Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 114-124

Effect of Enzymatic "Decapping" on Protamine mRNA Translation in Wheat Germ S-30

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Received September 14, 1978

SUMMARY

The cap structure from the 5'-ends of the deadenylated protamine mRNAs (PmRNA) was removed with tobacco acid pyrophosphatase. The penultimate 5'-nucleotides were labelled using T_4 polynucleotide kinase in the presence of [γ-^{32}P]-ATP and the labelled mRNA shown to be intact by electrophoresis on 6% polyacrylamide gels in 8M urea. These "decapped" and labelled PmRNAs do not form 80S initiation complexes and their translation in the wheat germ extract is greatly diminished. This effect may be attributable to the presence of an enzyme in the wheat germ extract with a 5'→3' exoribonuclease activity which degrades "decapped" PmRNAs, releasing the 5'-terminal ^{32}P-labelled mononucleotide, 5'-AMP.

INTRODUCTION

Most eukaryotic mRNAs contain at their 5'-ends a cap structure of the form $m^7G^{5'}ppp^{5'}X_m$ (1) which is added post-transcriptionally to the primary transcript. Many attempts have been made to elucidate the biological significance of this modification. Its functional importance in mRNA translation has been indicated using cap analogues such as m^7GMP or m^7GTP, as competitive inhibitors of translation (2-4) and from studies on the effect of chemical (5-7) and enzymatic (8, 9) decapping of mRNAs on their template activities. However, some results have been contradictory, for example, removal of the m^7G residue by β-elimination from native mRNAs led to a decrease in translation as well as ribosome binding ability (10-12) whereas decapping of mRNA with T_4 polynucleotide kinase which yields 5'-monophosphoryl ends, did not decrease its activity (13). Furthermore, decapping with either tobacco or potato pyrophosphatase, to yield 5'-monophosphate termini without apparent mRNA cleavage led to loss of mRNA activity (8, 9).

Recently we have employed tobacco acid pyrophosphatase to cleave the pyrophosphate linkage between 7-methyl guanylic acid and the 5'-phosphate

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at the 5'-termini of deadenylated PmRNAs (Gedamu et al., manuscript in preparation) and have demonstrated that the "decapped" mRNAs cannot form an 80S initiation complex and do not retain their full activity as templates in a wheat germ system.

MATERIALS AND METHODS

Preparation and labelling of poly $(A)^+$ PmRNA:

Poly $(A)^+$ PmRNA was purified from trout testes in milligram quantities by a procedure described previously (14) and was characterized extensively (15-19). This mRNA was labelled to a very high specific activity with $Na^{125}I$ by the modification of the method of Commerford (20) described by Tereba and McCarthy (21). The labelled PmRNA was separated from unreacted iodide by binding it to a small oligo(dT)-cellulose column (Type T-2, 2 ml bed volume) in high salt followed by elution from the column with water. Analysis on 6% polyacrylamide-8M urea gel shows that 80% of the label co-migrated with unlabelled poly$(A)^+$ PmRNA (data not shown).

Deadenylation of Poly$(A)^+$ PmRNA:

The poly(A) segment from both unlabelled and ^{125}I-labelled poly$(A)^+$ PmRNA was removed enzymatically by RNAse H treatment and the deadenylated PmRNA purified as described previously (22).

Enzymatic decapping and end group labelling of PmRNA:

Removal of 5'-end cap structures, dephosphorylation and subsequent end group labelling were performed in the same reaction mixture using slight modifications of the previously described procedure of Efstratiadis et al. (23). The reaction was performed with 1-2 µg PmRNA in 10 µl containing 25 mM sodium acetate, pH 6.0, 10 mM mercaptoethanol, 0.2 mM EDTA and 1 unit of tobacco acid pyrophosphatase. Incubation was at 37°C for 60 min at which time 2 µl of 0.5 M Tris-HCl, pH 8.3 and 0.1 unit of calf intestinal alkaline phosphatase[1] were added. The reaction was incubated for an additional 30 minutes at the same temperature and 6 µl of a solution containing 33 mM $MgCl_2$ and 33 mM DTT was added. The alkaline phosphatase was inhibited by addition of 2 µl of 25 mM potassium phosphate, pH 9.5 and [γ-^{32}P]-ATP was added to a final concentration of 10 µM in the final reaction volume of 25 µl. Phosphorylation was started by the addition of 1 µl (4 units) of polynucleotide kinase and the reaction mixture incubated at 37°C for 15 min at which time 220 µl ethanol, 60 µl H_2O, 10 µl 2.4 M ammonium acetate and 4 µl of yeast $tRNA^{Phe}$ (2 mg/ml) were added. The mixture was chilled at -80°C and centrifuged at 10,000 rpm in the Sorvall HB-4 rotor. The RNA pellet was dissolved in a small volume of distilled water and stored at -20°C.

For translational studies, the mRNA (usually 5 µg) after decapping was extracted twice with phenol-chloroform-isoamyl alcohol and the RNA was precipitated from the aqueous phase with 3 volumes of ethanol. After washing the precipitate twice with 70% ethanol, it was dried, redissolved in distilled water in 0.1 mg/ml aliquots and stored at -40°C.

Formation of 80S initiation complex:

Both ^{125}I and 5'-end [γ-^{32}P]-ATP labelled deadenylated PmRNAs were assayed for their ability to form an 80S initiation complex in a wheat germ cell-free system. The assay was performed essentially as described previously (15) except that cycloheximide at a final concentration of 1 mM was substituted for sparsomycin to inhibit the elongation step of protein syn-

1 Commercial calf intestinal alkaline phosphatase (Boehringer-Mannheim, grade I) was further purified by gel filtration on Sephadex G-100 and the purified enzyme was shown to be free of RNase activity (Chaconas, G., PhD thesis, University of Calgary, 1978).

thesis. Reaction mixtures were analyzed by centrifugation on 10 - 30% sucrose gradients in buffer containing 30 mM Hepes, pH 7.6, 50 mM KCl and 3 mM magnesium acetate. Fractions were collected and ^{125}I and ^{32}P-(Cerenkov) counts were estimated using Picker Nuclear Gamma and Beckman LS-350 liquid scintillation counters respectively.

Analysis of 5'-end mononucleotides:

During the formation of the 80S initiation complexes, it was observed that the terminal 5'-^{32}P-labelled mononucleotides were released from the mRNA in the wheat germ S-30 and aliquots were spotted onto Whatman 3 MM paper together with a marker of a mixture of unlabelled 5'-mononucleotides (0.4 A_{260}/ml) and dye markers of orange G and crystal violet. High voltage electrophoresis was performed in 50 mM citrate buffer, pH 3.5, for 3 hrs at 2 KV. The paper was dried and autoradiographed by exposure to either Dupont or Kodak Blue Brand X-ray film.

Translation of mRNA in the wheat germ S-30, isolation and characterization of cell-free products:

The wheat germ cell-free system was prepared as described by Roberts and Patterson (24) and mRNA activity was assayed as previously described (14, 16). mRNA was present at final concentrations between 4 to 20 µg/ml. The ^{3}H-arginine labelled polypeptides were isolated by acid extraction (14-16) and characterized by chromatography on a CM-52 column (1 x 60 cm) using a linear gradient ot (0.66 M to 1.10 M) lithium chloride/20 mM lithium acetate, pH 5.0 to separate the protamine components.

RESULTS

The poly(A) segment (65-110 A-residues, Iatrou et al., Manuscript submitted for publication) of poly(A)$^+$ PmRNAs can be removed from the 3'-end by RNAse H treatment (22) and the resulting deadenylated mRNAs have been employed to study the possible role of the 5'-end cap structures during translation in a wheat germ extract.

Figure 1A indicated that the deadenylated PmRNAs migrate as 3 major and one minor band (total of 4 bands) upon electrophoresis on a 6% polyacrylamide-8M urea denaturing gel system (18, 22). The total deadenylated mRNAs and more recently the four individual bands have been shown to be translated with equal efficiencies as the poly(A)$^+$ PmRNAs (22, Gedamu et al., manuscript in preparation).

In order to show the effect of removal of the 5'-end cap structures from these mRNAs on either their translation or their ability to form 80S initiation complexes in the wheat germ S-30, we performed the following experiments. Total deadenylated PmRNAs were treated with tobacco acid pyrophosphatase, under conditions (see Materials and Methods) which lead to the quantitative removal of the cap structure from globin mRNA, to give mRNAs terminated by a 5'-phosphoryl group. One portion was dephosphorylated and subsequently labelled with [γ-^{32}P]ATP using polynucleotide kinase. These 5'-^{32}P-labelled mRNAs were analyzed on 6% polyacrylamide gel in 8 M urea and shown to co-migrate with the unlabelled PmRNAs (Fig. 1B). After showing that the 5'-end labelled mRNAs remain intact (Fig. 1B), these samples were used to study the

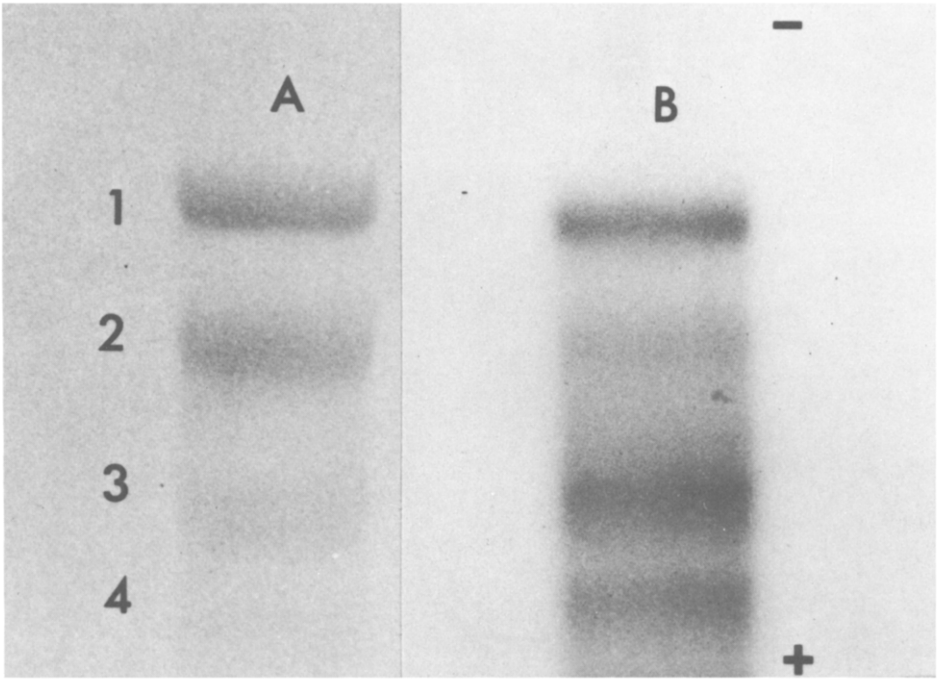

Figure 1: Analysis of deadenylated PmRNAs by electrophoresis on a 6% polyacrylamide gel in 8 M urea.

(A) 3 μg of unlabelled native end
(B) 1 μg of [α-^{32}P]ATP labelled "decapped" deadenylated PmRNAs were analyzed as described previously (18, 22) except that electrophoresis was performed at 800 volts for 5 hrs. Bands 1 to 4 (top to bottom) indicate the stained (A) and autoradiographed (B) portions of the four deadenylated PmRNAs.

effect of the removal of the cap structure on their ability to form an 80S initiation complex in the wheat germ S-30. A second portion of the decapped but not dephosphorylated mRNA was used for translational studies. Figure 2B shows that 5'-^{32}P-end-labelled total deadenylated PmRNAs are unable to form 80S initiation complexes. The entire ^{32}P-label remained unbound at the top of the sucrose gradient. A similar result was also obtained with the individual 5'-end-labelled deadenylated PmRNA components which have been shown to be intact by various criteria (Gedamu et al., manuscript in preparation) after purification by gel electrophoresis. On the other hand, ^{125}I-labelled total deadenylated capped PmRNA does form a true 80S initiation complex (Fig. 2A) and the formation of the complex is sensitive to inhibitors of initiation of protein synthesis such as edeine and aurinetricarboxylic acid (ATA) (unpub-

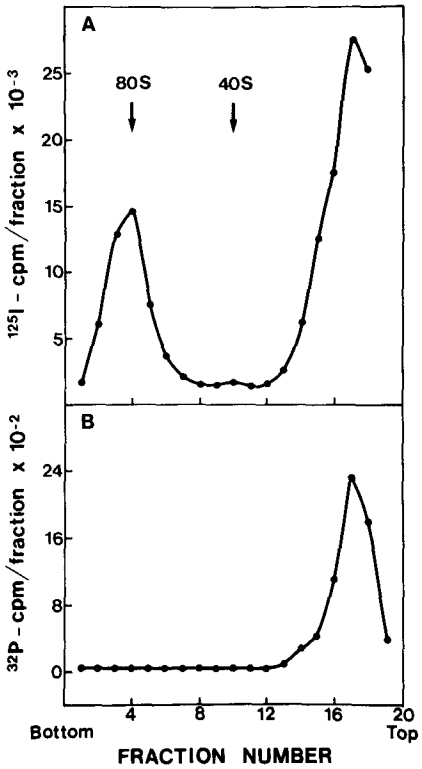

Figure 2: Initiation complex formation with "capped" and "decapped" PmRNAS: Equivalent amounts of ^{125}I-labelled capped (A), and ^{32}P-labelled decapped (B) PmRNAs were added to a wheat germ cell-free system, which was preincubated with 1 mM cycloheximide, to form an 80S initiation complex (15; Gedamu and Dixon, manuscript in preparation). The reaction mixtures were analyzed as described previously (15).

lished data). Thus the presence of an intact cap structure appears to be essential for the formation of the 80S initiation complex.

The fact that the 5'-^{32}P-label of the protamine mRNA remained at the top of the sucrose gradient illustrated in Fig. 2B could have several explanations. Three major possibilities exist; first, the 5'-end labelled mRNA could re in intact but be unable to bind because of the absence of the cap structure. Secondly, the 5'-^{32}P-end label might be removed by a monophosphatase present in the wheat germ S-30 or thirdly, the protamine mRNA might be degraded exonucleolytically releasing the 5'-terminal labelled mononucleotide. To examine these possibilities, an aliquot from the top of the sucrose gradient (Fig. 2B) was spotted on a DEAE-cellulose paper and descending chromatography was performed in 0.35 M ammonium formate buffer to separate oligonucleotides from

Table 1: Separation of oligonucleotides from mononucleotides

Aliquots from pooled fractions in Figure 2B, were spotted in DEAE-cellulose strips and descending chromatography was performed in 0.35 M ammonium formate buffer for 3 hrs. The strips were dried, radioactivity localized by a Geiger counter and counted in 10 mls of scintillant (15).

Experiment	^{32}P counts in the Origin	^{32}P counts in the Mononucleotides	% radioactivity in the mononucleotides
1	121	3136	96.3
2	101	2240	95.7

mononucleotides and free phosphate. The result in Table 1 showed that 96% of the radioactivity did not remain at the origin and could not therefore be oligonucleotide in nature. To distinguish between the second and the third possibilities, high voltage electrophoresis of a second aliquot was performed at pH 3.5 on Whatman 3 MM paper. The labelled material co-migrated (Fig. 3) predominantly with unlabelled adenosine 5'-monophosphate. We have observed previously that if the 5'-end-labelled deadenylated PmRNA components are degraded with penicillium nuclease, the nucleotide at the 5'-end of each component is adenosine 5'-monophosphate (Gedamu et al., manuscript in preparation). The wheat germ S-30, therefore, appears to contain an enzyme with a 5' → 3' exonuclease activity (8). Thus, one of the functions of the cap structure may be to protect mRNA from 5' → 3' exonuclease attack during translation.

The results presented in Figs. 2B and 3 do not, however, exclude the possibility that the remainder of the mRNA molecule which has lost an undefined number of nucleotides including the 5'-^{32}P-labelled adenylate could still form an initiation complex and be translated. To test this hypothesis, a second aliquot of "decapped" mRNA was assayed for its ability to stimulate incorporation of ^{3}H-arginine into hot TCA-tungstate acid precipitable material, in the wheat germ S-30. Table 2 shows that 85 - 90% of the mRNA activity was lost compared to the control. Secondly, the products of translation of the "capped" and "decapped" mRNAs were isolated and characterized by ion-exchange chromatography on CM-52 columns as shown in Fig. 4. All the protamine components described previously (14, 16) are synthesized when "capped" mRNAs are used as templates (Fig. 4A) and these protamine polypeptides elute from the column with authentic unlabelled protamines added as carrier [since the labelled protamines are presumably phosphorylated in

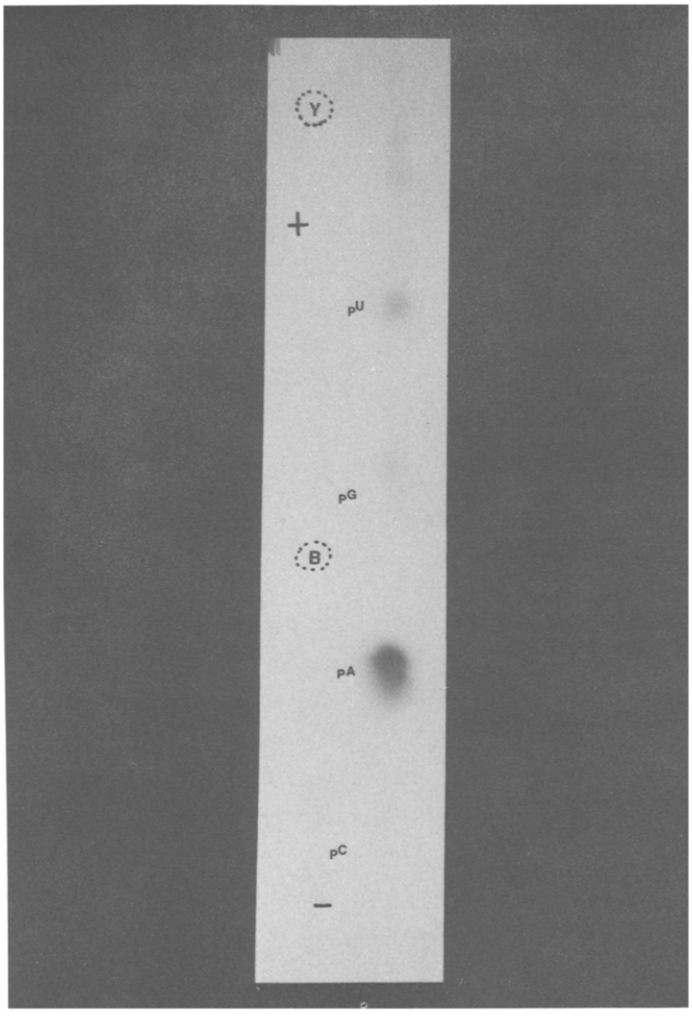

Figure 3: Analysis of the 5'-end mononucleotides by high voltage electrophoresis was performed as described in Materials and Methods. Orange G and crystal violet dye markers are indicated by Y and B respectively.

the wheat germ S-30, they are eluted earlier than their optical density markers (14)]. However, in the presence of an equal amount of "decapped" protamine mRNA as template, the incorporation of ^{3}H-arginine into protamine polypeptides diminishes by greater than 90%. The residual incorporation appears to be distributed between the three resolvable protamine components in the same ratio as in the control and may represent a small amount of mRNA that still retains its cap structure.

Table 2: Template activities of protamine mRNAs in a wheat germ cell-free system

mRNA	Amount in µg	^{3}H-Arginine incorporated (cpm)	Specific Activity cpm/µg
"Capped" deadenylated PmRNA	0.5	43,520	87,040
	0.6	60,060	100,100
	1.0	97,050	97,050
"Decapped" deadenylated PmRNA	0.2	2,320	11,600
	0.7	9,790	13,986

The standard incubation was carried out for 60 min at 28°C and hot TCA-tungstate precipitable radioactivity was estimated as described previously (14). In the absence of mRNA, 19285 cpm were incorporated and are subtracted.

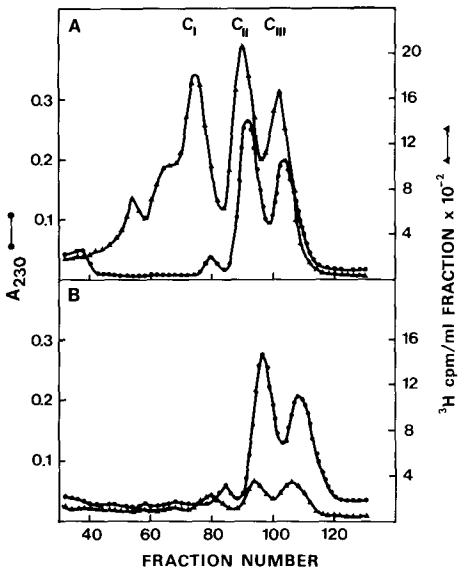

Figure 4: Translational products of "capped" and "decapped" protamine mRNAs:

After testing the template activity of the capped and decapped protamine mRNAs in the wheat germ cell-free system (Table 2) the products formed in the presence of 0.72 µg of each form of protamine mRNAs were analyzed by chromatography on CM-52 columns (14-16). Radioactivity was estimated in every second fraction. Fractions 1 - 40 which contained unincorporated ^{3}H-arginine are not included.

DISCUSSION

In order to understand the possible biological role of the 5'-cap structure of eukaryotic mRNAs, protein synthesis activity and the ability to form an 80S initiation complex in the wheat germ S-30 was compared for the native and decapped protamine RNAs.

Protamine mRNAs, in both the polyadenylated and the deadenylated forms, can support normal synthesis of the 3-4 protamine polypeptides (14, 16) (Fig. 4A). When the 5'-terminal "cap" structure had been removed by tobacco acid pyrophosphatase, each of the PmRNA components lost the ability to support protamine synthesis (Table 2, Fig. 4B). Since by labelling the 5'-ends of the "decapped" PmRNAs with T_4 polynculeotide kinase in the presence of [γ-^{32}P]ATP, the mRNAs are shown to co-migrate with unlabelled PmRNAs (Fig. 1B), the loss in template activity of the "decapped" messages in the wheat germ S-30 is not due to scission in other parts of the RNA molecules during the decapping step. Our results, therefore, indicate that "cap" structures are required for translation of mRNAs in wheat germ cell-free extracts.

Since protein synthesis proceeds in the 5' → 3' direction of the template mRNA, the 5'-terminal cap structure may be related to the formation of an 80S initiation complex in protein synthesis. Deadenylated PmRNA labelled with ^{125}I-Na, with the "cap" structure still present, binds to wheat germ 80S ribosomes (Fig. 2A). How ever, "decapped" PmRNAs labelled at the 5'-end with [γ-^{32}P]ATP lost the ability to form an initiation complex (Fig. 2B). Therefore, the 5'-terminal cap structure of the mRNA is necessary at the step of formation of the initial complex in protein synthesis.

When the cap structure is removed, the mRNA molecule appears to be degraded exonucleolytically from the 5'-terminal by an enzyme in the wheat germ extract (8) releasing the 5'-terminal labelled mononucleotide, adenosine 5'-monophosphate (Fig. 3), at the 5'-end of each PmRNA component. It appears, therefore, that the cap structure is necessary to protect mRNAs from 5'-exonucleolytic degradation (8).

The number of nucleotides removed from the 5'-end of the PmRNAs per unit time (degradation rate) by the putative wheat germ exonuclease during initiation complex formation and translation of the messages is not known at the moment. However, this could be determined by studying in the wheat germ S-30 the kinetics of degradation of PmRNA, labelled with either ^{125}I or at the 3'-end with ^{32}P in the presence of polynucleotide phosphorylase.

There are conflicting observations on the requirement of the cap structure for the translation of certain mRNAs in cell-free systems (25). Chemical decapping of mRNAs such as VSV, TMV, Reovirus and globin by β-elimination (5, 6, 26, 27) or enzymatically by treatment with tobacco acid pyro-

phosphatase (8), demonstrated that efficient translation of these mRNAs in a wheat germ cell-free system depends upon the presence of a 5'-terminal m^7G cap structure. Our results are in agreement with these findings. On the other hand, it has been reported that decapping of rabbit globin and light chain immunoglobulin mRNAs with T_4 polynucleotide kinase did not result in loss of activity of the messages when translated in a wheat germ S-30 (13). However, it is possible that the T_4 enzyme might not have completely removed the 5'm^7GDP from the mRNAs (23, Chaconas, unpublished observation). In reticulocyte lysates, however, it was reported that there was no difference in translation of "capped" and "decapped" mRNAs (10-12, 26). Moreover, not all eukaryotic mRNAs are capped, for example, Poliovirus RNA isolated from the polyribosomes of infected cells is not capped; its terminus is pUp . . . (28) and it also has a protein bound to the 5'-end of the virion RNA (29). Similarly, encephalomyocarditis (EMC) RNA and plant tobacco necrosis virus (STNV) RNA (30) do not have a 5'm^7G Cap and yet these mRNAs are translated in some eukaryotic cell-free systems (2, 3, 6). It is possible, therefore, that in a situation where the 5' → 3' exonuclease activity is low, uncapped mRNAs may be sufficiently stable for translation to occur but in the presence of significant exonuclease activity as, for example, in the wheat germ S-30, the 5'-end of an uncapped mRNA may be degraded so rapidly that the structural requirements for the formation of an initiation complex are lost before translation takes place. Thus a major function of the cap structure would be to stabilize the mRNA against exonuclease attack.

ACKNOWLEDGEMENTS

This work was generously supported by the Medical Research Council of Canada. Lashitew Gedamu is a postdoctoral fellow of the Medical Research Council of Canada, supported by a grant to Professor G. H. Dixon. Tobacco acid pyrophosphatase is a gift from Dr. Efstratiadis and polynucleotide kinase from Dr. G. Chaconas.

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