# STIMULATION BY CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE OF RNA SYNTHESIS IN A MAMMALIAN CELL-FREE SYSTEM

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## 1. Introduction

Cyclic 3′,5′-adenosine monophosphate (cyclic AMP) regulates the expression of genetic information in bacterial cells by activating the transcription of certain genes [1–3]. In animal cells, cyclic AMP is involved in the control of several metabolic pathways, through the regulation of the activity of many enzymes [4]. Presumably, as in the case of bacterial cells, some actions of cyclic AMP are mediated by changes in the rate of synthesis of RNA. This is in line with the fact that cyclic AMP and its analogue, dibutyryl cyclic AMP, have been shown to stimulate *in vivo* and *in vitro* the synthesis of RNA in many tissues [5–9].

In order to investigate how RNA synthesis is influenced by cyclic AMP, we have studied the action of this nucleotide upon an RNA synthesizing cell-free system. In this paper, we will describe the features of the stimulation of transcription by cyclic AMP, and discuss the possible site of its action.

## 2. Materials and methods

Calf thymus DNA, phosphoenolpyruvate, dithiothreitol and spermidine were from Calbiochem.; phosphoenolpyruvate kinase was from Sigma Chemical Co. Cyclic AMP, cyclic GMP, cyclic UMP and cyclic IMP were kindly supplied by Boehringer Mannheim, GmbH. $^3$H-UTP and $^3$H-ATP were obtained from Schwarz BioResearch Inc., and $\gamma$-$^{32}$P-ATP and $^3$H-uridine were from the Radiochemical Centre, Amersham, England.

For studies dealing with the $^3$H-uridine incorporation into RNA, rat liver slices were incubated at 37° in 2 ml of Eagle's medium containing 2 $\mu$Ci of $^3$H-uridine. The gas phase was 95% $O_2$–5% $CO_2$. After 60 min of incubation, the slices were withdrawn, chilled on ice, washed with 0.1 M KCl–0.01 M phosphate buffer (KCl–$PO_4$ buffer) pH 7.4 and homogenized in the same buffer. The homogenate was centrifuged 5 min at 10,000 *g* and the supernatant decanted. 50 $\mu$l aliquots of the supernatant were then precipitated with cold TCA (0.5 ml), containing 0.1 M sodium pyrophosphate. Five min later, the precipitate was filtered on Whatmann GF/A glass fiber discs and washed 7 times with 3 ml of the 5% TCA–Na pyrophosphate mixture. The discs were then dried and counted in Triton X-100 scintillation liquid (1 l of Triton X-100; 2 l of toluene; 165 ml of Liquifluor). The results were expressed as counts/min/mg of tissue.

Rat liver RNA polymerase was prepared according to the method of Roeder and Rutter [10]. The method of Walton and Garren [11] was followed for the preparation of the factor necessary for the stimulation of RNA synthesis by cyclic AMP and for the assay of the cyclic AMP binding activity. The fraction used corresponded to the component S-11 of the preparation of Walton and Garren [11].

The RNA polymerase assay was carried out according to Roeder and Rutter [12]. After the incubation, the reaction was stopped by adding 1 ml of ice-cold

Table 1

The effect of dibutyryl cyclic AMP and cyclic AMP on RNA synthesis by rat liver slices and in a cell-free system.

| | | System | |
|---|---|---|---|
| | | Rat liver slices | Calf thymus DNA rat liver RNA |
| Compound | Conc. (M) | $^3$H-uridine incorporated (cpm/mg tissue) | $^3$H-UMP incorporated (pmoles/0.125 ml) |
| None | – | 1470 | 23 |
| Cyclic AMP | $5 \times 10^{-4}$ | 1480 | 23 |
| Cyclic AMP | $5 \times 10^{-5}$ | 1460 | 22 |
| Cyclic AMP | $5 \times 10^{-6}$ | 1470 | 23 |
| Dibutyryl cyclic AMP | $5 \times 10^{-4}$ | 2500 | – |

Rat liver slices were incubated in 2 ml of Eagle's medium, containing 2 μCi of $^3$H-uridine at 37° for 60 min. After this period, the slices were washed in ice-cold 0.1 M KCl–0.01 M phosphate buffer (KCl–$PO_4$ buffer) pH 7.4 and homogenized in the same buffer. The homogenate was then processed as described in Materials and methods. The assay mixture for RNA polymerase activity contained in a volume of 0.125 ml: 10 μg native calf thymus DNA, 2.5 μg pyruvate kinase, 0.5 μmole phosphoenolpyruvate, 7 μmole of Tris-HCl buffer (pH 7.9), 0.2 μmole $MnCl_2$, 0.75 μmole NaF, 0.2 μmole dithiothreitol, 0.075 pmole of GTP, CTP, ATP, 0.0125 pmole of unlabelled UTP, 1 μCi of $^3$H-UTP (2 Ci/mmole), 0.046 μmole of ammonium sulfate and 50 μg of enzyme preparation. After the incubation at 30° for 20 min, the TCA-insoluble material was processed as described in Materials and methods.

TCA containing 0.1 M sodium pyrophosphate. Acid-insoluble material was collected on Whatmann glass-fiber discs and washed 7 times with 3 ml of the 5% TCA–Na pyrophosphate solution. Once completely dried, the discs were solubilized and counted in Triton X-100 scintillation liquid.

Protein concentration was determined by the biuret method [13].

## 3. Results and discussion

Dibutyryl cyclic AMP stimulates the synthesis of RNA in rat liver slices, whereas cyclic AMP does not (table 1). This discrepancy is probably due to the better solubility of the substituted derivative which facilitates its penetration into the cells. However, cyclic AMP, directly added to an RNA synthesizing cell-free system containing calf thymus DNA and rat liver RNA polymerase, fails to stimulate transcription. Among the several possible explanations, the lack of effect could be due to the hydrolysis of the cyclic AMP. However, the cyclic nucleotide is inactive also in conditions which normally prevent its hydrolysis, such as the presence of 8 mM theophylline.

Since it is well established that mammalian tissues contain a cyclic AMP binding protein which plays an important role in the action of the cyclic nucleotide, we looked for the possibility that a similar special factor was needed for the action of cyclic AMP on RNA synthesis. We found, indeed, that in the presence of a fraction of the cytoplasm of rat liver, which precipitates between 0–50% of a saturated solution of ammonium sulfate, cyclic AMP enhances the synthesis of RNA by calf thymus DNA (table 2). The minimal concentration effective in stimulating RNA synthesis was $2 \times 10^{-6}$ M. The same concentration of the nucleotide, in the absence of the stimulating factor, was inactive. Other nucleotides, such as cyclic GMP, cyclic UMP and cyclic IMP do not replace cyclic AMP (data not shown). It is noteworthy that the fraction by itself stimulates RNA synthesis and has cyclic AMP binding capacity. It is also important that, in order to be fully active, the stimulating fraction has to be incubated with the DNA at least 5 min before the reaction is started by the addition of the polymerase. It could be argued that the cytoplasmic factor is a type of nuclease which nicks the DNA, thus enhancing its template activity. However, DNA treated with the fraction and re-used after phenol extraction, shows the same

Table 2
The effect of cyclic AMP on RNA synthesis in a cell-free system.

| Addition | Concentration | Stimulating factor | $^3$H-UMP incorporated (pmoles/0.125 ml) | Stimulation (%) |
|---|---|---|---|---|
| None | – | minus | 22.8 | 100 |
| None | – | plus | 47.8 | 210 |
| Cyclic AMP | $2 \times 10^{-6}$ | minus | 23.0 | 102 |
| Cyclic AMP | $2 \times 10^{-6}$ | plus | 68.7 | 302 |

The RNA polymerase activity was measured as described in the legend to table 1. Calf thymus DNA was preincubated 5 min at 30° with 8 μg of the cytoplasmic fraction, with and without cyclic AMP, before the reaction was started by the addition of the enzyme. The cytoplasmic activating fraction was dissolved in 10 mM Tris-HCl buffer, pH 7.4, containing 6 mM 2-mercaptoethanol and 8 mM theophylline.

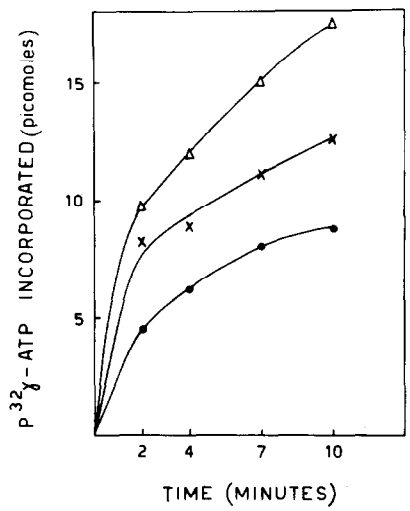


Fig. 1. The effect of cyclic AMP on the initiation step of RNA synthesis by calf thymus DNA. The RNA polymerase activity was measured as described in the legend to table 1, except that the concentration of unlabelled UTP and ATP were interchanged, and $\gamma$-$^{32}$P-ATP was used in the place of $^3$H-UTP. Calf thymus DNA was preincubated with the cytoplasmic fraction at 30° for 5 min, in the presence or absence of cyclic AMP, in the conditions indicated in the legend to table 2. The amount of the cytoplasmic fraction was 8 μg. (●—●—●): Control; (X—X—X): plus cytoplasmic fraction; (△—△—△): plus cytoplasmic fraction plus cyclic AMP.

unstimulated and stimulated levels of transcription as DNA which was not previously incubated with the cytoplasmic fraction. The characteristics of the stimulation of RNA synthesis by this cytoplasmic fraction will be described elsewhere (Ambesi-Impiombato et al., manuscript in preparation).

Another feature of the stimulatory effect of cyclic AMP is that it seems preferentially to activate the initiation step of the transcription. In the presence of the stimulating fraction, cyclic AMP enhances the incorporation of $\gamma$-$^{32}$P-ATP into RNA and this effect is evident from the onset of the reaction (fig. 1).

So far, most of the actions of cyclic AMP in mammalian cells have been related to the stimulation of protein kinase. One important action of the cyclic AMP protein kinases is their ability to phosphorylate histone. Furthermore, it has been recently shown that the skeletal muscle protein kinase stimulates the *E. coli* RNA polymerase, and cyclic AMP enhances this effect [14]. It has been suggested that the stimulatory effect would be mediated by a phosphorylation mechanism. In our experiments it seems unlikely that phosphorylation of histone is involved in the stimulating effect of cyclic AMP because no histones were present in the reaction. Very likely, cyclic AMP exerts its action directly on the DNA template and not on RNA polymerase, because the stimulation of RNA synthesis occurs only when cyclic AMP and the protein fraction have been in contact with the template before the addition of the polymerase.

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