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Regulation of transcription by cyclic AMP-dependent protein kinase

(phosphorylation/chorionic gonadotropin gene/cotransfection)

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ABSTRACT **cAMP-dependent protein kinase (PKA; ATP: protein phosphotransferase; EC 2.7.1.37) appears to be the major mediator of cAMP responses in mammalian cells. We have investigated the role of PKA subunits in the regulation of specific genes in response to cAMP by cotransfection of wild-type or mutant subunits of PKA together with cAMP-inducible reporter genes. Overexpression of catalytic subunit induced expression from three cAMP-regulated promoters (α-subunit, c-*fos*, E1A) in the absence of elevated levels of cAMP but did not affect expression from two unregulated promoters (Rous sarcoma virus, simian virus 40). Cotransfection of a regulatory subunit gene containing mutations in both cAMP binding sites strongly repressed both basal and induced expression from the cAMP-responsive α-subunit promoter without affecting expression from the Rous sarcoma virus promoter. These experiments indicate that cAMP induces gene expression through phosphorylation by the catalytic subunit and that the ambient degree of phosphorylation dictates the level of basal as well as induced expression of the cAMP-regulated α-subunit gene.**

In mammalian cells, cAMP is generated in response to ligand–receptor interaction at the cell surface, transducing that signal by binding to the regulatory subunits (R subunits) of protein kinase A (PKA; ATP:protein phosphotransferase; EC 2.7.1.37) (1). The PKA holoenzyme is an inactive tetramer of two catalytic subunits (C subunits) and two R subunits, and cAMP binding to the R subunits causes the release of active C subunits. Many of the biological effects of cAMP are thought to be caused by the phosphorylation of specific substrates by the C subunits (2).

cAMP can also influence gene expression. In *Escherichia coli*, cAMP acts as a messenger in regulatory pathways by binding directly to a transcription factor, catabolite gene activator protein (CAP), altering its ability to bind promoter sequences and activate gene transcription (3, 4). In *Dictyostelium*, cAMP regulates cellular events, in part, by binding to a cell surface receptor (5). The role of cAMP and PKA in regulating transcription in mammalian cells is not clearly defined. It has been postulated that the R subunit or some other cAMP binding protein might play a direct role in transcription analogous to the cAMP binding protein of bacteria (6). Alternatively, the C subunit of the kinase may regulate transcription through direct phosphorylation of transcription factors or activation of specific transcription factor kinases. The transcriptional response of several genes to cAMP has been localized to a specific DNA sequence termed a cAMP response element (CRE) (7–9). A protein that binds to this element has been identified and characterized as a 42-kDa phosphoprotein present in many cells and tissues that are known to respond (CREB) (10). This protein may be involved in regulating many cAMP-responsive genes (11–13), and the cDNA encoding this protein has recently been cloned (14, 15). Evidence that the C subunit of the kinase may directly modify this protein includes its phosphorylation *in vitro* by purified kinase (16). Though the holoenzyme may be cytoplasmic, treatment of bovine epithelial cells with cAMP has been shown to translocate C subunit immunoreactivity to the nucleus which would be required for direct phosphorylation of a nuclear protein (17). In addition, an inhibitor of the C subunit kinase activity has been shown to block cAMP induction of the enkephalin and prolactin genes (18, 19).

To further investigate the role of the individual subunits of the kinase in gene regulation, we have used cDNA expression vectors that encode R and C subunits to perturb the balance and function of the kinase in transfected cells. We demonstrate that overexpression of C subunit can induce expression of several known cAMP-responsive genes, completely substituting for elevated cAMP. In contrast, the R subunit failed to significantly induce gene expression, supporting the conclusion that phosphorylation plays a key role in mediating the effects of cAMP on gene expression. We have also examined the role of the basal level of kinase activity on gene expression in uninduced cells. R subunit expression vectors with mutated cAMP binding sites (20) blocked the cAMP response and also lowered the basal transcription level of the cAMP-responsive α-subunit promoter. This latter finding demonstrates that even in the absence of elevated cAMP, the ambient phosphorylation activity of the C subunit of PKA plays an important role in the maintenance of transcription levels of genes regulated by cAMP.

MATERIALS AND METHODS

Plasmid Constructions. Reporter plasmids with the promoter of the α subunit of the glycoprotein hormones (α promoter) contained sequences from −168 to +45 of the human gene (8) linked to the coding sequences of either the luciferase (α-luc) (21) or the chloramphenicol acetyltransferase (α-CAT) (22) genes. Expression vector plasmids contained the mouse metallothionein 1 promoter (Mt-1) (23) cloned onto the cDNAs for the α isoform of the mouse PKA C subunit (MtC) (24), or the mouse type I PKA R subunit [MtR(wt)] (20) with a human growth hormone segment to provide the poly(A) site (25). A control expression vector contained the same Mt-1 promoter fragment cloned onto a mouse–human hybrid β-globin gene (Mtglobin) (26). Mutant cDNAs of the RIα regulatory subunit [MtR(B), MtR(AB)] were cloned as described (20). MtR(B) contains two amino acid substitutions in the site B cAMP binding site at amino

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Abbreviations: CAT, chloramphenicol acetyltransferase; PKA, protein kinase A (ATP:protein phosphotransferase); R subunit, regulatory subunit; C subunit, catalytic subunit; CRE, cAMP response element; CREB, cAMP-responsive element binding protein; α promoter, promoter of the α-subunit gene of the human glycoprotein hormones; Mt-1, mouse metallothionein 1 promoter; RSV, Rous sarcoma virus; SV40, simian virus 40; CAP, catabolite gene activator protein.

acid positions 324 and 332, and MtR(AB) contains an additional substitution at position 200 in the site A cAMP binding site. E3CAT contained sequences between −487 and +65 of adenovirus type 5 E3 gene (27) and fosCAT contained 711 base pairs of the 5′ flanking DNA of human c-*fos* (12) and were kindly provided by P. Sassone-Corsi (The Salk Institute, La Jolla, CA). Other plasmids have been described: RSV-βgal (28), RSVluc and SVluc (21), RSVCAT (22).

Cell Culture and Transfections. JEG-3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 4.5 mg of glucose per ml. Transfections were performed by using calcium phosphate precipitates (29) containing 0.1–2.5 μg of reporter plasmid DNA, 7.5–10 μg of total Mt expression vector DNA (as stated for individual experiments), and 2 μg of the internal control plasmid, RSV-βgal (28). Cells were incubated for 5 hr followed by a medium change; they were harvested 36–48 hr later. For experiments using forskolin, the drug was added 16–18 hr prior to harvest, to a final concentration of 10 μM. For experiments using zinc, 80 μM zinc was added directly after the medium change.

Luciferase, β-Galactosidase, and CAT Enzymatic Assays. Protein extracts were prepared by freeze-thawing as described (ref. 22; except harvesting buffer was 1 mM dithiothreitol/100 mM potassium phosphate) and protein concentrations were determined using Bio-Rad protein assay reagent (8). CAT assays were performed as described (8, 22). Acetylated chloramphenicol was excised from the chromatogram and the radioactivity was quantitated by scintillation spectroscopy. Luciferase assays were performed as described (21). Background radioactivity was determined by performing luciferase or CAT assays without added extract, and the background values were subtracted from each experimental value. Assay values were always greater than five times background. β-Galactosidase assays were performed as described (28) using 12.5 μg of protein, and the acetylated [^{14}C]chloramphenicol radioactivity or luciferase units were normalized for transfection efficiency by dividing by β-galactosidase activity. Values shown are the average of two identical experiments except for Fig. 4, which is the average of five experiments.

RESULTS

To investigate the effect of overexpression of the individual subunits of PKA on gene transcription, we used a cotransfection assay that has been used extensively to study the effects of steroid receptors and oncogenes on specific gene expression (30, 31). Expression vectors were designed to produce individual subunits of PKA directed by the Mt-1 promoter, a moderately strong promoter that can be further induced with metal ions (23). Control transfections substituted an analogous plasmid that contained the Mt-1 promoter directing the expression of a β-globin gene (26), which had no effect on the expression of any of the reporter genes (data not shown). These vectors were cotransfected with either CAT or luciferase reporter plasmids directed by promoters known to be either responsive or unresponsive to elevation of intracellular levels of cAMP. The cell type chosen for the experiments was a human choriocarcinoma placental cell line, JEG-3, which has been characterized as responsive to elevated levels of cAMP using several inducible promoters (8, 12).

Effects of Overexpression of the C Subunit of PKA on Transcription. The human promoter for the α subunit of the glycoprotein hormones has been well-characterized as cAMP responsive in JEG-3 and other cell types (8, 32, 33). It contains two tandem CRE sequences and an element that confers tissue-specific expression in placenta (8). The reporter plasmid α-luc (containing α promoter sequences from −168 to +45) was used to determine the response of a cAMP-inducible gene to excess C subunit. Cotransfection with a Mt-1 promoter expression vector that produces the C subunit (Cα) of PKA (MtC) induced expression of the α-luciferase reporter gene by 6.5-fold, an effect that was increased to 15-fold when MtC was induced by treatment of transfected cells with zinc (Fig. 1). A control expression vector that expressed the neutral protein, β-globin, was used to equalize any effects of the Mt-1 promoter and showed no effect on α-luc activity. The induction by C subunit was equivalent to that produced by forskolin, which strongly stimulates adenylate cyclase to raise intracellular levels of cAMP (8, 34). The effect of excess C subunit was therefore not additive with that of elevated cAMP, indicating that the same pathway was operative for both mechanisms of stimulation. In contrast, the Rous sarcoma virus (RSV) long terminal repeat promoter (RSVluc) and the simian virus 40 (SV40) early promoter (SVluc), both with their viral enhancers intact, were not induced by cotransfection with the C subunit expression vector (MtC) in the presence or absence of forskolin (Fig. 2). RSVluc was also tested in the presence of zinc and remained unaffected by C subunit (data not shown). Uhler *et al.* (35) have also identified an isoform of the C subunit, termed Cβ, which is preferentially expressed in

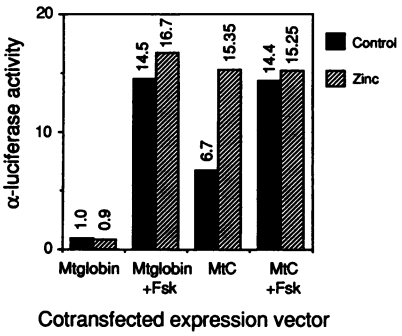

FIG. 1. Transcription of the α-subunit promoter is induced by the C subunit of PKA. One-tenth microgram of α-luc was cotransfected into JEG-3 cells with 10 μg of MtC, a Mt-1 expression vector that produces the C subunit of PKA, or Mtglobin, the same expression vector expressing a β-globin protein. Forskolin (Fsk) was added to increase cAMP, and zinc was added to increase expression of the C subunit as indicated. Values shown are the average of two identical experiments and are expressed normalized to the control expression level with Mtglobin in the absence of forskolin or zinc.

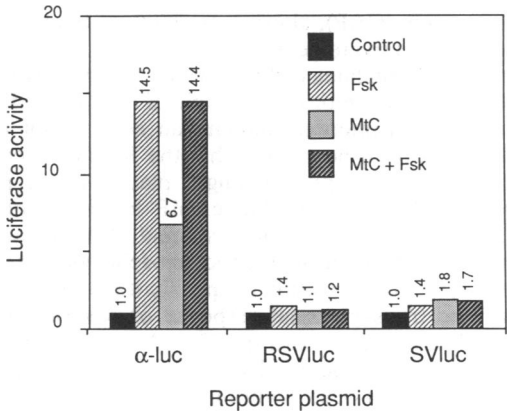

FIG. 2. Induction of transcription by the C subunit of PKA is specific to a cAMP-regulated gene. One-tenth microgram of the reporter plasmids was cotransfected into JEG-3 cells with 10 μg of MtC or Mtglobin and treated with forskolin (Fsk) as indicated. Values are the average of two identical experiments and are shown relative to the control expression of each reporter gene in the absence of forskolin and C subunit.

neural tissue. Cotransfection with an identical expression vector carrying the cDNA for the Cβ form produced stimulation of α-luciferase similar to that obtained with Cα (data not shown).

To determine whether the response to C subunit is general to cAMP-inducible promoters, we examined two additional cAMP-responsive promoters. The promoter of the adenovirus early region 3 (E3) contains the CRE consensus sequence and binds a factor common to the other inducible genes (13, 27), and the mouse c-*fos* gene promoter has also been shown to be cAMP inducible and to bind CREB (12). To detect the low levels of expression of these weaker promoters using the CAT reporter gene, it was necessary to increase the amount of reporter used from 0.1 μg to 1 μg. Both promoters respond quite strongly to excess C subunit (Fig. 3), though their response to elevated levels of cAMP (forskolin) is not as great as with the α-subunit promoter in this cell type. This effect may be due to the ability of transfection to elevate free C subunit levels beyond those attainable with forskolin. Thus, the limiting factor in this experiment may be free C subunit.

Effects of Overexpression of the R Subunit of PKA on Transcription of a cAMP-Responsive Promoter. The role of the R subunit of PKA in mediating gene expression was also investigated by cotransfecting JEG-3 cells with a Mt-1 expression vector containing the RIα cDNA [MtR(wt)] (20). Excess R subunit caused a 1.6-fold increase in α-subunit promoter activity either in the presence or absence of forskolin (Fig. 4). This small effect was not seen with RSV or SV40 promoters (data not shown). Its significance is difficult to explain but might involve an inhibition of phosphatase activity since the R subunit has been shown to interact with specific phosphatases (36).

Each R subunit protein contains distinct binding sites for cAMP termed A and B. Mutations in the B site of the R subunit prevent cAMP binding at that site and cause a shift in the K_a for cAMP activation of holoenzyme from 40 nM to >5 μM (37). The effect of overexpression of this mutated R subunit [MtR(B)] was tested in the cotransfection assay with the α-luciferase reporter plasmid (Fig. 4). Basal expression of this promoter was reproducibly inhibited by 2.5-fold and induction by cAMP was reduced from 24.2-fold to 8.1-fold. The residual induction may be due to remaining endogenous wild-type holoenzyme or a slight activation of the mutant holoenzyme.

The introduction of a second mutation that destroys cAMP binding at the A site in addition to the B site produces a R subunit [MtR(AB)] that does not bind cAMP. This mutated subunit has been shown to act as an irreversible inhibitor of the C subunit (unpublished data). Under the assay conditions

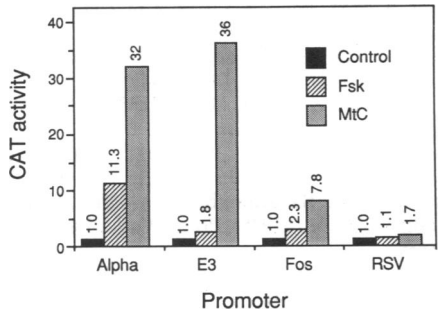

FIG. 3. Additional cAMP-regulated promoters can be induced by the C subunit of PKA. Two and one-half micrograms of the reporter plasmids was cotransfected into JEG-3 cells with 7.5 μg of MtC or Mtglobin. Cultures were incubated in the presence of zinc and alternate plates were treated with forskolin (Fsk) as indicated. CAT values are the average of two identical experiments and are shown relative to the control expression of each reporter gene in the absence of forskolin and C subunit.

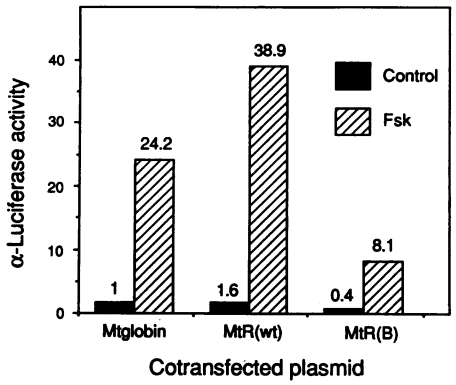

FIG. 4. Effects of cotransfection of the R subunit of PKA on α-luciferase in JEG-3 cells. One-tenth microgram of α-luc was cotransfected into JEG-3 cells with 10 μg of Mtglobin, MtR(wt), or MtR(B) and treated with forskolin (Fsk) as indicted. Values are the average of five identical experiments and are expressed relative to the level of expression with Mtglobin in the absence of forskolin.

shown in Fig. 4, expression of α-luciferase was reduced below detection. To accurately assess the effects on expression of this "irreversible inhibitor" of the C subunit, it was necessary to increase the quantity of reporter gene used by 10-fold to 1 μg per plate and to carry out a titration of the MtR(AB) expression vector using the neutral reporter plasmid, Mtglobin, to maintain the total DNA concentration. Introduction of excess MtR(AB) under these conditions, while having very little effect on the RSV promoter, progressively decreased basal expression of the α-subunit promoter to 10% of the control level (Fig. 5*A*). MtR(AB) completely

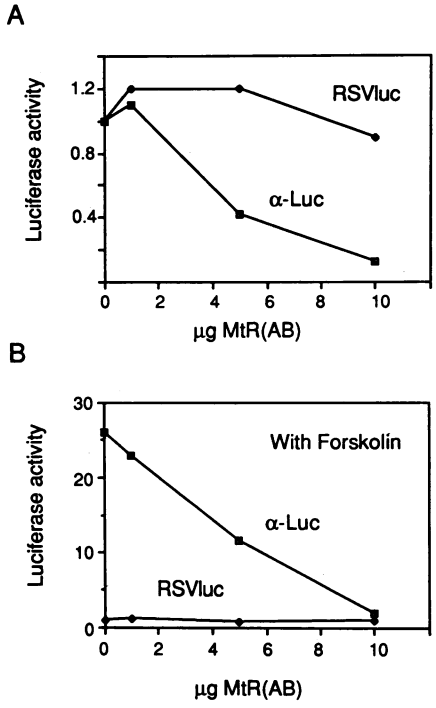

FIG. 5. The R subunit of PKA mutated in both cAMP binding sites blocks cAMP induction and lowers basal transcription from α-luciferase. (*A*) Effects of MtR(AB) on the basal expression of α-luciferase. (*B*) Effects of MtR(AB) on the cAMP-induced expression of α-luciferase. One microgram of α-luc or RSVluc was cotransfected into JEG-3 cells with 10 μg of total of Mt expression vector DNA that was composed of 0, 1, 5, or 10 μg of MtR(AB) and sufficient Mtglobin DNA to total 10 μg. Values are the average of two identical experiments and are normalized to the level of expression of each promoter transfected with 10 μg of Mtglobin in the absence of forskolin. Cells were treated with forskolin as described.

blocked induction of the α-subunit promoter in the presence of cAMP (forskolin; Fig. 5*B*) decreasing expression to the original uninduced level.

DISCUSSION

We have used gene transfection to assess the roles of the individual subunits of the cAMP-dependent protein kinase in the regulation of gene transcription. The overexpression of C subunit (either the Cα or Cβ isoform) was found to induce the expression of a cAMP-regulated promoter derived from the α-subunit gene of the human glycoprotein hormones. The maximal induction by C subunit was proportional to the level of expression of the C subunit and reached a level equivalent to or greater than that produced by induction of the α promoter by forskolin, a strong inducer of adenylate cyclase activity. This induction was not augmented further by the addition of forskolin, indicating commonality in the pathway mediating both inductions. Thus the C subunit can completely replace cAMP as a positive regulator of α-subunit gene transcription, indicating that protein phosphorylation mediates the transcriptional responses to cAMP in this system. In further support of this conclusion, a C subunit in which a lysine essential for kinase activity (Lys-72) was replaced by alanine does not activate α promoter transcription (W. Ran and G.S.M., unpublished observations).

Induction by C subunit was restricted to promoters that contain CREs. Neither the RSV nor the SV40 promoter was regulated. However, two additional known cAMP-regulated promoters, the adenovirus E3 promoter and the human c-*fos* promoter, were induced by the C subunit. The E3 promoter binds a protein termed ATF at a site homologous to the CRE sequence, and Lin and Green (13) have recently shown that ATF is equivalent to CREB. Though E3 responded 36-fold to excess free C subunit, it was induced only ≈2-fold by forskolin in this assay. In PC12 cells, this promoter responds by 8- to 10-fold to the addition of 20 μM forskolin (27). In addition, the c-*fos* promoter is induced by forskolin 15-fold in PC12 cells (12) and only 2.3-fold in JEG-3 cells but is induced almost 8-fold by excess C subunit. In this experiment, the α-subunit promoter also gave a 3-fold greater response to C subunit expression compared to forskolin treatment. These results indicate that under these conditions some genes are more strongly affected by high levels of free C subunit than by the levels of cAMP produced by forskolin treatment. Thus, it is likely that the free C subunit produced by transfection may exceed the endogenous free C subunit level generated by elevating the intracellular cAMP levels and that phosphorylation is the limiting step in the expression from these promoters.

Purified C subunit has been mechanically introduced to cells in culture by fusion with erythrocyte ghosts loaded with C subunit (38). In these studies, the activity of a cAMP-inducible enzyme, tyrosine aminotransferase, was found to increase by 2-fold. During the preparation of this manuscript, Riabowol *et al.* (39) showed that microinjection of C subunit increases endogenous c-*fos*-encoded protein and the immunoreactive protein from an integrated β-galactosidase gene driven by the vasoactive intestinal peptide gene promoter. These experiments support the finding that the C subunit can induce cAMP-dependent genes.

Since excess free C subunit can induce cAMP-regulated genes, overexpression of a mutant R subunit that can bind the C subunit in an inactivatable holoenzyme might be expected to block the induction of a cAMP-regulated gene. Introduction of cAMP binding site R subunit mutants into JEG-3 cells effectively inhibited the induction of the α-subunit promoter by cAMP and also produced a strong repressive effect on the basal transcription level of this promoter. Since deletion of the CREs from the α promoter destroys its transcriptional activity (8), the role of the CRE in transcription of these genes is thus not only as a modulated enhancer that responds to cAMP but also as a basal transcription element. The dependence of basal promoter activity on CREs is common with other cAMP-regulated genes as well (40). These results demonstrate that the basal transcription of cAMP-responsive genes depends on the ambient level of free C subunit in the cell and implies an important role for phosphorylation in maintaining the uninduced transcription of CRE-containing genes.

Posttranslational modification by phosphorylation of cellular proteins is a rapid reversible mechanism for the modulation of many processes in response to physiological stimuli. Our experiments support the model that specific phosphorylation of cellular proteins is the key step in the regulation of transcription by cAMP-mediated pathways. The simplest version of this model postulates that the protein that is phosphorylated to produce these effects is CREB. This model is supported by evidence that this protein can be phosphorylated *in vitro* and *in vivo* and that these phosphorylation events modify its activity (10, 16, 41). This phosphorylation does not, however, appear to modify the DNA binding affinity or dimerization of CREB (8, 16). It is also possible that the target protein for phosphorylation by the C subunit is another kinase that acts directly on CREB or another protein that can modify the activity of CREB. Recent studies have shown that a protein related to CREB that mediates the phorbol ester transcriptional response through the protein kinase C pathway (AP-1) must be bound to c-*fos* protein to produce its effects (42). In addition, the c-*fos* protein is itself phosphorylated in response to phorbol esters (43).

In conclusion, we have developed a rapid cotransfection assay to assess the role of the C and R subunits of PKA in the modulation of transcription by hormones. This assay will greatly facilitate the further study of the structure–function relationships involved in the formation of holoenzyme, the regulation of kinase activity and specificity, and the participation of kinase in gene transcription using *in vitro* mutagenesis of the kinase subunits.

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