Regulation of the Human Enkephalin Promoter by Two Isoforms of the Catalytic Subunit of Cyclic Adenosine 3′,5′-Monophosphate-Dependent Protein Kinase

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Cyclic AMP regulates a variety of cellular responses through activation of the catalytic subunit of cAMP-dependent protein kinase. The cDNAs for two protein isoforms of the catalytic subunit, Cα and Cβ, were placed into expression vectors, and their ability to stimulate cAMP-dependent transcription of the human enkephalin promoter was examined in transiently transfected CV-1 cells. Expression vectors for Cα and Cβ that were directed by the human cytomegalovirus promoter produced up to 350- and 200-fold increases in chloramphenicol acetyltransferase activity, respectively, when cotransfected with the ENKAT-12 reporter plasmid. Transcriptional activation was shown to be dependent upon functional kinase activity by point mutations in catalytic subunit vectors which eliminated activation. Transcriptional activation by Cα and Cβ was eliminated when the cAMP response elements (CREs) were deleted from the native enkephalin promoter, but activation was recovered when this region was replaced with an oligonucleotide containing two copies of the somatostatin CRE consensus TGACGTCA. Cα expression vectors were found to produce 2-fold greater transcriptional activation than Cβ expression vectors. These results were most likely due to the cellular kinase activity produced by the catalytic subunit expression vectors and did not appear to be dependent on CRE motif or substrate specificity. *In vitro* mutagenesis indicates that neither Cα nor Cβ requires N-terminal myristylation for transcriptional activation, but threonine-197 is critical to subunit function. (Molecular Endocrinology 5: 921–930, 1991)

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INTRODUCTION

Cyclic AMP regulates many aspects of eukaryotic cellular metabolism through the activation of cAMP-dependent protein kinase (PKA). Binding of two molecules of cAMP to each of the two regulatory (R) subunits of the inactive tetrameric holoenzyme causes dissociation and release of two catalytic (C) subunits. The active C-subunit then catalyzes the transfer of γ-phosphate from ATP to specific serine and threonine residues of substrate proteins. These phosphoproteins and their subsequent interactions mediate the cellular responses to elevated levels of cAMP.

Regulation of gene transcription by cAMP has been demonstrated for many eukaryotic genes, including somatostatin (1), glycoprotein hormone α-subunit (2, 3), phosphoenol pyruvate carboxykinase (4), and enkephalin genes (5). Fusion of the promoter regions of these genes to reporter genes, such as that for bacterial chloramphenicol acetyltransferase (CAT), has facilitated the identification of specific *cis*-acting DNA sequences that are required for the cAMP regulation of gene transcription. These DNA sequences, which generally conform to the consensus TGACGTCA, have been shown to behave as enhancers and have been termed cAMP response elements (CREs). Specific protein binding to the CRE has been demonstrated in cellular extracts, and several CRE-binding proteins (CREBs) have been purified, characterized, and cloned (6–9); Karpinski, B. A., G. D. Morle, J. I. Huggenvik, M. D. Uhler, and J. M. Leiden, submitted). One CREB has been shown to be a substrate for the catalytic subunit of PKA *in vitro* (11), and the phosphorylated CREB was critical for activation of gene transcription (12). In addition, overexpression of a C-subunit for PKA has been

shown to induce the expression of three cAMP-responsive genes (13).

A number of important questions remain unanswered with respect to the role of PKA in the regulation of gene transcription. Two distinct isoforms of the PKA C-subunit, termed Cα and Cβ, have been characterized in mouse and bovine tissues (14–16). These two proteins are 91% identical in amino acid sequence, and the few differences in their primary sequence show strong evolutionary conservation (15). While the Cα isoform appears to be expressed ubiquitously, the Cβ isoform is preferentially expressed in the central nervous system (15). Experiments on highly purified protein preparations have been unable to provide a clear indication of differences in substrate specificity (17) or cellular function. One reason for the evolutionary conservation of two C-subunit isoforms could be that one isoform may play a greater role in the regulation of gene transcription. Of additional interest is the role of C-subunit posttranslational modifications, such as myristylation and phosphorylation, in the regulation of gene transcription. The C-subunit of PKA was the first myristylated protein identified (18), and while a number of physiological roles have been postulated for the N-terminal hydrophobic moiety, its precise function remains unclear. The phosphorylated threonine at position 197 was also one of the first threonyl-phosphate groups to have been described (19). However, the importance of this amino acid to mammalian PKA function has not been addressed.

In this paper we describe the use of a transient cotransfection assay to characterize the efficacy of normal and mutant C-subunit isoforms in regulation of a cAMP-responsive promoter. For these experiments we selected the human proenkephalin promoter, which contains a well characterized CRE and has previously been shown to direct high levels of basal and cAMP-regulated transcription of the CAT reporter gene. We report here that both Cα and Cβ are able to stimulate transcription from the enkephalin promoter, that coexpression of CREB leads to further transcriptional activation by both isoforms, and that myristylation of Cα or Cβ is not required for stimulation.

RESULTS

Transcriptional Activation of ENKAT-12 by Cotransfection of Expression Vectors for the Cα and Cβ Subunits of PKA

Transcriptional regulation of the enkephalin promoter by the Cα and Cβ isoforms of PKA was examined by a transient transfection assay in CV-1 cells. Previously, a fragment of the human enkephalin gene which includes 193 basepairs (bp) of 5′ flanking genomic DNA was fused to the CAT gene to produce the ENKAT-12 reporter plasmid (5). This plasmid allows cellular transcriptional responses to be monitored by the CAT activity occurring in extracts of transfected cells (20). All of the data presented here have been normalized to the constitutive activity of the cotransfected SV2βGal plasmid (5). In addition, the data are expressed as relative CAT activity, which is the ratio of normalized CAT activities in expression vector transfected cells to the activity in cells transfected with a control plasmid (see *Materials and Methods*).

Since cAMP is thought to mediate its effects through cAMP-dependent protein kinase, we sought to determine if the ENKAT-12 construct was sensitive to cotransfection with the expression vectors for the C-subunits of PKA. Initial experiments used MtCα and MtCβ, expression vectors for the Cα and Cβ isoforms of the catalytic subunit, which are directed by the mouse metallothionein promoter (Mt). As shown in Fig. 1, cotransfection of CV-1 cells with ENKAT-12 and MtCα resulted in an 81-fold induction of relative CAT activity, while cotransfection with MtCβ resulted in a 32-fold induction. To demonstrate that this effect was specific for PKA, cells were also cotransfected with the plasmid MtCαK72M. This plasmid encodes a Cα protein identical to that of MtCα, except that the codon for lysine-72 has been altered by *in vitro* mutagenesis to a methionine codon (21). ATP binding by the catalytic subunit is necessary for phosphotransferase activity, and lysine-72 has been shown to be involved in this interaction (22). When cotransfected with ENKAT-12, the MtCαK72M vector showed no stimulation of relative CAT activity (Fig. 1).

The activity of the Mt promoter is weak to moderate in its basal state, and although it is inducible by heavy metals, we wanted to avoid the deleterious side-effects this treatment might have on cells. We thought it important to compare the transcriptional effects of MtCα and MtCβ to those of C-subunit constructs containing

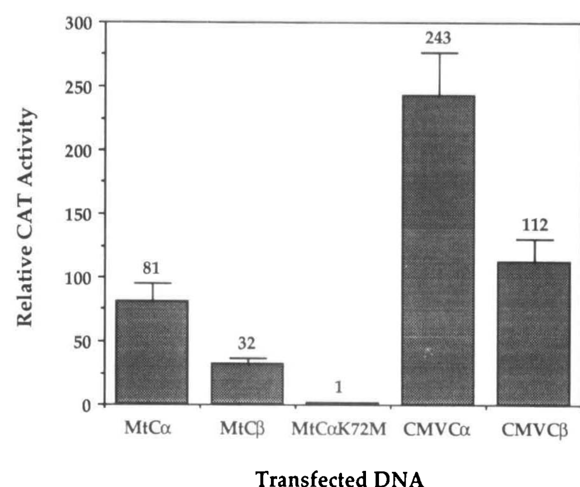

Fig. 1. Induction of CAT Enzyme Activity in CV-1 Cells Transfected with the ENKAT-12 Reporter Plasmid Containing the Human Enkephalin Promoter

Cells were transfected and analyzed as described in *Materials and Methods*. Cells were cotransfected with 2 μg ENKAT-12 and 10 μg MtCα, MtCβ, MtCαK72M, CMVCα, or CMVCβ. Results are the average ± SD of duplicate plates from five experiments.

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a more active promoter. For this pupose, the human cytomegalovirus immediate early promoter (CMV) was chosen, since it has previously been characterized as a strong constitutive promoter (23). The plasmids CMVCα and CMVCβ were constructed to be identical to MtCα and MtCβ, except that the Mt promoter had been replaced with the CMV promoter (see *Materials and Methods*). As shown in Fig. 1, cotransfection of ENKAT-12 with CMVCα resulted in a 243-fold increase in relative CAT activity, while CMVCβ produced a 112-fold increase. The CMV-directed C-subunit expression vectors were consistently 3–4 times more efficient at transcriptional activation than the corresponding Mt-directed vectors. In addition, we tested for PKA specificity by cotransfection of expression vectors that could repress catalytic subunit activity. A 19-fold molar excess of an antisense CMVCα construct produced 70% inhibition of the CMVCα induction of relative CAT activity. An expression vector for protein kinase inhibitor peptide has been shown to inhibit transcriptional activation by endogenous PKA (24, 25). The CMVCα and CMVCβ inductions of relative CAT activity were 80% and 86% inhibited, respectively, by a 19-fold excess of CMV-directed expression vector for a22 amino acid PKA inhibitor peptide (data not shown). When transcriptional initiation of mRNA at the induced enkephalin promoter was examined by RNase protection analysis, we found that initiation was identical to that described in previous studies (5, 26, 27) (data not shown). The apparent 2-fold preference of transcriptional activation by Cα expression vectors relative to the corresponding Cβ vectors is addressed in greater detail below.

Cα- and Cβ-Subunit Transcriptional Activation of the Enkephalin Promoter Is CRE Dependent

The enkephalin promoter displays a complex pattern of cAMP- and phorbol ester-inducible regulation (5, 26–28). Two CRE-like enhancer regions, termed ENKCRE-1 and ENKCRE-2 (Fig. 2), have been shown to be required for the regulation of basal and cAMP-inducible transcription of the enkephalin promoter (26). While the exact mechanisms have not been determined, enkephalin promoter regulation is likely to occur through the interaction of the CRE regions with one or more of the transcription factors AP-1, AP-4, ENKTF-1, and possibly CREB (26, 27). This indicates that cAMP regulation of the enkephalin promoter is likely to be more complex than that of other CRE-containing promoters and implies that specific transcription factors could be substrates of PKA. We, therefore, wanted to further evaluate the *cis*-acting DNA sequence required for Cα and Cβ transcriptional activation. A CAT reporter plasmid, ENK72CAT (5), which contained 72 bp of the human enkephalin promoter but lacked the up-stream CRE elements present in the plasmid ENKAT-12, was examined. A second plasmid, CRE-CAT, was identical to ENK72CAT, except that it contained a synthetic oligonucleotide coding for two copies of the somatostatin CRE consensus sequence TGACGTCA. The oligonucleotide was inserted just 5′ of position −72 in the minimal enkephalin promoter. Figure 2 shows that cotransfection of the ENK72CAT plasmid with CMV-directed expression vectors for Cα or Cβ resulted in no stimulation of CAT activity over basal levels. In contrast, CRE-CAT showed significant increases in CAT activity when cotransfected with CMVCα or CMVCβ. A greater than 2-fold preference of induction by Cα expression vectors was observed relative to Cβ expression vectors in transfections with ENKAT-12 and CRE-CAT. From these data, we concluded that activation of transcription by cotransfection with C-subunit expression vectors requires a functional CRE within the enkephalin promoter region. In addition, the Cα to Cβ preference of transcriptional activation is not likely to result from the complex nature of the native enkephalin CREs.

Comparison of Transcriptional Activation and Kinase Activity due to Cα- and Cβ-Subunit Isoforms

Throughout the previous studies, we had noted that transcriptional activation of the enkephalin promoter was consistently higher in cotransfections with Cα expression vectors than in those with Cβ expression vectors. A possible explanation for this difference could be that the Cα isoform of PKA has greater substrate specificity relative to Cβ in the phosphorylation of transcription factors required for activation of the enkephalin promoter. Alternatively, Cα expression vectors may simply produce a greater level of kinase activity relative to Cβ expression vectors. To address this question, we concurrently measured relative CAT activities and cellular kinase levels in CV-1 cells that had been transfected with increasing amounts of CMVCα or CMVCβ expression vectors. The total amount of DNA used in transfections (17 μg) was kept constant by balancing it with the appropriate amount of expression vector that lacked C-subunit cDNA sequences. Aliquots of cell lysates were then assayed for CAT activity and kinase activity, using a kinase assay specific for cAMP-dependent protein kinase (see *Materials and Methods*). The results of this study are shown in Fig. 3. Both CMVCα and CMVCβ produced a dose-dependent increase in CAT activity in transfections with the ENKAT-12 reporter (Fig. 3A). Transfections containing up to 6 μg C-subunit expression vectors displayed a linear increase in CAT activity, which appeared to plateau after 8 μg. As we had consistently observed in other experiments, CMVCα produced a larger increase in CAT activity than CMVCβ. At the maximum amount of expression vector used (10 μg), the ratio of CAT activity produced by CMVCα relative to CMVCβ was 1.8.

When cAMP-dependent protein kinase activity was examined in the cell lysates, a dose-dependent increase in kinase activity was observed for both CMVCα and CMVCβ expression vectors (Fig. 3B). The kinase levels produced by CMVCα were consistently higher than those produced by CMVCβ, paralleling their effects on transcriptional activation of the enkephalin promoter (Fig. 3A). At the maximum amount of expression vector

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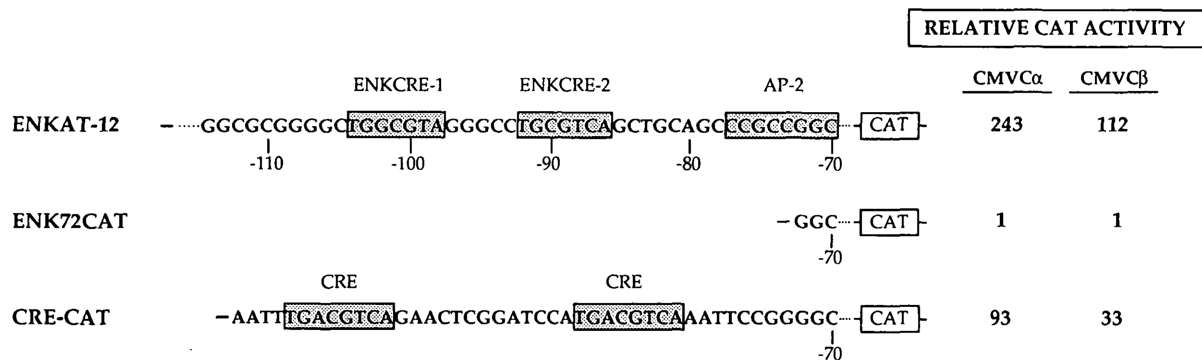

Fig. 2. CRE Dependence of Transcriptional Activation by PKA Catalytic Isoforms
Schematic representation of the plasmids ENKAT-12, ENK72CAT, and CRE-CAT. A portion of the 5′-flanking sequence of the human proenkephalin gene is shown for ENKAT-12. ENK72CAT is identical to ENKAT-12, except for the deletion of bases 5′ to position −72 of the promoter. The CRE-CAT contains two copies of the CRE consensus TGACGTCA and was constructed as described in *Materials and Methods*. The diagram uses a *dotted line* to indicate bases not shown and a *solid line* to indicate linkage to pUC sequences. CV-1 cells were cotransfected with 2 μg ENKAT-12, ENK72CAT, or CRE-CAT and 10 μg CMVCα, or CMVCβ. Relative CAT activities shown for ENKAT-12 are the averages of duplicate plates from five experiments, while ENK72CAT and CRE-CAT are the averages of duplicate plates from a representative experiment.

used, the ratio of kinase activity produced by CMVCα relative to CMVCβ (2.3) was very similar to their ratio for transcriptional activation of ENKAT-12. Collectively, these data suggest that the difference in transcriptional activation observed between CMVCα and CMVCβ is most likely due to the level of cellular kinase activity produced and is probably not due to CRE structure. Due to the high cross-reactivity of antibodies to Cα and Cβ, we are currently unable to assay for their respective protein levels in transfected cells; therefore, the precise reason for the difference in Cα and Cβ kinase activities remains to be determined.

CREB Increases Transcriptional Activation by Cα- and Cβ-Subunit Isoforms

CRE-dependent transcription of cAMP-responsive genes is thought to include regulation by a family of nuclear transcriptional factors referred to as CREBs (29). A model for transcriptional activation by these proteins has been proposed (30) in which the catalytic subunit of PKA is translocated to the nucleus of cells (31, 32), resulting in the phosphorylation of CREB, which is bound in dimeric form to the CRE(s) in the 5′ flanking regions of cAMP-responsive genes. It is further proposed that this phosphorylation results in a conformational change in CREB, which then allows interaction with other transcriptional components and leads to increased transcription. To test the effects of CREB in the transfection assay, we used an expression vector in which the coding sequence for the human placental isoform of this protein, CREB-327 (8), had been placed under regulation of the CMV promoter using a strategy similar to that used to produce CMVCα and CMVCβ expression vectors (Karpinski, B. A., G. D. Morle, J. I. Huggenvik, M. D. Uhler, and J. M. Leiden, submitted). The resulting construct, pCREB, was cotransfected with CMVCα and CMVCβ to determine their combined effects on the transcriptional activation of the ENKAT-12 reporter plasmid. The results from these experiments are shown in Fig. 4. Using 5 μg plasmid DNA, pCREB by itself produced a 28-fold increase in CAT activity. Cotransfection of pCREB with CMVCα or CMVCβ produced 2.8- and 3.8-fold additional increases in CAT activity, respectively, relative to transfections containing only the C-subunit expression vectors. Cotransfections with CREB did not produce any further increase in kinase activity relative to transfections with C-subunit expression vectors alone (data not shown). We conclude from these data that the coexpression of CREB with either of the isoforms for the catalytic subunit of PKA produces an interaction leading to increased transcriptional activation of the enkephalin promoter.

Mutation Analysis of Cα and Cβ Catalytic Subunits

After having determined that both Cα and Cβ inductions of ENKAT-12 transcription were correlated with kinase activity, the transfection assay was further used to study the effects of *in vitro* mutagenesis on C-subunit function. A first set of experiments involved constructs designed to generate truncated Cα proteins. Two amino-terminal truncation mutants were constructed by introducing in-frame *NcoI* sites (CCATGG), such that the lysine codon for the amino acid residue at position 23 or the leucine residue at position 49 was changed to a methionine residue and, thus, became the initiator methionine of the truncated proteins. MtCαΔ1–23 encodes a Cα protein in which the first 23 amino acids have been deleted, and MtCαΔ1–49 codes for a Cα protein lacking the first 49 amino acids. In addition, two Cα expression vectors containing premature termination (PT) codons were constructed, in which the lysine codon for amino acid residue 295 was altered from AAG to TAG (MtCαPT295), and the proline codon for amino acid residue 316 was changed from CCA to TGA

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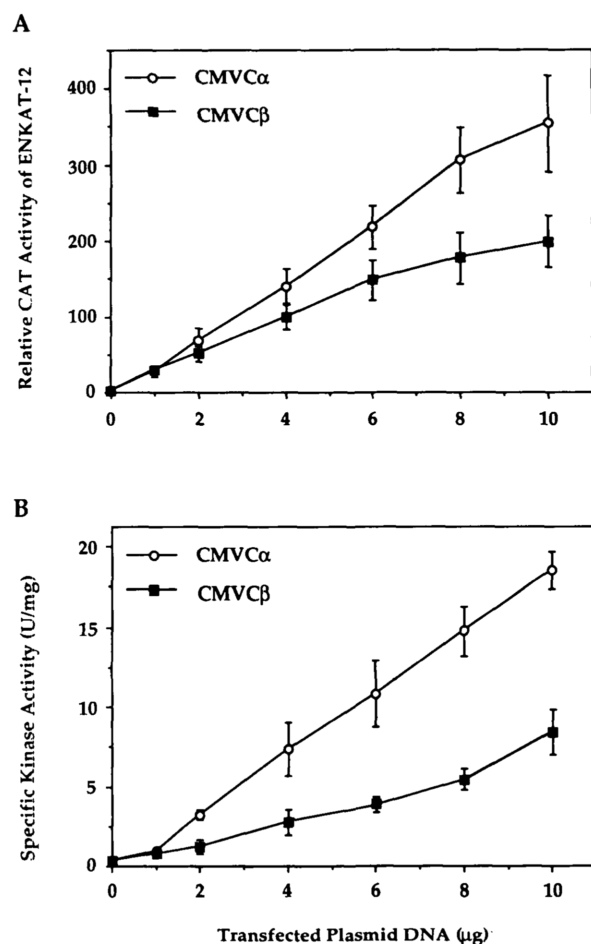

Fig. 3. Dose Response of CAT and Kinase Activity in CV-1 Cells Transfected with Increasing Amounts of C-subunit Expression Vectors

A, Cells were transfected with 2 µg ENKAT-12 and increasing amounts of CMVCα (O) or CMVCβ (■). CMVNeo was added to bring the total amount of DNA to 17 µg. Cells were sonicated in 200 µl kinase homogenization buffer, and 1-µl aliquots were measured for CAT activity. The results are the average ± SD of duplicate plates from three separate experiments. B, Two microliters of the same cell extracts as those shown in A were measured for phosphotransferase activity using the peptide substrate Kemptide, as described in *Materials and Methods*. The results are expressed as the average ± SD of triplicate measurements of each sample prepared from duplicate plates in three separate experiments.

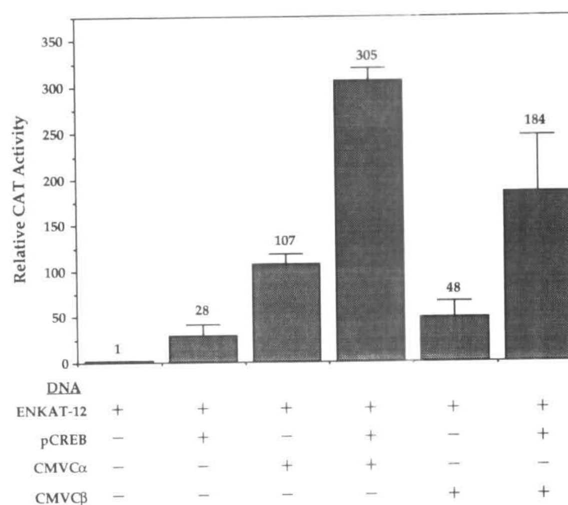

Fig. 4. Increased Transcriptional Activation by PKA Catalytic Isoforms by Cotransfection with a CREB Expression Vector

CV-1 cells were transfected with 2 µg ENKAT-12 and 5 µg of the indicated (+) plasmid. The total amount of plasmid DNA was held constant with the addition of 5 or 10 µg of the control plasmid CMVNeo. The results are the average ± SD from duplicate plates in two separate experiments.

(MtCαPT316). The effects of these various mutations on the ability of Cα to stimulate transcription from the ENKAT-12 reporter plasmid can be seen in Fig. 5. Deletion of the first 23 amino acids of Cα did not affect the ability of Cα to stimulate relative CAT activity. In contrast, deletion of the first 49 amino acids (MtCαΔ1–49), the last 55 amino acids (MtCαPT295), or the last 34 amino acids (MtCαPT316) completely eliminated the induction of relative CAT activity.

We also compared the induction of relative CAT activity for two specific point mutations of Cα and Cβ. The first of the mutations was the change in the codon for the amino-terminal glycine of each C-subunit isoform to an alanine codon. This mutation has been shown to block myristylation of several proteins, including the Cα-subunit (33). As shown in Fig. 5, MtCα produced an 81-fold increase in relative CAT activity, and the MtCαG1A expression vector produced a similar 88-fold increase in relative CAT activity. Similar results were obtained when the MtCβ expression vector (32-fold) and the MtCβG1A expression vector (37-fold) were compared. Thus, it appears that myristylation is not required of either Cα or Cβ for activation of enkephalin transcription.

The second point mutation that was analyzed changed a threonine residue at position 197 to an alanine for both the Cα- and Cβ-subunits. This threonine residue has been shown to be phosphorylated in the bovine heart (9, 19) and is proximal to cysteine 199, a residue that has been implicated in both substrate binding (34) and interactions with R-subunit (35). The conversion of the analogous threonine (threonine-241) in the yeast C-subunit to an alanine disrupts regulation by R-subunit and produces constitutive kinase activity (36). In contrast, the T197A mutations for both Cα and Cβ eliminated the ability of these expression vectors to stimulate transcription from the enkephalin promoter (Fig. 5). These data provide further evidence that phosphorylation of threonine-197 is likely to be important to C-subunit function and indicate that the mechanisms for PKA regulation in mammals and yeast are probably different. Also, in all of the mutations studied where transcriptional activation was lost, cellular kinase activities above endogenous levels were not observed. However, elevated kinase levels could be measured in transfections with MtCα, MtCαG1A, and MtCαΔ1–23 (data

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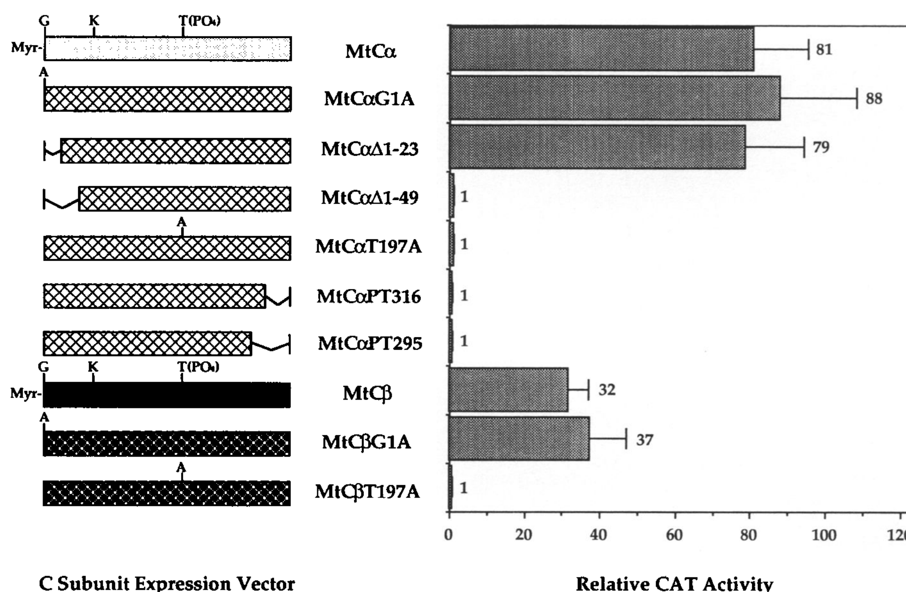

Fig. 5. Mutational Analysis of PKA Catalytic Isoforms

Schematic drawings depicting the wild-type and mutated Cα- and Cβ-subunit expression vectors constructed as described in *Materials and Methods*. CV-1 cells were cotransfected with 2 μg ENKAT-12 and 10 μg of the indicated plasmid. ◇, Mutations of the Cα expression vector; ♦, mutations of the Cβ expression vector. Relative CAT activities are the average ± SD of duplicate plates from four separate experiments.

not shown), again suggesting a correlation between functional kinase activity and transcriptional activation.

DISCUSSION

The large number of cellular responses produced by cAMP are dependent upon the release of active catalytic subunits from the regulatory subunits of PKA and the subsequent phosphorylation of substrate proteins. The existence of at least three evolutionarily conserved genes for the catalytic subunit (16, 37, 38) has suggested that the different protein isoforms produced by these genes might be involved in distinct regulatory events. To understand the role of the Cα and Cβ isoforms in the regulation of cAMP-dependent gene transcription, we have initiated studies that use a method in which the cloned cDNAs for each isoform can be overexpressed in a cellular system, and their effects on specific gene transcription can be ascertained. In this paper we describe a transient transfection assay in CV-1 cells, in which the expression of catalytic isoforms can be directed by weak and strong basal promoters, and their transcriptional effects can be monitored by the well characterized cAMP-dependent response of the human enkephalin promoter. In addition, due to the high efficiency with which CV-1 cells can be transfected, the direct measurement of kinase activity produced by the C-subunit expression vectors is possible. This has allowed correlation of transcriptional activation and kinase activity in the same cell extracts.

We observed that cotransfection of expression vectors coding for C-subunit isoforms could produce greater than 200-fold increases in relative CAT activity from a reporter plasmid containing the human enkephalin promoter. Transcriptional activation was shown to be dependent upon functional kinase activity. Point mutations and deletion mutations in the coding region of the C-subunit that abolished phosphotransferase activity resulted in the reduction of transcriptional activation to basal levels. These data provided evidence that active kinase is required for transcriptional activation of the enkephalin promoter.

Transcriptional activation by C-subunit was also shown to be dependent upon a functional CRE sequence within the enkephalin promoter. The endogenous enkephalin promoter contains two enhancer-like regions, termed ENKCRE-1 and ENKCRE-2, which have been shown to regulate cAMP- and phorbol ester-inducible transcription in conjunction with a downstream AP-2 element (5, 26, 27). The ENKCRE-1 element (TGGCGTA) occurs between nucleotides −104 and −98 (5′ of the transcription initiation site) and is required for a maximal cAMP and phorbol ester responses. ENKCRE-2 is defined by an unusual CRE sequence motif (TGCGTCA), which occurs at position −92 to −86 of the promoter and is absolutely required for basal and regulated transcription of the enkephalin gene. In addition to showing *in vitro* binding to the transcription factors AP-1, AP-4, and ENKTF-1, ENK-CRE-2 is also the most likely region for CREB binding (26, 27). Deletion of these regions from the ENKAT-12 plasmid (5′ to the −72 position) resulted in the complete

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loss of transcriptional activation in cotransfections with C-subunit expression vectors. However, replacement of the 5′ regions with a synthetic oligonucleotide containing two copies of the somatostatin CRE consensus (TGACGTCA) restored transcriptional activation by Cα and Cβ to levels approaching those observed for the endogenous enkephalin promoter. This indicates that both catalytic isoforms are capable of activating CRE-dependent transcription and that this activation is independent of the two types of CRE motifs studied.

The CRE dependence of transcriptional activation by the Cα and Cβ isoforms suggested that the mechanism of activation in the transient transfection assay was likely to occur through the phosphorylation of a transcriptional activator, such as CREB. We found that coexpression of Cα or Cβ with the human placental form of CREB resulted in greater relative CAT activities than expression of either C-subunit alone. This increase in CAT activity was not accompanied by any additional increase in kinase activity. This provides evidence that both Cα and Cβ can interact with a known transcriptional factor to regulate the enkephalin promoter, and that the transient transfection assay is likely to operate in a manner similar to endogenous regulation of cAMP-responsive gene transcription. We noted a small (<2-fold), but consistent, increase in the relative CAT activity produced by cotransfection of the Cα isoform of PKA relative to the Cβ isoform. When transiently transfected cell lysates were tested for PKA kinase activity, the level of kinase activity in Cα-transfected cells was also about 2-fold higher than what was observed in the Cβ-transfected cells. We conclude that the difference in activation of the enkephalin promoter by Cα and Cβ is highly dependent on and sensitive to the kinase activity within the cells and is probably not due to substrate specificities of the catalytic isoforms. This conclusion is supported by the data obtained by cotransfection with pCREB, where the relative CAT increases due to CMV-directed expression of Cα and Cβ (2.8- *vs.* 3.8-fold) were similar for each isoform, thus suggesting that Cβ is capable of phosphorylating CREB as efficiently as Cα. This conclusion would also be consistent with previous studies which showed that highly purified preparations of Cα- and Cβ-subunits display very similar activities during the *in vitro* phosphorylation of a substrate peptide (17).

The transient transfection assay allowed us to analyze a number of mutations in both the Cα- and Cβ-subunits in order determine their functional effects on transcriptional activation. We found that while deletion of up to 23 N-terminal amino acids in the Cα-subunit could be tolerated with no loss of function, deletions of 49 amino acids in the N-terminus or deletions of 34 or 55 amino acids in the C-terminus eliminated transcriptional activation. The amino acid consensus sequence Gly-X-Gly-X-X-Gly is highly conserved throughout the protein kinase superfamily and is thought to be necessary for ATP binding (39). This consensus occurs at position 50 in the Cα-subunit of PKA, and it seems likely that deletion of the N-terminal 49 amino acids in Cα may disrupt the binding of ATP to this region. It is apparent that the C-terminus of Cα is also highly sensitive to modification. Threonine-197 in both Cα and Cβ is phosphorylated in bovine heart (9), and the analogous residue has been shown to be important in the regulation of yeast C-subunit (36). Conversion of threonine-197 to an alanine using site-directed mutagenesis resulted in a total loss of function for both the Cα- and Cβ-subunits, demonstrating that this residue, or possibly its phosphorylation, is critical to C-subunit function. Cα and Cβ are also known to be myristylated at their N-terminal glycines (33). The function of this posttranslational modification is unclear, but previous reports have shown that conversion of the N-terminal glycine to an alanine prevents N-terminal myristylation with no loss of Cα-subunit function in stably transfected NIH 3T3 cells (33). Similarly, in our transient transfection assay, the conversion of N-terminal glycines to alanines for both Cα- and Cβ-subunits resulted in no loss of transcriptional activation by either of the isoforms.

The effects of overexpression of cloned C-subunits have been analyzed for several different cAMP-responsive genes in a number of cell expression systems (13, 21, 40, 41). Transient cotransfection of JEG-3 cells with MtCα has produced 32-, 36-, and 8-fold increases in transcription from the α-subunit glycoprotein, E1A, and *c-fos* promoters, respectively (13). Stable transfection of MtCα into L-929 cells produces a significant increase in endogenous alkaline phosphatase transcription upon induction with zinc (21), and transient transfection of PC-12 cells with MtCα produces a 12-fold increase in basal transcription of the vasoactive intestinal peptide promoter (41). The only previous analysis of transcriptional effects by both isoforms of C-subunit indicated that a 3-fold transcriptional activation of the PRL promoter was produced equally well by Cα and Cβ (40). In this study we have observed greater than 300-fold inductions of transcription from the enkephalin promoter and a 1.8-fold preference of transcriptional activation by Cα relative to Cβ. However, assays of PKA enzymatic activity indicate that this small activation preference is due to the lower kinase activity produced by transfection with Cβ expression vectors, and it seems likely that transcription from the enkephalin promoter is simply a sensitive reflection of kinase activity. Although endogenous regulation of any cAMP-responsive gene is likely to be a complex combination of events requiring the interaction of many components, it is expected that a number of common regulation themes are likely to occur. The ability to measure the transcriptional activation by both isoforms of C-subunit and to directly assay for kinase activity should greatly facilitate the future analysis of both C-subunit structure and function as well as specific mechanisms of C-subunit regulation of gene transcription.

MATERIALS AND METHODS

Cell Culture and Transfection

CV-1 cells were plated at a density of 300,000 cells/60-mm dish in Dulbecco's Modified Eagle's Medium containing 10%

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fetal bovine serum. Cells were transfected with CsCl-purified plasmid DNA by calcium phosphate precipitation (20) and harvested 36 h later (5). In all experiments, duplicate plates were cotransfected with 2 μg of the reporter plasmid, ENKAT-12 (5); 5 μg of the internal control plasmid, SV2βgal (5); and 10 μg of the plasmid(s) of interest. ENKAT-12 plasmid contains 193 bp of 5′ flanking sequence, exon I, and intron A and 53 bp of exon II of the human enkephalin gene fused with the CAT gene. In preliminary studies we observed that overexpression of C-subunit in CV-1 cells produced an altered morphology in which cells were rounded and could easily detach from tissue culture plates. To compensate for this potential loss of transfected cells and correct for transfection efficiency, all transfections contained the plasmid SV2βGal. This plasmid contains the simian virus early promoter region (SV40) fused to the coding region of bacterial β-galactosidase and is not induced in cotransfections with C-subunit (13).

CAT and Kinase Assays

In the initial experiments cell extracts were prepared in 100 μl 0.25 M Tris (pH 7.5) and 0.5% Triton X-100. CAT activity in 10 μl extract was assayed after incubation for 1 h at 37 C, and β-galactosidase activity in 5 μl extract was measured as previously described (5). CAT activity is expressed as the counts per min in the acetylated products, normalized to the β-galactosidase activity in each extract. In addition, the data are expressed as relative CAT activity, which is the ratio of CAT activity produced by a reporter plasmid cotransfected with a given expression vector divided by the CAT activity produced by the reporter plasmid (and control plasmid) alone. In experiments in which kinase activity was determined, cells were sonicated in 200 μl kinase homogenization buffer and assayed as previously described (42). Briefly, aliquots of cell extract were added to a reaction mixture containing buffers, [γ-^{32}P]ATP (200 cpm/pmol), the synthetic peptide substrate Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly; Sigma, St. Louis, MO), and 5 μM cAMP and incubated for 5 min at 30 C, and then half of the reaction was spotted onto phosphocellulose paper. Kinase activity is reported as nanomoles of phosphate transferred to the substrate per min/mg protein (units per mg). Preparation of the cell extracts in the kinase homogenization buffer increased CAT activity 2- to 3-fold over that in extracts prepared in Tris-Triton X-100 and allowed kinase activity to be quantitated in 2 μl extract.

Construction of Expression Vectors

The construction of MtCα, MtCβ, MtCαK72M, and pCREB has been described previously (10, 21). Template for mutagenesis was generated by subcloning the 580-bp *SacI/EcoRI* fragment of MtCα and the 930-bp *EcoRI/PvuII* fragment of pMCα (42) into *SacI/SmaI*-cut M13 mp18, such that the Cα-coding region was contiguous through the ligated *EcoRI* site. The single stranded DNA produced by this vector contained the sense strand of the Cα-coding region and was used for all *in vitro* mutagenesis of the Cα isoform. Construction of MtCαG1A used the oligonucleotide (GGCGGCGTTGG-CCATGGCGGC) to change the codon for the Gly residue (GGC) at position 1 to a codon for Ala (GCC) and to create a *NcoI* site (CCATGG) which includes the initiator methionine codon. The resultant mutant phage was sequenced and the 1.1-kilobase (kb) *SacI/BglII* fragment was substituted for the corresponding region of MtCα. MtCαT197A was generated by changing the Thr codon at residue 197 (ACC) to an Ala codon (CGG) using another oligonucleotide (ACACAGGGC-CCAAGTACG). The resulting mutation was sequenced and the 450-bp *BglII* fragment of the Cα coding region was substituted for the corresponding region of MtCα.

The amino terminal deletion expression vectors MtCαΔ1-23 and MtCαΔ1-49 were constructed using the respective synthetic oligonucleotides ATCTTCCATGGCTTTGGC and GGTGCCCATGGTCTTGAT to change the Lys codon (AAG) at position 23 and the Leu codon (CTT) at position 49 to Met codons (ATG). Each of these mutagenic oligonucleotides also created a unique *NcoI* site at the new Met codon. The corresponding *NcoI/ApaI* fragment of the mutagenized vector was then substituted for the *NcoI/ApaI* fragment of pMtCαG1A. The PT vectors, MtCαPT295 and MtCαPT316, were generated using the oligonucleotides AAACCACTAGTGGTTCTT and AAACTTTCATATGAAGGG, respectively, to alter the Lys codon (AAG) for residue 295 and the Pro codon (CCA) for residue 316 to termination codons. The *SacI/ApaI* fragments were sequenced from the mutagenized vectors and were substituted for the *SacI/ApaI* fragment of MtCα.

For construction of the MtCβG1A mutation, the 480-bp *SacI/PstI* fragment of MtCβ was subcloned into the corresponding sites of M13 mp18. Single stranded DNA from this vector was mutagenized using the oligonucleotide GCAGT-GTTCGCCATGGCCGGA to alter the codon for the Gly residue (GGC) at position 1 to a codon for Ala (GCC) and to create a *NcoI* site (CCATGG) which includes the initiator methionine codon. The 200-bp *SacI/XmnI* fragment containing this mutation was substituted for the corresponding fragment in MtCβ. For construction of the MtCβT197A expression vector the 2.1-kb *SacI/EcoRI* fragment of MtCβ was subcloned into M13 mp19 for generation of single stranded template for *in vitro* mutagenesis. The oligonucleotide GGTACCACACA-ATGCCCA was used to change the Thr codon (ACA) to an Ala codon (GCA), and the 440-bp *PstI/BglII* containing the mutation was exchanged for the corresponding fragment of MtCβ.

The CMVCα expression vector was constructed from the parental plasmids MtCα, pZEM3, and pSUB, a plasmid containing the immediate early promoter of human CMV (a generous gift from Dr. Michael Clarke, Univerisity of Michigan). A 700-bp *SpeI/FnuDII* fragment corresponding to nucleotides 153–834 of the published CMV promoter sequence (23) was used to replace the *XbaI/BglII* fragment of pZEM3 containing the mouse metallothionein promoter to generate CMV3. The *BamHI* fragment of pKONeo (42) containing the neomycin phosphotransferase gene driven by the SV40 promoter was subcloned into the *BamHI* site of CMV3 to generate the plasmid CMVNeo. CMVCα and CMVCβ were constructed from CMVNeo by ligating the same *BamHI*-linked cDNA fragments used previously to construct MtCα and MtCβ into the *BglII* site separating the CMV promoter sequences from the human GH transcription termination sequences.

ENKAT-12 and ENK72CAT (a *Bal*-31 deletion mutant of ENKAT-12) have been previously described (5). CRE-CAT was constructed using the oligonucleotides AATTATGACGTCA-GAACTCGGATCCATGACGTCA and AATTTGACGTCATGG-ATCCGAGTTCTGACGTCAT. These oligonucleotides were hybridized together to produce a double stranded DNA that contains two copies of the CRE consensus TGACGTCA. The DNA fragment was then ligated into the *EcoRI* site of ENK72CAT (5). This ligation produced mutations in the linker region of the oligonucleotides, and the final construct contained the DNA sequence shown in Fig. 2, as determined by DNA sequencing.

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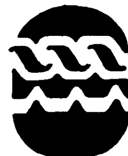

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