FORMATION OF ATP FROM THE POLY-ADENYLATED REGION OF EUKARYOTIC MESSENGER RNAs

Abraham K. ABRAHAM and Alexander PIHL

Biochemistry Department, University of Bergen, 5000 Bergen and *Norsk Hydro's Institute for Cancer Research, Montebello, Oslo 3, Norway*

Received 12 December 1977

1. Introduction

It is now firmly established that most, but not all, eukaryotic mRNAs possess a poly(A) sequence attached to the 3′ end [1,2]. The function of the poly(A) moiety of mRNA is not clearly understood. Its presence is not an obligatory requirement for translation of mRNA. However, evidence has been obtained that the stability of mRNAs, as measured by their template activity, is reduced when poly(A) is removed [3]. The precise mechanism whereby poly(A) confers stability on the mRNA is not known.

Poly(A) is synthesized [1] according to the reaction:

$$\text{Primer} + n\text{ATP} \longleftrightarrow \text{Primer}-(\text{AMP})_n + n\text{PP}_i$$

The enzyme involved, poly(A) polymerase, is present not only in the nucleus, but also in mitochondria, as well as in the microsomes and the cytosol [4]. Recently, we have shown that pure poly(A) in the presence of the purified enzyme and pyrophosphate can be converted to adenine nucleotides [5]. Moreover, it has been found that poly(A) undergoes turnover in the cytoplasm [6,7]. Here we report data indicating that the poly(A) moiety of normal mRNA may be degraded in a protein synthesizing system in the presence of pyrophosphate with the formation of ATP.

2. Materials and methods

2.1. *In vitro protein synthesis*

Translation of purified mRNA was carried out in a wheat germ extract as in [8].

2.2. *Purification of mRNA*

^{14}C-Labelled mRNA was isolated from mouse plasmacytoma cells (MPC-11), grown in the presence of radioactive adenosine for 24 h. RNA extraction and oligo(dT)-cellulose chromatography was performed as in [8,9].

2.3. *Extraction and separation of nucleotides after in vitro translation of mRNA*

Nucleotides were extracted with ice-cold perchloric acid (final conc. 0.4 M). After neutralization the extracted nucleotides were adsorbed to a suspension of activated charcoal in 0.01 N HCl. The adsorbed nucleotides were eluted with alkaline ethanol (25% ethanol adjusted to pH 10.5 with NH_4OH). The nucleotide mixture was separated by two dimensional chromatography on PEI-impregnated cellulose as in [10].

2.4. *Hybridization of globin mRNA to labelled cDNA*

Polyadenylated RNA, re-extracted from wheat germ extract after translation, was hybridized with excess tritium-labelled globin cDNA, synthesized as in [11]. Unhybridized cDNA was removed by treatment with S_1-nuclease, isolated from *Aspergillus oryzae* as in [12].

3. Results and discussion

Evidence is available that the length of the poly(A) sequence attached to the mRNAs may vary considerably [13]. In attempts to relate these variations to the level of the adenine nucleotides in the system we

have gauged the length of the poly(A) after incubation in an in vitro system at different concentrations of ATP and pyrophosphate. For this purpose a protein synthesizing system from wheat germ was used. Polyadenylated mRNA (primarily the light-chain immunoglobulin fraction [8]) labelled with [^{14}C]adenosine, isolated from a mouse plasmacytoma cell line (MPC-11), was incubated for 45 min at 30°C in this system. After incubation the length of the poly(A) sequences was studied by oligo(dT)-cellulose chromatography of the isolated RNA fraction. The chromatography was run at 20°C where mRNA molecules with long stretches of poly(A) are bound. The mRNA not bound at this temperature was then rechromatographed at 4°C where also mRNA containing shorter poly(A) stretches are bound [14]. It is seen (table 1) that after incubation in the presence of normal concentrations of ATP (1 mM), most of the mRNA (about 80–85%) became attached to oligo(dT)-cellulose at 20°C, showing that it contained mostly long stretches of poly(A). Pyrophosphate added at this level of ATP did not alter the results. However, when the ATP concentration was reduced to 0.1 mM and pyrophosphate (0.1 mM) was added, the fraction of mRNA bound at 20°C was decreased, while concurrently the fraction bound at 4°C increased strongly (from about 15–40%). The data indicate that in the presence of pyrophosphate and low levels of ATP the average size of the poly(A) moiety of the mRNA molecules is shortened.

Additional evidence that the poly(A) chain of mRNA can be degraded in the presence of pyrophosphate, is given in table 2. Here it is shown that in a protein synthesizing system at suboptimal concentrations of ATP, the amino acid incorporation was markedly stimulated by addition of pyrophosphate to the reaction mixture. Since no stimulation by pyrophosphate was seen when the ATP concentration was optimal the results indicate that, during the incubation with pyrophosphate, ATP was generated from the poly(A) region of mRNA. The effect of the pyrophosphate on protein synthesis could not be accounted for by the ability of pyrophosphate to complex magnesium, since magnesium chloride was added to compensate for this effect.

Evidence was obtained that the shortening of the poly(A) stretches of mRNA was not associated with degradation of the mRNA as such. This was shown by studying the integrity of added rabbit globin mRNA

Table 1
Ability of mRNA to bind to oligo(dT)-cellulose after incubation in a wheat germ extract with pyrophosphate and low concentrations of ATP

Conditions		mRNA bound to oligo(dT) cellulose at	
Pyrophosphate (mM)	ATP (mM)	20°C (cpm)	4°C (cpm)
0	1	31 350	5260
0.1	1	29 900	6100
0.1	0.1	19 660	11 360
0.2	0.1	17 570	13 120

The labelled mRNA (about 1.5 μg, 40 000 cpm) was incubated with 12 A_{260} units wheat germ extract [8] for 25 min at 30°C. The reaction mixture contained in 0.3 ml, 20 mM Hepes (pH 7.6), 70 mM KCl, 3 mM magnesium acetate, 1 mM dithiothreitol, 0.2 mM GTP and 10 mM creatine phosphate, 10 units creatine kinase and 10 μM each of 19 amino acids and 5 μM L-[^{3}H]leucine (0.5 μCi). The concentrations of ATP and pyrophosphate were as indicated. RNA was reextracted after incubation and submitted to oligo(dT)-cellulose chromatography at 20°C. The unbound mRNA was subsequently chromatographed at 4°C, as in section 2

Table 2
Stimulatory effect of pyrophosphate on protein synthesis in the presence of suboptimal concentrations of ATP

Conditions		Amino acid incorporation (cpm)
Pyrophosphate (mM)	ATP (mM)	
0	1.0	17 150
0.1	1.0	17 070
0	0.1	2360
0.1	0.1	3420
0.2	0.1	3330
0	0.3	3920
0.2	0.3	5120

Protein synthesis was measured in a wheat germ system as in table 1, except that 4 A_{260} units of wheat germ extracts and 3 μg globin mRNA/0.1 ml reaction mixture were used. In the samples containing pyrophosphate appropriate amounts of magnesium chloride were added to compensate for the chelating effect of the pyrophosphate. The mixtures were incubated for 45 min at 30°C. The samples were processed and counted as in [8]

Table 3
Degradation of mRNA in absence of protein synthesis

Additions	Pyrophosphate (mM)	ATP (mM)	Ribosomes/mRNA molar ratio	Labelled cDNA hybridized to mRNA (cpm)
None (Control)[a]	0	1	7	4970
None	0	1	7	4030
None	0.1	0.1	7	3980
None	0	1	2	940
None	0.1	0.1	2	910
Puromycin, 3 mM	0	1	7	880

[a] RNA extracted from the reaction mixture at zero time

Unlabelled rabbit globin mRNA was incubated with different amounts of wheat germ extract under conditions in table 1. After incubation the total RNA was extracted with phenol and poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose at 4°C. The isolated mRNA was hybridized with excess [^{3}H]CTP-labelled cDNA as in section 2

after incubation in the wheat germ system by hybridization of the added messenger to complementary DNA. The results in table 3 show that after incubation the amount of cDNA hybridized was slightly reduced, showing that the mRNA was degraded to some extent. However, this degradation was not increased under conditions of ATP and pyrophosphate concentrations which induced the formation of a adenine nucleotides from mRNA (table 4). The pyrophosphate-induced degradation of poly(A) thus appears to be specific and does not involve degradation of the mRNA proper. The data also demonstrate that, under conditions when the protein synthesis is reduced or abolished, the major fraction of mRNA was degraded. Thus, after incubation in the presence of puromycin, the amount of mRNA that still hybridized to cDNA was strongly reduced. Similar results were obtained when the ratio of ribosomes to messenger RNA was reduced, resulting in an excess of mRNA not engaged in protein synthesis. Clearly the data indicate that the ribosomes, as expected, protect mRNA and that mRNA actively engaged in protein synthesis is considerably more stable than free mRNA molecules.

Direct evidence that ATP and ADP may be formed from the poly(A) of mRNA is given in table 4, where

Table 4
Formation of adenine nucleotides from mRNA in the presence of pyrophosphate

Conditions		Radioactivity in adenine nucleotides			
Pyrophosphate (mM)	ATP (mM)	ATP (cpm)	ADP (cpm)	AMP (cpm)	Total (cpm)
0	0.1	22	10	22	54
0.1	0.1	108	382	273	763
0.2	0.1	226	392	201	819
0.1	0.2	137	445	145	727
0.2	0.2	253	523	144	920

mRNA (about 2 μg, 18 500 cpm) was incubated for 45 min in the wheat germ system under conditions as in table 1. The reaction mixture was precipitated with ice-cold perchloric acid. After extraction and separation of the nucleotides, the radioactivity in the adenine nucleotides was determined

it is shown that when adenosine-labelled mRNA was incubated under similar conditions as above, substantial amounts of these labelled nucleotides were liberated. In the absence of pyrophosphate virtually no adenine nucleotides were formed, showing that the results could not be accounted for by the presence of ribonucleases. Probably the primary reaction product is ATP. The presence of ADP and AMP is readily explained by the fact that the system was actively synthesizing protein at suboptimal ATP concentrations. Hence, ATP formed from poly(A) would be expected to be consumed under these conditions.

The present results do not elucidate the role of poly(A) for the stability of mRNA in long term experiments [15]. However, they suggest that poly(A) may serve as reservoir of adenine nucleotides which under certain conditions can be drawn upon to regenerate ATP. The physiological significance of such a mechanism is not clear. Thus, the data in table 2 suggest that the globin mRNA could increase ATP by only approx. 30 μM. However, the amount of ATP that can be formed will depend on the concentration of mRNA. Also it should be realized that free poly(A) is present in several cell types [16].

Acknowledgements

This work was supported by the Norwegian Cancer Society, The Norwegian Research Council for Science and Humanities, and by the European Molecular Biology Organization (EMBO). A.K.A. would like to thank Dr B. Mach, Dept. Pathology, The University of Geneva for his help with the cDNA experiments.

References

[1] Adesnik, M., Salditt, M., Thomas, W. and Darell, J. E. (1972) J. Mol. Biol. 71, 29–30.
[2] Williamson, R., Crossby, J. and Humphries, S. (1974) Biochemistry 13, 703–707.
[3] Soreq, H., Nudel, U., Solomon, R., Revel, M. and Littauer, U. Z. (1974) J. Mol. Biol. 88, 223–245.
[4] Jacob, S. T. and Rose, K. M. (1978) in: Methods in Cancer Research, (Busch, H. ed) vol. XIV, in press.
[5] Abraham, K. A. and Jacob, S. T. (1977) Proc. Natl. Acad. Sci. USA. In press.
[6] Dicz, T. and Brawerman, G. (1974) Proc. Natl. Acad. Sci. USA 71, 4091–4095.
[7] Dolechi, G. J., Roger, F. and Humphreys, T. (1977) Cell 11, 339–344.
[8] Abraham, K. A. and Pihl, A. (1977) Eur. J. Biochem. 77, 589–593.
[9] Abraham, K. A. and Eikhom, T. S. (1975) Biochem. J. 149, 669–674.
[10] Randerrath, K. and Randerath, E. (1967) in Methods Enzymol. 12, 323–329.
[11] Rougeon, F. and Match, B. (1976) Proc. Natl. Acad. Sci. USA 73, 3418–3422.
[12] Vogt, V. M. (1973) Eur. J. Biochem. 33, 192–200.
[13] Mendecki, J., Lee, S. Y. and Brawerman, G. (1972) Biochemistry 11, 792–798.
[14] Nudel, U., Soreq, H. and Lillaner, U. Z. (1976) Eur. J. Biochem. 64, 115–121.
[15] Huez, G., Marbaix, G., Hubert, E., Leclereq, M., Nudel, U., Soreq, H., Solomon, R., Lebleu, B., Revel, M. and Littauer, U. Z. (1974) Proc. Natl. Acad. Sci. USA 71, 3143–3146.
[16] Edmonds, M. and Winters, M. A. (1976) Proc. Nucl. Acad. Res. Mol. Biol. 17, 149–179.