EFFECT OF N^6,$O^{2'}$-DIBUTYRYL CYCLIC AMP TREATMENT OF LYMPHOMA CELLS ON THE TRANSLATION OF GLOBIN mRNA BY CELL-FREE EXTRACTS

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1. Introduction

The presence of cAMP-dependent protein kinase in many tissues in which cAMP functions as a second messenger has led Kuo and Greengard [1] to propose that cAMP may act in eukaryotes exclusively by activating a protein phosphokinase. According to this hypothesis the biological activity of cellular proteins is modified via specific phosphorylations, a mechanism demonstrated for the regulatory effects of cAMP in glycogen metabolism and in lipolysis [2–5]. Recent studies bring evidence for a possible role of protein kinase also in the regulation of cell growth [6] and the induction of specific enzymes by cAMP [7,8]. Although the site of regulation by cAMP was not definitively demonstrated, experiments indicate that the cyclic nucleotide stimulates the phosphorylation of both nuclear [9,10] and ribosomal proteins [11–14]. The fact that the eukaryotic ribosomal proteins are phosphorylated in vivo and in vitro by cAMP-dependent protein kinase implies that the function of the ribosomes might be regulated by the reversible phosphorylation of their proteins. In vitro studies in a cell-free protein synthesizing system did not, however, bring evidence as to a functional role for ribosomal protein phosphorylation [15].

As an approach to the study of the effects of cAMP on the protein synthesizing machinery of an eukaryotic cell we have used a mouse lymphoma cell line in which db cAMP induces growth inhibition and cytolysis [6]. By growing these cells in the presence of increasing concentrations of db cAMP, a population of cAMP-resistant lymphoma cells was derived [6]. Both endogenous cAMP and exogenous db cAMP inhibited uptake of precursors of macromolecular synthesis and induced synthesis of cAMP-phosphodiesterase in the parental cell-line, but not in the cAMP-resistant mutant [7,16]. It was found that, compared to the wild type lymphoma, the resistant cells had reduced activity of cAMP-binding protein and cAMP-dependent protein kinase [6]; due to a mutation in the regulatory subunit of protein kinase, this enzyme presents a 100-fold lower affinity for cAMP [17].
In the present report we have studied the protein synthesizing capacity of cell free extracts prepared from cAMP-sensitive (S) and resistant (R) lymphoma cells exposed in culture to db cAMP. To avoid any effects of cAMP at the transcriptional level which could affect the mRNA content of the cells, we have studied the translation of an exogenous mRNA, globin mRNA.

It was found that exposure of cAMP-sensitive lymphoma (S) cells to db cAMP resulted in a stimulation of globin synthesis by the derived cell-free extracts. The lack of a similar effect in the cAMP-resistant cells (R) suggests that the cAMP action on the protein synthesizing machinery of the cell may involve the phosphorylation of a component by the cAMP-dependent protein kinase. The synthetic glucocorticoid dexamethasone, which induces growth inhibition and cytolysis of lymphoma cells by a mechanism independent of that of cAMP [18,19], was found to differ in its action from that of db cAMP and to produce cell-free extracts with a reduced ability to translate globin mRNA.

Abbrevations: cAMP, cyclic 3′,5′-adenosine monophosphate; db cAMP, N^6,$O^{2'}$-dibutyryl cAMP; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonate; SDS, sodium dodecyl sulfate.

2. Materials and methods

2.1. *Materials*

ATP, GTP, db cAMP, dexamethasone, creatine phosphate and creatine kinase were from Sigma Chemical Co.; [^{35}S]methionine from Radiochemical Centre, Amersham, and Sephadex G-25 from Pharmacia.

2.2. *Cells and growth conditions*

The mouse lymphoma cell line S49.1TB4 obtained from Ms R. Epstein at the Salk Institute was propagated in Dulbecco's modified Eagle's medium containing 5% heat-inactivated fetal calf serum (Grand Island Biological Co.). 2 mM db cAMP-resistant cells were selected from the sensitive S49.1TB4 lymphoma line as previously described [6].

2.3. *Preparation of cell-free system from lymphoma cells*

Lymphoma cells, washed three times in 35 mM Tris-HCl buffer pH 7.5 containing 140 mM KCl, were suspended in buffer A (10 mM Hepes buffer, pH 7.6, 20 mM KCl, 1.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol) and homogenized in a Dounce homogenizer. The homogenate was then adjusted to 20 mM Hepes buffer, 120 mM KCl, 5 mM $MgCl_2$, 7 mM mercaptoethanol and 10% glycerol (buffer B), and was centrifuged at 10 000 *g* to produce the supernatant S10. The cell-free extract S10 was then filtered through a Sephadex G-25 column equilibrated with buffer B. For the assays using exogenous mRNA the lymphoma cell-free extracts were preincubated for 60 min under the conditions of protein synthesis and were then filtered through Sephadex G-25.

2.4 *Protein synthesis*

Reactions were carried out in 50 μl containing 20 mM Hepes buffer pH 7.5, 3 mM $MgCl_2$, 70 mM, KCl, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.25 mM GTP, 5 mM creatine phosphate, 5 μg creatine kinase, 50 μM each of 19 unlabeled amino acids, 10 μCi [^{35}S]methionine (510 Ci/mmol), 0.3 to 0.7 A_{260} cell-free extract, 0.3 μg globin mRNA and 5 μl of reticulocyte ribosomes 0.5 M KCl wash fluid.

2.5. *Analysis of cell-free products*

After 60 min of incubation at 37°C the reaction was stopped by the addition of 15 μl of a solution containing 0.15 M Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate, 2 M 2-mercaptoethanol and 30% glycerol and heating at 100°C for 10 min. Aliquots of the samples were then subjected to electrophoresis at 150 V for 4 h through 10 cm slab gels containing 10–20% polyacrylamide gradient prepared as described by Maizel [20]. Gels were stained with Coomassie brilliant blue, dried and autoradiographed.

2.6. *Isolation of globin mRNA*

Globin mRNA was prepared from rabbit reticulocyte polysomes by extraction with a mixture of phenol-chloroform, isoamylalcohol followed by chromatography on oligo-(dT)-cellulose [21].

2.7. *Preparation of reticulocyte ribosomal wash fluid (RWF)*

Preparation of 0.5 M KCl wash fluid from reticulocyte ribosomes was as described by Shafritz and Anderson [22].

3. Results

3.1. *Effect of cAMP and dexamethasone on lymphoma cell growth.*

Exposure of cAMP and steroid sensitive-lymphoma (S) cells to 10^{-4} M db cAMP 5×10^{-7} M dexamethasone results in growth inhibition and cytolysis (table 1). It was previously observed that the inhibition of cell growth is accompanied by a decrease in the ability to incorporate [^{3}H]leucine [16]. The cAMP-resistant (R) cells, which were independently selected also as steroid-resistant, were not inhibited in their growth or amino acid incorporation and were not killed by db cAMP or dexamethasone (table 1). Since a 12 h exposure to db cAMP or dexamethasone did not decrease the number of viable lymphoma S cells, although it had a marked effect on amino acid incorporation, we have chosen this period of exposure for the study of protein synthesis in lymphoma cells.

3.2. *Translation of globin mRNA in lymphoma cell-free extracts*

The inhibition of amino acid incorporation in lymphoma S cells as a result of db cAMP or dexamethasone treatment may be due to a block in membrane transport or to changes in the protein synthe-

Table 1
Effect of cAMP and dexamethasone on lymphoma cell growth

Cell type	Time (h)	Control Cells X 10^6/ml live	Control dead	db cAMP live	db cAMP dead	Dexamethasone live	Dexamethasone dead
S cells	0	1.5	–	1.5	–	1.5	–
	12[a]	2.2	0.12	1.86	0.15	1.56	0.24
	34	3.75	0.31	1.56	1.3	1.3	1.46
R cells	0	1.5	–	1.5	–	1.5	–
	10	1.9	0.12	2.1	0.21	2.55	0.27
	34	4.9	0.52	4.2	0.46	4.9	0.55

At zero time 1.5×10^6 S or R lymphoma cells were exposed to 10^{-4} M db cAMP and 2×10^{-4} M theophylline or 5×10^{-7} M dexamethasone. Samples were taken at the specified period of exposure and cell counts were determined in a hemocytometer using trypan blue exclusion as a criterion for cell viability.

[a][^{3}H] leucine incorporation in S cells exposed for 12 h to db cAMP or dexamethasone was 60 and 50% of control cells respectively. Amino acid incorporation was measured as previously described [16].

sizing machinery of the cell. To avoid complications due to variability in amino acid transport, protein synthesis was studied using lymphoma cell-free extracts prepared from cells exposed for 10–12 h to db cAMP or dexamethasone. The incorporation of [^{35}S]methionine by lymphoma S cell-free extracts, translating endogenous mRNA, was found to be reduced by 30 and 50% after a 10 h period of treatment with db cAMP or dexamethasone respectively. Since the reduction in translation of endogenous mRNA may reflect an effect of db cAMP or dexamethasone on mRNA synthesis or degradation in lymphoma cells we have used an exogenous mRNA to study the protein synthesizing capacity of the system. Fig.1 shows that purified rabbit globin mRNA can

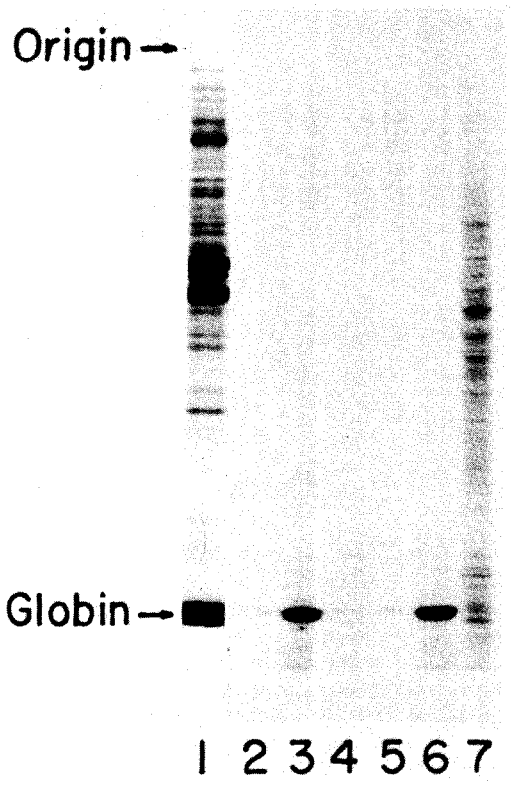

Fig.1. Autoradiography of [^{35}S]methionine-labeled polypeptides synthesized in the lymphoma cell-free system. Protein synthesis and analysis of the products of SDS-polyacrylamide gel electrophoresis were as described in Materials and methods. Gel (1) represents Coomassie brilliant blue staining of the proteins from a reaction mixture containing globin. For autoradiographed gels the reaction mixtures contained: (2) globin mRNA); (3) globin mRNA plus reticulocyte RWF; (4) reticulocyte RWF (5) globin mRNA and 100 mM KCl; (6) globin mRNA plus reticulocyte RWF and 100 mM KCl; (7) not preincubated lymphoma S10 extract.

be translated in the lymphoma cell-free system. Analysis by polyacrylamide gel electrophoresis of the product obtained demonstrates that globin mRNA directs the exclusive synthesis of globin chains. The efficiency of globin production by the lymphoma cell-free extract was markedly increased by the addition of 0.5 M KCl wash fluid from reticulocyte polyribosomes (fig.1 gels 3 and 6), probably due to the initiation factors contained in the latter fraction [22,23].

3.3. *Effect of db cAMP and dexamethasone treatment of lymphoma cells on the globin synthesizing capacity of the cell-free extracts*

The translation of globin mRNA by cell-free extracts prepared from lymphoma S cells exposed for 10–12 h to 10^{-4} M db cAMP or 5×10^{-7} dexamethasone is illustrated in fig.2A. The figure clearly shows that db cAMP treatment of lymphoma S cells resulted in a marked stimulation of the capacity of the cell-free extract to translate globin mRNA. In a similar experiment, in which the cAMP-resistant lymphoma R cells were exposed to 10^{-4} M db cAMP, the capacity of the cell-free extracts to synthesize globin was not affected (fig.2B). For a more quantitative evaluation of globin mRNA translation by the different cell extracts, densitometer tracings of the autoradiograms were compared and the relative globin production is presented in table 2. Treatment of cAMP-sensitive (S) cells by db cAMP induces a 3- to 4-fold stimulation of globin mRNA translation in the cell-free extracts prepared from these cells. The effect of dexamethasone on lymphoma S cell protein synthesis differs from that induced by db cAMP and seems to be rather inhibitory for globin mRNA translation (table 2). The lymphoma R cells, which are resistant to the in vivo effects of both cAMP and dexamethasone, yielded cell-free extracts which were not affected in their protein synthesizing capacity (table 2).

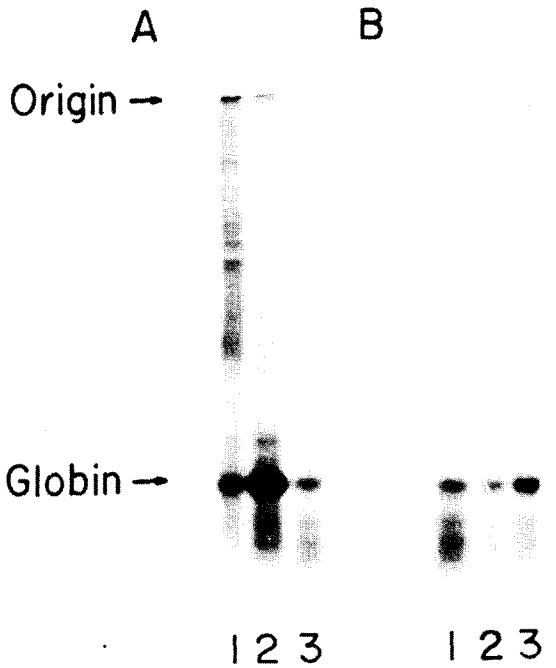

Fig.2. Translation of globin mRNA in cell-free systems prepared from: (A) lymphoma S cells and (B) lymphoma R cells exposed for 12 h without any addition (1) with the addition of 10^{-4} M db cAMP and 2×10^{-4} M theophyline (2) or 5×10^{-7} M dexamethasone (3). The [^{35}S]methionine-labeled polypeptides presented by the autoradiograms of SDS-polyacrylamide gel electrophoresis were products of 0.140 A_{260} and 0.070 A_{260} cell-free extracts from lymphoma S and R cell preparations respectively.

4. Discussion

An increase in intracellular cAMP levels, caused by exposure of cAMP-sensitive S49 lymphoma cells to

Table 2
Globin synthesis in cell-free extracts of S and R lymphoma cells exposed to db cAMP and dexamethasone

Extract	Cell treatment	Relative globin production
S cells	None	100
	db cAMP	305
	Dexamethasone	50
S cells	None	100
	db cAMP	430
	Dexamethasone	77
R cells	None	100
	db cAMP	80
	Dexamethasone	137

Densitometer tracings of globin bands from autoradiograms as described in fig.2 were cut out and weighed. The synthesis of globin is compared relatively to that obtained in extracts from untreated cells taken as 100%.

isoproterenol or db cAMP and theophylline, was previously found to inhibit membrane transport of uridine, leucine and 2′-deoxyglucose [19]. These treatments also cause a depression of protein and RNA synthesis [16] which may reflect a starvation for macromolecular precursors, due to the block in membrane transport, or an inhibition in the nucleic acid and protein synthesizing systems. Cell-free extracts prepared from cells exposed to db cAMP provide therefore a means to study the effects of the cyclic nucleotide without the complications of precursor transport. The present experiments demonstrate that the inhibition of amino acid incorporation by cAMP-sensitive S49 lymphoma cells exposed to db cAMP or isoproterenol is not due to an inactivation of the protein synthesizing machinery. Although protein synthesis in cell-free extracts using the endogenous mRNA was reduced, probably due to an effect of cAMP on mRNA synthesis, the translation of exogenous globin mRNA was actually stimulated (table 2).

In support for a possible regulatory role of cAMP at the level of translation many reports have indicated that proteins of eukaryotic ribosomes can be phosphorylated in vivo and in vitro by cAMP-activated protein kinases [11–15]. In lymphoma S49 cells the effects of cAMP seem to be mediated by the cytoplasmic cAMP-dependent protein kinase, the only cAMP-regulated protein kinase observed in these cells. An alteration in the cAMP-binding, regulatory subunit, of this protein kinase resulted in a loss of cAMP functional [6,17]. The protein kinase activity associated with lymphoma cell ribosomes was found to be a cAMP-independent protein kinase and since it does not interact with the separated regulatory subunit of the cytoplasmic protein kinase we concluded that it is not related to this enzyme (unpublished results). The cytoplasmic cAMP-dependent protein kinase may therefore be responsible for the stimulation of globin mRNA translation in extracts from cells previously exposed to db cAMP, possibly via a cAMP-stimulated phosphorylation of the ribosomes or another component involved in protein synthesis. This hypothesis is supported by the results obtained with cell-free extracts from cAMP-resistant lymphoma (R) cells exposed to db cAMP, which were not affected in their capacity to translate globin mRNA (table 2).

Since exposure of lymphoma cells to db cAMP was found to produce a block in membrane transport and a cell starvation for amino acid [19] it may be argued that this treatment activates a cytoplasmic factor responsible for enhanced rates of protein initiation similar to that observed in HeLa cells [23]. To clarify this question we have compared the effect produced by db cAMP treatment of lymphoma cells to that produced by dexamethasone. Glucocorticoids were shown to induce cytolysis of lymphoid cells [24], producing at the early stages of treatment a block in membrane transport [25]. A 10 to 12 h treatment of lymphoma S49 cells with dexamethasone, similarly to db cAMP treatment, induces a decrease in protein synthesis probably as a result of starvation for amino acids. The cell-free extracts prepared from the dexamethasone-treated cells present however a decreased capacity (50–70% of control) in translating globin mRNA (table 2). These results suggest that the stimulation of globin mRNA translation by extracts of lymphoma cells exposed to db cAMP does not result from an enhanced rate of initiation due to the arrest of protein synthesis in intact cells. A specific inhibitory effect of dexamethasone on the lymphoma protein synthesizing system cannot however be excluded.

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