Removal of 5'-terminal m7G from eukaryotic mRNAs by potato nucleotide pyrophosphatase and its effect on translation

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ABSTRACT

The procedure for isolation of nucleotide pyrophosphatase (E.C. 3.6.1.9.) from potato has been modified to yield an endonuclease-free preparation purified 2300-fold. The enzyme was used for specific cleavage of pyrophosphate linkages in the 5'-terminal cap (m7GpppN) of several eukaryotic messenger RNAs. Enzymatic removal of 5'-terminal pm7G from reovirus, rabbit globin and Artemia salina mRNAs resulted in an almost complete loss (>80%) of their template activities in a cell-free protein synthesizing system from wheat germ. Incubation with nucleotide pyrophosphatase did not decrease the translation of phage f2 RNA in an Escherichia coli cell-free system.

INTRODUCTION

All eukaryotic mRNAs studied so far and most messenger RNAs of animal and plant viruses are terminated at their 5'-ends with a unique methylated structure m7G5'ppp5'N, generally referred to as a "cap" (1). Several lines of evidence suggest that the m7G cap plays an important role in initiation of mRNA translation (2-18). Most of this evidence comes either from experiments in which translation of unmethylated reovirus or vesicular stomatitis virus mRNAs, synthesized in vitro in the presence of S-adenosylhomocysteine (AdoHcy), was compared with their methylated counterparts, or from data on the activity of messengers submitted to β-elimination, a chemical procedure which removes terminal m7G from mRNA (2-11). However, both of these approaches have certain limitations. The availability of methylated and unmethylated messengers is limited to those viral messengers which can be synthesized in vitro by viral cores containing the necessary enzymes (2,8, 19-24). On the other hand, β-elimination, which includes periodate and aniline treatment of RNA, apart from its effect on the 5'-end of mRNA, also alters its 3'-terminus and may result in some non-specific damage (7). It therefore seemed desirable to seek an alternative procedure for removal of m7G from any cap-terminated mRNA without affecting the rest of the molecule.
Isolation from potato of a nucleotide pyrophosphatase (E.C. 3.6.1.9.) catalyzing the cleavage of the pyrophosphate linkage in \(^7\text{GpppG}^m\)-terminated fragments of reovirus mRNA has been described (25), but its ability to remove \(^7\text{G}\) from intact mRNA was not unequivocally established due to contamination with traces of endonucleolytic activity. A similar enzyme was also purified from cultured tobacco cells (26) and shown to be effective in removing the 5'-'terminal \(^7\text{G}\) from mRNA (27,28).

This study describes a modified procedure for the isolation of nucleotide pyrophosphatase and its use for specific removal of \(^7\text{G}\) from mRNAs. Translational activity of several "decapped" mRNAs obtained by treatment with potato nucleotide pyrophosphatase was assayed in a wheat germ cell-free protein synthesis system.

**MATERIALS AND METHODS**

**Chemicals.**

Thymidine-5'-(p-nitrophenylphosphate), NAD, adenyl-(3'-5')adenosine and snake venom pyrophosphatase were obtained from Sigma; E. coli alkaline phosphatase, BAPC, from Worthington; and CM-Sephadex G-50 and Sepharose 6B from Pharmacia. DE-52 cellulose and GF/C filters were purchased from Whatman, TLC DC-cellulose F plates from Merck, and bentonite from Fischer Scientific. All chemicals used for translation studies were analytical grade reagents from Sigma. \(^7\text{G}\), \(^7\text{CMP}\), \(^7\text{GDP}\) and \(\text{G}^m\) were from P-L Biochemicals. L-\(^{14}\text{C}\)-leucine (sp. act. 59 \(\mu\text{C}/\mu\text{mole}\)) and \(^{35}\text{S}\)-methionine (sp. act. 200 \(\text{C/mmole}\)) were from Radiochemical Centre, Amersham.

**Messenger RNAs.**

*Artemia salina* polyadenylated mRNA was isolated directly from the post-nuclear supernatant of developing *A. salina* cysts (29) by affinity chromatography on oligo-dT-cellulose in the presence of sodium dodecylsulfate (SDS) with a proteinase K digestion step (30). Capped methylated non-radioactive and \(^3\text{H}\)-methyl-labeled reovirus mRNAs were synthesized in vitro by reovirus cores in the presence of cold and \(^3\text{H}\)-methyl-S-adenosylmethionine (Adomet), respectively (4). The specific activity of reovirus \(^3\text{H}\)-mRNA was 14,500 cpm/\(\mu\text{g}\), with the radioactivity evenly distributed between the 5'-'terminal \(^7\text{G}\) and penultimate 2'-0-methylG. Phage f2 RNA was prepared as previously described (31). Purified rabbit globin mRNA was obtained from Searle Laboratories, High Wycombe, England.

**Assays of nucleotide pyrophosphatase activity.**

Nucleotide pyrophosphatase activity was routinely assayed against thymidine-5'-(p-nitrophenylphosphate) according to the method of Razzell and
Khorana (32). The reaction mixture (0.1 ml) containing 5 mM thymidine-5'- (p-nitrophenylphosphate), 0.1 M Tris-HCl buffer pH 7.0 and enzyme was incubated for 15 min at 37°. One unit of nucleotide pyrophosphatase is defined as the amount of enzyme which liberates 1 µmole of p-nitrophenol from thymidine-5'- (p-nitrophenylphosphate) in 1 min at 37°. A molar extinction coefficient of 12,000 at 400 nm was used in the calculations.

The nucleotide pyrophosphatase activity against NAD and 5'-AMP was assayed in 0.1 ml incubation medium containing 0.1 M sodium acetate buffer pH 5.2, 10 mM substrate and enzyme. Following incubation for 30 min at 37°, samples containing NAD were incubated for an additional 10 min with 1.5 units alkaline phosphatase in 0.25 ml 0.1 M Tris-HCl buffer pH 8.0. The enzymatically liberated phosphate was assayed according to Lowry and Lopez (33).

Treatment of mRNAs with nucleotide pyrophosphatase.

mRNAs to be used for translation studies were incubated in 0.1 ml of 0.02 M sodium acetate buffer pH 5.2, 0.1 M KCl, 1.75 units of bentonite-adsorbed enzyme or bentonite alone and the following amounts of mRNA: 35 µg reovirus mRNA, 44 µg A. salina mRNA, 30 µg globin mRNA, and 100 µg f2 RNA. Following incubation at 37°C for 30 min, bentonite-adsorbed enzyme was removed by centrifugation and the RNA precipitated from the supernatant by addition of 2.5 vol. of ethanol, was washed with ethanol and dissolved in water. In some experiments when the initial concentration of RNA was high enough, it was used directly for translation experiments. Low amounts of pyrophosphatase which, in such instances, may have been added inadvertently with RNA to the protein synthesis system were found not to interfere.

Analysis of reovirus 3H-methyl-mRNA on DMSO-sucrose denaturing gradients.

2.7 µg of reovirus 3H-methyl-mRNA were incubated in 25 µl of 0.02 M sodium acetate pH 5.2, 0.1 M KCl containing 0.35 unit of bentonite-adsorbed enzyme or bentonite alone (control). After incubation at 37°C for 30 min and removal of bentonite-adsorbed enzyme by centrifugation, samples were diluted with 0.2 ml of 99% dimethylsulfoxide (DMSO) containing 1 mM sodium EDTA, pH 7.0, and 10 mM LiCl and subsequently heated at 60°C for 2 min. Each sample was then divided into two 0.1 ml portions and loaded onto 4.5 ml sucrose gradients (5-20%, w/v) in DMSO-EDTA-LiCl solution as above (34). Centrifugation was for 24 hr at 48,000 rpm in a Beckman SW 50.1 rotor at 27°C. Fractions from one experimental and one control gradient were counted directly in Bray's scintillant; to fractions from the remaining experimental and control gradients, 200 µg of yeast RNA were added as a carrier and the RNA was precipitated with cold 10% trichloroacetic acid (TCA). Precipitates were collected
on Whatman GF/C filters which were subsequently washed with 5% TCA, ethanol-ether, ether and counted in toluene-based scintillant.

Polyacrylamide gel electrophoresis of mRNA in 98% formamide.

Reovirus mRNA was incubated as for the DMSO-sucrose gradients, precipitated with ethanol in the presence of carrier rabbit liver tRNA and dissolved in 5 mM barbital-formamide pH 6.5. Polyacrylamide gels (4% - 8 x 0.5 cm) in 98% formamide (35) were loaded with 5 μg of mRNA and 15 μg tRNA, and electrophoresis was for 4-5 hr at 1.25 mg/gel. Gels were stained for ~16 hr with Stainsall (Serva), destained with H2O and scanned at 600 mp with a Joyce Loebl microdensitometer.

Preparation of extracts used for protein synthesis.

Commercial "untoasted" wheat germ (General Mills Inc., Minnesota, USA) S-23 extracts were prepared essentially by the method of Roberts and Paterson (36) with the following modifications (W. Zagorski, personal communication). Wheat germ (5 g) was ground with an equal weight of sea sand (Fisher Scientific) and 25 ml of a solution containing 20 mM Hepes-KOH pH 7.6, 50 mM KCl, 3 mM Mg acetate and 1 mM dithiothreitol. Directly after centrifugation, the S-23 supernatant was dialysed for 24 hr against 4 changes (500 ml each) of extraction solution. S-30 extract from E. coli Q 13 was prepared essentially according to Webster et al. (37), with the exception that grinding with alumina was used for cell disintegration, and dialysis of the S-30 supernatant was preceded by preincubation for 15 min at 37°C under translation conditions.

Translation assays.

Assays of protein synthesis in the wheat germ cell-free system contained, in a final volume of 25 μl, 10 μl of wheat germ S-23 extract (64.5 mg of protein/ml), 20 mM Hepes pH 7.6, 95 mM potassium acetate, 4 mM Mg acetate, 80 μM spermine, 2.5 mM ATP, 0.375 mM GTP, 0.5 mM dithiothreitol, 10 mM creatine phosphate, 1.5 μg creatine phosphokinase, 42 μM each of the 19 L-amino acids without leucine and 50 μM 14C-leucine. Samples containing varying amounts of mRNAs were incubated at 30°C for 60 min.

Assays of protein synthesis in the E. coli system contained, in a final volume of 25 μl: 4 μl of S-30 extract (23 mg protein/ml), 60 mM Tris-HCl pH 7.6, 40 mM NH4Cl, 30 mM KCl, 3.6 mM β-mercaptoethanol, 14.5 mM Mg acetate, 6 mM ATP, 0.2 mM GTP, 10 mM PEP, 0.4 μg phosphoenolpyruvate kinase, 80 μM each of the 19 L-amino acids without leucine and 80 μM 14C-leucine. Samples were incubated at 37°C for 30 min.

Incorporation of amino acids into protein was measured as hot TCA-insoluble radioactivity by the Whatman 3 MM paper disc method of Mans and
Protein was determined by a modified Lowry procedure (39). Polypeptide products synthesized with 3.5 μM $^{35}$S-methionine instead of $^{14}$C-leucine as the radioactive precursor were analyzed by polyacrylamide slab gel electrophoresis as described previously (29).

Reovirus mRNA binding to 80S ribosomes.

Reovirus $^{3}$H-methyl-mRNA binding to wheat germ 80S ribosomes was assayed essentially as described by Both et al. (4). The incubation medium (50 μl volume) contained the same concentrations of all components as in wheat germ translation assays, except that 200 μM sparsomycin was included to prevent polypeptide chain elongation, and all 20 amino acids present were non-radioactive. Assays contained 0.5 μg of $^{3}$H-methyl-labeled mRNA incubated with or without pyrophosphatase under standard conditions for 30 min. Enzyme-treated $^{3}$H-mRNA was added to the assay without being freed from the liberated pm$^{-7}$G. Incubation was for 10 min at 25°C. Samples were then diluted 4-fold with 20 mM Tris-HCl pH 7.6, 70 mM KCl and 3 mM Mg acetate, and loaded onto 4.5 ml glycerol gradients (10-30%, v/v) made up in the same buffer. After centrifugation for 95 min at 47,000 rpm in an SW 50.1 rotor at 4°C, fractions were collected and counted directly in Bray's scintillant.

RESULTS

Purification of enzyme.

All steps (Table I) were carried out at 6°C, starting with 10 kg of a local variety of potato. Steps 1-5 were essentially those of Kole et al. (25).

Step 6. 580 ml (69 mg of protein) of the enzyme solution from step 5 was concentrated by adding solid ammonium sulfate to 60% saturation. After centrifugation, the precipitate was dissolved in 10 ml of 0.1 M Tris-HCl buffer pH 7.6 containing 0.2% sodium deoxycholate, and applied to a Sepharose 6B column (90 x 1.5 cm) previously equilibrated with the same buffer. The column was eluted with the deoxycholate-containing buffer at a flow rate of 7 ml/hr. Seventy percent of the total nucleotide pyrophosphatase activity, contaminated with traces of nucleolytic activity, was recovered in peak I, between 70 and 160 ml of eluate. Peak II, appearing between 180 and 280 ml of eluate, contained over 90% of total nuclease activity and about 20% of nucleotide pyrophosphatase activity.

Step 7. Beginning with this step, the enzyme exhibited a tendency to adsorb to glass surfaces, and plastic ware was used in all subsequent manipulations. Peak I (90 ml) from step 6 was diluted 2-fold with distilled water and applied to a DE-52 cellulose column (40 x 2.5 cm) pre-equilibrated with 0.02 M Tris-HCl buffer pH 7.6, at a flow rate of 30 ml/hr. Over 80% of the
activity was recovered in the breakthrough and subsequent washing with equili-
brating buffer, total eluate 200 ml. The most active fractions were pooled
(100 ml).

Although the enzyme no longer released any acid-soluble products from
B. subtilis $^3$H-DNA or from phage f2 $^3$H-RNA, polyacrylamide gel electrophoresis
of enzyme-treated $^3$H-labeled f2 RNA, or a mixture of 28S and 18S rRNA from
chick embryos, revealed appreciable endonucleolytic activity. Attempts at its
removal by gel filtration through Sephadex G-100 and Sephadex G-200 in 0.2 M
NaCl, by affinity chromatography on 5'-AMP-Sepharose 4B and chromatography on
DE-52 cellulose in 7 M urea, were unsuccessful.

Step 8. 100 ml of the enzyme solution was stirred overnight with 2 mg
bentonite suspended in 0.1 ml water. Following centrifugation, the sediment
with the adsorbed enzyme was suspended in an appropriate volume of water
(2 ml), so that 1 µl contained about 0.07 activity units.

Bentonite was prepared for enzyme adsorption by stirring it three times
for 60 min at 37° in 25 ml of water, with centrifugation after each wash. The
exact amount of bentonite in the final suspension was estimated by dry weight
analysis. The ratio of bentonite to enzyme protein was maintained at 1:1
(w/w) because excess bentonite caused up to 5-fold inhibition of activity
against NAD.

Alternatively, the effluent from DE-52 cellulose was concentrated, with
40% loss in activity, by adding solid ammonium sulfate to 70% saturation. The
precipitate was collected by centrifugation and dissolved at 0.5 mg protein/ml
water. Before use with polynucleotides, it was diluted about 50-fold and
adsorbed onto bentonite as above.

The results of a typical purification procedure are summarized in Table
I. The enzyme was purified 2300-fold with a yield of 9%. The activity
adsorbed on bentonite or in concentrated aqueous solution remained stable for
over 3 months at 0-4°. Its general properties and specificity were basically
similar to those given by Kole et al. (25), with the exception that the rate
of hydrolysis of NAD, erroneously reported previously as 4300, was 800 µmoles
hydrolyzed per 30 min at 37°. However, with the modified purification proce-
dure, the enzyme exhibited an increased tendency to adsorb to glass, dialysis
tubing and diaflo-ultrafilters, which could not be overcome by addition of
non-ionic detergents.

Specific cleavage of pyrophosphate linkage in capped mRNA.

Lack of endonucleolytic activity in bentonite-adsorbed nucleotide pyro-
phosphatase was initially ascertained by showing no change in mobility of 28S
Table I

Purification scheme for potato nucleotide pyrophosphatase

| Purification Step | Volume (ml) | Total Activity (units) | Protein (mg) | Specific Activity (units/mg protein) | Yield (%) |
|-------------------|-------------|------------------------|--------------|-------------------------------------|-----------|
| (1) 30% saturated ammonium sulfate extract | 3325 | 1,480 | 34,510 | 0.04 | 100 |
| (2) Ammonium sulfate to 60% extract | 300 | 1,430 | 20,000 | 0.07 | 98 |
| (3) Calcium phosphate I | 246 | 797 | 1,033 | 0.77 | 54 |
| (4) Calcium phosphate II | 870 | 451 | 198 | 2.3 | 30.5 |
| (5) CM-Sephadex G-50 | 580 | 273 | 69 | 4 | 18.5 |
| (6) Sepharose 6B, 0.2% sodium deoxycholate | 90 | 192 | - | - | 13 |
| (7) DE-52 cellulose | 100 | 146 | 2 | 73 | 9.8 |
| (8) Bentonite adsorption | 2 | 140 | 1.5 | 93 | 9.4 |

*Protein could not be estimated in the presence of sodium deoxycholate.*

and 18S chick embryo rRNA or reovirus $^3$H-methyl-mRNA in polyacrylamide gel electrophoresis (40) following treatment with enzyme. In order to examine more rigorously whether the nucleotide pyrophosphatase preparation is completely devoid of endonucleolytic activity, profiles of enzyme and mock-treated reovirus mRNA were compared by DMSO-sucrose gradient sedimentation. Under the denaturing conditions employed, any hidden breaks in the RNA chain caused by enzyme treatment should result in a change in the mRNA sedimentation profile. Comparison of the radioactivity distribution in gradients of enzyme-treated and control samples of reovirus mRNA reveals that the pyrophosphatase treatment causes a shift of about 50% of the $^3$H-methyl counts to the top of a gradient, with little effect on the sedimentation profile of the remaining radioactivity, which moves with the macromolecular material (Fig. 1A). When two parallel gradients were analyzed for TCA-precipitable radioactivity (Fig. 1B), it became evident that all the counts at the top of the gradient in Fig. 1A were acid-soluble. Profiles of acid-precipitable RNA were again found to be similar, the only difference between the two gradients being that acid-precipitable radioactivity in the enzyme-treated sample was approximately half that of the control RNA.

Although not resolved in density gradients (Fig. 1), the 1, m and s size classes in another preparation of reovirus mRNA were separated by polyacrylamide gel electrophoresis in 98% formamide (Fig. 2A). Treatment with pyrophos-
Fig. 1. DMSO-sucrose density gradient analysis of reovirus $^3$H-methyl-mRNA incubated with (O---O) or without (●●●) nucleotide pyrophosphatase. Radioactivity distributions from two separate gradients, representing enzyme-treated and control RNA, are superimposed in each panel. Although the three classes of mRNA in the mixture are not resolved here, in other experiments the separated l, m and s classes sedimented with peaks at fractions $\sim$6, 7 and 10, respectively. (A) total radioactivity, (B) cold TCA-precipitable radioactivity.

Fig. 2. Electrophoretic analysis of reovirus mRNA in polyacrylamide-formamide gels. Viral mRNA was incubated in the absence (A) or presence (B) of nucleotide pyrophosphatase and analyzed as described in the methods section.

Phatase again did not alter the migration profile (Fig. 2B). In other experiments, TMV RNA migrated as a single band in the same position (mol. wt. $\sim$2x10$^6$) before and after enzyme treatment, and the mobility of rabbit globin mRNA, which separated into α and β chains in polyacrylamide-formamide gels, was unchanged by incubation with nucleotide pyrophosphatase. These results indicate that the enzyme preparation is devoid of endonucleolytic activity and support the assumption that the material cleaved from reovirus $^3$H-methyl-mRNA corresponds to 5'-terminal pm$^7$G, which should comprise half the radioactivity of the viral mRNA (19).
This conclusion was further supported by TLC analysis of nucleotide pyrophosphatase treated reovirus mRNA. At a low enzyme to substrate ratio (Fig. 3A and B), when 90% and 77% of radioactivity remained at the origin, the only product of hydrolysis exhibiting any mobility migrated in a position corresponding to the pm7G marker. At a higher enzyme to substrate ratio, ensuring acid-solubilization of about 50% of RNA radioactivity (see below and Fig. 4),

![TLC analysis of products cleaved from reovirus mRNA.](image)

Fig. 3. TLC analysis of products cleaved from reovirus mRNA. 0.6 μg reovirus 3H-methyl-mRNA was incubated in 20 μl of 0.1 M acetate buffer pH 5.2 containing 0.0007 (A), 0.007 (B) and 0.33 (C) units of bentonite adsorbed nucleotide pyrophosphatase. After incubation for 30 min at 37°C, the reaction mixtures were spotted on TLC cellulose plates and developed in isobutyric acid 0.5 N NH4OH (10:6, v/v), with m7GDP, pm7G and m7G as markers. For (D), ~0.3 μg mRNA was digested with pyrophosphatase as in (C), ethanol precipitated, washed with ethanol three times, dissolved in 50 μl of 5 mM sodium acetate pH 6 containing 25 μg of P1 nuclease and incubated at 37°C for 1 hr. The pH was then adjusted to 8 in 10 mM Tris-HCl and incubation continued for 1 hr with 1 unit of BAPC and 0.25 unit of snake venom nucleotide pyrophosphatase. Samples were analyzed in 2-propanol:1% (NH4)2SO4 (2:1 v/v) with Gm and m7G as markers. For (E), material at the origin in (C) was incubated and analyzed as in (D) and the same procedure was used for (F) except that the mRNA was mock-treated during the pyrophosphatase digestion. After drying, the plates were divided into areas of 1x2 cm from which the cellulose layer was quantitatively removed and counted in a toluene-based scintillant.
two hydrolysis products became detectable: 21% of the counts migrated with pm$^7$G and 26% with 7-methylguanosine (Fig. 3C). The remaining ~50% of the counts (at the origin) correspond to mRNA with radioactivity in the 2'-O-methylguanosine (Figs. 3D-F).

These results indicate that the products cleaved from reovirus $^3$H-methyl-mRNA by treatment with nucleotide pyrophosphatase correspond to pm$^7$G and m$^7$G, the latter presumably resulting from phosphatase activity in the enzyme preparation. The relatively high activity of nucleotide pyrophosphatase towards nucleoside diphosphates (25,41) precludes the possibility of detecting $^3$H-m$^7$GDP, should it constitute one of the primary products of cap hydrolysis.

Additional support for the lack of endonucleolytic activity in the enzyme preparation may be derived from the experiments on the effect of increasing enzyme concentrations on the amount of TCA-soluble counts released from reovirus $^3$H-methyl-mRNA. Fig. 4 shows that, despite the excess of enzyme, only about 50% of the radioactivity, corresponding to the total radioactivity associated with m$^7$G, became acid-soluble. The same results were obtained with reovirus mRNA alone, or in the presence of excess capped Artemia salina mRNA.

It should be stressed that reovirus $^3$H-methyl-mRNA analyzed on DMSO-sucrose gradients and polyacrylamide-formamide gels (Figs. 1 and 2), and all mRNAs used in subsequent experiments (Figs. 5-7), were incubated with the pyrophosphatase at a concentration of 0.35 units of enzyme per 20 μl incubation mixture (Fig. 4). These activities are well in excess of those which ensure complete removal of 5'-terminal m$^7$G from mRNA (cf. Fig. 4) without causing endonucleolytic degradation.

**Effect of removal of pm$^7$G on the translation of mRNAs.**

Fig. 5 shows that removal of pm$^7$G by treatment of reovirus $^3$H-methyl-mRNA with potato nucleotide pyrophosphatase resulted in almost complete loss of binding to wheat germ ribosomes. While in the control experiments with capped reovirus $^3$H-mRNA about 70% of radioactivity was recovered in the 80S region, little, if any, enzyme-treated mRNA bound to the ribosomes. Most of the $^3$H-methyl radioactivity remained at the top of the gradient suggesting that "decapped" mRNA is degraded by a 5'-exonuclease activity as described previously (10). Comparison of the translation of enzyme-treated and control reovirus mRNA similarly showed that removal of 5'-terminal pm$^7$G from reovirus mRNA destroys its template activity in a wheat germ system (Fig. 6C).

Translation of two other eukaryotic cellular messenger RNAs was also found to be strongly affected by incubation with potato nucleotide pyrophosphatase. Removal of terminal pm$^7$G by enzyme treatment decreased the template
Fig. 4. Effect of nucleotide pyrophosphatase activity on acid-soluble products released from reovirus $^3$H-methyl-mRNA (O---O), and from $^3$H-methyl viral mRNA in the presence of A. salina mRNA (A---A); controls incubated without enzyme (O---O, A---A). The percentage of radioactivity acid-solubilized with nucleotide pyrophosphatase was assayed in incubation mixtures (20 μl) containing 0.02 M Na acetate buffer pH 5.2, 0.1 M KCl, $^3$H-mRNA (2000 cpm) and either 5 μg A. salina or 2.5 μg reovirus non-radioactive mRNA, and bentonite-adsorbed enzyme or corresponding amounts of enzyme-free bentonite. Addition of cold mRNA served to increase the substrate concentration to values comparable to those used in incubations of mRNA with nucleotide pyrophosphatase for translation experiments. After 30 min at 37° the precipitable radioactivity was estimated by the method of Bollum (42).

activity of rabbit globin and Artemia salina mRNAs more than 10-fold and 4-5-fold, respectively (Fig. 6A and B).

The polypeptide products directed by enzyme-treated and untreated globin mRNA in both cases migrated in polyacrylamide gels in the position of authentic rabbit globin (Fig. 7). In addition, the products stimulated in wheat germ extract by TMV RNA and by reovirus mRNA were the same before and after incubation with pyrophosphatase.

In contrast to the eukaryotic system, incubation of bacteriophage f2 RNA with the nucleotide pyrophosphatase preparation did not decrease its translation in an E. coli cell-free system (Fig. 6D). These results provide further evidence for the absence of endonuclease in the purified pyrophosphatase.

Enzyme treatment of TMV RNA had a smaller effect (10-30% decrease) on its translation in wheat germ S-23. This is consistent with the observation that under the translation conditions employed the cap analog pm$^7$G at a concentration of 0.5 mM inhibited TMV RNA translation by 10-20% as compared to 80% for Artemia salina mRNA (data not shown). Translation of enzyme-treated TMV RNA
Fig. 5. Formation of initiation complexes between wheat germ 80S ribosomes and reovirus 3H-methyl-mRNA (A) before, and (B) after treatment with nucleotide pyrophosphatase.

Fig. 6. Translational activity of different mRNAs after treatment with (O---O) or without (●-●) nucleotide pyrophosphatase. Translation of rabbit globin mRNA (A), A. salina mRNA (B) and reovirus mRNA (C) was assayed in wheat germ S-23 system; translation of f2 RNA was tested in E. coli S-30 system (D). Blanks without mRNA (5-8 pmoles for wheat germ and 4 pmoles for the E. coli system) were subtracted.

was not accompanied by recapping of RNA in the wheat germ protein synthesis system since addition of the methylation inhibitor, Adohcy, did not result in a decrease of TMV RNA translation. Similarly, the presence of either Adomet
Fig. 7. Polypeptide products directed by globin mRNA. Rabbit globin mRNA was incubated with or without nucleotide pyrophosphatase and translated in wheat germ extract as described in the methods section. Products (one-fourth of total) were analyzed in 12.5% polyacrylamide gels (29), and the autoradiogram was obtained after one day exposure with Agfa Structurix FW D10 film. Lane (1): 1.2 ug untreated mRNA, total incorporation = 250,000 cpm. Lane (2): 1.2 ug pyrophosphatase-treated mRNA, total = 25,000 cpm. Marker polypeptides were β-galactosidase (135 K), bovine serum albumin (68 K), ovalbumin (43 K), aldolase (40 K) and rabbit globin (16 K).

(4 μM) or AdoHcy (160 μM) did not affect the levels of translation of pyrophosphatase-treated or control A. salina mRNAs.

DISCUSSION

The modification of previous procedures (25,41) for isolation of nucleotide pyrophosphatase has resulted in a preparation free of nucleolytic activity. This has been accomplished by a combination of two techniques: (I) introduction of deoxycholate to gel filtration and subsequent DEAE-cellulose chromatography, which led to a preparation releasing no acid-soluble products from polynucleotides, and (II) adsorption on bentonite which resulted in total inhibition of residual endonuclease activity. Aside from its inhibitory effect on traces of endonuclease(s), bentonite not only offers the advantage of concentrating the nucleotide pyrophosphatase with no concomitant loss of activity, but also allows for simple removal of the predominant part of the activity from the reaction mixture. The extremely high affinity of nucleotide pyrophosphatase for bentonite is no doubt related to its tendency to adsorb to solid surfaces like glass or dialysis membranes and to form aggregates. This property, troublesome in enzyme isolation, probably accounts for the relatively low yields of the present and previous (25,41) isolation procedures. It also partially explains the nonhomogeneity of preparations obtained so far, regrettable in view of the extremely broad specificity of the enzyme (25,41,43).

Potato nucleotide pyrophosphatase is strikingly similar in most of its properties to nucleotide pyrophosphatase from cultured tobacco cells (26). The
similarities in specificity include not only cleavage of the pyrophosphate linkage in capped mRNA and nucleotide coenzymes, but also hydrolysis of aryl esters of nucleoside 3'- and 5'-phosphates, of nucleoside 2':3'-cyclic phosphates and nucleoside 3':5'-cyclic phosphates. The use of bentonite in treatment of mRNA with tobacco nucleotide pyrophosphatase (27) suggests that the similarities between the two activities may extend also to endonucleolytic contamination copurifying with the pyrophosphatase. Minor dissimilarities in specificity concern cleavage of 5'-AMP. The tobacco enzyme was reported to be inactive against 5'-AMP while the potato enzyme cleaved it a rate of 18 nmoles per activity unit per 30 min, i.e. at 0.06% the rate of cleavage of thymidine 5'-p-(nitrophenyl)-phosphate. Since the latter value represents a 12-fold decrease in rate relative to that reported by Kole et al. (25), we feel that the nucleoside monophosphatase activity is not an inherent property of the nucleotide pyrophosphatase molecule.

Our results demonstrate that potato nucleotide pyrophosphatase can be used successfully for specific and quantitative removal of 5'-terminal pm7G from mRNAs of eukaryotic cells and viruses. Several lines of evidence indicate that the enzyme preparation used in these studies is free from endonucleolytic activity: (I) The enzyme did not introduce internal scissions into reovirus, TMV or rabbit globin mRNA; (II) pm7G and mg7G were the only radioactive products cleaved from 3H-methyl-labeled reovirus mRNA even at exceedingly high enzyme concentrations; (III) Translation of f2 RNA in an E. coli cell-free system, which has no cap requirement, was not reduced by pyrophosphatase treatment; and (IV) The limited effect of enzyme treatment on TMV translation in the wheat germ system correlated well with the effect of cap analogs on this process.

Results of this work indicate that removal of 5'-terminal pm7G from reovirus mRNA by pyrophosphatase treatment destroys its template activity in a wheat germ system. The importance of the 5'-terminal cap for reovirus mRNA translation at the level of ribosome binding was previously extensively documented (2-5,9,10), and our results confirm these findings. The loss of ability to bind to ribosomes following nucleotide pyrophosphatase treatment (Fig. 5) provides additional evidence that the decrease in template activity of "decapped" reovirus mRNA is not caused by internal scissions in the polynucleotide chain but results from hydrolysis of the 5'-terminal cap. Binding of 5'-terminally labeled reovirus mRNA to ribosomes should not be affected by internal nicks in mRNA, since 5'-terminal viral mRNA fragments 30-40 nucleotides in length bind efficiently (44) to wheat germ ribosomes. If the decapped mRNA is
rapidly degraded by an exonuclease in the cell extract (10), the loss of ribosome binding and translation of pyrophosphatase-treated mRNA may be secondary to its decreased stability.

Translation of two other eukaryotic cellular mRNAs used in these studies, rabbit globin and *Artemia salina* mRNAs, was also found to be strongly decreased after enzymatic treatment with pyrophosphatase. Both these mRNAs contain caps (3,5,45) and previous work in which these messengers were subjected to the β-elimination procedure indicated that the 5'-terminal cap is important for their efficient translation in wheat germ (3,5) and *Artemia salina* (5) systems. Additional support for the functional role of 5'-terminal caps in reovirus, globin and *Artemia salina* mRNAs comes also from experiments in which cap analogs were shown to inhibit translation of these messengers in several different protein synthesis systems (14,16-18,45).

Apart from potato nucleotide pyrophosphatase, tobacco nucleotide pyrophosphatase (27) also has the ability to cleave the cap from mRNA. The tobacco enzyme has already been used to establish that the presence of m\(^7\)G in TMV RNA is essential for RNA infectivity, but not for reconstitution with the coat protein (28). With regard to the potential physiological role of plant nucleotide pyrophosphatases, their broad specificities make it unlikely that they are of special significance in cap metabolism. By contrast, the m\(^7\)G(5')pppN-pyrophosphatases from HeLa cells (46,47) and other sources (16), which cleave the cap in oligonucleotides but not in intact mRNA, exhibit high specificity for the pm\(^7\)G structure and may play some role in mRNA turnover.

These results were presented at the International Symposium on Translation in Poznán, Poland, May 10-12, 1977 where similar results were reported by Miura et al. ("Importance of 7-methylguanylic acid-blocking structure for stabilizing mRNA in eukaryotic protein synthesis" by K. Miura, K. Shimotohno, Y. Kodama and I. Hashimoto).

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REFERENCES

1. Shatkin, A.J. (1976) Cell 9, 645-653
2. Both, G.W., Banerjee, A.K. and Shatkin, A.J. (1975) Proc.Nat.Acad.Sci.USA 72, 1189-1193
3. Muthukrishnan, S., Both, G.W., Furuichi, Y. and Shatkin, A.J. (1975) Nature 255, 33-37
4. Both, G.W., Furuichi, Y., Muthukrishnan, S. and Shatkin, A.J. (1975) Cell 6, 185-195
5. Muthukrishnan, S., Filipowicz, W., Sierra, J.M., Both, G.W., Shatkin, A.J. and Ochoa, S. (1975) J.Biol.Chem. 250, 9336-9341
6. Shih, D.S., Dasgupta, R. and Kaesberg, P. (1976) J.Virol. 19, 637-642
7. Rose, J.K. and Lodish, H.F. (1976) Nature 262, 32-37
8. Lodish, H.F. and Rose, J.K. (1977) J.Biol.Chem. 252, 1181-1188
9. Muthukrishnan, S., Morgan, M., Banerjee, A.K. and Shatkin, A.J. (1976) Biochemistry 15, 5761-5768
10. Furuichi, Y., LaFiandra, A. and Shatkin, A.J. (1977) Nature 266, 235-239
11. Kemper, B. (1976) Nature 262, 321-323
12. Both, G.W., Furuichi, Y., Muthukrishnan, S. and Shatkin, A.J. (1976) J.Mol. Biol. 104, 637-658
13. Toneguzzo, F. and Ghosh, H.P. (1976) J.Virol. 17, 477-491
14. Hickey, E.D., Weber, L.A. and Baglioni, C. (1976) Proc.Nat.Acad.Sci.USA 73, 19-23
15. Roman, R., Brooker, J.D., Seal, S.N. and Marcus, A. (1976) Nature 260, 359-360
16. Filipowicz, W., Furuichi, Y., Sierra, J.M., Muthukrishnan, S., Shatkin, A.J. and Ochoa, S. (1976) Proc.Nat.Acad.Sci.USA 73, 1559-1563
17. Shafritz, D.A., Weinstein, J.A., Safer, B., Merrick, W.C., Weber, L.A., Hickey, E.D. and Baglioni, C. (1976) Nature 261, 291-294
18. Canaani, D., Revel, M. and Groner, Y. (1976) FEBS Lett. 64, 326-331
19. Furuichi, Y., Morgan, M., Muthukrishnan, S. and Shatkin, A.J. (1975) Proc. Nat.Acad.Sci.USA 72, 362-366
20. Furuichi, Y. and Shatkin, A.J. (1976) Proc.Nat.Acad.Sci.USA 73, 3448-3452
21. Wei, C.M. and Moss, B. (1975) Proc.Nat.Acad.Sci.USA 72, 318-322
22. Furuichi, Y. and Miura, K. (1975) Nature 253, 374-375
23. Urushibara, T., Furuichi, Y., Nishimura, C. and Miura, K. (1975) FEBS Lett. 49, 385-389
24. Rhodes, D.P. and Banerjee, A.K. (1976) J.Virol. 17, 33-42
25. Kole, R., Sierakowska, H. and Shugar, D. (1976) Biochim.Biophys.Acta 438, 540-550
26. Shinshi, H., Miwa, M., Kato, K., Noguchi, M., Matsushima, T. and Sugimura, T. (1976) Biochemistry 15, 2185-2190
27. Shinshi, H., Miwa, M., Sugimura, T., Shimotohno, K. and Miura, K. (1976) FEBS Lett. 65, 254-257
28. Ohno, T., Okada, Y., Shimotohno, K., Miura, K., Shinshi, H., Miwa, M. and Sugimura, T. (1976) FEBS Lett. 67, 209-213
29. Sierra, J.M., Filipowicz, W. and Ochoa, S. (1976) Biochem.Biophys.Res. Commun. 69, 181-189
30. Wiegers, U. and Hilz, H. (1972) FEBS Lett. 23, 77-82
31. Wodnar-Filipowicz, A., Filipowicz, W. and Szafranski, P. (1975) Acta Biochim.Polon. 22, 11-24
32. Razzell, W.E. and Khorana, H.G. (1961) J.Biol.Chem. 236, 1144-1149
33. Lowry, O.H. and Lopez, J.A. (1946) J.Biol.Chem. 162, 421-426
34. Lindberg, U. and Persson, T. (1972) Eur.J.Biochem. 31, 246-254
35. Orkin, S.H., Swan, D. and Leder, P. (1975) J.Biol.Chem. 250, 8753-8760
36. Roberts, B.E. and Paterson, B.M. (1973) Proc.Nat.Acad.Sci.USA 70, 2330-2334
37 Webster, R.E. and Zinder, N.D. (1969) J.Mol.Biol. 42, 425-439
38 Mans, R.J. and Novelli, G.D. (1961) Arch.Biochem.Biophys. 94, 48-53
39 Shatkin, A.J. (1969) in Fundamental Techniques in Virology, Habel, K. and Salzman, N.P., eds., pp. 234-237, Academic Press, New York
40 Bishop, D.H.L., Claybrook, J.R. and Spiegelman, S. (1967) J.Mol.Biol. 26, 373-387
41 Kornberg, A. and Pricer, W.E. (1950) J.Biol.Chem. 182, 763-778
42 Bollum, F.J. (1966) in Procedures in Nucleic Acid Research, Cantoni, G.L. and Davies, D.R., eds., pp. 296-300, Harper and Row, New York
43 Razzell, W.E. (1968) Can.J.Biochem. 46, 1-7
44 Kozak, M. and Shatkin, A.J. (1976) J.Biol.Chem. 251, 4259-4266
45 Groner, Y., Grosfeld, H. and Littauer, U.Z. (1976) Eur.J.Biochem. 71, 281-293
46 Nuss, D.L., Furuichi, Y., Koch, G. and Shatkin, A.J. (1975) Cell 6, 21-27
47 Nuss, D.L. and Furuichi, Y. (1977) J.Biol.Chem. 252, 2815-2821