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Requirement for 7-methylguanosine in translation of globin mRNA in vivo

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ABSTRACT

The 7-methylguanosine (m^7G) residue present in the $m^7G^{5'}ppp^{5'}X$- "CAP" structure of rabbit globin mRNA was removed quantitatively by periodate oxidation followed by β-elimination in the presence of cyclohexylamine. The RNA thus treated was intact and exhibited no signs of degradation as examined by polyacrylamide gel electrophoresis in formamide. Assay for protein synthesis using a wheat germ cell-free system showed that the globin mRNA lacking m^7G had lost most of its messenger activity. Identical treatment of satellite tobacco necrosis virus (STNV) RNA, which does not contain the 5 - terminal "CAP" structure, resulted in no loss of its mRNA activity. Since the importance of the m^7G residue in eukaryotic mRNA has not yet been shown essential for translation in vivo, both untreated and treated globin mRNAs were injected into frog oocytes and their translation into globin was measured at intervals over a ninety-six hour period. Globin mRNA either treated with periodate alone or lacking in m^7G altogether were both found to have lost more than 90% of their activity in vivo.

INTRODUCTION

"CAP" structures of the type $m^7G^{5'}ppp^{5'}X$- (1) have been found at the 5'-termini of several viral and non-viral eukaryotic mRNAs (1,2) and have been implicated in the formation of mRNA-ribosome initiation complex (3-7) and/or in the protection of mRNAs towards nucleolytic degradation (8,9). The most extensive studies have been done with viral mRNAs, and both the efficiency of the ribosome binding and translation activity of methylated (m^7GpppX----) versus unmethylated (GpppX----) (3,10) or "capped" (m^7GpppX----) versus "decapped" (pppX----) mRNAs (11,12) have been compared in vitro. Such studies have indicated that for mRNAs which normally contain the "CAP" structure, the contribution of m^7G towards ribosome binding and translational efficiency varies between the different viral mRNAs examined (11,12) and for a given mRNA, between the protein synthesizing systems used in vitro (10-13. Additional evidence for the role of the "CAP" structure in mRNA function has come from studies using analogues of "CAP" such as pm^7G which were found specifically to inhibit the translation in vitro of mRNAs containing the "CAP" struc-

ture (4,5,10,11).

Removal of the m^7G residue by treatment with periodate followed by β-elimination with aniline has till now been used as one of the approaches to obtain a mRNA lacking the "CAP" structure. It has, however, been reported (6,8,11) that such treatment can often cause degradation of the RNA as well as non-specific modification affecting mRNA activity. In the absence of appropriate control experiments, results obtained with β-eliminated RNA prepared by this method must, therefore, be treated with caution.

Alternative methods described recently for the removal of the $m^7G^{5'}ppp^{5'}$X---- "CAP" structure involve the use either of polynucleotide kinase (14) or pyrophosphatases isolated from tobacco cells or potato extracts (15,16).

We have recently developed a procedure for quantitative removal of m^7G from eukaryotic mRNA "CAP" structures that results in neither detectable degradation of the mRNA nor any non-specific modifications affecting their translation. We have used such a procedure to remove m^7G from rabbit globin mRNA. By a comparison of the translational activity of globin mRNA containing the 5'-terminal m^7G with that of the mRNA lacking the m^7G, we show for the first time the important contribution made by this nucleoside for the functioning of a non-viral eukaryotic mRNA *in vivo*.

MATERIALS AND METHODS

General: Sodium periodate was purchased from Fisher Scientific Co. Cyclohexylamine, tetramethylglycinamide and "Stains All" were obtained from Eastman Kodak Co., Rochester, N.Y. ^{35}S-methionine (400-500 Ci/mmole) was purchased from New England Nuclear, Boston, Mass., and ^{3}H-histidine (40 Ci/mmole) from the Radiochemical Centre, Amersham, England. Rabbit globin mRNA was prepared as described previously (2). Satellite Tobacco Necrosis Virus (STNV) RNA was a kind gift from Dr. John M. Clark, Jr., Dept. of Biochemistry University of Illinois. Uniformly ^{32}P-labeled VSV mRNA was a kind gift from Dr. John K. Rose, Dept. of Biology, M.I.T.

Removal of 5'-Terminal m^7G from Eukaryotic mRNA: The detailed procedure describing periodate treatment of mRNA followed by β-elimination with cyclohexylamine has been published elsewhere (2,17). The extent of m^7G removal using this procedure was assayed by the method of Rose and Lodish (6) using uniformly ^{32}P-labeled VSV mRNA.

Acrylamide Gel Electrophoresis: Electrophoretic analysis of globin mRNA was carried out on 5.5% polyacrylamide tube gels run in 98% formamide (18). Gel samples consisted of 4 μg of RNA dissolved in 98% formamide (containing xylene cyanol and bromophenol blue), and were electrophoresed for 11 hours at

1 ma/gel. The gels were stained overnight at 4°C in 0.01% "Stains All" in 50% formamide, destained overnight at 4°C in distilled water, and then scanned at 600 nm using a Gilford 2400-S spectrophotometer.

Electrophoresis of proteins synthesized in cell-free systems (19) or frog oocytes (20) was performed on 12.5% polyacrylamide slab gels. Radioactive bands on the gels were located by fluorography (21), and the amount of radioactive protein synthesized was measured by densitometric tracing of the fluorogram on a linear portion of the exposure curve.

Cell-Free Protein Synthesis: The wheat germ system was prepared and used for *in vitro* translation as described by Roberts and Paterson (22).

Oocyte Microinjection and Analysis: Frogs (*Xenopus laevis*) were obtained from the South African Snake Farm (Fish Hoek, Cape Province, South Africa). Batches of 10 oocytes were injected with globin mRNA, cultured and labeled for 3.5 hours with ^{3}H-histidine (23). mRNA samples were injected at a constant concentration (100 µg/ml) and at an approximately constant volume (60-100 nl/cell), within the roughly linear portion of the dose response curve. Corrections were made for slightly different volumes injected (<10% variation per batch). Oocytes were washed and stored at -17°C before being assayed for their globin content (24).

RESULTS

Removal of 7-Methylguanosine from 5'-Terminus of Rabbit Globin mRNA. A direct approach for studying the role of $m^{7}G$ in eukaryotic mRNA is to compare the translational efficiencies of two mRNAs which differ only in the $m^{7}G$ residue. Since most non-viral eukaryotic mRNAs as usually isolated are already "capped", use of such an approach requires first a method for the quantitative removal of $m^{7}G$ residue from the 5'-terminal "CAP" structure of mRNAs.

We have recently developed (2,17) such a method: it involves treatment of the RNA with periodate followed by β-elimination with cyclohexylamine. Figure 1 shows the results of an experiment in which rabbit globin mRNA was treated with periodate or cyclohexylamine, or with both reagents, and then analyzed by electrophoresis on 5.5% acrylamide gels run in 98% formamide. Nucleotidic material was detected by staining. The two closely spaced peaks are due to the separation of α- and β-globin mRNAs. It can be seen that neither treatment alone nor in combination caused any noticeable degradation of the mRNA. Additional treatment with alkaline phosphatase and subsequent 5'-terminal labeling with ^{32}P using γ-^{32}-P-ATP and T4 polynucleotide kinase also showed the β-eliminated RNA to be intact and usable for nucleotide

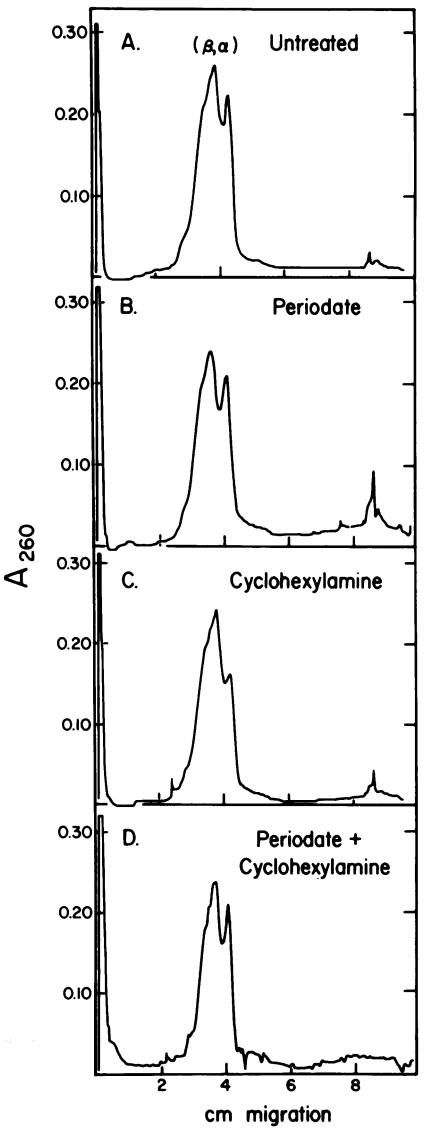

Figure 1. Polyacrylamide gel electrophoretic analysis in 98% formamide of untreated and treated rabbit globin mRNA. Gels were run, stained overnight with "Stains All" and then scanned at 600 nm (Materials and Methods). A variable peak at 9 cm migration is due to residual xylene cyanol marker dye. A, untreated; B, periodate treated; C, cyclohexylamine treated; D, periodate + cyclohexylamine treated.

sequencing of its 5'-termini (2,17,25). The removal of m^7G is essentially quantitative (>95%) as found (data not shown) by a similar treatment of uniformly labeled ^{32}P-VSV mRNA and analysis by the method of Rose and Lodish (6). Though the extent of 5'-end labeling of globin mRNA with T4 kinase is not quantitative due to the hypermodified nature of the 5'-terminus, up to 70% labeling is observed suggesting essentially quantitative removal of caps from globin mRNA as well. Hence, the procedure for the removal of m^7G

residue from globin mRNA using periodate and cyclohexylamine is gentle, efficient and reproducible.

Effect of Removal of 7-Methylguanosine on Globin mRNA Translation in Vitro. Limiting concentrations (2.5 µg/ml) of treated and untreated globin mRNA were used for in vitro translation in a mRNA dependent wheat germ cell-free system, as shown in Figure 2A. Untreated mRNA was carried through both steps of the procedure used for the removal of m^7G, but with the omission of both periodate and cyclohexylamine. Cyclohexylamine treatment alone with no previous periodate treatment gave essentially control level synthesis, while periodate treatment alone resulted in a greater than 60% loss of translatability. The mRNA which had been treated with both periodate and cyclohexylamine was found to have lost greater than 90% of its translational activity. Neither periodate treated nor β-eliminated mRNA affected the translation of untreated globin mRNA when mixed in equal molar amounts, indicating that the loss of mRNA translatability was not due to the presence of a residual contaminant.

When the various treated mRNAs were assayed over a range of concentrations, as shown in Figure 2B, the periodate + cyclohexylamine treated RNA showed no significant stimulation even at levels well above saturation, suggesting that negligible amounts of mRNA remain functional after removal of m^7G. Protein synthetic activity of periodate treated mRNA appears to saturate at a higher concentration (15-20 µg/ml) than untreated mRNA, yet is only one third as active. This result suggests that the periodate treated mRNA is a much less efficient template for protein synthesis. In fact, a gradual decrease of mRNA activity could routinely be observed during the periodate treatment with a 30-35% loss of translatability only after five minutes of incubation with sodium periodate (data not shown). Loss of translatability of mRNA after treatment with periodate alone has also been noted with reovirus and vesicular stomatitis virus mRNA (6,12) and parathyroid hormone mRNA (26), but not for brome mosaic virus RNA (27). Treatment with periodate converts the ribose cis-diol of m^7G to the corresponding dialdehyde; failure to recover the activity thus lost by reduction of the RNA with sodium borohydride to the corresponding primary alcohols (data not shown) suggests that there may exist a fairly rigid structural requirement for the ribose cis-diol in the m^7G moiety of the "CAP" structure(28-30). Thus, the results obtained in the wheat germ cell-free system suggest a specific requirement for both the presence and integrity of the m^7G moiety of rabbit globin mRNA for its translation in vitro. β-elimination of the mRNA will also remove an adenosine residue from the poly A tract at the 3' terminus, leaving the mRNA with a 3'-phosphate, as well as the 5'-triphos-

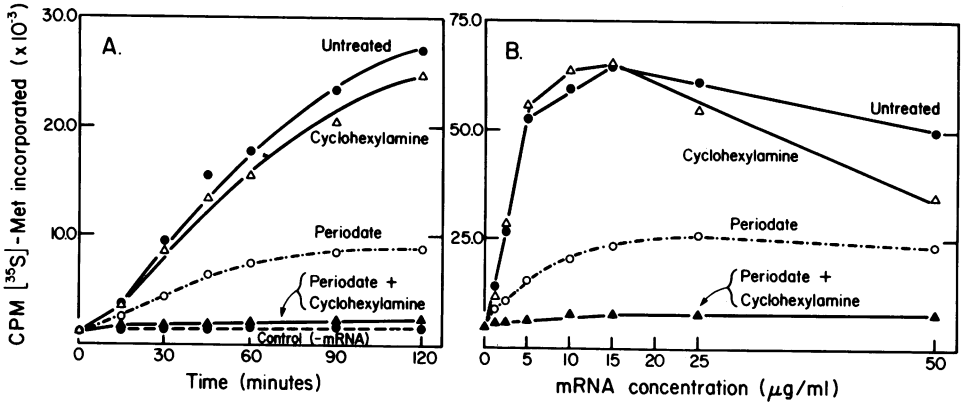

Fig. 2 Translation of untreated and treated mRNA in a wheat germ cell-free system. A) Kinetic analysis of untreated and treated mRNA at subsaturating amounts (2.5 μg/ml) of messenger. B) Saturation curve comparing treated and untreated mRNA. Incorporation of ^{35}S-methionine into protein was determined by removing 4 μl aliquots from reaction mixtures and precipitating with trichloroacetic acid (22). A direct relationship was observed between the relative precipitable CPM's and the relative amounts of globin synthesized as determined by polyacrylamide gel electrophoresis (19). (●——●) untreated; (Δ——Δ) cyclohexylamine treated; (o. – .o) periodate treated; (▲——▲) periodate + cyclohexylamine treated; (●---●) control (– mRNA).

phate group. After treatment with alkaline phosphatase, however, the translatability fo the mRNA is not restored indicating that production of residual phosphate moieties after β-elimination is not responsible for the loss of mRNA activity.

A possible explanation of the above results is that the conditions used for the removal of m^7G cause non-specific modifications within the globin mRNA resulting in loss of its translational activity. This possibility was ruled out by subjecting another eukaryotic mRNA, satellite tobacco necrosis virus (STNV) RNA, which does not contain the m^7GpppX---- "CAP" structure (31), to an identical treatment as used on globin mRNA and comparing the translational activities of the treated STNV RNA to untreated STNV RNA.

STNV RNA is a monocistronic messenger which codes for a 22,000 molecular weight viral coat protein. When assayed over a wide mRNA concentration range, as indicated in Figure 3, neither treatment alone nor in combination resulted in any noticeable loss of messenger activity. Thus, the pronounced loss of translational activity of globin mRNA upon treatment with periodate followed by cyclohexylamine can be attributed to the removal of m^7G residue of the 5'-terminal "CAP" structure rather than due to a non-specific modification of the RNA.

Translational Studies in Vivo of Globin mRNA Lacking m^7G. Although results

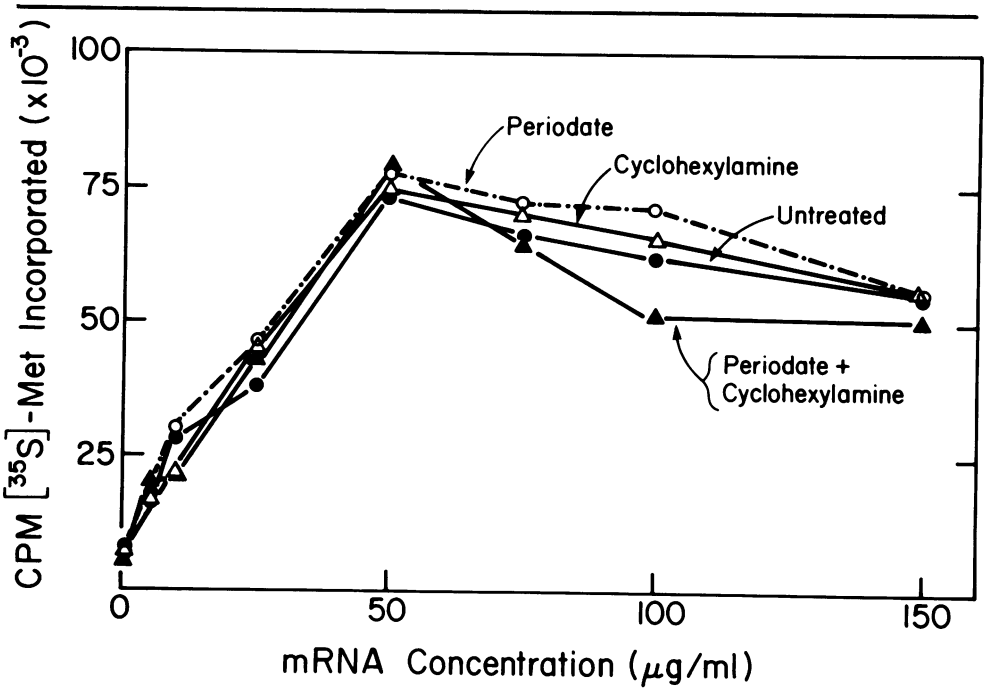

Figure 3. Translation of treated and untreated STNV RNA in a wheat germ cell-free system. Incorporation of ^{35}S-methionine into protein was measured and analyzed as described in Figure 2. (●——●) untreated; (Δ——Δ) cyclohexylamine treated; (o. -.o) periodate treated; (▲——▲) periodate + cyclohexylamine treatment.

obtained in cell-free systems suggest a requirement for m^7G in mRNA function, in view of the possible artifacts of in vitro systems (10,13), a more direct study would be to evaluate the role of m^7G for mRNA translation in an in vivo system. An ideal in vivo system for such an experiment is the oocytes of Xenopus laevis. Rabbit globin mRNA injected into oocytes is both functionally stable and efficiently translated for several weeks (32). The injected mRNA appears to be as stable, in terms of activity, as the average endogenous oocyte mRNA. In an attempt to determine the role of globin mRNA "CAP" structure for its translation in vivo, batches of oocytes were injected with control and treated globin mRNA and the rate of rabbit globin synthesis was measured at intervals over a 96 hour time period. Figure 4A shows a polyacrylamide gel analysis of globin synthesized at 5, 24, and 48 hours as determined after a 3.5 hour pulse-labeling with ^{3}H-histidine (23). The amount of globin synthesized was measured by densitometric scanning of the autoradiogram, and the data obtained for the whole 96 hour period is summarized in Figure 4B. Uninjected oocytes served as controls for the very low level of proteins synthesized on endogeneous mRNA which migrate in the globin region

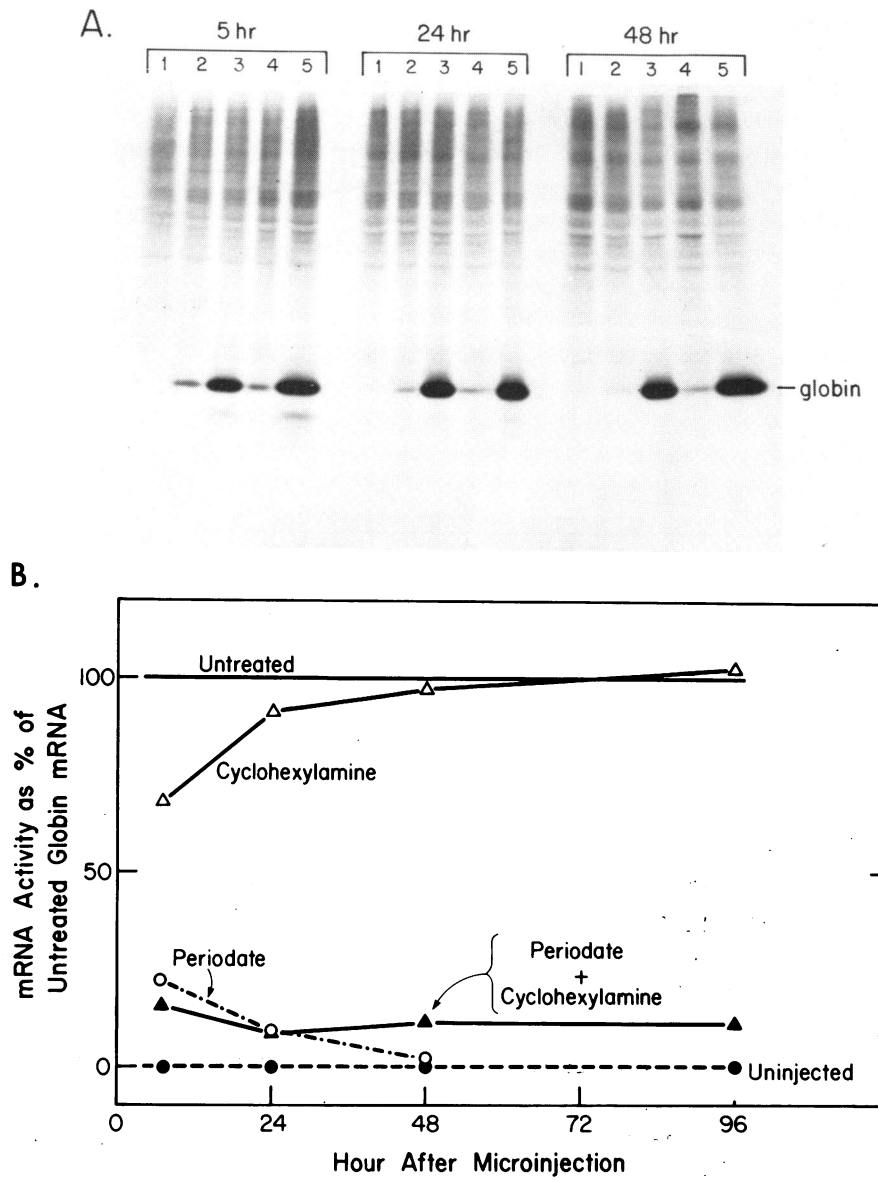

Figure 4. Translation of treated and untreated globin mRNA microinjected into Xenopus oocytes. A) Polyacrylamide gel electrophoretic analysis of globin synthesized in oocytes at 5, 24 and 48 hours after microinjection. Gels were electrophoresed and fluorographed (Materials and Methods). 1. Uninjected; 2. periodate treated; 3. cyclohexylamine treated; 4. periodate + cyclohexylamine treated; 5. untreated. B) Summary of electrophoretic data on globin synthesis 5, 24, 48 and 96 hours after microinjection. (——) Untreated; (●---●) uninjected; (Δ——Δ) cyclohexylamine treated; (o. - .o) periodate treated; (▲——▲) periodate + cyclohexylamine treated.

of the polyacrylamide gel; other experiments (data not shown) have shown that injection of buffer has no significant effect on this or any other region of the gel profile. Figure 4B shows that cyclohexylamine treated RNA is almost as active as the native mRNA; however, periodate treated RNA and RNA lacking the m^7G residue both appear to have lost 90% of the translational activity in the oocyte.

DISCUSSION

An unequivocal analysis of the role of m^7G in mRNA translation requires that the two mRNAs whose translational efficiencies are being compared differ only in the m^7G residue. Thus, for a cellular mRNA which can normally be isolated only in the form of a m^7GpppX---- "CAP" structure, conditions used for removal of m^7G must be nearly quantitative, and must not cause either significant degradation or non-specific modification of the molecule. We have shown (2,17) that periodate oxidation of mRNA followed by β-elimination using cyclohexylamine rather than aniline (6,8) satisfies these criteria. Results of translational studies using β-eliminated globin mRNA in a cell-free system show a strong requirement for the m^7G moiety in agreement with similar studies by others using either globin mRNA (9,16) or viral mRNAs (6,7,12). Periodate treatment alone is enough to reduce translatability by 30-60%, reflecting the sensitivity *in vitro* to the integrity of the nucleoside. More important are results obtained with *Xenopus* oocytes which are known to translate injected globin mRNAs for several weeks (32). In view of the possible artifacts which can be generated in assembling *in vitro* protein synthesizing systems (10,13,33), we undertook to determine the role of the m^7G moiety *in vivo*. Assayed from time of injection to five hours after microinjection, both β-eliminated and periodate treated mRNAs had lost 90% of their translatability, and this low level of activity persisted for the next four days. Though we speculate that the residual activity of β-eliminated mRNA is the result of inefficient translation *in vivo*, we cannot absolutely rule out residual "recapping" of some portion of the treated mRNA in the oocyte (34). Studies *in vitro* indicate that the primary effect of removal or modification of the m^7G moiety is on ribosome binding (3-13), but, although possible, such studies have not previously been extended to the oocytes. Based on the relative rates of degradation within oocytes of injected reovirus mRNAs carrying different 5'-termini such as m^7GpppX--- "CAP", GpppX--- and pppX---, Furuichi *et al.* (8) suggest that the presence of a m^7GpppX--- or a GpppX--- structure at the 5'-terminus protects mRNAs against 5'-exonucleolytic degradation. However, until the translatability within the oocyte of native reovirus mRNA

carrying the m^7GpppX--- "CAP" structure is compared to that of a highly stable eukaryotic mRNA such as the globin mRNA, it is difficult to evaluate the effect of 5'-exonucleases on the translatability in oocytes of periodate treated or "decapped" globin mRNA. Even though wheat germ extracts appear to contain a very significant amount of a similar nucleolytic activity (8,9), the primary effect of "CAP" removal appears to be on the rate and extent of ribosome binding (3-13). We are presently investigating the chemical stability of both native vs. "decapped" globin mRNA within the oocyte.

Recently, Abraham and Pihl (14) have reported that when the m^7G residue of rabbit globin mRNA is removed enzymatically using polynucleotide kinase, no loss of translational activity is seen in the wheat germ system. This result is in direct conflict with our data using both chemically and enzymatically "decapped" (see below) globin mRNA, and is difficult to reconcile with several studies on viral mRNAs (3-6,9-12,15). One possible interpretation proposed to explain their results is that when polynucleotide kinase removes m^7GDP from the 5'-termini, it leaves behind a residual 5'-phosphate with a net negative charge of 2 at physiological pH, similar to the net charge on a "CAP" strcture. This possibility is unlikely, since we have more recently cleaved the triphosphate linkage between the m^7G residue and the remainder of the globin mRNA using a pyrophosphatase isolated from tobacco cells (15). This method also leaves behind a residual 5'-phosphate and causes no detectable scission of the mRNA as examined by polyacrylamide gel electrophoresis. When pyrophosphatase treated globin mRNA as well as ovalbumin mRNA were assayed for activity in both a homologous mRNA dependent reticulocyte lysate (35) and a wheat germ cell-free system, the translational activity of these mRNAs was similarly abolished and identical to that of mRNA which had been treated with periodate and then with cyclohexylamine (Chu, Lockard, RajBhandary and Rhoads, submitted for publication).

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