ENZYME CLEAVING THE 5′-TERMINAL METHYLATED BLOCKED STRUCTURE OF MESSENGER RNA

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Received 5 April 1976

1. Introduction

Recently we purified a novel phosphodiesterase from cultured tobacco cells to an apparently homogeneous state by polyacrylamide gel electrophoresis [1]. The enzyme has a pH optimum at around 6, and is fully active in the presence of EDTA. This tobacco acid phosphodiesterase also shows pyrophosphatase activity, and hydrolyzes various phosphodiesters and pyrophosphate bonds, including those in cyclic AMP, ATP, ADP and NAD^+. However, it does not hydrolyze polynucleotides.

The recent discovery of the 5′-terminal methylated blocked structure of various viral and mammalian mRNA [2–13], prompted us to determine the susceptibility of the pyrophosphate bond in this structure to this novel phosphodiesterase. This paper reports that the tobacco phosphodiesterase preferentially cleaves the 5′-terminal blocked structure of cytoplasmic polyhedrosis (CP) virus mRNA releasing pm^7G from it, but does not degrade the polynucleotide chain of the mRNA.

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Abbreviations: pm^7G, 7-methylguanosine-5′-phosphate; pAm, 2′-*O*-methyladenosine-5′-phosphate.

2. Materials and methods

CP virus [methyl-^{3}H]mRNA with the 5′-terminal structure $m^7G^{5'}ppp^{5'}Am$ was synthesized in vitro in the presence of *S*-adenosyl[methyl-^{3}H]methionine as described before [2,4]. Analysis by paper electrophoresis following digestion of the CP virus [methyl-^{3}H] mRNA with snake venom phosphodiesterase, showed that 50% of the radioactivity of the mRNA was in the m^7G moiety, and the other 50% in the AM moiety. [Methyl-^{3}H]$m^7G^{5'}ppp^{5'}Am$ was prepared from the CP virus [methyl-^{3}H]mRNA, by digestion with *Penicillium* nuclease P_1 (Yamasa Shoyu Co.) and alkaline phosphatase (Worthington Biochemical Corp.) and then separation by anion exchange column chromatography on Bio-Rad AG-1.

Tobacco phosphodiesterase was purified from tobacco cells grown in suspension culture. The purification procedure is described in detail elsewhere [1]. The enzyme was incubated with substrates at 30°C in 0.05 M sodium acetate buffer (pH 6.0) containing 1 mM EDTA. When CP virus mRNA was used as substrate, bentonite was added to the reaction mixture at a final concentration of 200 μg/ml.

Products of hydrolysis were analyzed either by paper electrophoresis is on Whatman 3 MM paper in 5% acetic acid–morpholine buffer (pH 3.5) containing 5 mM EDTA at 2500 V for 100 min in cooled hexane [4], or by column chromatography on Bio-Rad AG-1

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using a linear concentration gradient of NaCl as described in the legend to fig.3.

The incubation mixture of mRNA and tobacco phosphodiesterase was analyzed by centrifuging the mixture at 34 000 rev/min on a 10–30% (v/v) glycerol gradient containing 20 mM Tris-HCl buffer (pH 7.5), 0.1 M NaCl, 5 mM EDTA and 0.5% (w/v) sodium lauroyl sarcosinate for 17 h at 4° C in an SW-41 swinging-bucket rotor of a Beckman-Spinco ultracentrifuge.

Radioactivity was counted in methyl cellosolve and Kinard's scintillation fluid [14].

3. Results

3.1. *Cleavage of $m^7G^{5'}ppp^{5'}Am$ by the tobacco phosphodiesterase*

An incubation of the tobacco phosphodiesterase with [methyl-^{3}H] $m^7G^{5'}ppp^{5'}Am$ two main products pm^7G and pAm were formed as shown by paper electrophoresis (fig.1). The substrate was completely hydrolyzed within 1 min, and incubation for 60 min yielded the same products, indicating that the enzyme is completely free from 5′-nucleotidase

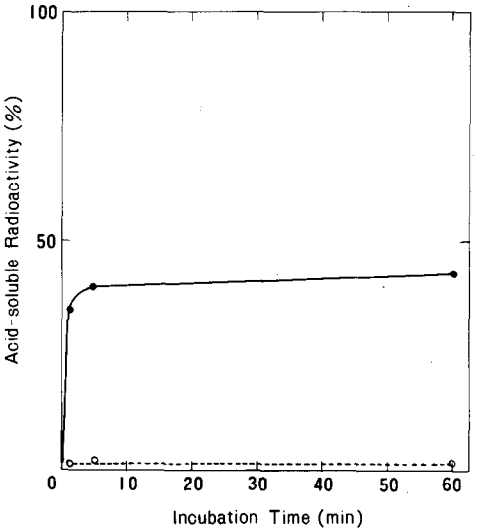
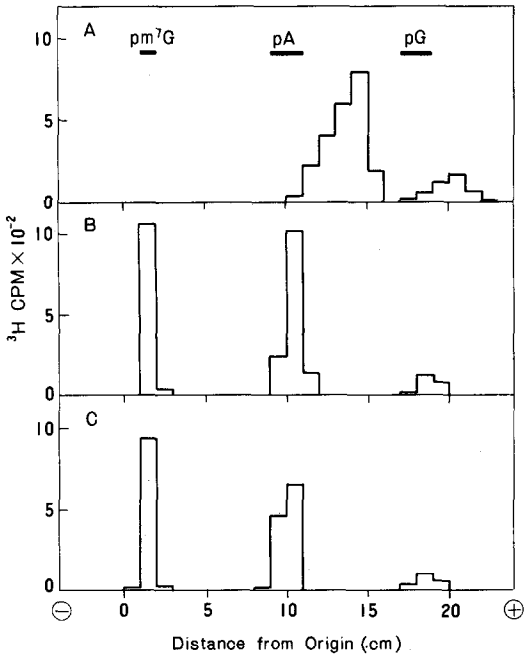


Fig.2. Time course of production of acid-soluble material during digestion of CP virus mRNA by the tobacco phosphodiesterase. CP virus [methyl-^{3}H]mRNA was incubated with 0.013 unit of the enzyme under the standard condition. The reaction was stopped by adding 150 μg of *E. coli* ribosomal RNA and trichloroacetic acid to a concentration of 10%. The mixtures were stood in ice water for 30 min, and then acid-soluble radioactivity was counted (○) No enzyme; (●) with enzyme.



activity. As the phosphodiesterase also hydrolyzes nucleoside diphosphates [1], it is understandable that pm^7G and pAm were identified as cleavage products.

As shown in fig.1A, the sample of $m^7G^{5'}ppp^{5'}Am$ contained a small amount of radioactive material migrating faster than $m^7G^{5'}ppp^{5'}Am$ on electrophoresis, and this seemed to be ring-opened $m^7G^{5'}ppp^{5'}Am$. The results in fig.1 suggest that this was also hydrolyzed by the enzyme to yield ring-opened pm^7G and pAm.

Fig.1. Paper electrophoresis of cleavage products formed by incubation of $m^7G^{5'}ppp^{5'}Am$ with the tobacco phosphodiesterase. [Methyl-^{3}H]$m^7G^{5'}ppp^{5'}Am$ was incubated with 0.013 unit of enzyme under the standard conditions. The reaction was stopped by cooling the mixture in ice water and samples were taken for analysis by paper electrophoresis. (A) Incubated without enzyme for 60 min; (B) incubated with enzyme for 1 min; (C) incubated with enzyme for 60 min.

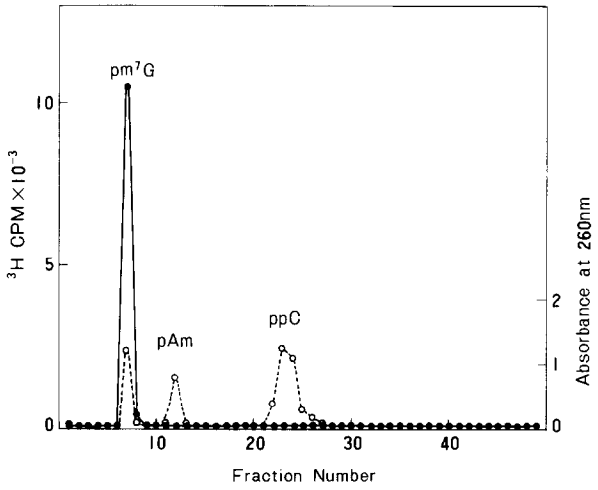


Fig.3. Analysis of cleavage products formed on incubation of CP virus mRNA with the tobacco phosphodiesterase by column chromatography on Bio-Rad AG-1. CP virus [methyl-^{3}H]mRNA was incubated for 5 min with the enzyme under the standard conditions and then the incubation mixture was applied to a column (0.6 X 20 cm) of Bio-Rad AG-1. The column was washed with water and then eluted with a linear concentration gradient formed with 0.005 N HCl (50 ml) and 0.01 N HCl in 0.1 M NaCl (50 ml). pm^{7}G, pAm and CDP were added as internal markers. (•) Radioactivity; (○) absorbance at 260 nm.

3.2. *Cleavage of m^{7}G$^{5'}$ppp$^{5'}$Am at the 5'-terminus of the intact CP virus mRNA by the tobacco phosphodiesterase*

If the enzyme preferentially hydrolyzed the pyrophosphate bond of the methylated blocked structure at the 5'-terminus of intact CP virus mRNA, after incubation of the enzyme with [methyl-^{3}H] mRNA, 50% of the radioactivity should be acid-soluble and 50% acid-insoluble. The results in fig.2 show that the enzyme released 43% of the radioactivity of mRNA as acid-soluble material during incubation for 60 min.

The radioactive product released was analyzed by column chromatography on Bio-Rad AG-1. As shown in fig.3 the acid-soluble product containing 45% of the radioactivity of mRNA was pm^{7}G and no radioactive material was detected in the position of pAm.

To see whether the phosphodiester bonds of the mRNA were hydrolyzed by the phosphodiesterase action of this enzyme, CP virus [methyl-^{3}H]mRNA was incubated with the enzyme and then the mixture was analyzed by glycerol density gradient centrifugation. As shown in fig.4B, approximately half the radioactivity remained at the top of the gradient, while the other half was found in macromolecules with the same distribution as that of untreated materials (fig.4A), which consisted of ten molecular species being different in size [15,16]. The fractions from the gradient were also analyzed for acid-insoluble radioactivity. As seen in fig.4C and 4D, the sedimentation profiles of acid-insoluble radioactivity were similar in enzyme treated (fig.4D) and control (fig.4C) samples. However the acid-insoluble radioactivity of the former was half that of the latter, presumably owing to release of pm^{7}G. This indicates that the radioactivity remaining at the top of the gradient (fig.4B) was acid-soluble and did

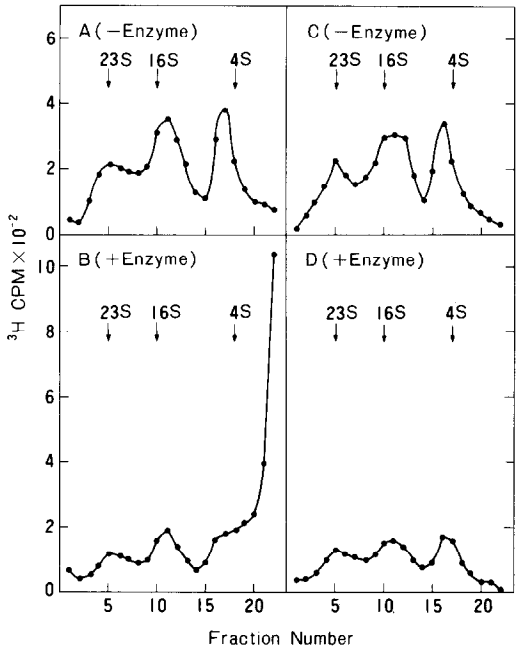


Fig.4. Glycerol density gradient analysis of CP virus mRNA after incubation with the tobacco phosphodiesterase. CP virus [methyl-^{3}H]mRNA was incubated with or without the enzyme under the standard conditions for 5 min. The mRNA was analyzed by glycerol density gradient centrifugation. (A and B) Samples of the fractions of the gradient were taken for counting radioactivity. (C and D) Samples of the gradient fractions were mixed with 10% trichloroacetic acid and 500 μg of yeast RNA as a carrier, and the precipitates were filtered on a Whatman GF/F filter, dissolved in 0.4 N NaOH overnight, neutralized with HCl and counted. The arrows indicate the positions of *E. coli* ribosomal RNA and tRNA markers determined from the absorbance at 260 nm.

not represent degraded oligonucleotide fragments of the mRNA. These results suggest that incubation of the CP virus mRNA with tobacco phosphodiesterase did not cause any change in the size of the mRNAs.

4. Discussion

The present results show that the tobacco acid phosphodiesterase preferentially cleaves the pyrophosphate bond of the 5′-terminal methylated blocked structure of CP virus mRNA and releases pm^7G from it. However, no detectable degradation of the mRNA was observed by glycerol gradient centrifugation. Nuss et al. [17] recently reported the presence of m^7G specific enzyme activity in HeLa cell extracts causing cleavage of $m^7G^{5'}ppp^{5'}Gm$ to pm^7G and ppGm, but not cleavage of the blocked structure of intact reovirus mRNA. The tobacco phosphodiesterase reported here does not seem to be specific for m^7G, but it cleaves only the 5′-terminal blocked structure in the intact CP virus mRNA.

The methylated blocked structure has been shown to be required for translation of reovirus and vesicular stomatitis virus mRNAs [18,19]. This modified structure at the 5′-terminus of mRNA has been found not only in animal systems [2–9], but also in plant systems, such as that of the tobacco mosaic virus RNA [11,12] and in other plant virus RNAs [6,10,13]. The tobacco phosphodiesterase may be involved in regulation of the activity and metabolism of mRNA in the tobacco plant by splitting the 5′-terminal structure of mRNA.

The tobacco phosphodiesterase can easily be purified to a homogeneous form, and so it may be a useful tool in studies on the structures and biological roles of 5′-terminal modification of nucleic acids.

Acknowledgements

The authors wish to thank Drs Kunio Kato and Masao Noguchi of Central Research Institute, the Japan Tobacco and Salt Public Corporation for valuable advice.

This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan.

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