ENZYMATIC CLEAVAGE OF m^7 GDP FROM EUCARYOTIC mRNA

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1. Introduction

It has been found recently that viral and eucaryotic mRNA contain different abnormal structures at their 5′-termini [1]. The biological significance of this so called cap structure is not clearly understood. A general feature of the approach made by several groups to determine the function of cap structure is to modify a specific mRNA by removing the inverted nucleoside at the 5′-terminus. The translational efficiency of the modified mRNA is then compared with that of normal mRNA in a reconstituted system.

Periodate oxidation and aniline treatment has been employed to eliminate the terminal ribose moietes containing free-OH groups in both 2 and 3 positions (β elimination) [2,3]. Such chemical modification of mRNA is rather drastic and results in mRNA with abnormal termini at both ends of the molecule. In this paper we report a novel method for the removal of the blocking nucleotide m^7 GDP from the 5′-terminus of eucaryotic mRNA.

2. Experimental

2.1. *Preparation of mRNA*

Total RNA was extracted essentially as described earlier [4] from membrane-bound ribosome fractions of a kappa chain producing mouse plasmacytoma cell line, MPC-11, grown in the presence of carrier free $[^{32}P]P_i$ for 16 h. Polyadenylated RNA was separated by chromatography on oligo(dT)-cellulose columns [5].

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2.2. *Isolation of cap structures*

Capped structures were isolated from labelled mRNA by mild alkaline hydrolysis [6], followed by chromatography on a DEAE–cellulose column [7]. The broad radioactive peak eluted from the column immediately after UTP marker was freeze dried and dissolved in H_2O.

2.3. *Enzymatic cleavage of the cap structure*

Bacteriophage T_4-induced polynucleotide kinase isolated according to Panet et al. [8] was used to cleave the cap structure. The reaction mixture contained per 0.2 ml: 65 mM Tris–HCl (pH 7.8), 15 mM mercaptoethanol, 9 mM magnesium chloride, 30 μg mRNA or cap structures isolated from 50 μg mRNA, and 3 units of highly purified polynucleotide kinase. After 30 min at 30°C, the reaction was stopped by adding EDTA [9].

3. Results

Cap structure of microsomal mRNAs from myeloma cells contain a minimum of 27 different sequences of the 32 possible ones [10]. All of them contain 7 methyl guanosine attached to the rest of the molecule through a 5′–5′ triphosphate linkage [10]. The present method for the cleavage of m^7 GDP involves the use of polynucleotide kinase. The enzyme normally transfers the terminal phosphate of a triphosphate to the free hydroxyl

group of the ribose moiety of a polynucleotide. Under suitable conditions, the enzyme can also catalyze the reverse reaction. Thus when 5′-[^{32}P]-labelled oligonucleotide is incubated with ADP and polynucleotide kinase, [γ-^{32}P] ATP is formed [9]. Since it seemed likely that the forward and the backward reactions might be catalysed through the formation of an intermediate analogous to that found in the capped mRNA, we studied the action of polynucleotide kinase on cap structures. Hence it would be expected that cap structure may be split between β and γ phosphate groups by the enzyme, producing m^7 GDP.

Treatment of [^{32}P] P_i-labelled cap structure with polynucleotide kinase produced cleavage products that comigrated with GDP and GMP on paper chromatography (fig.1). 7-Methylguanosine containing compounds are alkali labile [11]. Prolonged treatment with strong alkali produces the open ring structure that has lost the extra positive charge of 7-methyl-guanine [11]. A partial conversion to the open ring form would be expected to occur during the isolation of the cap structure [6]. It is not surprising, therefore, that the cleavage products produced migrated as broad peak (fig.1).

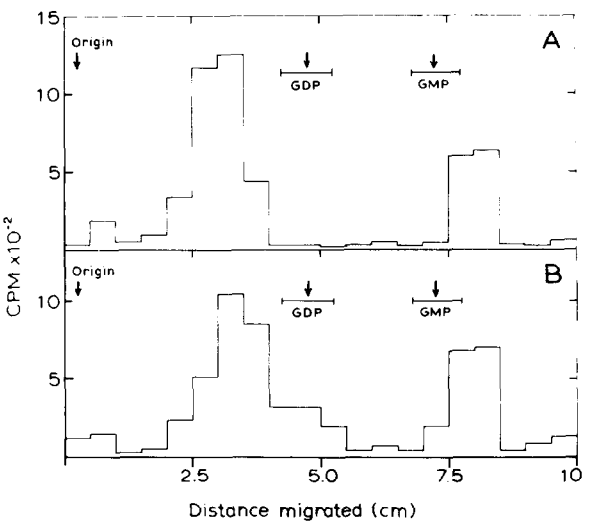

Fig.1. Paper chromatography of polynucleotide kinase treated cap structure. Isolation of cap structure and treatment with polynucleotide kinase was as described in the experimental section. At the end of the reaction material was analysed by descending paper chromatography in isobutyric acid/0.5N NH_4OH (10:6; v/v), using GDP and GMP as optical markers. (A) Control, (B) polynucleotide kinase treated.

3.1. *Cleavage of m^7 GDP from Intact mRNA*

[^{32}P] P_i-labelled mRNA treated with polynucleotide kinase also produced acid soluble material (table 1). The radioactivity released reached a maximum value after 25 min incubation. The amount of acid soluble material produced varied between 0.1–0.15% of the total mRNA radioactivity. Assuming uniform labelling and an average chain length of 1000 nucleotides for the poly-(a) containing microsomal mRNA from myeloma cells, this would suggest that the enzyme has a cleaving efficiency of at least 50–75%. These calculations are based on the fact that all polyadenylated microsomal RNA are capped [10].

Very often, mRNA isolated by oligo(dT)-cellulose chromatography contains traces of rRNA of high specific activity [12]. Therefore it seems reasonable to assume that the actual efficiency of removal of the cap is higher.

In order to confirm the identity of the cleavage product, the acid soluble material produced was subjected to DEAE–cellulose chromatography [7]. As expected 97% of the radioactivity eluted together with m^7 GDP used as an optical marker (fig.2). By thin layer chromatography on PEI cellulose [13] the radioactivity was shown to comigrate with standard m^7 GDP kindly provided by Dr A. J. Shatkin (results not shown).

Table 1
Time course of the cleavage reaction

Incubation period (min)	Radioactivity recovered in the acid soluble fraction (%)
5	0.04
10	0.07
15	0.11
20	0.13
25	0.14
30	0.14

Polyadenylated microsomal RNA labelled with [^{32}P]P_i was treated with polynucleotide kinase as described in the experimental section. The reaction was stopped at different time intervals by adding 100 μg rRNA and cold perchloric acid to 4% final concentration.

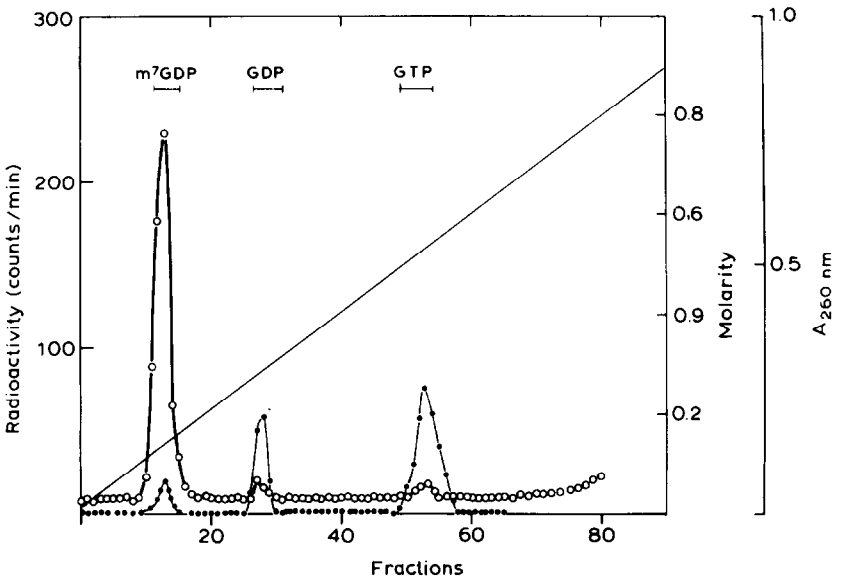

Fig.2. DEAE–cellulose chromatography of cold perchloric acid soluble material produced on polynucleotide kinase treatment of mRNA. [^{32}P] P_i-labelled polyadenylated microsomal RNA (500 000 cpm) was treated with kinase as described in the experimental section. Cold perchloric acid soluble material was neutralized with KOH and subjected to chromatography on a DEAE–cellulose column together with optimal markers as described by Nomoto et al. [7] The horizontal bars indicate the positions of optical markers. m^7 GDP, GDP and GTP respectively (o——o) radioactivity (•——•) A_{260} nm.

4. Discussion

Our results show that bacteriophage T_4 induced polynucleotide kinase can cleave the 5′–5′ triphosphate linkage of the cap structure producing m^7 GDP.

Recently two other enzymes able to cleave the 5′-terminal blocking nucleotide have been reported [14,15]. The enzyme activity present in HeLa cells [14] was specific for the cap structure. The tobacco phosphodiesterase [15] is less specific and was shown to cleave the pyrophosphate bond of NAD^+ as well as the cap structure: The cleavage product produced from mRNA by both these enzymes was m^7 GMP. Polynucleotide kinase in contrast, cleaves off m^7 GDP from mRNA. Like mRNAs of prokaryotes and poliovirus, decapped mRNAs produced by polynucleotide kinase have a single phosphate group at the 5′-terminus. The enzyme can be purified to homogeneity free of nucleases [8]. Therefore this enzyme can be a useful tool for comparing the biological activity of capped and decapped mRNA.

Acknowledgements

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