Induction of Alkaline Phosphatase in Mouse L Cells by Overexpression of the Catalytic Subunit of cAMP-dependent Protein Kinase*

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Mouse L929 cells were used to study the mechanism of cAMP induction of alkaline phosphatase (AP) activity. Following treatment with 200 μM 8-chlorophenylthio-cAMP (CPT-cAMP), alkaline phosphatase enzyme activity was observed to increase 80-fold after 24 h. The CPT-cAMP dose response of the alkaline phosphatase enzyme activity correlated well with the CPT-cAMP activation of cAMP-dependent protein kinase (AP) in L cells. A DNA clone for the alkaline phosphatase was isolated and used to demonstrate a 10-fold increase in alkaline phosphatase mRNA levels after a 24-h treatment of L cells with CPT-cAMP. Increased mRNA levels were first detected 4–6 h after CPT-cAMP treatment, and the level of alkaline phosphatase mRNA decreased rapidly after removal of CPT-cAMP. In vitro nuclear transcription studies showed that a 3-fold increase in alkaline phosphatase gene transcription was detectable 6 h after CPT treatment, and this increase was blocked by cycloheximide.

In order to determine if the catalytic (C) subunit of cAMP-dependent protein kinase was able to mediate the induction of AP, L cells were transfected with expression vectors containing the metathionine promoter and coding for the Ca isoform of the catalytic subunit of cAMP-dependent protein kinase or for a catalytic subunit in which lysine 72 had been mutated to methionine (CaK72M). Zinc treatment of stably transfected cells expressing the wild-type C subunit showed an increase in protein kinase activity and an increase in AP activity. Zinc treatment of cells containing the mutant C subunit expression vector produced an increase in the amount of a protein which was recognized by C subunit antibodies on Western blots, but these cells showed no increase in protein kinase activity or in AP activity. We conclude that the C subunit is sufficient for transcriptional induction of the AP gene and that the phosphotransferase activity of the C subunit is required for this induction.

A preponderance of experimental evidence suggests that the mechanisms by which cAMP regulates gene transcription are distinct in eukaryotic and prokaryotic organisms (Roesler et al., 1988). In prokaryotes, cAMP binds to catabolite activator protein, which is itself a DNA-binding protein (Weber et al., 1982). While cAMP is bound to catabolite activator protein, it enhances transcriptional initiation by interacting with specific promoter sequences and RNA polymerase. In eukaryotes, however, the major receptor for cAMP is the regulatory (R) subunit of cAMP-dependent protein kinase (Beebe and Corbin, 1986; Taylor et al., 1988). cAMP-dependent protein kinase exists as a catalytically inactive tetramer of two regulatory subunits and two catalytic (C) subunits. The binding of two molecules of cAMP to each R subunit results in the dissociation of the holoenzyme and release of catalytically active C subunit. The release of C subunit from the holoenzyme results in phosphorylation of serine and threonine residues in many cellular proteins. Although multiple isoforms of both the R and C subunits have been characterized, their functional significance is currently uncertain (Beebe and Corbin, 1986; Chrivia et al., 1988). The majority of evidence to date would support a model for cAMP regulation of eukaryotic gene transcription where the C subunit released from the inactive holoenzyme is translocated to the nucleus (Boney et al., 1983; Riabowol et al., 1988). There the C subunit is thought to phosphorylate proteins important in the regulation of gene transcription (Montminy and Bilezikjian, 1987; Grove et al., 1987). In most cases these phosphorylated proteins are themselves thought to be DNA-binding proteins (Mitchell and Tjian, 1985). The expression of many eukaryotic genes has been reported to be induced by cAMP, but one of the most dramatic inductions reported to date has been the induction of AP activity in mouse L cells (Firestone and Heath, 1981). Alkaline phosphatase is a membrane-bound glycoprotein that exists in several isoforms that can be experimentally distinguished by differences in antigenicity and inhibitor sensitivity (Gum and Raetz, 1985). Alkaline phosphatase activity in bone and other tissues is thought to play a role in extracellular phosphate metabolism (Stigbrand and Fishman, 1984). Dibutyryl-cAMP treatment of mouse L cells resulted in a 2000-fold increase in the specific activity of AP in cell extracts. Furthermore, in vitro translation of mRNA from control and cAMP-treated cells suggested that this induction was due to an increased abundance of the mRNA coding for AP (Firestone and Heath, 1981). Although the mechanism of induction of enzyme activity was not demonstrated clearly, this system offers several advantages for the analysis of cAMP-dependent protein kinase function in the regulation of gene expression. The availability of expression vectors that allow the over...
production of the C subunit of cAMP-dependent protein kinase (Uhler and McKnight, 1987) enabled us to test whether the C subunit has a direct effect on AP gene transcription. In this paper we present evidence that the induction of AP activity is at least partially due to AP gene transcription. Furthermore, we show that overexpression of the C subunit is sufficient to induce AP enzyme activity and mRNA levels. Finally, we have tested a mutant C subunit in which a lysine residue thought to be involved in ATP binding has been altered to methionine by in vitro mutagenesis. This mutant C subunit is catalytically inactive and unable to induce AP activity when overexpressed in mouse L cells.

MATERIALS AND METHODS

Cell Culture and Transfection of Mouse L Cells—NCTC clone 929 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium containing 10% horse serum. The cells were split 1:20 the day before transfection. Confluent mouse L cells were split 1:20 the day before transfection. Furthermore, we show that overexpression of the C subunit is sufficient to induce AP enzyme activity and mRNA levels. All protein concentrations were determined spectrophotometrically using a dye binding assay (Bio-Rad).

Enzyme Assay—For quantitation of AP enzyme activity, the medium was aspirated from 10-cm culture plates, and the plates were washed twice with cold Tris-buffered saline (0.01 M Tris (pH 7.3), 0.15 M NaCl). The cells were scraped off into cold Tris-buffered saline and pelleted at 800 rpm for 5 min. The pellet was then resuspended in cold Tris-buffered saline and sonicated. This cell extract was assayed for AP activity using p-nitrophenyl phosphate as the substrate as described (Firestone and Heath, 1981). Activity is reported as units/g total protein, where 1 unit represents 1 μmol of p-nitrophenol phosphate hydrolyzed per min. All protein concentrations were determined spectrophotometrically using a dye binding assay (Bio-Rad).

Kinase Assay—Cell pellets were resuspended in homogenization buffer (10 mM NaPO4, pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose) and the protein concentration adjusted to 2 mg/ml. Kinase assays were performed as described previously (Uhler and McKnight, 1987). Briefly, protein extract was added to a reaction mixture containing buffer, [γ-32P]ATP (200 cpm/pmol), and the synthetic peptide substrate Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly; Sigma) in the presence or absence of cAMP. After a 5-min incubation at 30 °C, half of the reaction mix was spotted onto phosphocellulose paper, and this was washed in dilute phosphoric acid. Enzymatic activity was measured by the incorporation of 32P into Kemptide. One unit of activity is defined as 1 pmol of phosphate transferred to the substrate per min. Endogenously active kinase was determined in the absence of any added cAMP in the kinase assay, and total kinase activity was determined in the presence of 10 μM cAMP.

Cloning of the Mouse L Cell Alkaline Phosphatase cDNA—Twenty 15-cm plates of L cells were treated for 48 h with 1 μM CPT-cAMP. Poly(A) RNA was isolated from these plates using guanidinium thiocyanate for isolation of total RNA and oligo(dT)-cellulose for poly(A) RNA purification as described (Uhler et al., 1986b). A cDNA library of 400,000 independent clones in λgt10 was constructed as described previously (Uhler et al., 1986b) and screened using an oligonucleotide (ATGATCTCACCATTTTTAGTACTGGCCATCG-GCACCTGCCTTACCAAC) corresponding to the first 48 coding nucleotides of the published mouse placental AP cDNA (Teran and Mintz, 1987). Two positively hybridizing plaques were identified, and the corresponding recombinant phage were purified. Both phage contained cDNA inserts approximately 2.5 kb in length, and the cDNA inserts from these phage were then subcloned into M13mp18 and sequenced by dideoxy sequencing (Messing, 1983).

SP6 Quantitation of Messenger RNA—The cDNA fragments were subcloned into pGEM-4 (Promega Biotec), and 32P-labeled antisense transcripts for both the alkaline phosphatase cDNA and the C subunit of cAMP-dependent protein kinase were generated using SP6 polymerase as described (McKnight et al., 1988). The radiolabeled antisense transcripts were used for detection of mRNA by hybridization, and M13mp18 constructs containing the sense strand were used as standards for mRNA quantitation in a solution hybridization assay (McKnight et al., 1988).

In Vitro Nuclear Transcription—L cells were fed with fresh DMEM/horse serum 4 h before treatment. Nuclei were isolated as described (McKnight and Palmiter, 1979) from three 10-cm plates of L cells for each treatment. Nuclei (about 50 μg of DNA equivalent) were incubated for 1 h at 26 °C with 300 μM of [32P]TPP (800 Ci/mmol, Du Pont-New England Nuclear) (McKnight and Palmiter, 1979). 32P-Labeled RNA was isolated and hybridized to nitrocellulose filters adsorbed with pGEM-4 DNA containing AP, γ-actin cDNA, or no insert. Insertion rates were determined from the amount of [32P]RNA hybridized to each filter. Calf alkaline phosphatase was used at a concentration of 30 μM for hybridization. 35S-labeled cDNA for the C subunit was used to determine the efficiency of hybridization.

Western Blot Analysis—Cell extracts prepared as above for the kinase assay were boiled for 5 min after adding sample buffer (Uhler and McKnight, 1987). The denatured samples were cooled on ice, electrophoresed on a 10% SDS-PAGE gel (Laemmli, 1970), and the proteins were transferred to nitrocellulose paper, and this was washed in dilute phosphoric acid. Incubation at 30 °C, half of the reaction mix was spotted onto phosphocellulose paper, and this was washed in dilute phosphoric acid. Enzymatic activity was measured by the incorporation of 32P into Kemptide. One unit of activity is defined as 1 pmol of phosphate transferred to the substrate per min. Endogenously active kinase was determined in the absence of any added cAMP in the kinase assay, and total kinase activity was determined in the presence of 10 μM cAMP.

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RESULTS

Time Course of CPT-cAMP Induction of AP—We observed a dramatic increase in L cell alkaline phosphatase enzyme activity after treatment with the cAMP analogue CPT-cAMP (Fig. 1A). This cAMP analogue was chosen for these studies because it has previously been shown to be more resistant to degradation by phosphodiesterases and is a more potent activator of cAMP-dependent protein kinase than other cAMP analogues such as dibutyryl cAMP (Miller et al., 1975). In addition, its metabolism does not generate butyrate, which has been shown in some systems to induce alkaline phosphatase (Gum et al., 1987). The first
increase in enzyme activity was detected between 6 and 8 h after treatment. An 80-fold increase was observed at 24 h after CPT-cAMP treatment from 0.14 unit/g total cellular protein to 10.6 units/g.

CPT-cAMP Activation of cAMP-dependent Protein Kinase and Induction of AP Activity—To assess the possible correlation between alkaline phosphatase induction and cAMP-dependent protein kinase activation, dose-response curves for both alkaline phosphatase activity and cAMP-dependent protein kinase activity were generated after treatment with various concentrations of CPT-cAMP. For kinase activity determinations, L cells were harvested after 1 h of treatment since this time point represented peak activity of the kinase activity (data not shown). For alkaline phosphatase assays, the experiment was performed at 24 h of treatment when alkaline phosphatase activity was still linearly increasing, even at maximal CPT-cAMP concentration. As shown in Fig. 1B, there is a correlation between the degree of kinase activation and the induction of alkaline phosphatase enzyme activity in mouse L cells at higher levels of CPT-cAMP (>30 μM CPT-cAMP). At the lowest CPT-cAMP (10 μM) concentrations, the basal level of kinase activity represented 15% activation of total kinase whereas at the highest concentration (3 mM) more than 90% of the kinase was activated. Neither kinase activation nor alkaline phosphatase induction was saturated at 3 mM CPT-cAMP. Concentrations of CPT-cAMP greater than 3 mM were difficult to obtain because of the low solubility of CPT-cAMP in DMEM.

Down-regulation of cAMP-dependent Protein Kinase by CPT-cAMP—The kinase activities shown in Fig. 1B were determined after 1 h of CPT-cAMP treatment because long term treatment of L cells with CPT-cAMP caused a significant decrease in the total amount of cellular cAMP-dependent protein kinase present. It can be seen (Fig. 1C) that 3 mM CPT-cAMP treatment for 24 h caused a 45% reduction in the total amount of protein kinase activity when compared with the total kinase activity in control L cells. This decrease in kinase activity was dependent on the dose of CPT-cAMP used and was even more pronounced at 48 h than at 24 h. An analogous decrease in total cAMP-dependent protein kinase activity has also been seen in porcine LLC-PK cells after elevation of cellular cAMP levels (Hemmings, 1986). The loss of activity in LLC-PK was shown to be due to specific proteolysis of the C subunit and may represent one cellular mechanism for down-regulating the activated kinase.

Isolation of an AP cDNA Clone from Mouse L Cells—In order to determine if the increase in alkaline phosphatase enzyme activity was because of an increase in the amount of mRNA coding for alkaline phosphatase, an AP cDNA clone was isolated. Using an oligonucleotide probe based on the first 48 nucleotides of the coding region of the published mouse placental alkaline phosphatase cDNA sequence (Terao and Mintz, 1987), we screened a cDNA library from CPT-cAMP-treated L cells and isolated two alkaline phosphatase cDNA clones after screening 400,000 recombinant phage. Sequencing of these cDNAs showed them to be 98% identical to the published placental cDNA except in the 5′-noncoding region. The first 92 nucleotides of the 5′ end of the mouse L cell AP cDNA clone showed no homology to the placental cDNA (Fig. 2). However, within the region beginning at a position 105 bases upstream of the translational initiation codon and continuing through to the poly(A) tail, the mouse L cell and placental cDNAs showed nearly complete identity (data not shown). Sequencing of a large number of rat liver cDNA clones for alkaline phosphatase as well as the rat alkaline phosphatase gene has suggested that two independent promoters within the rat alkaline phosphatase gene are functional in rat liver (Toh et al., 1989). The two promoters are immediately upstream of exons 1 and 2 of the rat bone/liver/kidney gene. As shown in Fig. 2 the first 92 nucleotides of the 5′-noncoding region in the mouse L cell cDNA which are not highly homologous to the mouse placental cDNA nucleotide sequence (37% identity) show a high degree of homology with the rat exon 2 (89% identity). Conversely, the mouse placental sequence shows a high degree of homology (85% identity) to rat exon 1 in this region. These nucleotide homologies and the fact that two independent L cell alkaline phosphatase cDNA clones contained the sequence homologous to rat exon 2 suggest that in mouse L cells the promoter for exon 2 is inducible with cAMP.
Regulation of Alkaline Phosphatase Gene Transcription

Fig. 2. Nucleotide sequence comparison of mouse L cell and placental alkaline phosphatase cDNAs with exons 1 and 2 of the rat alkaline phosphatase gene. The 5'-untranslated region of the mouse L cell alkaline phosphatase cDNA (M L cell) and the mouse placenta alkaline phosphatase cDNA (M placenta) are aligned to show homology with exons 2 and 1, respectively, of the rat alkaline phosphatase gene. Sequencing of the mouse L cell alkaline phosphatase cDNA was performed by subcloning in M13mp18 and M13mp19. Numbering of nucleotides is relative to the adenine residue of the initiator methionine. Sequences that are not identical are shown in lower cases, and gaps that have been introduced for maximal alignment are indicated with a dash (-).

Fig. 3. Northern blot analysis of RNA isolated from control and CPT-cAMP-treated L cells. A, samples of poly(A) RNA from control and 24-h CPT-cAMP-treated L cells were prepared. B, L cells were treated for 0, 12, 24, 48, or 72 h with 1 mM CPT-cAMP and total RNA isolated. C, L cells were treated for 0, 2, 4, 6, 8, 10 or 12 h with 1 mM CPT-cAMP, and total RNA isolated. D, L cells were treated for 16 h with 1 mM CPT-cAMP, and the medium was changed to DMEM with no added CAMP. Incubation was continued for 0, 2, 4, 6, 8, or 10 h, and total RNA was isolated. RNA samples were subjected to electrophoresis and Northern blot hybridization analysis. In all cases the 2.5-kb mouse L cell alkaline phosphatase cDNA in pGEM-4 was used to generate a radiolabeled antisense RNA probe for hybridization. The size of the hybridizing band in the CAMP-treated lane was determined by using RNA sizing standards and staining the gel before transfer to nitrocellulose. The arrow in A indicates the heterogenous high molecular weight species seen in control cells.

The mouse L cell alkaline phosphatase cDNA was used for Northern blot analysis of poly(A) RNA from control and CPT-cAMP treated mouse L cells (Fig. 3A). The Northern blot indicates a substantial induction of the 2.5-kb alkaline phosphatase mRNA in the poly(A) RNA from CPT-cAMP-treated cells compared with control cells. Densitometric scanning of the autoradiogram indicated at least a 10-fold increase in the amount of radiolabeled cDNA probe hybridizing to this 2.5-kb band after CPT-cAMP treatment, and this level of induction was confirmed independently by a solution hybridization assay (data not shown). Further analysis showed that the induced level of alkaline phosphatase mRNA was sustained for at least 72 h of CPT-cAMP treatment (Fig. 3B), that the increased level of alkaline phosphatase mRNA was
first detectable 4–6 h after CPT-cAMP treatment (Fig. 3C), and that following removal of CPT-cAMP the level of alkaline phosphatase mRNA rapidly declined with a half-life of approximately 2 h (Fig. 3D). Quantitation of amount of AP mRNA present in these RNA samples by a specific solution hybridization assays confirmed the results by Northern blot analysis and showed that even at maximal induction the AP mRNA represented less than 0.001% of the total mRNA present (data not shown).

**CPT-cAMP Induction of AP Gene Transcription**—The alkaline phosphatase cDNA was also used to determine whether this increase in mRNA was due to increased alkaline phosphatase gene transcription. Nuclei were isolated from both control L cells and from L cells treated with 3 mM CPT-cAMP. The nascent RNA transcripts were elongated in vitro in the presence of [32P]UTP (McKnight and Palmiter, 1979). The labeled RNA was hybridized to pGEM-4 plasmid containing the alkaline phosphatase cDNA, a mouse γ-actin cDNA fragment, or pGEM-4 alone. The mouse γ-actin gene has been shown to be expressed at a low level in mouse L cells (Tokunaga et al., 1988). As shown in Table I, alkaline phosphatase gene transcription increased from 5.8 ppm in control L cells to 15.5 ppm after 6 h of CPT-cAMP treatment. In the same samples the γ-actin transcription rate remained unchanged. In other experiments (data not shown) the basal AP gene transcription rate in L cells was as low as 3.2 ppm, and CPT-cAMP treatment resulted in a 5-fold induction of the AP gene transcription. In addition, the increase of alkaline phosphatase transcription rate measured at 6 h was almost completely blocked by treatment of the L cells with cycloheximide (Table I), suggesting that synthesis of an intermediate protein is required for alkaline phosphatase induction.

**Stable Transfection of Mouse L cells with C Subunit Expression Vectors**—To determine directly if cAMP-dependent protein kinase was involved in the transcriptional response of the AP gene to cAMP, L cells were co-transfected with an inducible expression vector for the Ca subunit of CAMP-dependent protein kinase and an expression vector for the selectable marker, neomycin phosphotransferase. The expression vector for the Ca subunit of cAMP-dependent protein kinase has been demonstrated previously to code for a catalytically active protein in mouse NIH3T3 cells (Uhler and McKnight, 1987). G-418-resistant clones were isolated and characterized for their expression of the Ca subunit mRNA in the presence and absence of ZnSO4. L cell clones expressing a mutated form of the Ca subunit in which the lysine at position 72 was changed to methionine were also isolated by transfection with an alternate expression vector. This lysine residue has been demonstrated by affinity labeling experiments to be involved in ATP binding by the catalytic subunit (Zoller et al., 1979). In vitro mutations that change the analogous lysine to methionine in other protein kinases have shown to inactivate those protein kinases (Chou et al., 1987; Weinmaster et al., 1986; Snyder et al., 1985; Kamps and Sefson, 1986). In both transfections, clones that showed induced Ca subunit mRNA in response to ZnSO4 treatment were characterized with respect to induction of kinase activity and AP activity. Although results for only one or two clones of each class of transfectant, normal or mutant C subunit, are shown, similar results were obtained for at least five clones of each class of transfectant.

**Effects of Overexpression of C Subunit mRNA Levels on Kinase Activity**—A study of ZnSO4 induction of C subunit mRNA and kinase activity was performed by comparing the effect of ZnSO4 on wild-type L cells, the Ca2-transfected L cell clone containing the Ca subunit expression vector, and the CaK72M13 clone containing the mutant Ca subunit expression vector in which the lysine at position 72 has been changed to methionine. Representative results are shown in Fig. 4. Fig. 4A shows the level of Ca subunit mRNA in these three different cell types in the absence or presence of ZnSO4. Wild-type L cells contain approximately 50 molecules of Ca mRNA per cell, and ZnSO4, treatment has no effect on the level of Ca mRNA in these cells. The Ca2 cells, which have been transfected with the Ca expression vector, contain approximately 80 molecules of Ca mRNA per cell in the absence of ZnSO4, but this increases to 400 molecules/cell after ZnSO4 treatment. In the CaK72M13 clone, harboring the mutant Ca subunit expression vector, the Ca subunit mRNA level increases from 500 molecules/cell under basal conditions to 7000 molecules/cell after ZnSO4 treatment. The effect of these Ca subunit mRNA levels on kinase activity in the three different cell types is shown in Fig. 4B. There is no effect of ZnSO4 on kinase activity in wild-type L cells where endogenously active kinase activity is 50 units/mg protein, and total kinase activity is 800 units/mg ZnSO4 treatment of the Ca2 cells, however, increases the endogenously active kinase activity 3-fold from 80 to 240 units/mg. This same treatment increases the total kinase from 900 units/mg in the basal state to 2100 units/mg after ZnSO4 induction of transcription from the Ca expression vector. In the CaK72M13 cells, however, the endogenously active kinase activities are 50 units/mg protein in the absence or presence of ZnSO4, and total kinase activities are 450 units/mg protein independent of whether or not the cells have been treated with ZnSO4. Thus, even though the CaK72M13 cells are expressing a 140-fold excess of the mutant Ca mRNA, they

### Table I

| cDNA            | Duration of CPT-cAMP treatment (h) |
|-----------------|-----------------------------------|
|                 | 0   | 2   | 4   | 6   | 8   | 8+ Cycloheximide* |
| Alkaline Phosphatase* | 5.8 ± 0.5 | 7.7 ± 0.6 | 7.9 ± 0.7 | 15.5 ± 1.5 | 9.9 ± 0.8 | 7.7 ± 1.6 |
| -fold induction  | 1.0 | 1.3 | 1.4 | 2.7 | 1.7 | 1.3   |
| γ-Actin         | 99 ± 11 | 107 ± 9 | 102 ± 9 | 106 ± 8 | 102 ± 10 | 73 ± 9 |
| -fold induction | 1.0 | 1.1 | 1.0 | 1.1 | 1.0 | 0.7   |

* Cycloheximide was used at a concentration of 30 μM.

* The RNA was hybridized to nitrocellulose filters adsorbed with pGEM-3 DNA containing the AP cDNA, γ-actin cDNA, or no insert. Transcription rates were determined from the amount of 32P RNA hybridized to each filter and expressed in ppm.
Regulation of Alkaline Phosphatase Gene Transcription

A) Confluent cultures of mouse L cells, Co2, or CaK72M13 were grown in DMEM containing 10% fetal calf serum in the absence (unfilled bars) or presence (filled bars) of 140 μM ZnSO4 for 24 h prior to harvesting for total nucleic acid isolation or for assay of cAMP-dependent protein kinase. Ca mRNA levels were measured as described under “Materials and Methods” using a C-specific 32P-labeled SP6 RNA probe in a solution hybridization assay. CaK72M13 columns were grown in DMEM containing 10% fetal calf serum in the absence (white and hatched bars) or presence (black and stippled bars) of 140 μM ZnSO4 for 24 h prior to harvesting for assay of cAMP-dependent protein kinase. Kinase activity was determined as described under “Materials and Methods” in the absence (black and white bars) or presence (hatched and stippled bars) of 10 μM cAMP. Each value represents the mean ± S.D. for triplicate determinations.

show no increase in kinase activity. We conclude from these experiments that the C subunit containing the lysine to methionine change at position 72 possesses little if any kinase activity.

Effect of Increased C Subunit on AP Activity in L Cell Transfectants—The ability of each of these L cell clones to induce AP in response to ZnSO4 and CPT-cAMP was tested. As shown in Fig. 5, L cells do not show increased AP in response to ZnSO4 treatment but did respond to CPT-cAMP treatment with a 40-fold increase in AP activity from 0.11 units/g in the control cells to 4.4 units/g in the CPT-cAMP-treated cells. In contrast to the L cells, the Co2 cells responded to ZnSO4 treatment with a 20-fold increase in AP activity from 0.06 to 1.2 units/g. These same cells showed an 80-fold increase in AP activity when cells treated with CPT-cAMP (5.0 units/g) are compared with untreated cells (0.06 units/g). The CaK72M13 cells did not show an increase in AP activity with ZnSO4 treatment (0.28 units/g in control cells and 0.16 units/g in ZnSO4-treated cells) but did respond to CPT-cAMP with a 10-fold increase in AP activity (3.3 units/g in CPT-cAMP-treated cells).

Northern Blot Analysis of AP mRNA Induced by ZnSO4 and cAMP—In order to test the possibility that CPT-cAMP and ZnSO4 treatment of Co2 cells caused induction of mRNAs for the different isoforms of AP, Northern blot analysis of these cells was performed using conditions of high stringency. Poly(A) RNA from the Co2 cells was isolated after no treatment or after ZnSO4 or CPT-cAMP treatment and subjected to Northern blot analysis using the AP cDNA as radiolabeled probe. As is shown in Fig. 6, both the ZnSO4 and CAMP treatments increased the level of 2.5-kb AP mRNA in these cells although CAMP treatment resulted in 2-3-fold higher levels of AP mRNA than ZnSO4 treatment.

Measurement of Alkaline Phosphatase Gene Transcription Rates in Transfected L Cells—The AP gene transcription rates were measured in L cells and in Co2 cells treated with CPT-cAMP or ZnSO4 to test whether the increase in AP mRNA levels was due to an increase in AP gene transcription (Fig. 7). Whereas cAMP treatment of both mouse L cells and Co2 cells resulted in a 2.5-fold increase in gene transcription, ZnSO4 treatment increased AP gene transcription only in the Co2 cells. Thus, overexpression of the C subunit induced by ZnSO4 treatment resulted in an increased rate of AP gene transcription. Furthermore, since the basal transcription rate of the AP gene is low in L cells and near the level of sensitivity for this assay (approximately 0.5–2.0 ppm), the fold induction determined by this assay may be an underestimate of the actual degree of induction.
Regulation of Alkaline Phosphatase Gene Transcription

FIG. 6. Northern blot analysis of RNA isolated from control, ZnSO4-treated, and CPT-cAMP-treated Co2 cells. Samples of poly(A) RNA from untreated Co2 cells, Co2 cells treated with 200 µM ZnSO4 for 24 h, or Co2 cells treated with 3 mM CPT-cAMP for 24 h were subjected to electrophoresis and hybridization analysis as described under "Materials and Methods." The 2.5-kb mouse L cell AP cDNA was used to generate 32P-radiolabeled probe. The size of the hybridizing band in the CAMP-treated lane was determined by using RNA size standards and staining the gel before transfer to nitrocellulose.

FIG. 7. Alkaline phosphatase gene transcription rates in mouse L cells and Co2 transfectants. Nuclei were prepared from confluent 10-cm tissue culture dishes that had been cultured in DMEM with 10% fetal calf serum in the presence or absence of 3 mM CPT-cAMP or 110 µM ZnSO4. The nuclei were incubated with 15P-UTP and radiolabeled RNA transcripts isolated. The RNA was hybridized to nitrocellulose filters adsorbed with pGEM-4 DNA containing AP or no insert. Transcription rates were determined from the amount of 32P RNA specifically hybridized to each filter and expressed in ppm. Transcription rates for mouse L cells (white bars) and Co2 transfectants (black bars) are shown.

Western Blot Analysis of C and RI Subunits of cAMP-Dependent Protein Kinase in Transfected L Cell—In order to assess if the CaK72M expression vector was able to produce C subunit protein in the stably transfected cells, cell extracts were prepared from Co2 and CaK72M18, a cell line expressing levels of mutant C subunit similar to those CaK72M13 cells. These extracts were subjected to Western blot analysis to study the protein produced by the expression vectors (Fig. 8). ZnSO4 treatment of Co2 cells produced an increased level of C subunit that co-migrates on SDS-PAGE with the endogeneous C subunit in L cells. Quantitation of the C subunit present in Co2 cell extracts using 125I-protein A (Uhler and McKnight, 1987) showed that ZnSO4 treatment of Co2 cells produced a 3-fold increase in immunoreactive C subunit. There was also an increase in immunoreactivity of a band that co-migrates with the RI subunit. This is consistent with a similar compensatory increase in RI protein in response to C subunit overexpression which was described previously for transfected mouse NIH/3T3 cells (Uhler and McKnight, 1987). ZnSO4 treatment of CoK72M15 or CaK72M18 cells, however, caused an increase in the amount of a protein immunologically related to the C subunit but which migrated slightly faster in SDS-PAGE than endogenous C subunit. Quantitation of the C subunit induction in CoK72M18 cells showed an 8-10-fold induction of total C subunit protein. Since identical ZnSO4 concentrations produced 15-fold higher levels of Cc mRNA in CaK72M cells than in Co2 cells (Fig. 4A), the CaK72M protein can be estimated to be 5-fold less stable than the wild-type C subunit. Although small increases in RI subunit were observed occasionally after ZnSO4 treatment of CaK72M18 cells, the RI subunit compensation was never as large as that seen in Co2 (Fig. 8).

Thus, cells expressing the CaK72M subunit did show induction of both C and Ca subunit mRNA and Cc subunit protein as shown in Figs. 4A and 8, respectively, but this mutant protein lacks kinase activity (Fig. 4B). Furthermore, the mutant C subunit protein in the CaK72M13 cells appears to be incapable of inducing AP activity in response to ZnSO4 (Fig. 5) although the endogenous Cc subunit gene product is still able to induce the AP gene in response to CPT-cAMP.

DISCUSSION

Our results demonstrate that the previously reported induction of alkaline phosphatase by dibutyryl cAMP can also be mimicked by CPT-cAMP. This lends support to the notion that induction of alkaline phosphatase occurs through a cAMP-dependent mechanism and not through the effect of butyrate. The observation that induction of alkaline phosphatase enzyme activity correlates with activation of cAMP-dependent protein kinase suggested that effect of cAMP may be mediated by activation of the kinase. It is interesting to note that even at 3 mM CPT-cAMP the induction of alkaline

Fig. 8. Western blot analysis of C and RI subunit proteins in Co2 and CaK72M18 cells. Two hundred micrograms of protein extract from Co2 or CaK72M18 was electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. Twenty nanograms of purified bovine heart C subunit was also run for size comparison. The Western blot was incubated with a polyclonal antibody against the C subunit followed by an AP-coupled second antibody as described under "Materials and Methods." After color development and identification of the C subunit, the blot was further incubated with a polyclonal antibody against the RI subunit.
phosphatase activity has not reached a maximum (Fig. 1B). This is in contrast to many other cellular responses to CAMP which are maximally induced at concentrations of CAMP which activate a much smaller fraction of the total cellular CAMP-dependent protein kinase. For example, in perfused liver, phosphorylase is maximally stimulated by epinephrine concentrations that activate only 30–50% of the total CAMP-dependent protein kinase (Keely et al., 1975). Maximal stimulation of ACTH release occurs at concentrations of corticotropin releasing factor which activate only 50% of the type I kinase whereas type II kinase is not activated at all by corticotropin releasing factor (Litvin et al., 1984). It is possible that this difference in response to CAMP-dependent protein kinase activation between alkaline phosphatase induction and other cellular responses reflects differences in the kinase isoforms which mediate these various cellular CAMP responses or that these cellular functions occur in different cellular compartments.

The present finding that the cDNA for the alkaline phosphatase induced in mouse L cells is identical to the mouse placental alkaline phosphatase cDNA was not unexpected. Three isoforms of human alkaline phosphatase have been described: an intestinal isoform, a placental isoform, and a bone/liver/kidney isoform. In humans it is the bone/liver/kidney isoform which is CAMP inducible. Genetic evidence suggests, however, that only two forms of alkaline phosphatase exist in the mouse (Terao et al., 1988), a placental and an intestinal isoform. Furthermore, the placental cDNA was used to show that the placental isoform is expressed in tissues other than the placenta. Biochemical characterization of the potency of various peptide inhibitors has also suggested that in mouse the placental and L cell alkaline phosphatase are very similar (Gum and Raetz, 1983). Thus, in mouse it appears as if the bone/liver/kidney and placental alkaline phosphatases are encoded by the same gene and that this form of alkaline phosphatase is expressed in L cells.

It was reported previously that the amount of mRNA coding for alkaline phosphatase as determined by in vitro translation increases with dibutyryl CAMP treatment (Firestone and Heath, 1981). This is in complete agreement with present experiments, which determined that alkaline phosphatase mRNA levels increased at least 10-fold as determined by Northern blot hybridization. Furthermore, a modest (3-fold) and transient increase in alkaline phosphatase gene transcription was detected by in vitro nuclear transcription assay. It appears from data presented here that this CAMP induction of alkaline phosphatase gene transcription is a secondary event and that alkaline phosphatase may belong to a small group of genes that are known to be transcriptionally regulated at a secondary level by CAMP. Further study of alkaline phosphatase gene promoter structure and particularly the promoter for exon 2 may help to characterize the mechanism of secondary effects of CAMP on gene transcription. It is interesting to note, however, that the rat alkaline phosphatase promoter region upstream of exon 2 does not contain a classic CAMP response element (Toh et al., 1989).

Although AP enzyme activity was observed to increase 80-fold over 24 h of CPT-cAMP treatment (Fig. 1A), the AP mRNA levels were observed to increase only 10–20-fold (Fig. 3A). Some of this discrepancy may be due to the inaccuracy of measuring the basal level of AP mRNA in uninduced L cells. The level of AP mRNA after maximal induction by CPT-cAMP was estimated to be 0.001% of the total mRNA by both RNA solution hybridization assay and the relative abundance of the AP cDNA in the cDNA library. However, it is possible that CAMP exerts some effect at the level of translation of AP mRNA or at the level of the AP protein stabilization. A much larger discrepancy is seen when comparing the 3-fold induction of AP gene transcription with the 10–20-fold increase in AP mRNA. Here again, the basal level of AP gene transcription was difficult to determine exactly, and the actual fold induction by CAMP may be higher than that determined in our assay. However, in poly(A) RNA from control cells, we consistently observed low amounts of heterogeneous high molecular weight RNAs that hybridized to the AP cDNA (Fig. 3A). Furthermore, CPT-cAMP treatment consistently reduced the amount of hybridization to these high molecular weight RNAs. This observation might suggest that CPT-cAMP increases the formation of mature AP mRNAs from unprocessed RNA transcripts. A precedent for this type of regulation has been reported for the regulation of α1-acid glycoprotein gene by glucocorticoids in a rat hepatoma cell line (Vannice et al., 1984). Alternatively, the apparent discrepancy between AP gene transcriptional induction and AP mRNA induction may represent stabilization of the AP mRNA by CPT-cAMP. However, although other modes of regulation most probably affect AP gene expression, the results of in vitro transcription assays (Table I) demonstrate that CAMP does regulate transcription of the AP gene.

Since the induction of overexpression of the Ca subunit in Co2 cells by ZnSO4 treatment was sufficient to induce AP enzyme activity and AP mRNA levels, the major effect of CAMP on AP gene expression appears to be to regulate the kinase activity of the C subunit. Several other Co overexpressing cell lines were generated during the course of these experiments, and zinc induction of Co mRNA levels had similar effects on alkaline phosphatase activity, but the Co2 cells were convenient for the studies presented here because of their low basal level of Co mRNA expression. Although overexpression of the Ca subunit does lead to increased levels of R subunit protein, this R subunit results from an increased stability of the R subunit in the holoenzyme complex as compared with the free R subunit (Steinberg and Agard, 1981) and is not due to an increase in the mRNA for the R subunit (Uhler and McKnight, 1987). In addition, the compensating R subunit protein is complexed in an inactive holoenzyme complex with C subunit as determined by kinase activity (Uhler and McKnight, 1987).

From the experimental data presented here, induction of alkaline phosphatase activity requires the kinase activity of the Ca subunit. Expression of a mutant Ca subunit in which the lysine residue at position 72 in the protein has been changed to methionine is incapable of inducing alkaline phosphatase activity. An analogous lysine residue has been shown by affinity labeling to be involved in ATP binding to the porcine C subunit (Zoller et al., 1979). This lysine is part of a sequence motif strictly conserved among protein kinases, and mutagenesis of this lysine residue has been shown to abolish kinase activity for other protein kinases including the human insulin receptor (Chou et al., 1987), the fps oncogene (Weinmaster et al., 1986), and v-srC (Snyder et al., 1985; Kamps and Selton, 1988). The present finding that the analogous mutation in the Ca subunit abolishes kinase activity is therefore not surprising. However, the fact that this protein is unable to induce alkaline phosphatase activity in intact cells suggests that protein phosphorylation by the Ca subunit plays a central role in CAMP regulation of alkaline phosphatase gene expression. Several differences were noted between the CaK72M mutant protein and the wild-type Ca protein. First, the CaK72M mutant protein consistently migrated more rapidly than the wild-type protein on SDS-PAGE. Second, the CaK72M protein appeared to be less stable than the wild-
type protein in that much higher levels of the CaK72M mRNA as compared with wild-type Ca mRNA were required to see similar amounts of the two proteins (see Figs. 4A and 8). Finally, the CaK72M mutant was not able to stabilize the RI subunit as well as the wild-type Ca subunit. Since the mutated lysine residue has been shown to play a role in ATP binding (Zoller et al., 1979), it is possible that autophosphorylation of the C subunit has been affected and that lack of autophosphorylation may in turn affect conformation as seen by altered migration in SDS-PAGE, proteolytic degradation, and holoenzyme formation of the Ca subunit. Alternatively, it may be the simple change in charge from positively charged lysine to neutral methionine which alters the rate of migration of the mutant protein.

The conclusion that phosphorylation by the C subunit of cAMP-dependent protein kinase is required for transcriptional regulation of the alkaline phosphatase gene by cAMP is consistent with recently reported results in other systems. An expression vector for the protein kinase inhibitor peptide has been shown to inhibit the cAMP stimulation of transcription of the human enkephalin promoter in a transient expression assay system (Grove et al., 1985), and C subunit expression is able to stimulate cAMP-responsive transcription in a similar transient expression assay system (Mellon et al., 1989). In addition, microinjection of the C subunit has been shown to stimulate transcription from the vasoactive intestinal peptide gene promoter and the c-fos promoter whereas microinjection of the R subunits did not stimulate transcription from these promoters (Ribawol et al., 1988). Although these studies clearly implicate the kinase activity of the C subunit in regulation of gene transcription, in neither of these cases was it possible to assay for cAMP-dependent protein kinase in the cell and directly correlate kinase activity with the transcriptional response. The mouse L cell alkaline phosphatase system presented here possesses some advantages over other gene transcription systems and has facilitated the demonstration that the kinase activity of the C subunit of cAMP-dependent protein kinase is sufficient and necessary for transcriptional induction of the mouse L cell alkaline phosphatase gene.

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