses. The fingerprint patterns of the two bound RNA 4 samples were identical to those shown in Fig. 1. No trace amount of $m^7GpppGp$ could be detected.

Translation and ribosome-binding properties of modified RNA 3. Figure 6 shows the incorporation activities of modified and unmodified RNA 3 at a concentration of 100 μg/ml. The activity of modified RNA 3 is about 50% of that of unmodified RNA 3. The difference between modified and unmodified RNA 3 in leu-

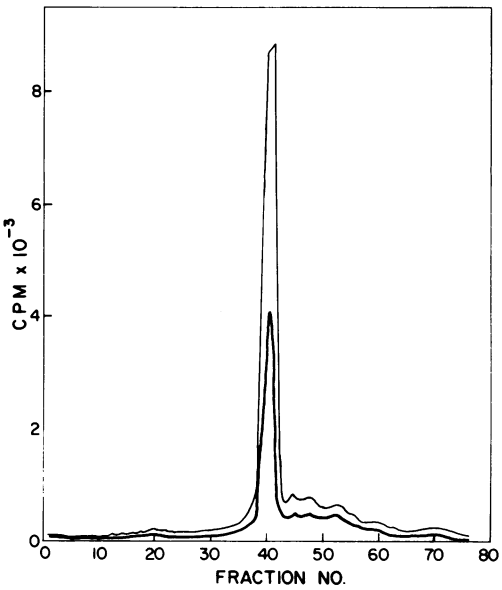

FIG. 4. *Polyacrylamide gel electrophoretic patterns of the products synthesized from modified and unmodified RNA 4. Protein synthesis was performed under the amino acid incorporation assay conditions. The radioactive samples were treated and analyzed as previously described (13). Fifty-microliter samples were analyzed. Electrophoresis was from left to right. Thin line, Products from unmodified RNA 4; thick line, products from modified RNA 4.*

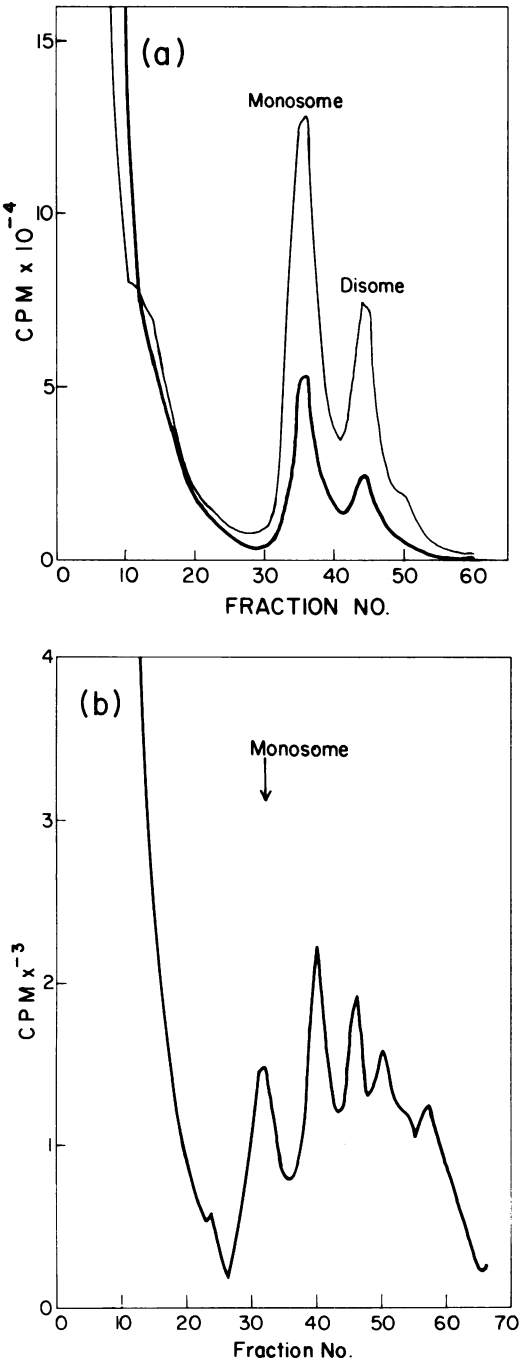

FIG. 5. *Sucrose density gradient centrifugation patterns of RNA-ribosome complexes. (a) Binding assays were performed as described in Materials and Methods. The binding reaction mixtures (400 μl) contained 25 μg of ^{32}P-labeled RNA (5 × 10^6 cpm). Thin line, Unmodified RNA 4; thick line, modified RNA 4. (b) The reaction mixture (100 μl) contained 5 μg of ^{32}P-labeled modified RNA 4 (2 × 10^5 cpm).*

cine incorporation was larger when the RNAs were translated at lower RNA concentrations. For example, at 20 μg/ml, the incorporation promoted by modified RNA 3 was about 8% of that promoted by unmodified RNA 3. These results indicate that, like modified RNA 4, modified RNA 3 can be translated, but at reduced efficiency. Figure 7 shows the gel electrophoretic patterns of the products synthesized. Normal protein 3a is made from modified RNA 3. The binding properties of modified RNA 3 are similar to those of RNA 4.

DISCUSSION

It is not clear how m^{7}G is involved in RNA translation. However, we believe that the terminal dinucleotide m^{7}GpppGp by itself is not sufficient for ribosome recognition because the structure isolated from the T_1 digest did not bind to wheat embryo ribosomes under our usual assay conditions (4). Our incorporation data of the phosphatase-treated sample indicated that the terminal triphosphate structure is probably not involved in RNA translation.

The dependence of RNA translation on m^{7}G seems to be different for different messengers. Encephalomyocarditis virus RNA does not contain the methylated structure (11), but nevertheless it is an efficient messenger. BMV RNA

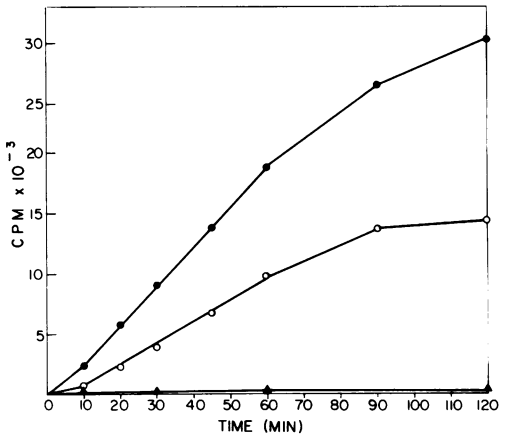

FIG. 6. *Comparison of the incorporation activities of modified and unmodified RNA 3. Amino acid incorporation assays were performed as described in Materials and Methods. Reaction mixtures contained 100 μg of RNA per ml. Ten-microliter samples were counted. Symbols: (△) no RNA added; (○) modified RNA 3; (●) unmodified RNA 3.*

Anisomycin was omitted from the reaction mixture to allow peptide chain elongation and the mixture was incubated for 20 min. The gradient was centrifuged for 3 h.

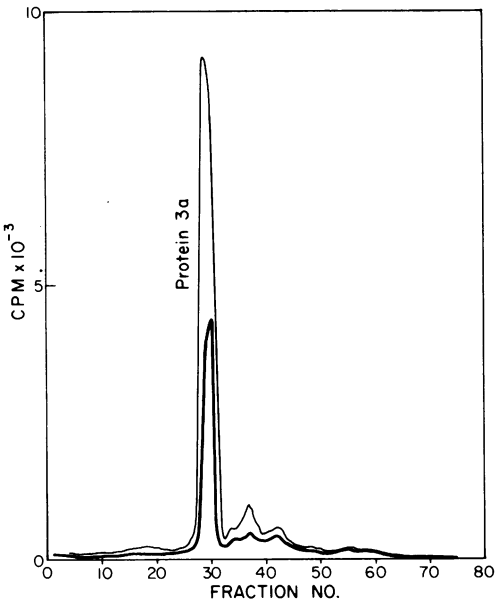

FIG. 7. *Polyacrylamide gel electrophoretic patterns of the products synthesized from modified and unmodified RNA 3. Protein synthesis was performed under the amino acid incorporation assay conditions. The radioactive samples were treated and analyzed as previously described (13). Fifty-microliter samples were analyzed. Electrophoresis is from left to right. Thin line, Products from unmodified RNA 3; thick line, products from modified RNA 3.*

and brine shrimp messengers (10) are only partly dependent on the presence of the methylated structure for their translation. On the other hand, the structure appears to be an obligatory requirement for translating in vitro-synthesized reovirus and vesicular stomatitis virus RNAs in the wheat embryo extracts (9). Obviously, RNA translation is a complex process and many determining factors must be involved. Factors such as m^7G, the location and structural surroundings of the initiation AUG codon, and the nucleotide sequence of the initiation region may all participate in the initiation process of RNA translation. The importance of each individual factor may vary, depending on the properties of a certain RNA and the nature of the translation system.

The sequence of the first 13 bases of RNA 4 is m^7GGUAUUAAUAAUG. The sequence $-$UAAUAAUG can form several base pairings with a GAUCAUUA$_{OH}$, which is the common 3′ end sequence of all sepcies of 18S rRNA that have been analyzed (14). The 3′ end sequence of wheat embryo 18S rRNA has not been determined. However, occurrence of base pairing between RNA 4 and wheat embryo rRNA's is very likely because formation of rRNA-RNA 4 complexes was observed during isolation of initiation ribosome-binding fragments (4). Thus, RNA-RNA interaction of this type may be responsible for ribosome recognition and the initiation of protein synthesis. In some direct way, yet to be elucidated, m^7G also participates.

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