Enhancement of *in-vitro* Translation of Eukaryotic RNAs by Cyclic AMP

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Addition of cyclic AMP to normal rabbit reticulocyte lysate brings about substantial increase in protein synthesis programmed by both nuclear and polysomal eukaryotic RNAs. The increase in synthesis is largely nonspecific and the *in-vitro* translation system is rendered viable for longer periods of time than in the absence of cyclic AMP. This stimulation of translation could be due to the inhibition of protein kinases that are initially activated by secondary structure of RNAs. However, the ability of cyclic AMP to further stimulate methyl mercury or heat denatured RNA indicates that additional mechanisms may be involved in the enhancement of translation.

Introduction

The translational efficiencies of many eukaryotic RNAs in *in-vitro* systems such as rabbit reticulocyte lysate and wheat germ extract are poor. This problem is further complicated by the existence of secondary structures and/or RNA aggregates that inhibit protein synthesis [1–3]. Recent findings from many eukaryotic organisms show that heterogeneous RNA contains transcripts that carry complementary repetitive sequence elements that are capable of forming double stranded regions (reviewed in [4]). Unlike nuclear RNAs, polysomal mRNAs show only very limited repetitive sequence contents and thus do not form double stranded regions. RNA fractions enriched for molecules containing single copy transcripts interspersed with repetitive sequences have much lower translational efficients compared to single copy transcripts [5]. Compounds such as cyclic AMP and adenine, that are known to remove the inhibition of protein synthesis produced by double stranded RNA of viral origin in hemin deficient lysates, are also found to be effective in enhancing synthesis programmed by eukaryotic RNAs. More interestingly mRNAs that contain no double stranded RNA-RNA regions also register substantial increase in protein synthesis in normal lysates upon addition of cyclic AMP.

Material and Methods

Rabbit globin mRNA was obtained from Bethesda Res. Labs. (BRL). Poly(A)$^+$ RNA was isolated by poly(U) Sepharose (Pharmacia chemicals) [6] and oligo(dT) cellulose chromatography (Collaborative Research Inc.). Cell-free translation of RNA was carried out in nuclease treated rabbit reticulocyte lysate from Amersham containing 20 μM hemin. Cyclic AMP (Serva) was adjusted to pH 7.2 with free Tris and stored frozen. Similarly ATP, GTP, purine and glucose were prepared. Hemin chloride was dissolved in dilute KOH and neutralized with HEPES. Adenine (Sigma) was used without adjusting the pH. All compunds were used at a final concentration of 5 mM, unless specified, and were added to RNA samples before freeze drying. The highly toxic methyl mercury hydroxide (CH_3HgOH) (Thiokol/Ventron Division Alfa) was handled only in fume hood and added to RNA samples at 2.5 mM before diluting with lysate to a final concentration of 0.25 mM. Electrophoretic separation of protein products was achieved by one dimensional polyacrylamide gel electrophoresis system of Laemmli [7]. RNA-RNA hybrids were visualized by electron microscopy (Siemens IA) by spreading poly(A)$^+$ RNA from a hyperphase of 80% formamid, 0.1 M Tris (pH 8.0) 5 mM EDTA and 100 μg ml^{-1} cytochrome c at room temperature. Single and double stranded RNA molecules were separated by cellulose/ethanol chromatography as described [8].

Results and Discussion

Existence of secondary structure and or double stranded regions could lead to reduced translational efficiency of RNAs. Electron microscopic examinations of poly(U) Sepharose isolated nuclear poly(A)$^+$ RNA reveal short regions of double strands (RNA-

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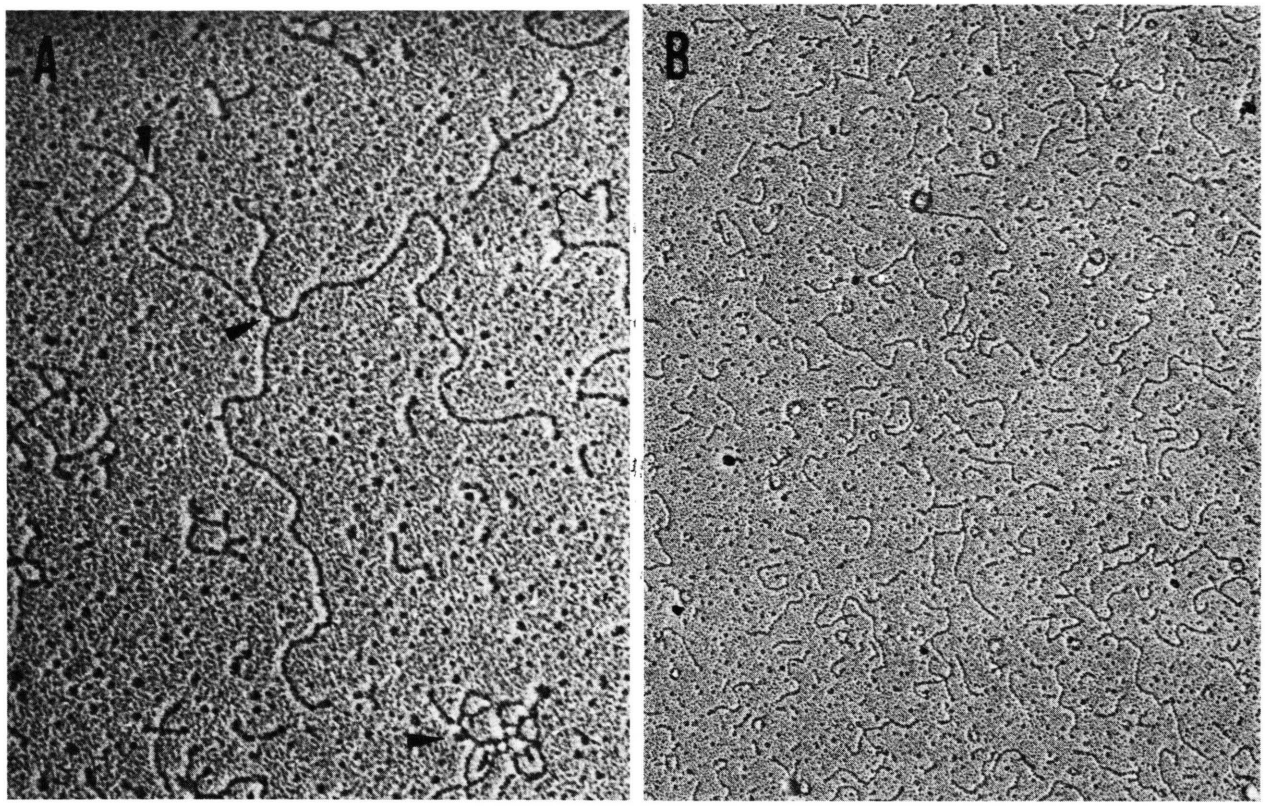

Fig. 1 A and B. Electron micrograph of A.) poly(A)$^+$ RNA from *Xenopus laevis* tadpole nuclei, isolated by poly(U) Sepharose chromatography. Double stranded regions are marked by pointers. B.) Liver polysomal poly(A)$^+$ RNA isolated by poly(U) Sepharose chromatography.

RNA hybrids) among transcripts while polysomal RNA is mostly single stranded (Fig. 1 A and B). Cellulose ethanol chromatography can effectively separate these single and double stranded RNA molecules. We and others have demonstrated that the single stranded RNA separated by cellulose/ethanol chromatography is largely devoid of double stranded RNA molecules [5, 9]. As shown in Table I, removal of double stranded RNA results in increased translational efficiencies of single stranded RNA which are further stimulated by addition of cyclic AMP. In a control experiment poly(A)$^-$ RNA obtained after poly(U) Sepharose chromatography when subjected to cellulose/ethanol chromatography eluted as 95% single stranded and 4% as double stranded, demonstrating that ribosomal RNA is not the major constituent of double stranded material. The table also shows the significant difference in double strand content of nuclear and polysomal poly(A)$^+$ RNAs. Our previous work has shown that single and double stranded RNA separated by cellulose/ethanol chromatography have similar protein product profiles and cDNA-RNA hybridization characteristics [5]. A draw back of cellulose chromatography is the loss of RNA (ranging from 10 to 40% of input) on the columns. To circumvent this problem we used unfractionated RNA and applied compounds that are known to remove inhibition due to double stranded RNAs. Among the compounds tested only cyclic AMP, adenine, hemin and methyl mercury (in that order) showed stimulatory effect while ATP and GTP (5 mM) were found to inhibit the system. Some of these results are shown in Table II. A number of factors such as storage condition and age of lysate influence the rate of stimulation mentioned in Table II and therefore these values can be taken only as an approximation. In some experiments freshly prepared mRNAs exhibited substantially higher control values but approximately the same cpm values upon addition of

Table I. Effect of cellulose/ethanol chromatography on translation of RNAs.

poly(A)$^+$ RNA	Fractionation by cellulose/ethanol		Translation CPM/µl		
	single st. %	double st. %	total	single	double
Xenopus laevis tadpole nuclear	66	34	21 256	35 990	15 465
Mouse liver polysomal	96	4	3 425 (16 239)	5,528 (22 502)	3 405 (4 816)
No RNA	–	–	1 450		

Poly(A)$^+$ RNAs were applied to cellulose columns and eluted with varying concentrations of ethanol containing buffers [8]. "Total" refers to RNA before chromatography. Single and double stranded RNAs are eluted with 15% and 0% ethanol containing buffers, respectively. Percentages given are relative amounts of eluted RNA. Quantitative recovery was not possible. The majority of molecules in double stranded RNA is in duplex formation as judged by electron microscopic analysis (see Ref. [5, 9]). Translations were done in rabbit reticulocyte lysates. Numbers in brackets refer to CPM/µl obtained upon addition of 5 mM cAMP.

cyclic AMP as aged RNA did. Therefore in these instances fold stimulation was less significant. While we have no explanation for this observation, lower control values with aged RNA cannot be due to self annealing and formation of secondary structures, since heating of this RNA only slightly enhances amino acid incorporation (see Table II). None of the above compounds were effective in the wheat germ system (not shown). In order to investigate whether the enhancement of translation is specific for any subsets of RNA population, we have analysed the newly synthesized protein products on one dimensional polyacrylamide gels (Fig. 2). The stimulation of synthesis of proteins of wide molecular weights indicates that the effect is not specific for low or high molecular weight RNA molecules. Also there does not appear to be any significant specific stimulation or inhibition of particular proteins except for one or two proteins in the low molecular weight region which may show higher synthesis (Fig. 2, lanes C, D). Cyclic AMP is also effective even after the initiation of protein synthesis. This is shown in Fig. 3 A and B. In homologous systems such as rabbit reticulocyte lysate and rabbit globin mRNA, the protein synthesis proceeds in a linear fashion for about 60 min (Fig. 3B), while in a heterologous system the time span for active synthesis is much shorter (Fig. 3A). In both systems when added at zero time, cyclic AMP brings about maximal stimulation. More importantly it is seen that cyclic AMP treated lysates are able to maintain active synthesis beyond the time points at which untreated lysates cease synthesis (Fig. 3A and B), while actinomycin D or CH_3HgOH have no effect on the time span of synthesis [2].

It has been reported that cyclic AMP can overcome the inhibition of translation in hemin deficient lysates [10]. The lysates that we used contain 20 µM exogenous hemin (similar to many other commercially available lysates which also contain exog-

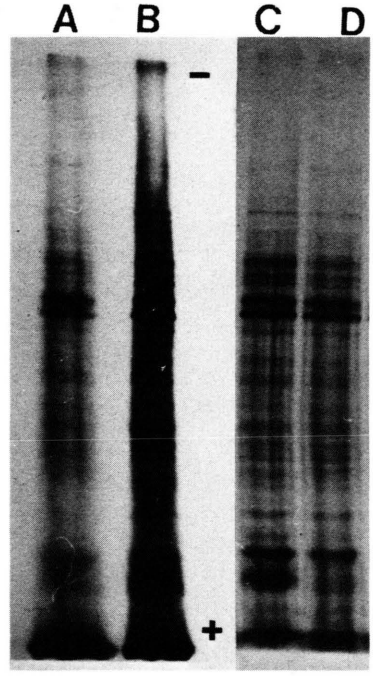

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel (10%) electrophoresis of [^{35}S]methionine labelled protein products of *Xenopus* nuclear poly(A)$^+$ RNA in the absence and presence of cyclic AMP. Following synthesis equal aliquotes were removed from cyclic AMP treated (lane B; 29 720 counts) and nontreated (lane A; 8,668 counts) lysates and electrophoresed. In lane C (cyclic AMP treated; 15 000 counts) and lane D (nontreated 15 000 counts) equal number of counts were applied. Cyclic AMP (5 mM) containing samples cannot be used for two dimensional gel analysis and its removal (*e.g.* by dialysis) is essential before isoelectric focusing.

Table II. Variation in the enhancement of translation of various RNAs in rabbit reticulocyte lysate by cyclic AMP and other compounds.

RNA	Translation CPM/µl		Fold stimulation[a]
	Control	Compound added	
Xen. oocyte p(A)$^+$	6 832	cAMP (5 mM) 17 789	3.0
Xen. gastrula nucl. p(A)$^+$	11 380	cAMP (5 mM) 34 538	3.3
Xen. liver nucl. P(A)$^+$	6 327	CH_3HgOH (0.25 mM) 11 415	2.0
Xen. liver nucl. p(A)$^+$	6 327	CH_3HgOH (0.25 mM) + cAMP (5 mM) 16 337	3.1
Mouse polysomal mRNA	3 425	cAMP (5 mM) 16 239	7.5
Rabbit globin mRNA	32 630	cAMP (5 mM) 188 244	6.0
Rabbit globin mRNA	32 630	heated (65 °C, 70 s) 40 375	1.3
Rabbit globin mRNA	32 630	heated + cAMP (5 mM) 193 311	6.1
Rabbit globin mRNA	32 630	GTP (1 mM) 38 113	1.2
Rabbit globin mRNA	32 630	GTP (5 mM) 9 707	–
Rabbit globin mRNA	32 630	Hemin (40 µM) 91 364	2.9
No RNA (blank)	1 450	cAMP (5 mM) 1 898	1.3[b]

RNAs were translated in the presence of [^{35}S] methionine (150 µCi/ml; 1440 Ci/mmol) for 60 min at 30 °C. TCA precipitable counts/min from 1 µl aliquots are shown. Maximal stimulation was seen in freshly obtained lysates. Lysates stored in liquid nitrogen over long periods of time did not respond to cAMP.

[a] Calculated by comparison of the assays in the absence and presence of compounds after substracting the blank value.

[b] Without substracting the blank.

enous hemin in the range of 20 to 25 µM). A severe hemin deficiency would have been detected by a significant enhancement of synthesis upon addition of GTP [11]. This does not happen (Table II). Smaller amounts (10 µM) of hemin is found to have no stimulatory effect (not shown). While 40 µM hemin increases the synthesis by 2.9 fold (Table II) increasing the hemin concentration to 80 µM reduces the stimulatory effect (not shown). Restoration of protein synthesis by cyclic AMP in heme deprived or in lysates where double stranded RNA has been added is known to be related to the inhibition of protein kinases which phosphorylate the initiation factors [10, 12]. In these instances cyclic AMP merely restores protein synthesis to near normal values. We find that cyclic AMP also substantially increases protein synthesis directed by eukaryotic RNAs such as rabbit globin mRNA and mouse polysomal mRNA or cellulose selected single stranded RNA, which are mostly devoid of double strands (Tables I and II, Figs. 2 and 3). This also agrees with our observation, that heating of these RNAs before addition to lysate did not significantly alter the rate of protein biosynthesis. On the other hand, cyclic AMP is able to enhance stimulation of heat denatured RNA to the same extent as nondenatured (see Table II). Moreover it is shown, that upon addition of cAMP to methyl mercury denatured RNA, protein synthesis is further stimulated (Table II). Thus the ability of cAMP to further enhance protein synthesis after addition of methyl mercury or heat denatured RNAs indicates that in addition to the known mechanism of inhibition of protein kinase, which phosphorylates the initiation

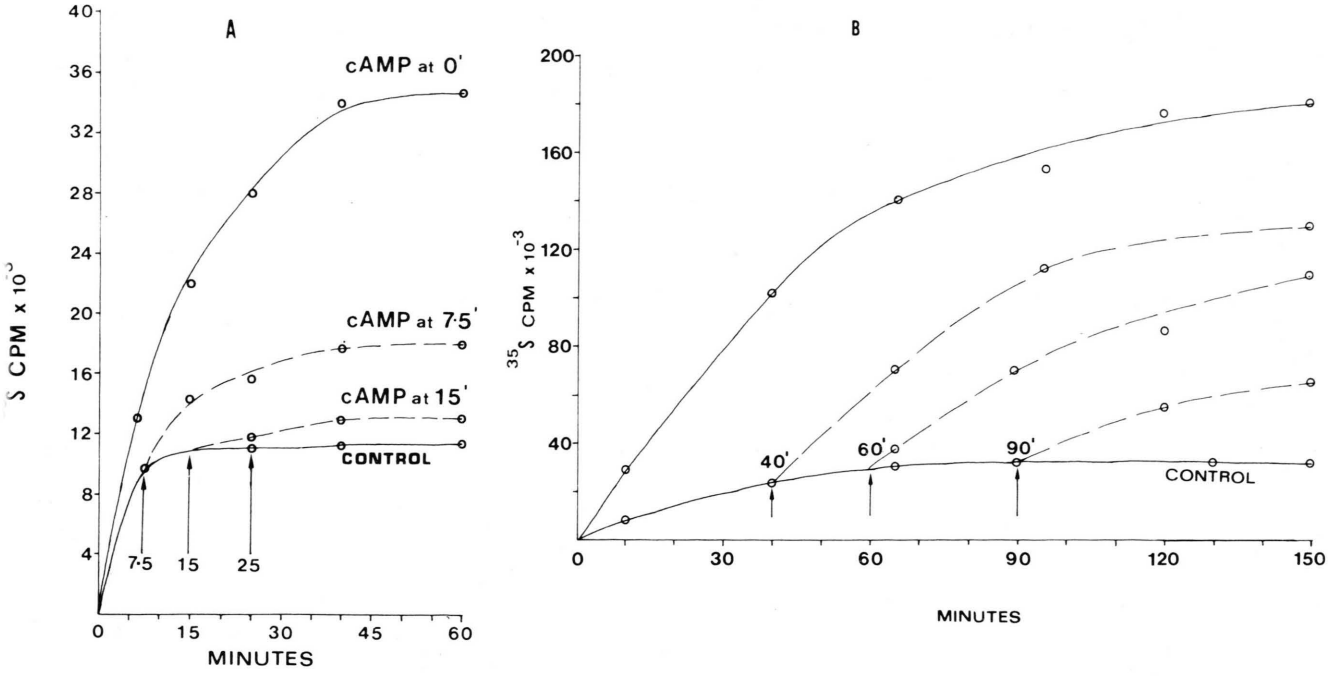

Fig. 3A and 3B. Effect of cyclic AMP on prolongation of protein synthesis directed by *Xenopus* nuclear poly(A)$^+$ RNA (gastrula stage), 3A, and rabbit globin mRNA, 3B. *Xenopus* poly(A)$^+$ RNA (80 µg/ml) was incubated with [^{35}S]methionine (150 µCi/ml; 1440 Ci/mmol) in RNA dependent rabbit reticulocyte lysate at 30 °C. At indicated times (zero, 7.5, 15 and 25 min) 15 µl of lysate were withdrawn and transfered to tubes containing freeze dried cyclic AMP (5 mM final concentration). Incubation was continued at 30 °C for up to 210 min 1 µl of reaction mixture was measured for TCA precipitable counts. Similar measurements were done for rabbit globin mRNA (15 µg/ml).

factors, additional factors may be involved in the cAMP induced enhancement. Although at present there are no proofs, a more complex process such as stabilization of RNA sequences or modification of the protein synthesizing machinery itself [13, 14] cannot be ruled out. In summary it is shown that removal of double stranded RNA by cellulose chromatography or addition of cyclic AMP to nuclear or polysomal RNAs brings about enhancement of translation. Due to its largely non selective stimulation of protein synthesis and its nontoxicity compared to methyl mercury, cyclic AMP could have wide spread application in translation experiments.

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