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CYCLIC AMP AS A TRANSCRIPTIONAL INHIBITOR OF UPPER EUKARYOTIC GENE TRANSCRIPTION

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Recently, glucagon and its second messenger, cyclic AMP, have been shown to stimulate the transcription rate of several upper eukaryotic genes (1-5).

We show here that glucagon can also block gene transcription. Both glucagon and cyclic AMP were found to inhibit the transcription of the genes encoding three liver glycolytic enzymes, including L-type pyruvate kinase and aldolase B. Thus, cyclic AMP proves to be not only an activator but also an inhibitor of gene transcription in eukaryotes. © 1984 Academic Press, Inc.

Cyclic AMP is a well-known regulator of gene expression in E. Coli, via its specific binding to the catabolite gene activator protein (CAP). The CAP-cAMP complex binds to specific DNA sequences and enhances the transcription rate of several operons (6). In eukaryotes, cyclic AMP stimulates the synthesis of several proteins, including gluconeogenic (7) and aminoacid metabolizing enzymes (8-9).

Recently, cyclic AMP has been shown to alter the transcription rate of several eukaryotic genes : phosphoenolpyruvate carboxykinase (1-2), thyroglobulin (5) and prolactin (3). However, all the modifications reported consisted in an activation of gene transcription. To date, the only known inhibition of an eukaroytic gene transcription by cyclic AMP concernes the discoidin gene of the amoeba Dictyostelium Discoideum (10). We show here that cyclic AMP can also block the transcription of upper eukaryotic genes.

Using differential screening, we have isolated several cDNA clones complementary to rat liver mRNAs which are induced by feeding fasted

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animals a carbohydrate-rich diet. Among them, several clones were found to be complementary to liver L-type pyruvate kinase and aldolase B mRNAs (11), and one clone recognized a 5.4 kb mRNA species encoding a 36,000 Mr unidentified protein (12).

We have recently shown that cytoplasmic RNAs complementary to the three probes were positively regulated by carbohydrates and negatively by glucagon and cyclic AMP (13). Other hormones, including insulin, exerted a permissive effect on the dietary induction of liver glycolytic enzymes (13).

In order to elucidate the mechanisms involved in the glucagon inhibition of glycolytic enzyme mRNA accumulation in the liver, we measured the rate of glycolytic enzyme gene transcription on isolated nuclei from carbohydrate-fed rats given regular glucagon.

MATERIALS AND METHODS

a-Preparation of nuclei

Nuclei were extracted from livers after sacrifice as described (14). Isolated nuclei were resuspended in 20 mM Tris pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50 % glycerol and stored at -70° C.

b-RNA synthesis in vitro

For transcription, 8.10^6 nuclei were incubated in 200 µl reaction mixture containing 20 % glycerol, 20 mM Tris pH 7.9, 50 mM NaCl, 4 mM $MnCl_2$, 0.5 mM aurintricarboxylic acid, 0.35 M $(NH_4)_2SO_4$, 1.2 mM DTT, 0.1 mM PMSF, 10 mM creatine phosphate, 1 mg/ml heparin sulfate, 500 units/ml RNAsine, 1 mM of ATP, GTP and CTP and 250 µCi of (α-^{32}P) UTP (410 Ci/mmol) at 30° C for 30 min (14). Then, 0.03 mg/ml proteinase K and DNAse 1 were added and the sample was incubated at 37°C for 30 min.

c-Isolation of RNA

RNAs were extracted in 0.7 ml (3 volumes) of 8 M guanidium HCl pH 5 containing 20 mM sodium acetate, 10 mM iodoacetate, 0.5 % (w/v) lauroyl sarcosine, and precipitated by addition of 0.6 volumes of ethanol for 12 h at -20° C. After centrifugation at 15,000 rpm at -10° C for 30 min, the pellet was redissolved twice in 0.5 ml guanidum HCl 7 M pH 7, and allowed to precipitate in 0.6 volumes of ethanol at pH 5 for 2 h at -20° C (15). After centrifugation, the pellet was dried then dissolved in the hybridization mixture and the RNAs were denatured for 10 min at 65° C.

d- Dot blot hybridization of nuclear RNA

Transcripts containing the glycolytic enzyme sequences were quantitated by hybridization to "gene screen plus" (New Engl. Nuclear) filter disks containing recombined pBR plasmids.

Plasmids (6 µg) complementary to L-type pyruvate kinase, aldolase B and 5.4 kb mRNAs were immobilized on 5 mm diameter filters. Hybridization mixture of 200 µl contained 50 % formamid, 1 % glycine, 2.4 % SDS, 10 x Denhardt's, 3 x SSEP, 100 µg/ml poly A, 200 µg/ml salmon DNA, 0.5 mg/ml A^- muscle RNA and from 1.5-2.5 x 10^7 cpm of the labeled RNA sequences. The hybridization mixture was overlayered with mineral oil and incubated at 42° C for 48 h. The filters were washed three times with 1 x SSC 1 %SDS at 65° C for 45 min each, treated for 1 h at 37° C in 1 x SSC containing 60 µg/ml proteinase K, then washed again with 0.5 x SSC 1 % SDS for 1 h at 65° C. After autoradiographic exposure, the filters were washed twice with 1 x SSC for 30 min at 65° C, digested for 30 min at 37° C in 1 x SSC

containing 10 µg RNAse A, then washed twice with 1 x SSC 30 min at 37° C. After a last autoradiographic exposure, radioactivity was eluted from the filters with 0.1 x SSC 0.1 % SDS, for 3 min at 100° C and counted by liquid scintillation.

Data are per cent of the zero time values. Using pBR 322 recombined plasmids as probes, the initial values, expressed in cpm specifically hybridized/10^7 cpm were 100-120, 250-350, 380-420 and 2000-2200 for L-type pyruvate kinase (cDNA insert : 670 bp), aldolase B (cDNA insert : 1020 bp), 5.4 kb mRNA (cDNA insert : 640 bp) and albumin mRNA respectively (cDNA insert : 1000 bp) (11,12,16). Using single-strand M13 phage as probe, the initial value in cpm for L-type pyruvate kinase mRNA was 120 when the transcribed cDNA strand was hybridized with 10^7 cpm total RNAs. All the values are given after substraction of the blank, i.e. non specific hybridization to non-recombined plasmids or phages. As shown in fig. 2, the level of the blank is usually very low, less than 50 cpm (open circle : hybridization of labeled RNAs with single-strand M13 phage).

RESULTS

12 h-refed rats were given glucagon intravenously for different periods of time, ranging from 10 to 120 min. Labeled RNAs, transcribed in vitro in the "run-off" transcription system, were hybridized with an excess of specific cDNAs fixed onto filters, using either double-strand pBR 322 recombinant plasmids or single-strand M13 phages (17) as probes. The L-type pyruvate kinase and 5.4 kb RNA synthesis were reduced to near background levels in the liver of glucagon-treated animals (fig. 1-2), while aldolase B gene transcription was reduced to only 40 % of fed controls, as expected from its persistence in fasted rat livers. The level of total RNA synthesis was essentially unaffected. When labeled RNA transcripts were submitted to

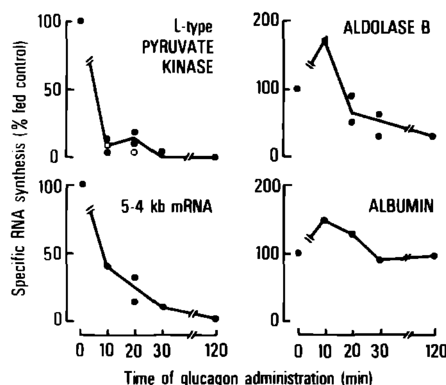

Fig. 1 : **Time course of glucagon effect on glycolytic enzyme RNA synthesis in liver nuclei of maltose-fed rats.**

48-h-fasted animals were refed the maltose-rich diet for 12 h. They were given regular glucagon (250 µg) intravenously for the time indicated (0 time point, no glucagon), through the femoral vein, cannulated four days prior to experiment (27).

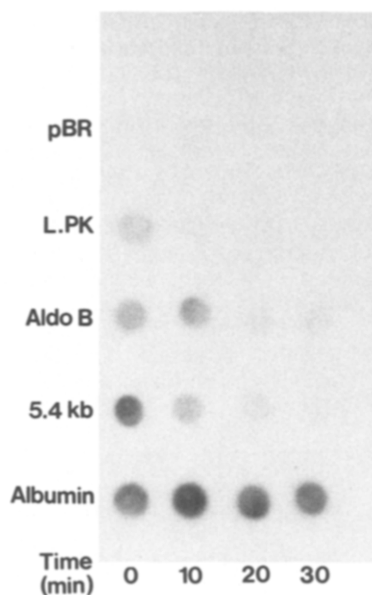

Fig. 2 : Time course of glucagon effect on glycolytic enzyme RNA synthesis in liver nuclei of maltose-fed rats.
Autoradiography of a typical experiment, performed as described in fig. 1.

electrophoresis and autoradiography, a smear ranging from 6 S to 23 S was observed (not shown). Analysis of the time-course of glucagon effects demonstrated that the inhibition of glycolytic enzyme gene transcription was rapid (fig. 1). After 10 min of glucagon treatment, the L-type pyruvate kinase and 5.4 kb RNA synthesis were reduced to 10 % and 40 % of carbohydrate-fed controls respectively and no RNA transcript was detectable after 30 min (fig. 1-2). Both strands of L-type pyruvate kinase cDNA were separately hybridized with in vitro labeled transcripts. Only the strand complementary to the RNA transcripts gave a significant signal, the kinetics of glucagon inhibition being similar to that of the double-strand probe. Cyclic AMP mimicked the effects of glucagon when administered intravenously as dibutyryl cyclic AMP (Bt_2cAMP) at a constant flow-rate over the 12 h-period of the refeeding (not shown). The inhibition of L-type pyruvate kinase gene transcription clearly preceded the changes in cytoplasmic RNA levels, which decreased much more slowly (105 % and 98 % of fed controls at 15 min and at 25 min of glucagon administration respectively), α-amanitin, a specific

inhibitor of RNA polymerase II, reduced overall transcription by 65-80 %, and totally blocked the specific transcription of the genes studied here (not shown).

Finally, the level of albumin gene transcription was found to be largely unaffected by glucagon administration (fig. 1-2).

DISCUSSION

In the isolated nuclei transcription system we use here, RNA production is due to the elongation of RNA chains whose synthesis was initiated in the living animals, and there is no in vitro initiation of new chains (18-19). Thus, when isolated nuclei are allowed to resume RNA synthesis in vitro, the transcripts produced accurately reflect RNA synthesis which occurred in vivo at the time of sacrifice.

The rapid decrease of specific hybridization following glucagon administration most likely indicates that cyclic AMP, the second messenger of glucagon, blocks the in vivo initiation of new transcripts, so that a much lower amount of nascent RNA chains is available for subsequent in vitro elongation. Since the rate of specific in vitro transcription (i.e the number of nascent RNA chains bound to the gene) is already very low after 10 min of glucagon treatment, the inhibition of initiation of gene transcription by glucagon/cyclic AMP may be a very early phenomenon, as is the cyclic AMP-induced stimulation of prolactin (3) and phosphoenolpyruvate carboxykinase gene transcription (1-2). The basic mechanism involved in the regulation of eukaryotic gene transcription by cyclic AMP remains unknown, but some recent data on the cyclic AMP-induced stimulation of tyrosine aminotransferase synthesis (20) favours the role of a cAMP-dependent protein kinase, phosphorylating a specific protein rather than the hypothesis of a mechanism similar to that occurring in E. Coli (namely a direct interaction between DNA regulatory sequences and a cyclic-AMP binding protein) (6). In fact, cyclic AMP is not the only agent that proves to either stimulate or inhibit gene transcription in eukaryotes.

Glucocorticoids, for instance, are activators of integrated murine mammary tumor virus (MMTV) gene transcription in rat hepatoma cells (21) and

inhibitors of the transcription of other genes, such as the proopiomelanocortin gene (22). In prokaryotes, the major role of the CAP-cAMP complex is to activate gene transcription, but CAP-binding can also repress transcription in some systems. In genes whose transcription is activated by cyclic AMP, the CAP-cAMP complex binds to a consensus sequence located upstream from the promotor, while for the genes whose transcription is inhibited by cyclic AMP (23), it binds to the same consensus sequence, within the promotor site. In the latter case, a steric hindrance of RNA polymerase binding to the promotor may be involved (23). The question of whether or not this model could be relevant to eukaryotes and especially mammals remains to be investigated. The availability of cDNA probes complementary to genes whose transcription is either stimulated or inhibited by cyclic AMP will be an essential tool for this study.

In conclusion, we have shown that cyclic AMP is both an activator and an inhibitor of gene transcription in eukaryotes and proves to be a major regulator of gene expression. Its effects on carbohydrates-metabolizing enzymes can be regarded as an integrated regulation at both the transcriptional and the post-translational level, since it both promotes protein phosphorylation (24-26) and modifies the synthesis of the enzymes, so as to enhance the gluconeogenic pathway and block the glycolytic pathway in the liver.

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