12-1991

Differential effects of cAMP on two hepatoma genes

Junseo Oh

To the Graduate Council:

I am submitting herewith a thesis written by Junseo Oh entitled "Differential effects of cAMP on two hepatoma genes." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Wesley D. Wicks, Major Professor

We have read this thesis and recommend its acceptance:

J. Koontz, D. Roberts

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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and recommend its acceptance:

John Koontz

Daniel P. Bloch

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

DIFFERENTIAL EFFECTS OF cAMP

ON TWO HEPATOMA GENES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Junseo Oh

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Acknowledgments

I would like to thank my wife and family for all the support they have given me. I would also like to thank my major professor Dr. Wesley D. Wicks, and the members of my committee, Dr. J. Koontz and Dr. D. Roberts.

I would also like to express my appreciation to Dr. Doris Schlichter for her patience and guidance.

ABSTRACT

It is known that the transcriptional rate of two of the cAMP-responsive genes, phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (TAT), is stimulated by cAMP in rat hepatoma cells and consequently their mRNA levels are increased, reaching a maximum within 2 hours. However, in both cases the mRNA levels decline after the peak (deinduction) due to unknown mechanisms. The kinetics of induction of $mRNA^{PEPCK}$ and $mRNA^{TAT}$ by cAMP have been studied in the present report, and it was demonstrated that, in spite of the superficially similar effects of cAMP on both genes, the mechanisms of deinduction are not the same; that is, the decline in the $mRNA^{TAT}$ level is due to cAMP metabolism, whereas the decline in $mRNA^{PEPCK}$ is caused by some kind of desensitization process which appears to involve delayed inhibition of the initial stimulation of PEPCK gene transcription.

Considering the multiplicity of CREB(CRE binding) / ATF (activating transcription factor) proteins and the important differences found in the location and sequences of the PEPCK and TAT CRE's (cAMP regulatory element), the differences in the mechanism of deinduction raises a question as to whether or not different CREB's might mediate the effects of cAMP on these two different genes in the same cell. From analysis of the effects of cycloheximide on cAMP inducibility, it appears that the effects of cAMP on $mRNA^{PEPCK}$ and $mRNA^{TAT}$ are direct and

that the protein factor mediating cAMP action on PEPCK differs from that regulating TAT, at least in terms of its stability.

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CHAPTER I

INTRODUCTION

It has been reported that cAMP modulates the transcriptional rate of a number of genes either positively [e.g. phosphoenolpyruvate carboxykinase (PEPCK) (Sasaki et al., 1984), tyrosine aminotransferase (TAT) (Hashimoto, Schmid and Schutz, 1984) and tyrosine hydroxylase (Lewis, Harrington and Chikaraishi, 1987) etc.] or negatively [e.g. pyruvate kinase (Meienhofer et al., 1987) and c-sis (Harsh et al., 1989) etc.], apparently as a result of direct action of the catalytic subunit of the cAMP-dependent protein kinase (PKA) (Mellon et al., 1989). These effects are presumably due to phosphorylation of specific DNA binding proteins, as will be described below.

In addition to transcriptional regulation, cAMP also exerts post-transcriptional effects. These include effects on mRNA stability (Jungmann et al., 1983; Hod and Hanson, 1988), the enhanced decline in the rate of TAT synthesis (Lewis et al., 1982), potential stimulatory effects on the rate of specific protein elongation in cultured hepatoma cells (Roper and Wicks, 1978) and possible increases in the *in vitro* translatability of TAT mRNA in intact rat liver (Noguchi et al., 1982).

The regulation of gene transcription by cAMP is initiated through the activation of adenylate cyclase upon

binding of poorly diffusible peptide hormones to receptors on the plasma membrane. The resulting intracellular production of cAMP leads to the activation of PKA. The inactive holoenzyme is composed of two catalytic(C) and two regulatory(R) subunits, and cAMP binding to the R subunits causes the release of active C subunits (Flockhart and Corbin, 1982). The activated PKA, in turn, presumably phosphorylates and activates specific DNA-binding proteins, resulting in the interactions of DNA-binding proteins with specific DNA promoter elements (Lee et al., 1988). In spite of the impressive progress made recently in delineating the site of action of cAMP and in characterizing components involved in the cAMP-induced regulatory cascade (described below), the exact mechanism responsible for cAMP regulation of gene expression is still largely unknown (Roesler et al., 1990; Habener, 1990).

The transcriptional response of several genes to cAMP has been localized to a specific DNA sequence within the promoter-regulatory regions termed a cAMP regulatory element (CRE). The CRE has been found to contain the palindromic core motif 5'-TGACGTCA-3' and to have the properties of an enhancer in that it can regulate transcription in a manner largely independent of position and orientation (Roesler, Vandenbark and Hanson, 1988). This element can confer cAMP-responsiveness on reporter genes, but the contextual sequences, which are DNA sequences around the CRE core motif, may exert a great influence on its effectiveness (Deutsch et

al., 1988). However, there are cases where some of the genes regulated by cAMP lack a CRE core motif, such as metallothionein II_A (Imagawa, Chiu and Karin, 1987), and a different cAMP-responsive element has been identified, termed the Activator Protein 2 (AP-2) binding site (Mitchell, Wang and Tjian, 1987) which is suggested to mediate induction by both cAMP and phorbol esters (Imagawa, Chiu and Karin, 1987).

The TGACGTCA-containing CRE is recognized by a family of related proteins referred to as CREB's or CREB/ATF's. The CREB proteins are related to the ATF's which are activated by the E1A protein encoded by adenovirus and also bind to CRE-like elements in the adenovirus promoters (Lin and Green, 1988). Recently, there have been at least twelve distinct CREB/ATF's reported in different laboratories from various sources, which differ in sequence and physical properties; e.g. CREB from human placenta (Hoeffler et al.,1988) and rat brain (Gonzalez et al., 1989), CRE-BP1 from human fetal brain (Maekawa et al., 1989), ATF-1, 2, 4, 7, 8 from human osteosarcoma cells and ATF-3, 5, 6 from HeLa cells (Hai et al., 1989) (for a review, see Habener, 1990). CREB's bind as a homodimer to CRE sequences, and their binding is apparently unaffected by cAMP (Habener, 1990; Montminy et al., 1990) in contrast to their activity as CRE-mediated transcription stimulators, which is augmented by phosphorylation (Yamamoto et al., 1988; Lee et al., 1988).

The CREB/ATF proteins appear to contain two distinct functional domains: an amino-terminal one involved in

transcriptional activation and a carboxyl-terminal DNA binding/dimerization region (Busch and Sassone-Corsi, 1990). The transcription activation domains of the CREB/ATF's have begun to be characterized, in particular with respect to the role of phosphorylation in mediating the transactivation functions (Yamamoto et al., 1990). The DNA binding/ dimerization domain, called a bZIP motif, contains two subdomains: a carboxy-terminal leucine zipper made up of heptad repeats of leucine residues that is involved in protein dimerization via the formation of a parallel coiled-coil stabilized by hydrophobic interactions and an upstream region with a high density of basic amino acid residues that is responsible for DNA binding with recognition specificity (Landschultz, Johnson and McKnight, 1988; O'Neil, Hoess and DeGrado, 1990).

There is a dimerization code that has not been elucidated as yet, by which some but not all combinations of bZIP proteins form stable heterodimers. A possible consequence of dimer formation concerns DNA binding specificity, in other words, the choice of a dimerization partner can influence binding specificity (Hai and Curran, 1991). The c-Jun protein, which can heterodimerize with c-Fos, can also efficiently dimerize with particular members of the CREB/ATF family, e.g. CRE-BP1 (or ATF2), ATF3 and ATF4. The binding specificity of the two heterodimers is different, however; unlike the Jun/Fos dimer, which preferentially recognizes an AP-1 site over the related CRE, the reverse is

apparently true with the Jun/CRE-BP1 complex (Benbrook and Jones, 1990; Macgregor, Abate and Curran, 1990).

As mentioned earlier, cAMP has been shown to stimulate the transcriptional rate of two genes, PEPCK and TAT; in both cases the peak accumulation of mRNA levels is reached within 2 hours (Nelson et al., 1980; Noguchi et al., 1978; Spielholtz et al., 1988). However, the mRNA levels of both genes decline after the peak (deinduction) due to unknown mechanisms. Since the mechanistic basis of the deinduction has not been explored, it is the purpose of this work to begin a study of this phenomenon.

For some of the cAMP-responsive genes the effects of cAMP are found to be indirect, that is, the synthesis of an intermediary protein is required, as has been suggested for thyroglobulin and βhCG (Gerard et al., 1989; Milsted, Cox and Nilson, 1987). Thus, it was also of interest to test whether cAMP effects on both PEPCK and TAT are direct or indirect using cycloheximide (CHX), an inhibitor of protein synthesis. It has been reported that in the absence of any hormonal stimulus, CHX increases the level of $mRNA^{PEPCK}$ (Nelson et al., 1980) and $mRNA^{TAT}$ (Ernest, 1982), possibly by protecting mRNA from degradation by stabilizing the polysome structure (Ernest, 1982). Therefore, analysis of the change in the $mRNA^{PEPCK}$ and $mRNA^{TAT}$ levels in cultured cells treated with both inducer and CHX must take into consideration potential effects of CHX itself. A brief description of the

transcriptional regulation of these two genes, PEPCK and TAT, is discussed next.

Phosphoenolpyruvate Carboxykinase (PEPCK)

PEPCK is a key regulatory enzyme in the gluconeogenic pathway which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (EC 4.1.1.32), and its synthesis rate is acutely regulated by a number of hormones (Granner et al., 1986). Glucocorticoids and cAMP increase transcription of the PEPCK gene, leading to an increase in the $mRNA^{PEPCK}$ level and the rate of enzyme synthesis (Sasaki et al., 1984; Beale, Katzen and Granner, 1981). Conversely, insulin and phorbol esters inhibit transcription of the PEPCK gene and thus result in a reduction of $mRNA^{PEPCK}$ (Sasaki et al., 1984; Chu and Granner, 1986).

The mRNA for PEPCK is 2624 nucleotides in length, and its half life has been reported to be about 40 minutes in FTO-2B hepatoma cells (Hod and Hanson, 1988). Analysis of a series of PEPCK promoter deletions in transfection experiments revealed a CRE located at -96 to -77 (Quinn et al., 1988). The sequence of the core motif in this CRE is 5'-TTACGTCA-3'. However, the precise locations of promoter sequences that mediate the effect of insulin, tissue specificity, and developmental regulation have not been identified.

Tyrosine Aminotransferase (TAT)

TAT, the first enzyme in the pathway of tyrosine catabolism in the liver and also a gluconeogenic enzyme, catalyzes the transfer of the amino group of tyrosine to α-ketoglutarate using pyridoxal 5'-phosphate as a cofactor (EC 2.6.1.5). The resultant *p*-hydroxyphenylpyruvate is then metabolized to fumarate and acetoacetate through four enzymatic steps.

The transcription of TAT is stimulated by glucocorticoids and cAMP (Hashimoto et al., 1984), and the incubation of Fao hepatoma cells with insulin resulted in an increase in TAT activity and mRNA level due to an increase in the transcriptional rate (Crettaz, Muller-Wieland and Kahn, 1988). Combined dexamethasone and cAMP treatment leads to higher $mRNA^{TAT}$ levels than are seen with each inducer alone, which implies that dexamethasone and cAMP act by distinct mechanisms. Analysis of the TAT promoter revealed a CRE located over 3.6 kbp upstream of the transcription start site (Boshart et al., 1990). The sequence of the CRE core motif is 5'-TGCGTCA-3', in which the third nucleotide (A) is missing from the perfect palindromic motif 5'-TGACGTCA-3', and the context around the motif is not the same as that of the PEPCK CRE. The mRNA for TAT, 2362 nucleotides in full length, is also short-lived with a half life of 2 hours in KRC-7 hepatoma cells (Spielholz et al., 1988).

As mentioned above, there are important differences between PEPCK and TAT, such as: 1) location of the CREs; 2) sequences of the CRE core motifs; 3) contextual sequences of the CREs; and 4) responses to insulin. These differences raise a question as to whether or not different CREB's might mediate the effects of cAMP on these two different genes in the same cell. Therefore, the goal of my research initially has been to investigate this possibility by more detailed analysis of the kinetics of induction to see if the basis for similar kinetics of the cAMP effects are same or not and by the analysis of possible differential effects of CHX on cAMP action.

CHAPTER II

MATERIALS AND METHODS

Materials

8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate and dexamethasone were purchased from Sigma (St. Louis, MO), and 8-Br-adenosine 3',5'-cyclic monophosphate was prepared from bromine and cAMP (Muneyama et al., 1971). Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum were from Sigma, and calf serum and horse serum from Whittaker M. A. Bioproducts (Walkersville, MD) and Gibco (Grand Island, New York), respectively. Diethylpyrocarbonate(DEPC), 5,6-dichlororibofuranosyl-benzimidazole (DRB), cycloheximide (CHX) and guanidinium thiocyanate were supplied by Sigma (St. Louis, MO). Gene Screen, from New England Nuclear, Boston, MA was used for immobilization and probing of RNA. The random priming kit and α-[^{32}P]-dCTP used for the radioactive labeling of oligonucleotides were from Promega (Madison, WI) and Du Pont Co. (Boston, MA), respectively.

Cell Culture

A clone, Fao, of the rat hepatoma H35 cell line (Deschatrette and Weiss, 1974) was grown in plastic cell culture plates with DMEM supplemented with 5 % calf serum and

5 % fetal bovine serum at 37°C in a humidified CO_2 incubator. A clone, KRC-7, of the Reuber H35 hepatoma cell line (Moore and Koontz, 1989) was grown with DMEM supplemented with 5% calf serum and 5% fetal calf serum. Cells were grown to 80~90% confluency for use and were switched to medium lacking serum for 48 hours before being treated with inducing agents or CHX.

RNA Isolation

Total cellular RNA was isolated by the method of Chirgwin et al. (1979) with modifications adapted from Maniatis et al. (1989). Cells were first washed twice with cold PBS (0.9% NaCl, 0.2% KCl, 20mM K_2HPO_4, pH 7.2) and then lysed in a solution of 4M guanidinium thiocyanate, 2% sodium lauryl sarcosine, 0.05M Tris (pH 7.6), 0.025M EDTA and 0.15M β-mercaptoethanol. The lysate was layered onto a cushion of 5.7M cesium chloride and centrifuged in a Beckman SW50.1 rotor at 20°C at 36,000 rpm for 20 hours. The clear RNA pellet was then dissolved in DEPC-treated H_2O, ethanol precipitated and suspended in DEPC-treated H_2O for storage at -20°C. RNA was quantitated by measuring the optical density at 260 nm, using an absorbance of 1 equal to 40 μg/ml. The ratio between the readings at 260 nm and 280 nm, which provides an estimate for the purity of RNA, is typically 1.8-1.9, in good agreement with the theoretical value of 2.0 indicating pure preparation of RNA.

Northern blot analysis

Various amounts of heat-denatured (60°C for 5~10 minutes) total RNA were separated by electrophoresis in a 1.0% agarose gel containing 6% formaldehyde. After staining with ethidium bromide to visualize the 28S and 18S bands and to check the integrity of RNA (for a typical example, see page 16), RNA in the agarose gel was then transferred to Gene Screen paper using 20X SSC (3M NaCl and 0.3M sodium citrate, pH 7.0) as the transfer buffer by the capillary method (Thomas, 1980) and immobilized to the membrane by UV crosslinking. The RNA blot was prehybridized at 42°C for at least 12 hours in 50% formamide, 0.25M NaH_2PO_4 (pH 7.2), 0.25M NaCl, 1mM EDTA, 7% SDS and 100μg/ml denatured salmon sperm DNA (Amasino, 1986).

DNA sequences complementary to regions of the TAT and PEPCK mRNA's were radiolabeled with [α-^{32}P]-dCTP by the random priming method. The plasmid, pcTAT3 (Scherer et al., 1982), which contains cDNA complementary to nucleotides 773 to 1373 of the full length TAT mRNA (2362 nucleotides in length) was used as a probe for this mRNA. A DNA sequence, referred to as BH1.2, which contains 547 nucleotides of 5' flanking sequence, two introns and two exons (140 and 240 nucleotides each) was used as a probe for PEPCK (Yoo-Warren et al., 1983). The labeled DNA probes were purified by passage through a Biogel P-6 column with a molecular weight

exclusion limit of 6,000 daltons to remove the free deoxynucleotides.

The labeled probe was then denatured by boiling and added to the prehybridization mixture. Hybridization was allowed to occur at 42°C for at least 12 hours, and then the blot was washed in decreasing concentrations of SSC (Maniatis et al., 1989). Autoradiograms were developed by exposing the blot to Kodak film at -70°C using an intensifying screen. The relative intensities of the bands on the autoradiograms were quantitated by scanning densitometry using an LKB model 2400 Gel Scan XL densitometer. Some of the blots probed for either $mRNA^{PEPCK}$ or $mRNA^{TAT}$ were washed in 0.02X SSC, 0.1% SDS at 50°C and reprobed with a cDNA made from the α-tubulin mRNA (Lemischka and Sharp, 1982) as an internal control. Although the usual procedure was to prepare two different blots, one for each mRNA, some of the blots probed for $mRNA^{PEPCK}$ were also washed and reprobed for $mRNA^{TAT}$ in order to verify the integrity of the data obtained. It showed that the results obtained from the reprobe with the TAT cDNA were essentially identical with those from the original probe.

Validation of the Northern blot assay

Each of PEPCK and TAT probes labels only one major band located between the 18S and 28S rRNA markers and a plot of the distance migrated versus log M shows that each RNA is ~2400-2600 nucleotides in length, which indicates that the

probes used are specific (the $mRNA^{PEPCK}$, $mRNA^{TAT}$, and the 18S and 28S rRNA are 2624, 2362, 1874 and 4718 nucleotides in length, respectively). A photograph of an ethidium bromide-stained gel and the corresponding autoradiogram are shown on page 16.

Data obtained from the Northern blot assay have been validated through four different steps:

1) The different absorbance values from scanning densitometry obtained for two different amounts of the same RNA (e.g. 4 and 8μg) loaded on 1% agarose gel were compared to ensure the validity of the transfer and scanning process. The ratio of the two scanning values is 1.85 $\pm$ 0.05 (n=124) in good agreement with the expected value of 2.0.

2) The data obtained from two different transfers using the same RNA applied to 1% agarose gel were compared, as a check on the efficiency of transfer. The average deviation from the mean is 4.6% (n=216). An example is shown on page 31.

3). The data obtained from two or more experiments using different preparation of RNA have been compared in most cases to determine the biological reproducibility of the data. The average deviation from the mean value is 6.5% (n=102). Results are shown on page 18, 19, 21, 23, 24, 26 and 33.

4). In order to compensate for possible differences in the amounts of total RNA loaded on the gel or transferred to the membrane, some of the RNA blots were reprobed subsequently with a cDNA to the α-tubulin gene. The average deviation of

individual $mRNA^{\alpha\text{-tubulin}}$ densities from the mean value is 12% (n=35).

TAT activity measurement

In order to confirm that the cycloheximide concentration used was sufficient to block protein synthesis, its ability to inhibit the effect of a cAMP analog on TAT activity was measured using a modification of the assay method of Diamondstone (1966) as described by Wicks (1969).

CHAPTER III

RESULTS

Induction of PEPCK by cAMP in KRC-7 Cells

The ability of cAMP to increase the activity of PEPCK was found to be apparently lost during passage of KRC7 cells while that of TAT was not (Wicks, W.D., unpublished observations). It was considered possible that this differential change in phenotype could be due to the selective loss of a cAMP-mediating protein factor (CREB etc.) that was different from the one responsible for regulation of TAT.

In order to investigate this possibility, KRC-7 cells were treated with 2mM 8-Br-adenosine 3',5'-cyclic monophosphate (8-Br-cAMP) for 3 hours and total cellular RNA was extracted as described in Methods. Figure 1 shows an autoradiogram generated from Northern blot analysis. Each of the PEPCK and TAT probes labels only one major band which is located between the 18S and 28S rRNA markers as expected. Scanning confirmed that the density value is directly proportional to the amount of RNA loaded on the agarose gel (data not shown).

In contrast to expectations, cAMP was found to increase the levels of both $mRNA^{PEPCK}$ and $mRNA^{TAT}$. However, the activity of PEPCK was not measured along with its mRNA level,

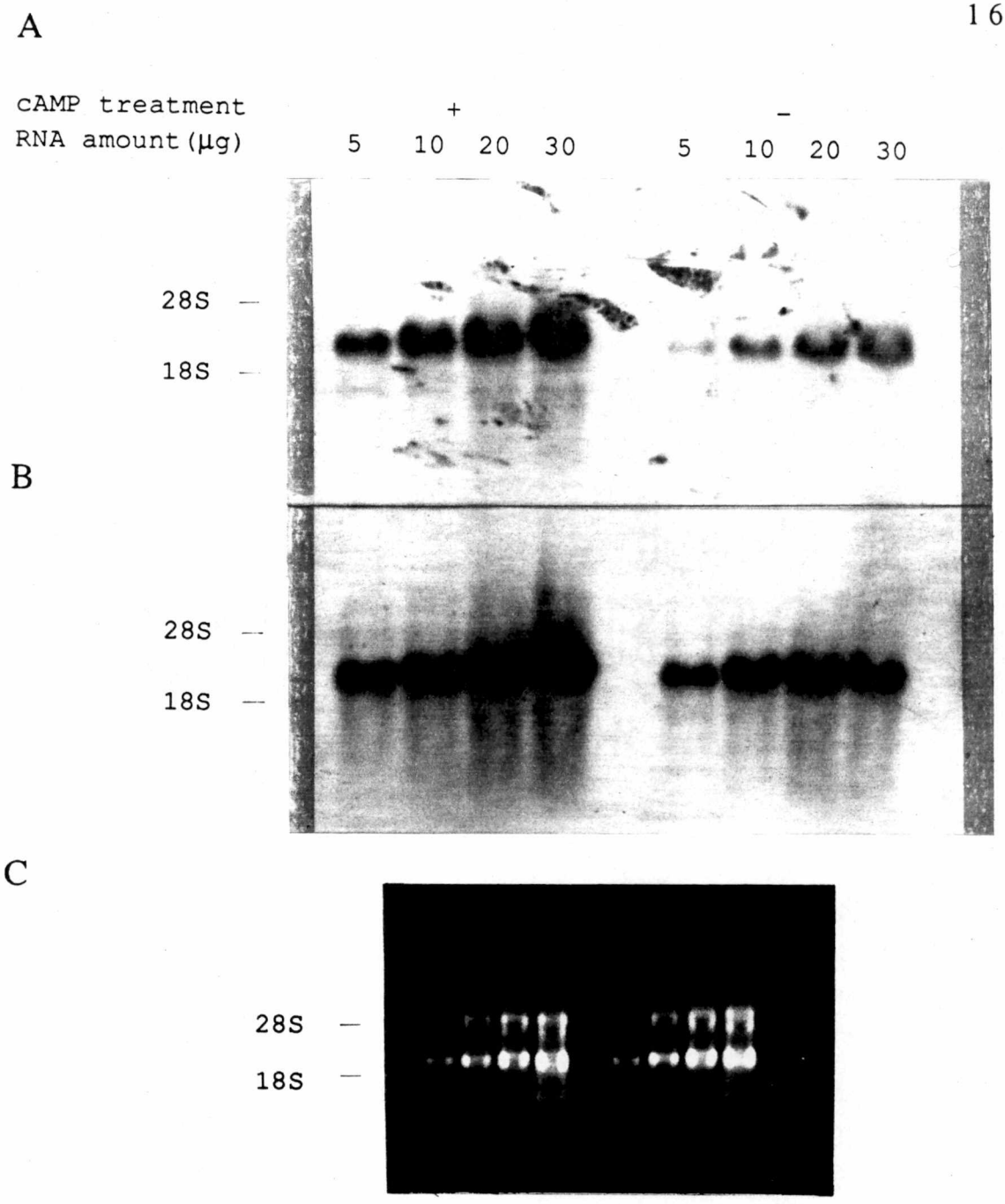


Figure 1. **cAMP induction of PEPCK and TAT in KRC-7 cells**. KRC-7 cells were treated with and without 2mM 8-Br-cAMP for 3 hours, and 5, 10, 20, 30μg of total extracted RNA were loaded on 1% agarose gel containing formaldehyde as described in the Methods. Autoradiograms generated from the northern blot assay are shown for $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B). A photograph of the ethidium bromide-stained gel corresponding to the autoradiogram (A) is shown in (C).

and so it is possible that cAMP reduces the translatability of $mRNA^{PEPCK}$. However, since I was interested only in effects at the transcriptional level, this line of investigation was dropped.

Analysis of the Induction Kinetics of $mRNA^{PEPCK}$ and $mRNA^{TAT}$ by cAMP in Fao cells

Fao cells were chosen for the studies of cAMP induction, since basal levels of $mRNA^{PEPCK}$ in KRC-7 cells were too low to permit accurate quantitation (Figure 2). Subsequently, Fao cells were treated with 2mM 8-Br-cAMP and the kinetics of change in the levels of both $mRNA^{PEPCK}$ and $mRNA^{TAT}$ were measured by Northern blot analysis. This analysis revealed that the levels of both $mRNA^{PEPCK}$ and $mRNA^{TAT}$ reach a maximum within 2 hours after cAMP addition, at which time both mRNA levels are increased ~4 fold (Figure 3). After the peak, the level of both mRNA's decreased gradually. This transient induction and ensuing decline (deinduction) have been reported previously for the response of both genes to cAMP in cultured hepatoma cells (Nelson et al., 1980; Noguchi et al., 1978; Spielholtz et al., 1988)

If cAMP is metabolized and the resulting loss of inducer is the cause of the decline in the $mRNA^{PEPCK}$ and $mRNA^{TAT}$ levels after the peak, then replenishment of lost inducer should prevent such a decline. In order to investigate this possibility, Fao cells were divided into two groups; one

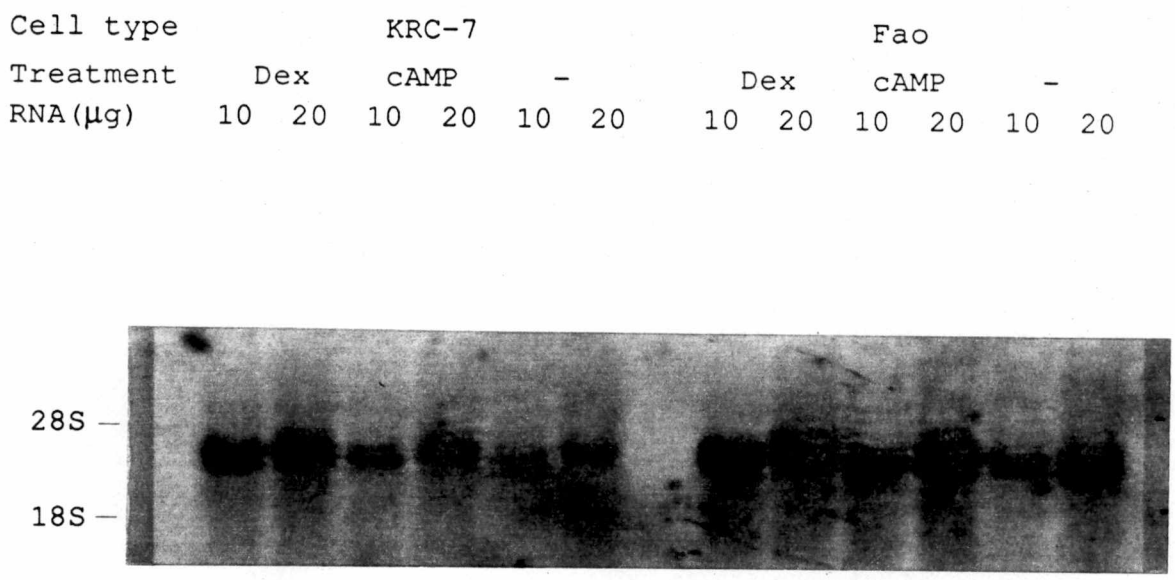
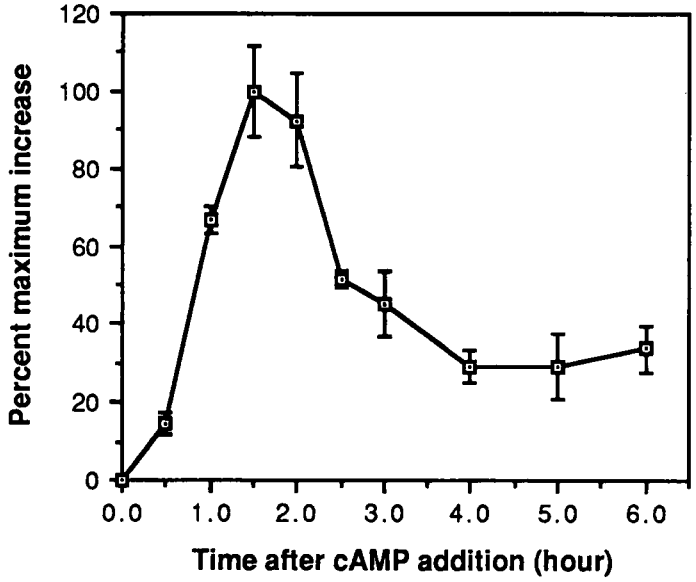


Figure 2. **Comparison of cAMP induction of PEPCK in KRC-7 and Fao cells.** KRC-7 and Fao cells were treated ± 2mM 8-Br-cAMP or ± 1µM Dex for 3 hours, and total RNAs were extracted as described in the Methods. 10 and 20µg of total RNAs were loaded on 1% agarose gel as described in the Methods. Autoradiograms generated from a northern blot assay are shown for $mRNA^{PEPCK}$.

A



B

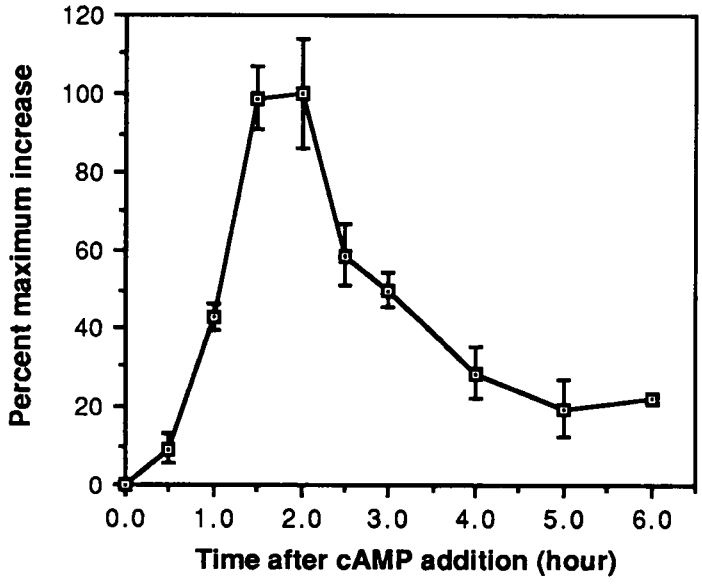


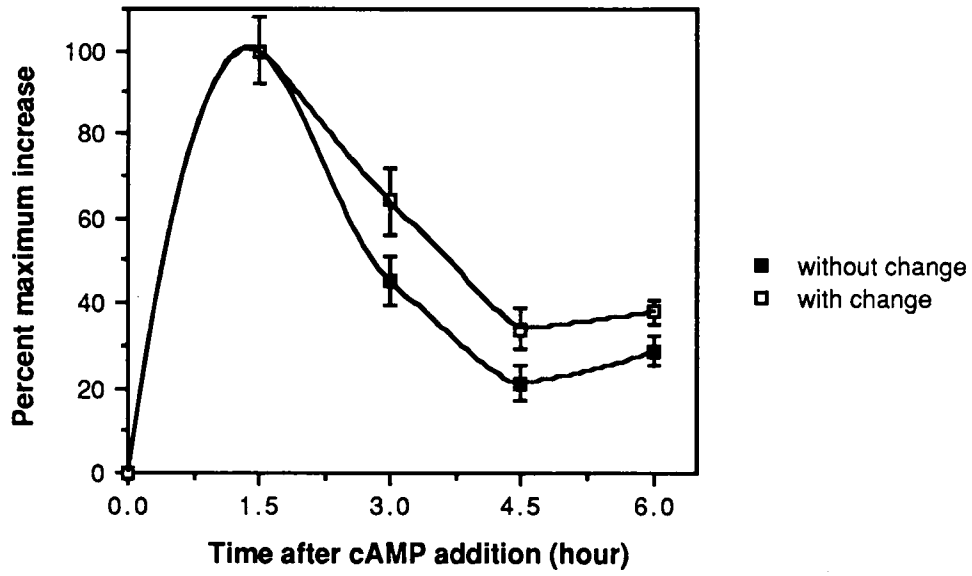
Figure 3. **Time course of increases in mRNA^{PEPCK} and mRNA^{TAT} levels after cAMP addition in Fao cells.** Fao cells were treated with 2mM 8Br-cAMP for the times indicated, and the levels of both mRNA^{PEPCK} (A) and mRNA^{TAT} (B) were determined by northern blot assay. Values are expressed as a percent of the average maximum increase (4.4 and 4.2 fold-increase for PEPCK and TAT, respectively) taking the average obtained from two independent experiments. Each error bar represents the deviation from the mean in two independent experiments.

group was exposed to 0.2mM 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (CPT-cAMP) for 1.5, 3, 4.5, 6 hours with the medium changed every 1.5 hours to one containing fresh cAMP analog. The other group was treated for the same period of time without any medium change. CPT-cAMP was used since it had been reported to be more resistant to cAMP phosphodiesterase than 8-Br-cAMP (Miller et al., 1975).

A similar decline in the $mRNA^{PEPCK}$ level is seen under both conditions indicating that replacing the CPT-cAMP every 1.5 hours did not prevent the decline (Figure 4A). In contrast to the case of PEPCK, the level of $mRNA^{TAT}$ remains elevated when the medium is sequentially changed to one containing fresh cAMP analog (Figure 4B). These results suggest that the decline in the $mRNA^{TAT}$ level after the peak of induction is likely to be due to the fact that cAMP analog used was metabolized, whereas the decline of $mRNA^{PEPCK}$ cannot be due to the same mechanism.

In order to test this possibility further, a different experiment was performed. If the decline in the mRNA levels after the peak is due to cAMP metabolism, then we would expect to see the mRNA levels remain elevated as long as a high level of cAMP is maintained in the medium. Fao cells were treated with 0.2mM CPT-cAMP for various periods of time with the medium being either unchanged or changed to fresh medium containing cAMP analog every 1.5 hours. Each time these treated cells were harvested, the medium was

A



B

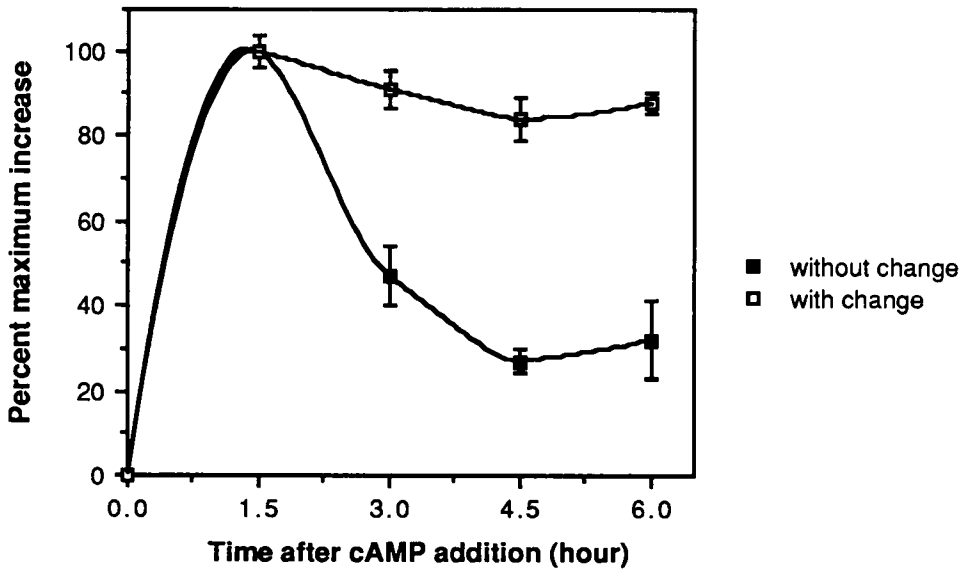


Figure 4. **Effect of changing the medium.** Fao cells were treated with 0.2mM CPT-cAMP for the time indicated with (open square) or without (filled) the medium being changed every 1.5 hours to one containing fresh cAMP analog, and the levels of both $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) were determined by northern blot assay. Values are expressed as a percent of the average maximum increase (5.2 and 4.6 fold-increase for PEPCK and TAT, respectively) taking the average obtained from two independent experiments. Each error bar represents the deviation from the mean in two independent experiments.

transferred to untreated cells, and then 1.5 hours later these cells were harvested.

Northern blot analysis demonstrates that without a medium change the $mRNA^{PEPCK}$ level of the transferred samples after the peak generally declines similar to that of the original samples, except the group at 6 hours (Figure 5A). With a medium change the $mRNA^{PEPCK}$ level of the original samples still appears to decline with time, even though there was enough cAMP analog present in the medium to fully induce PEPCK in the samples to which the medium was transferred (Figure 6A). The reason for the discrepant point seen in Figure 5A is unclear, but the results obtained with a medium change clearly suggest that the decline in the $mRNA^{PEPCK}$ level is not due to cAMP metabolism.

In the case of TAT, without a medium change the $mRNA^{TAT}$ level of both sets of samples does decline (Figure 5B) after the peak but does not do so with a medium change (Figure 6B). This indicates that the $mRNA^{TAT}$ level is dependent upon the amount of cAMP analog present (Figure 5B and 6B). Thus, this experiment also supports the conclusion that the decline in the $mRNA^{TAT}$ level is due to cAMP metabolism, whereas the similar decline seen in case of $mRNA^{PEPCK}$ appears to be due to some other mechanism.

The two experiments presented above involve medium changes which might alter the environment of the cells. For example, if an inhibitor of TAT transcription accumulated in the medium which is responsible for the decline in the $mRNA^{TAT}$

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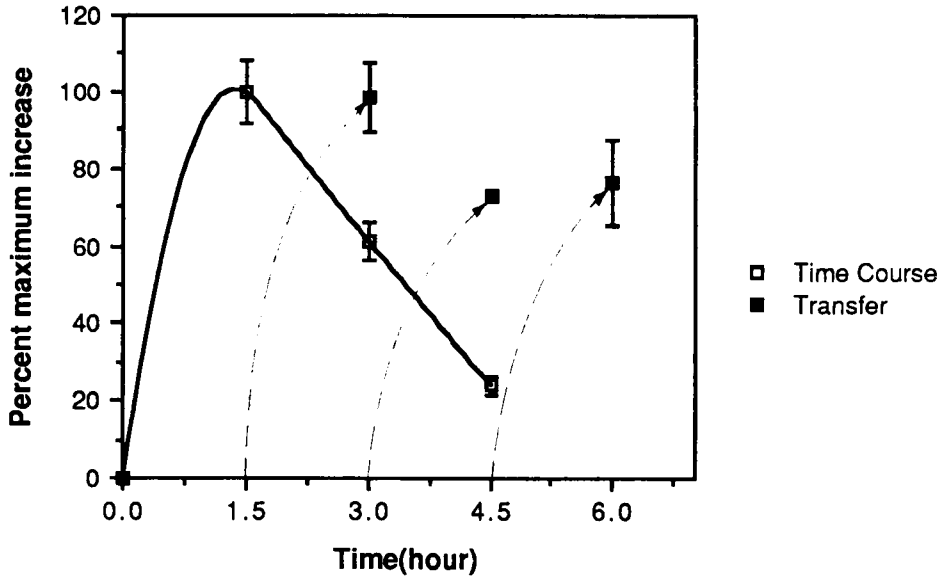

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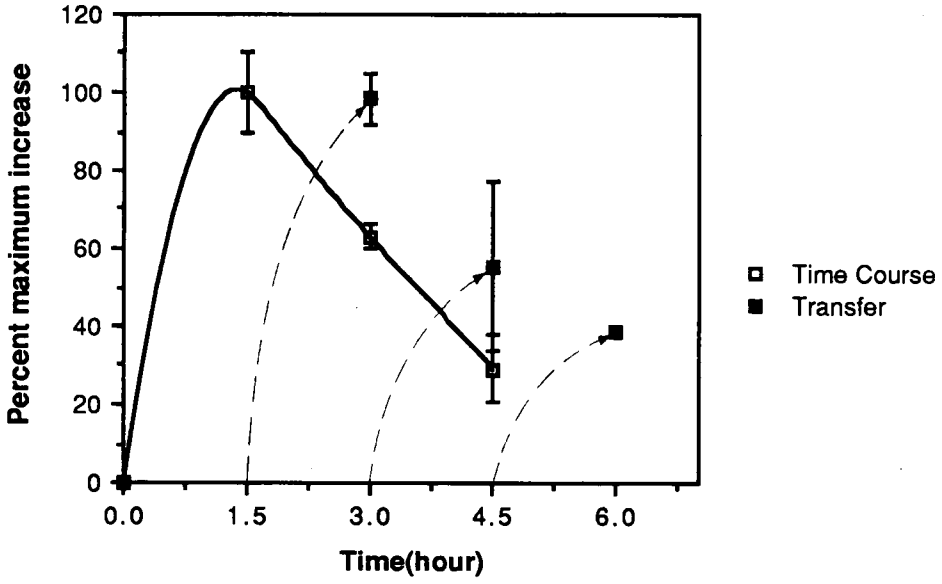

Figure 5. **Effect of transferring the medium without a change.** Fao cells were treated with 0.2mM CPT-cAMP up to 4.5 hours *without* the medium being changed. Each time that Fao cells were harvested (open square), the medium was transferred to untreated cells, and 1.5 hours later these cells were harvested (filled). Scanning values for the levels of both $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) are expressed as a percent of the average maximum increase (5.6 and 5.7 fold-increase for PEPCK and TAT, respectively) taking the average obtained from two independent experiments. Each error bar represents the deviation from the mean in two independent experiments.

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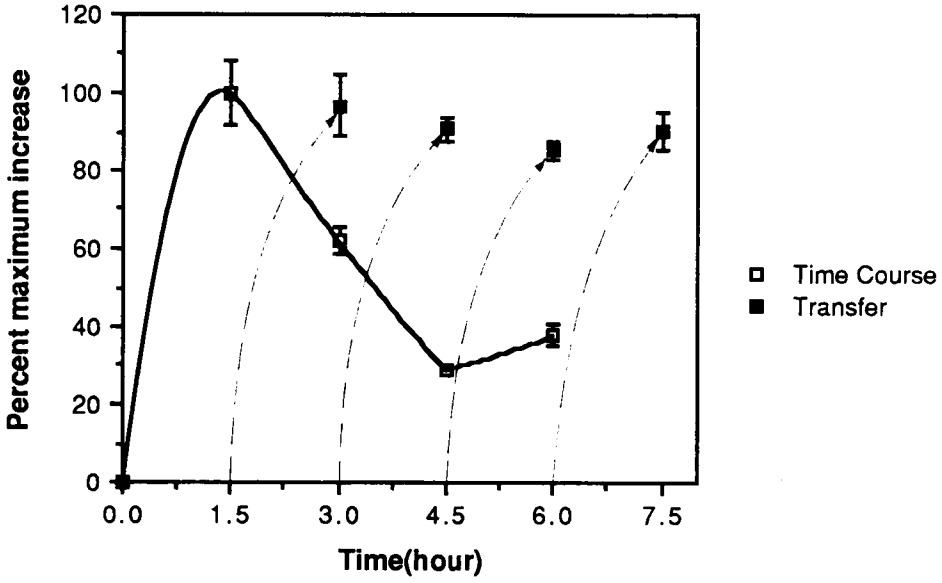

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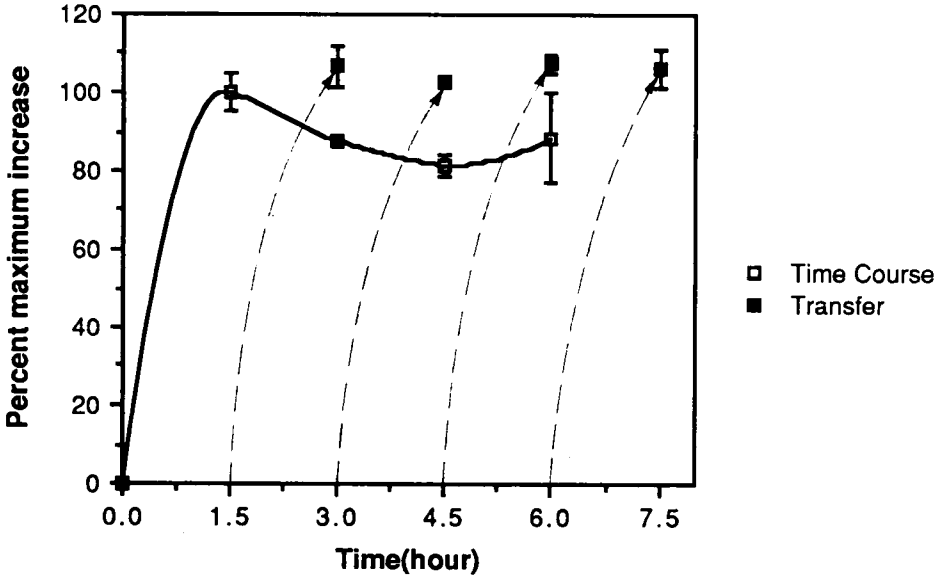

Figure 6. **Effect of transferring the medium with a change.** Fao cells were treated with 0.2mM CPT-cAMP up to 6 hours *with* the medium being changed every 1.5 hours. Each time that Fao cells were harvested (open square), the medium was transferred to untreated cells, and 1.5 hours later these cells were harvested (filled). Scanning values for the levels of both $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) are expressed as a percent of the average maximum increase (5.4 and 4.7 fold-increase for PEPCK and TAT, respectively) taking the average obtained from two independent experiments. Each error bar represents the deviation from the mean in two independent experiments.

level after the peak of induction, then a medium change would remove the inhibitor and prevent the decline in the level of $mRNA^{TAT}$. In order to check this possibility, another experiment was performed without a medium change. Fao cells were treated with 0.2mM CPT-cAMP for 1.5, 3, 4.5, 6 hours with or without sequential addition of fresh cAMP analog every 1.5 hours to replenish any lost inducer in the medium.

Northern blot analysis indicates that even with sequential addition of cAMP analog, the $mRNA^{PEPCK}$ still declines (Figure 7A). In contrast, the level of $mRNA^{TAT}$ remains elevated (Figure 7B). Therefore, this experiment clearly demonstrates that the decline of $mRNA^{TAT}$ after the peak of induction is, in all likehood, due to cAMP metabolism rather than the formation of a transcriptional inhibitor. Indeed, rat hepatoma H35 cells (from which Fao cells are derived) have been reported to have a high degree of phosphodiesterase activity, which could explain the need to replenish cAMP analog in the medium (Leichtling et al., 1978). The fact that the $mRNA^{PEPCK}$ level declines with comparable kinetics under all of the conditions tested suggests that a clear difference in the mechanism of deinduction of these two mRNA's must exist.

Since changes in the mRNA content were measured in the experiments described so far rather than changes in transcriptional rates, the possibility exists that a delayed change in the half life of $mRNA^{PEPCK}$ (e.g. a 4~5 fold-decrease) could be responsible for the observed deinduction.

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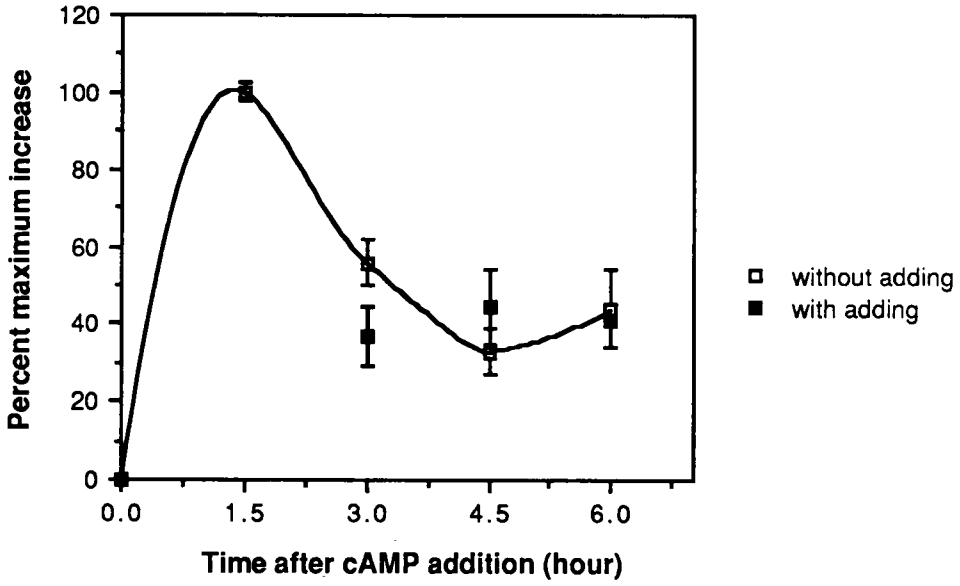

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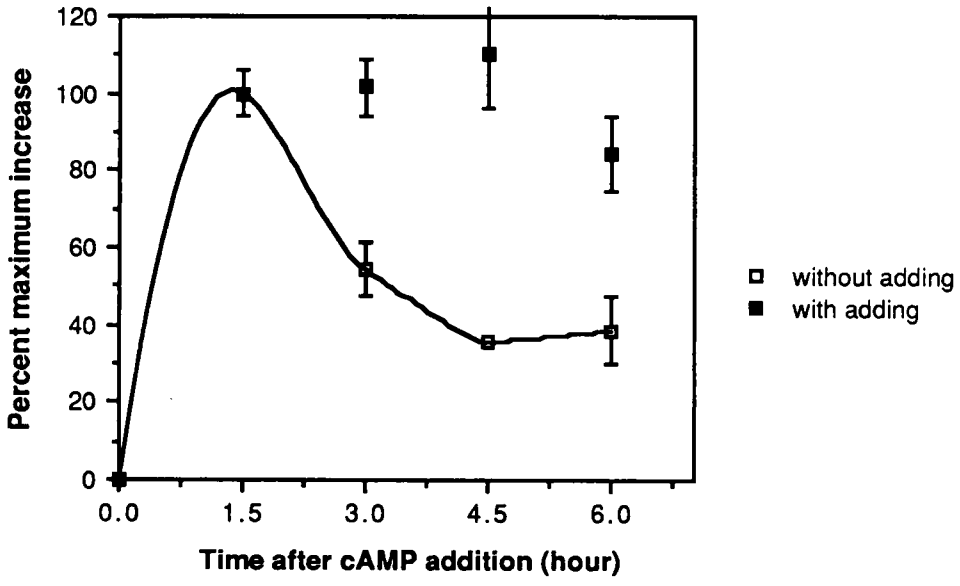

Figure 7. **Effect of addition of fresh cAMP.** Fao cells were treated with 0.2mM CPT-cAMP up to 6 hours with (filled square) or without (open) sequential addition of fresh cAMP analog every 1.5 hours. Scanning values for the levels of both $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) are expressed as a percent of the average maximum increase (4.5 and 4.4 fold-increase for PEPCK and TAT, respectively) taking the average obtained from two independent experiments. Each error bar represents the deviation from the mean in two independent experiments.

Therefore, this possibility had to be investigated in order to distinguish between transcriptional and post-transcriptional explanations for the decline seen after the peak of induction. Consequently, Fao cells were divided into four groups; the first group was not exposed to any inducer, the second treated with 0.2mM CPT-cAMP for 1.5 hours, the third treated with 1μM dexamethasone, a synthetic glucocorticoid which is a well-known transcriptional activator of both genes (Figure 2), for 3 hours, and the fourth treated with dexamethasone for 3 hours in the presence of 0.2mM CPT-cAMP for the last 1 hour. After each treatment, Fao cells in each groups were treated with 5,6-dichlororibofuranosyl-benzimidazole (DRB), the transcriptional inhibitor, in the presence of the previous inducer for different periods of time (Spielholz, 1988).

The results of northern blot analysis show that cAMP does not appear to affect the stability of either $mRNA^{PEPCK}$ or $mRNA^{TAT}$ and that the half-lives of PEPCK and TAT are ~90 and ~110 minutes, respectively (Table 1). Therefore, it appears that the decline in the $mRNA^{PEPCK}$ level cannot be due to a change in its half-life but more likely reflects a delayed inhibition of transcription.

Although the kinetics of the effects of cAMP on both the PEPCK and TAT genes appear to be virtually identical at first glance (Figure 3), the results obtained from the experiments described above indicate that the mechanisms behind the superficially similar effects of cAMP are not the same; the

Table 1. Half-Life Determinations(minutes) of $mRNA^{PEPCK}$ and $mRNA^{TAT}$.

mRNA	PEPCK		TAT	
Treatment	Exp 1	Exp 2	Exp 1	Exp 2
Dex	81 (100%)	108 (100%)	90 (100%)	120 (100%)
cAMP	88 (+9%)	N.D.	95 (+6%)	N.D.
Dex + cAMP	85 (+5%)	117 (+8%)	104 (+16%)	128 (+7%)

The half-life values were calculated from semi-log plots of the mRNA levels determined versus time, using least-square analysis. The value in each parenthesis represents the percentage difference in the half-life compared to that observed with Dex alone.

decline in the $mRNA^{TAT}$ level seen after the peak at about 1.5 hours after addition of cAMP analog is due to cAMP metabolism, whereas the decline seen in $mRNA^{PEPCK}$ is caused by some kind of desensitization process which appears to involve delayed inhibition of the initial stimulation of PEPCK gene transcription caused by cAMP.

The Effect of Cycloheximide on cAMP Inducibility in Fao cells

As described in the Introduction, at least twelve distinct CREB/ATF's have been reported and there are differences between the PEPCK and TAT genes in the: 1) location of CREs; 2) sequences of CRE core motifs; and 3) contextual sequences of CREs. Considering these findings, the observations made showing differences in the mechanism of deinduction of these two mRNA's in the kinetic experiments suggest that it is reasonable to consider the possibility that different CREB's are responsible for the regulation of these two genes by cAMP.

As another way of investigating this possibility, cycloheximide (CHX), an inhibitor of protein synthesis, was added with or a without a cAMP analog. The rationale behind using CHX is that it could provide some valuable information such as: 1) are the observed cAMP effects on both PEPCK and TAT transcription direct?; 2) are there differences in the apparent stability of the CREB(s) involved?; and 3) is protein synthesis involved in the mechanism by which the

$mRNA^{PEPCK}$ level declines after the peak of induction?

In order to examine the first two of these possibilities, Fao cells were treated with 5μM CHX for 2 to 6 hours ± with 0.2mM CPT-cAMP for the additional 1.5 hours in the continued presence of CHX, and then harvested. CHX pre-treatment could reveal possible differences, if any, in the half-life of CREB(s) mediating cAMP action on PEPCK and TAT, since the CREB with the shorter half-life would disappear more rapidly. The concentration of CHX used was chosen because it has been shown to effectively block TAT induction by cAMP (data not shown) and has been reported to inhibit protein synthesis by >95% (Cox et al., 1990). Data presented in Figure 9A and 9B were derived by subtracting the effect seen with CHX pre-treatment alone from that obtained with both CHX and cAMP treatment [i.e. mRNA level with CHX and cAMP - mRNA level with CHX], in order to ascertain the effect of cAMP alone under the conditions used.

Northern analysis shows that treatment with CHX alone increases the mRNA levels of both PEPCK (Figure 8A) and TAT (Figure 8B) by two fold at 2hr, after which the mRNA levels decline gradually. This increase in mRNA levels is consistent with what were reported by Nelson et al., (1980) and Ernest et al., (1982) and, as they have suggested, may be due to protection of these mRNA's from degradation by stabilizing the polysome structure.

When treated with both CHX and cAMP, Fao cells still show cAMP inducibility of PEPCK although the extent of

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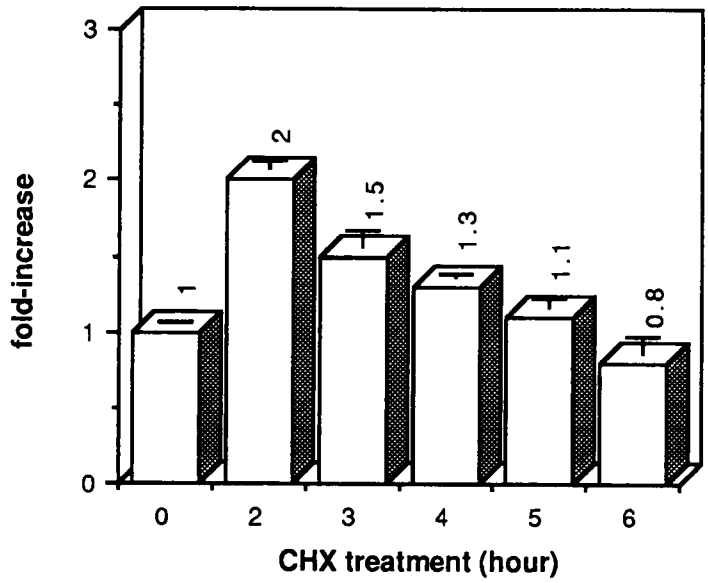

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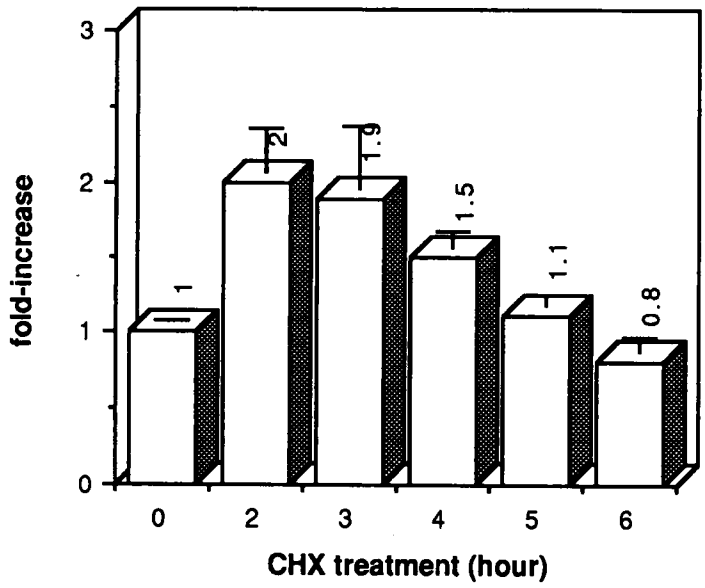

Figure 8. **Effect of CHX on basal mRNA levels.** Fao cells were treated with 5μM CHX alone for up to 6 hours, and the levels of $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) were determined by nothern blot assay. Each bar represents the average of data obtained from two independent experiments whose deviations are indicated by the error bar.

induction is slightly lower than the control group exposed to cAMP alone for 1.5 hours (Figure 9A). The reason why less than a full additive effect on $mRNA^{PEPCK}$ is seen with both CHX and cAMP [i.e. 73 and 60% seen instead of 100% with 2 and 3 hours of CHX pre-treatment, respectively] is not clear, but could be due to the gradual loss of the CREB involved (as is suggested by the fact that the induced $mRNA^{PEPCK}$ level gradually declines). These results suggest that; 1) the effect of cAMP on the PEPCK gene appears to be direct, i.e. the protein mediator (CREB) can generate an effect without requiring the synthesis of another protein(s), and 2) the protein factor mediating cAMP action on PEPCK probably has a reasonably long half life (a rough estimate of 4~5 hours is suggested by the decay kinetics in Figure 9A).

In contrast to PEPCK, no effect of cAMP on the level of $mRNA^{TAT}$ is seen after 2 to 3 hours of CHX pre-treatment, although a slight increase is seen at later intervals (Figure 9B). The meaning of this slight increase is unclear, but prolonged exposure of cells to CHX may well produce non-physiological effects. It seems reasonable to argue that the shorter the period of CHX treatment, the more likely it is that the cellular response to cAMP will be normal (i.e. the spectrum of proteins present will be closer to those in untreated cells). The complete inhibition of cAMP inducibility of $mRNA^{TAT}$ by a short period of CHX treatment, regardless of modest later increases, suggests either that the protein factor mediating the cAMP effect on TAT has a

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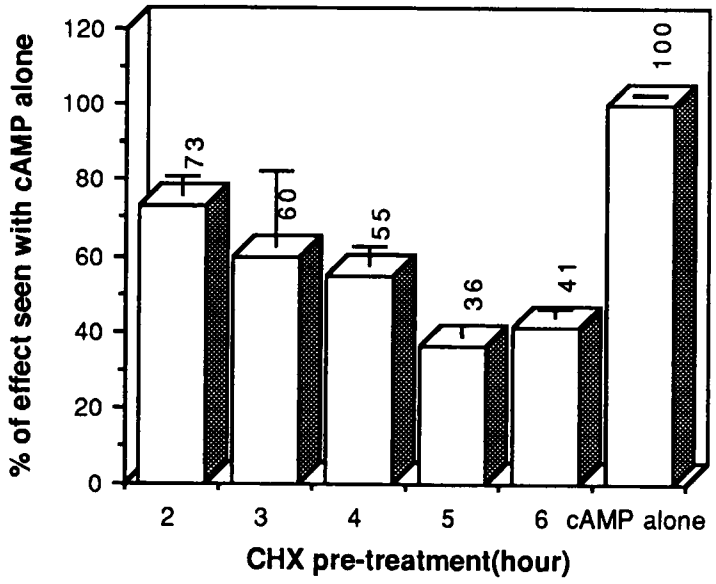

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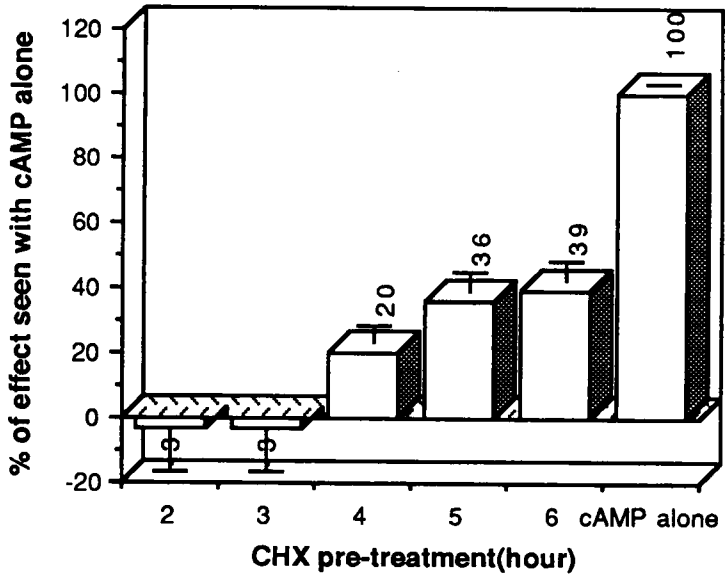

Figure 9. **Effect of CHX pretreatment on cAMP induction.** Fao cells were treated with 5μM CHX for 2 to 6 hours and harvested after treatment with 0.2mM CPT-cAMP for the additional 1.5 hours in the continued presence of CHX. Then, $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) were determined. Data presented are derived by subtracting the % increase in the mRNA level with CHX pre-treatment alone from the % increase seen with both CHX and cAMP treatment. Each bar represents the average of data obtained from two different transfers using the same RNA whose deviation are indicated by the error bar.

very short half-life or that the cAMP effect on TAT is indirect and requires the synthesis of another protein(s).

These results are consistent with the possibility that the protein factor(s) and mechanism mediating cAMP induction on TAT are different from those for PEPCK.

The Effect of Cycloheximide on the cAMP Time Course

Recently a number of protein factors have been discovered which have been shown to inhibit the activities of trans-activating factors like CREB1, either by blocking their binding to DNA or forming transcriptionally inactive heterodimers with active transcription factors, e.g. cAMP-responsive element modulator (CREM) (Foulkers, Borrelli and Sassone-Corsi, 1991), inhibitory protein (IP-1) (Auwerx and Sassone-Corsi, 1991) and ΔFosB (Nakabeppu and Nathans, 1991). These observations suggest the possibility that PEPCK transcription is stimulated initially by the binding of an active CREB homodimer to PEPCK-CRE and, after a delay of 30~60 minutes, it could become inhibited by a newly formed negative protein factor. The putative negative factor could either block DNA binding of the active CREB homodimer or form transcriptionally inactive heterodimers with the active CREB factor, which could then lead to the decrease in the $mRNA^{PEPCK}$ level after the peak of induction.

Cycloheximide (CHX) was also used in order to investigate such a possibility, since if the decline in the

$mRNA^{PEPCK}$ level after the peak indeed requires the synthesis of negative protein factor, CHX treatment could prevent induction of that factor, resulting in a blockade of the decline. Fao cells were divided into three groups; the first group was treated with 0.2mM CPT-cAMP alone for 1.5, 4, 6 hours, the second treated with 5μM CHX alone for 2, 4.5, 6.5 hours, and the third treated first with 5μM CHX and 30 minutes later with 0.2mM CPT-cAMP in the continued presence of CHX for additional 1.5, 4, 6 hours..

Northern blot analysis shows that CHX pre-treatment does not block PEPCK induction by cAMP as seen in the previous experiment (Figure 9A) and the $mRNA^{PEPCK}$ level did indeed remain elevated in the presence of CHX. That is, the decline in the $mRNA^{PEPCK}$ level seen after the peak appears to be blocked by CHX treatment (Figure 10A). Although not proven, these results are consistent with the possibility that CHX prevents the induction of a negative protein factor and thus blocks the decline in the $mRNA^{PEPCK}$ level.

In the case of TAT, some induction by cAMP is still seen even with CHX pre-treatment for 30 minutes in contrast to the previous experiment (Figure 9B). The previous experiment involved CHX pre-treatment for more than 2 hours and CHX was found to block TAT induction. The presence of TAT induction in this experiment by cAMP even with full inhibition of protein synthesis indicates that it is unlikely that the cAMP effect on TAT is actually indirect as suggested by the previous experiment. A possible explanation for this

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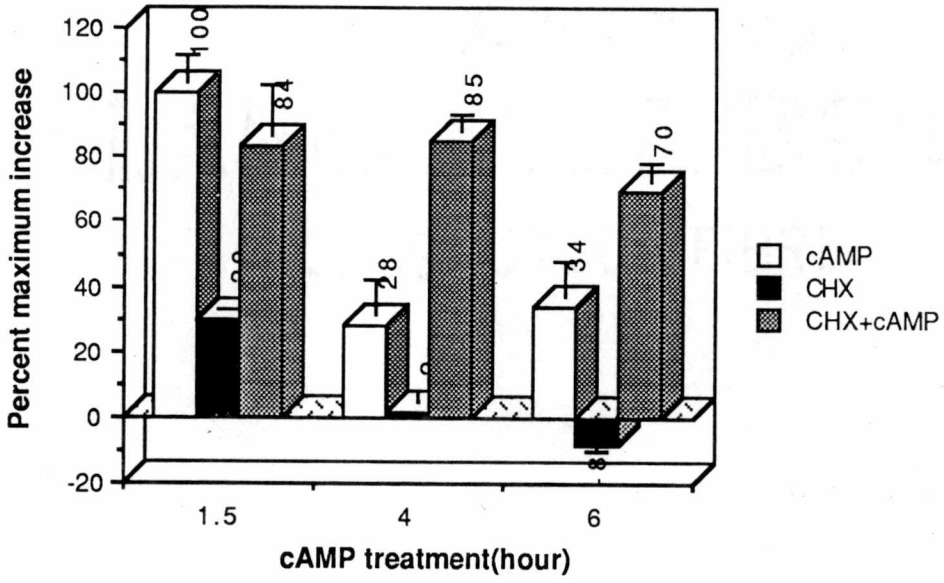

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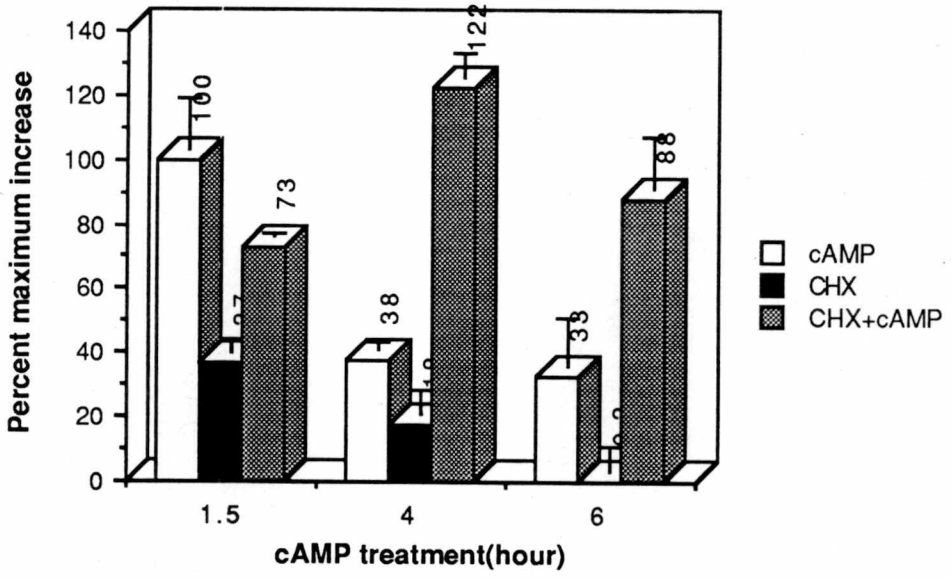

Figure 10. **Effect of CHX on deinduction process.** Fao cells were divided into three groups; 1. treated with 0.2mM CPT-cAMP (open bars), 2. treated with 5μM CHX for 2, 4.5, 6.5 hours (black), and 3. treated with 5μM CHX and 30 minutes later with cAMP analog in the continued presence of CHX (hatched). Values for the levels of both $mRNA^{PEPCK}$ (A) and $mRNA^{TAT}$ (B) are expressed as a percent of the average maximum increase (4.7 and 4.5 fold-increase for PEPCK and TAT, respectively) taking the average obtained from two independent experiments. Each error bar represents the deviation from the mean in two independent experiments.

discrepancy could be that the half life of the CREB regulating TAT is relatively short (e.g. 30 minutes or so) so that after the CHX pre-treatment for >2 hours, there would be little or no factor left for cAMP to act on (<5%). In contrast, after only 30 minutes sufficient factor might be available for substantial TAT induction (~50%). In addition, the $mRNA^{TAT}$ level also does not decline as was the case with PEPCK (Figure 10B). The reason for the lack of the decline is not clear at the present time, but it does suggest that the explanation for the lack of decline in $mRNA^{PEPCK}$ must be qualified.

Based on the two experiments involving CHX (Figure 9 and 10), it appears that the effects of cAMP on both $mRNA^{PEPCK}$ and $mRNA^{TAT}$ are direct and the protein factor mediating the cAMP effect on PEPCK may differ from that regulating TAT at least in terms of its stability.

CHAPTER IV

DISCUSSION

The regulation of genes by cAMP is thought to be mediated through the interactions of the activated DNA-binding proteins, CREB/ATF's, (by phosphorylation) with specific DNA promoter elements (CRE) which are located in the 5'-flanking regions of the genes upstream from the transcription start sites. However, the multiplicity of CREB/ATF's and the important differences found in the location and sequences of the PEPCK and TAT CRE's (as described in the Introduction) led us to investigate the possibility that different mechanisms may exist for the effects of cAMP on both genes.

First, analysis of the kinetics of induction of $mRNA^{PEPCK}$ and $mRNA^{TAT}$ by cAMP shows that the decline in the level of $mRNA^{TAT}$ after the peak of induction is due to cAMP metabolism, that is, the decay of activator. In contrast, the decline seen in the level of $mRNA^{PEPCK}$ is caused be some kind of desensitization process which could involve delayed inhibition of the cAMP stimulation of PEPCK gene transcription.

How might such a desensitization mechanism occur? Recently there have been at least four different inhibition systems discovered which are reported to down regulate induced transcription by various transcriptional activators.

First, the so-called cAMP response element modulator (CREM), which shows high sequence homology to the C-terminal portion of CREB but lacks the N-terminal glutamine-rich domain, shows a dominant negative effect on cAMP induced transcription, presumably by forming transcriptionally inactive heterodimers with active CREB1 (Foulkers, Borrelli and Sassone-Corsi, 1991). Second, the inhibitory protein (IP-1) is reported to be a negative modulator of AP-1 activity (activator protein-1), whose inhibitory effect is reversible through changes in phosphorylation state and probably results from blocking DNA binding of AP-1 (Auwerx and Sassone-Corsi, 1991). Third, an inducible negative modulator, ΔFosB, which is a truncated form of FosB, has a dominant negative effect on the activating function of Fos proteins, presumably by competing with the latter at both the steps of dimerization with Jun and binding of Fos-Jun heterodimers to an AP-1 site (Nakabeppu and Nathans, 1991).

Another well characterized example of a delayed transcriptional down regulation is that of tyrosine hydroxylase (TH) in PC12 cells exposed to nerve growth factor (NGF) (Gizang-Ginsberg and Ziff, 1990). TH transcription is induced at a maximum rate within less than 1 hour after treatment with NGF by a mechanism dependent on new protein synthesis. c-fos, an immediate early gene in NGF-induced differentiation of PC12 cells, is transiently transcriptionally induced, and the expressed c-fos protein has been found to form an active heterodimer with pre-

existing c-Jun. The Fos-Jun complex formed (AP-1) then binds to the AP-1 site in the TH promoter (Angel et al., 1987) and results in stimulation of TH transcription. As the duration of NGF stimulation is extended, the relative levels of Fos and Fra change. The level of Fos declines, most likely due to its autoregulation and short half-life, and Fra becomes the predominant product. Thus, the Fos-Jun complex at the AP-1 site appears to be gradually replaced by a complex consisting of a Fra-Jun complex which does not stimulate TH transcription, thus leading to a time delayed repression of stimulated TH transcription.

These recent findings suggest how complicated the molecular mechanism of the deinduction of PEPCK might be. However, considering the four inhibition systems described above together with the observation that another factor, ATF1, can form a heterodimer with CREB1 and the resulting heterodimer exerts much weaker effects on CRE-dependent transcription (Hurst, Totty and Jones, 1991), it seems reasonable to suggest that upon treatment with a cAMP analog, PEPCK transcription is stimulated initially by the binding of an active CREB homodimer to the PEPCK-CRE. As the duration of cAMP induction is extended, however, a protein factor which is either a negative factor (like CREM or ATF1) or one required for activating a pre-existing negative factor is induced, resulting in the induction or activation of a negative protein factor on a delayed basis. This factor, presumably by way of forming a heterodimer with the active

CREB, could either block DNA binding or reduce the stimulatory activity of CREB, and which would lead to the time delayed repression of cAMP stimulation on PEPCK transcription. This would be somewhat similar to the case of TH with NGF, where the active Fos in a Fos-Jun complex is replaced with the inactive Fra leading to the delayed lack of stimulation of TH transcription.

Consistent with the possibility mentioned above, the decline in the $mRNA^{PEPCK}$ level seen after the peak appears to be blocked by CHX treatment. Furthermore, the approximate half-life of $mRNA^{PEPCK}$ calculated from the deinduction kinetics (98 $\pm$ 11 minutes) is similar to the value obtained from the experiment using the transcriptional inhibitor, DRB (i.e. 80~110 minutes). It indicates that the stimulation of PEPCK transcription must be subject to maximum inhibition by the time the peak mRNA levels are reached since DRB completely inhibits transcription. Another thing that should be pointed out is that under all the conditions tested, the $mRNA^{PEPCK}$ level even after deinduction remains 20~30% over the basal level. A possible explanation might be that the heterodimer of CREB with a putative negative factor mentioned above might be able to exert weak stimulating activity. A similar result has been seen with ATF1, wherein an ATF1-CREB heterodimer shows 20~30% of the transcriptional activity obtained with CREB homodimer whereas the activity of the ATF1 homodimer is negligible.

Unexpectedly, the effects of CHX on the deinduction of $mRNA^{TAT}$ look similar to the case of PEPCK, that is, CHX blocks the decline in the mRNA level. There may be several explanations for this phenomenon and it could indicate that the explanation for the deinduction of PEPCK needs to be qualified. As seen with PEPCK, the $mRNA^{TAT}$ level after deinduction is also 20~30% over the basal level. A possible explanation is that even though the amount of cAMP analog is not enough to activate all the CREB present in the cell, it may be sufficient to activate this much.

Although the study of the effects of CHX on cAMP inducibility and cAMP time course is admittedly indirect, the results suggest that: 1) the effect of cAMP on PEPCK appears to be direct, based on the presence of a cAMP effect even after CHX pre-treatment; and 2) the protein factor mediating cAMP action on PEPCK probably has a reasonably long half-life (i.e. >4 hours), whereas the protein factor for TAT appears to have a relatively short half-life.

Therefore, although the kinetics of the effect of cAMP on both the PEPCK and TAT genes appears to be virtually identical at first glance, it does seem likely that a difference in the mechanisms of cAMP action exists for the deinduction phase and there may well also be a difference in the molecular process involved in the induction phase as well.

In order to elucidate the mechanism of the cAMP-induced changes in transcription further, more experiments are needed

to clarify whether or not the protein factors mediating the effects of cAMP on PEPCK and TAT do, in fact, differ from each other and how the desensitization process generates the decline in the level of $mRNA^{PEPCK}$ after the peak of induction. To characterize the protein factors further, southwestern analysis, UV crosslinking or gel-shift assays can be done with Fao cell nuclear extracts using the respective CREs of PEPCK and TAT as probes. Subsequently, after a number of purification steps *in vitro* transcription studies could provide information about their functional properties. In the case of the desensitization process, whether DNA binding of a transcriptional activator is blocked or whether transcriptionally inactive complexes form could be tested by binding assays and *in vitro* transcription done using nuclear factors isolated from cells during the deinduction phase.

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