Molecular cloning of human CREB-2: An ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element

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ABSTRACT The cAMP response element (CRE) is an octanucleotide motif (TGACGTCA) that mediates diverse transcriptional regulatory effects. In this report we describe the isolation and characterization of a full-length cDNA that encodes a CRE binding protein called CREB-2. Like other ATF/CREB transcription factors, the 351-amino acid CREB-2 protein contains a COOH-terminal leucine-zipper motif and an adjacent basic domain. CREB-2 mRNA is expressed ubiquitously in human tumor cell lines and mouse organs suggesting that it is involved in regulating transcription in a wide variety of cell types. Overexpression of CREB-2 resulted in a consistent and significant repression of CRE-dependent transcription in CV-1 cells. Deletional analyses localized the transcriptional repression activity of CREB-2 to a 102-amino acid COOH-terminal region (amino acids 249-351) that contains the leucine-zipper and basic domains of the molecule. These results demonstrate that CRE-dependent transcription can be both positively and negatively regulated by structurally related members of the ATF/CREB family.

The transcription of many eukaryotic genes is regulated by the binding of sequence-specific transcription factors to modular cis-acting promoter and enhancer elements. The cAMP response element (CRE) is among the best studied of the cis-acting transcriptional enhancer motifs. This palindrome octanucleotide (TGACGTCA) has been identified (1-5) in the transcriptional regulatory regions of a large number of eukaryotic genes and has been shown to mediate diverse transcriptional effects including (i) conferring responsiveness to cAMP (1, 2); (ii) binding of a cellular factor, ATF, and thereby conferring Ela1 responsiveness on several adenovirus genes (3); and (iii) modulating the basal activity of eukaryotic transcriptional enhancers including the human T-cell leukemia virus type I (4) and the c-fos protooncogene enhancers (5).

Recent studies have resulted in the cloning of several CRE binding proteins that form the ATF/CREB family (6-11). These proteins share highly related COOH-terminal leucine-zipper dimerization and basic DNA binding domains, but each contains a distinct NH2-terminal region. Although each of the ATF/CREB proteins appears to be capable of binding to the CRE as a homodimer, some of these proteins also bind to DNA as heterodimers (6, 8, 9). One of these proteins, CREB, has been shown to be a transcriptional activator that requires phosphorylation by protein kinase A (PKA) for its activity (12). A second protein, CRE-BP1 (also called HB16 and ATF-2) (6, 9, 10) binds to CRE sites as a heterodimer in conjunction with the JUN protein (9) and also interacts with the adenovirus Ela1 protein to activate transcription from CRE sites (13). Although a number of additional ATF/CREB proteins have been cloned (6), many of these clones represent partial-length cDNAs, and the transcriptional activities of most of these proteins remain unknown.

In this report we describe the isolation and characterization of a cDNA clone that encodes an ubiquitously expressed CRE-binding protein that we have called CREB-2. Overexpression of CREB-2 in monkey CV-1 cells caused a significant and specific repression of CRE-dependent transcription. Thus, CRE-dependent transcription can be subject to both positive and negative regulation by different members of the ATF/CREB family of transcription factors.

MATERIALS AND METHODS

Plasmids. The pENKAT-12 plasmid containing two copies of the human enkephalin enhancer and one copy of the human enkephalin promoter immediately 5' of the bacterial chloramphenicol acetyltransferase (CAT) gene and the pCMVC* plasmid in which the expression of the catalytic subunit of PKA is under the control of the cytomegalovirus promoter (14) have been described. The pH110 (15) and pMSV*gal (16) plasmids contain the B-galactosidase gene under the control of the simian virus 40 promoter/enhancer and the Moloney sarcoma virus promoter, respectively. The pCDM7 eukaryotic expression vector (17) was the generous gift of Brian Seed (Harvard Medical School, Boston). The full-length human CREB cDNA (7) was generously provided by Joel Habener (Harvard Medical School). CREB-2 (6, 29-31) (CREB-2) encoding amino acids 249-351 of CREB-2 was made by PCR amplification (18) of the CREB-2 cDNA with the synthetic oligonucleotide primers 5' primer (CCA AGC TTA TGG ATC CTC CTG GAG AGA AGA TGG) and 3' primer (GCC TCG AGA AGC ACA TGT AGC CTG AC).

DNase I Footprint Analysis. DNase I footprint analyses were performed as described (19).

Electrophoretic-Mobility-Shift Assay (EMSA). An EMSA using Jurkat nuclear extracts or Agt11 or 6A2 crude lysogen extracts was performed as described (19) except 5 µl of lysogen extract (corresponding to 50 µl of bacterial culture) was used in place of nuclear extract and the binding buffer lacked NaCl. The synthetic oligonucleotide probes used in the EMSA were Tad1 (20) (CTCCCATTTCCATGACGTCAAGA AGC TTA TGG ATC CTC CTG GAG AGA AGA TGG).

Abbreviations: TCR, T-cell receptor; CRE, cAMP response element; EMSA, electrophoretic-mobility-shift assay; PKA, protein kinase A; PKC, protein kinase C; CAT, chloramphenicol acetyltransferase.

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CATGTTTACC), mutant Ta1 (mTa1) (CTCggATTTgt-TcACCaCATGgTCAc), somatostatin CRE (SOM) (21) (GCCTCCTGAGGTGAGTCATAG), mutant somatostatin CRE (mSOM) (GCCTCCTGAGGTGAGTCAc), c-fos CRE (c-fos) (22) (TGGTGGACCGGTCGAGTTTACCT), AP-1 (23) (AGCTTGTAGGTTGAGTTCCCGG).

Preparation and Screening of a Jurkat Agt11 cDNA Library. A Agt11 Jurkat T-cell cDNA library was screened for CRE-binding proteins using a radiolabeled Ta1 probe as described (24).

DNA Sequencing. DNA sequence analysis was performed by the dideoxynucleotide chain-termination method.

Northern Blot Analysis. RNA prepared from human tumor cell lines (25) and from mouse organs (18) was subjected to Northern blot analysis using a radiolabeled CREB-2 cDNA probe (26).

Transfections and CAT Assays. Monkey CV-1 cells (10⁶ cells per 100-mm dish) were transfected with calcium phosphate (18). One to 5 μg of a β-galactosidase containing plasmid (either pCH110 or pMSVβgal) was included in each transfection. Cell extracts equalized for protein content were used for CAT and β-galactosidase assays (19).

Preparation of CREB-2-Specific Antiserum. Rabbit anti-CREB-2 antiserum was prepared by three sequential immunizations with 10 μg of immunoaffinity-purified CREB-2-β-galactosidase fusion protein (26). This serum immunoprecipitated in vitro-translated CREB-2 protein but failed to immunoprecipitate CREB or CRE-BP1 proteins (data not shown). CREB-specific antiserum was the generous gift of Mark Montmey (The Salk Institute, La Jolla, CA).

RESULTS

The Ta1 Site of the Human T-Cell Receptor α Chain (TCRα) Enhancer Contains a CRE. We (20) and others (27) have identified and characterized a T-cell-specific transcriptional enhancer element located 4.5 kilobases (kb) 3′ of the α chain constant region gene in the human and murine TCRα loci. The minimal TCRα enhancer contains two nuclear protein binding sites, Ta1 and Ta2, both of which are necessary for basal enhancer activity (20). The Ta1 binding site contains a consensus CRE, TGACGTCA (1, 2). Similar CRE motifs have been identified in the promoter regions of all murine and human TCR β chain variable region genes (28) and in the minimal TCRβ transcriptional enhancer (29). These TCRβ promoter and enhancer CREs bind a set of nuclear proteins that is identical to that bound by the Ta1 element of the TCRα enhancer (29).

The human TCRα enhancer CRE motif (Ta1) was protected from DNase I digestion by both Jurkat T-cell and KS62 chronic myelogenous leukemia cell nuclear proteins (Fig. 1A). An EMSA using a radiolabeled Ta1 oligonucleotide and Jurkat nuclear extracts demonstrated at least three specific protein-DNA complexes of altered mobility (Fig. 1B, arrows). These complexes represented specific binding of nuclear proteins to the CRE motif because they were efficiently competed by both the wild-type unlabeled Ta1 competitor and by an unlabeled competitor containing the CRE from the human somatostatin gene (SOM) but not by mutant Ta1 (mTa1) or somatostatin competitors containing nucleotide substitutions within the CRE motifs of these elements (Fig. 1B). Moreover, the formation of these complexes was inhibited efficiently by CRE sequence motifs from the adenovirus E2 promoter and, to a lesser extent, by the CRE-like motifs from the human c-fos (Fig. 1B) and human T-cell leukemia virus type 1 promoters (data not shown) but not by the closely related AP-1 oligonucleotide from the collagenase promoter (Fig. 1B). Finally, the pattern of nuclear protein binding to a radiolabeled somatostatin promoter CRE motif was identical to that seen using the radiolabeled Ta1 oligonucleotide (Fig. 1C). Thus, these results demonstrated that Jurkat T cells contain several CRE binding activities.

Molecular Cloning of CREB-2 cDNAs. A CDNA expression approach (23) was used to isolate CREB-2 CDNAs from a Agt11 CDNA library prepared from Jurkat T-cell poly(A)+ RNA. One clone, 6A2, was identified that specifically bound multimerized radiolabeled Ta1 probe. This clone also bound a multimerized somatostatin CRE probe but not probes containing mutated CREs. To examine the specificity of binding of the recombinant fusion protein produced by this clone, lysogen extracts were used in an EMSA with radiolabeled Ta1 and somatostatin CRE probes. With both probes, the 6A2 lysogen extract produced a band of altered mobility that was not seen in control experiments using Agt11 lysogen extracts (Fig. 2). This band was competed specifically by excess unlabeled Ta1 (Fig. 2A and C) and somatostatin (Fig. 2B) CRE sites but not by mutated CRE binding sites (mTa1 or mSOM; Fig. 2A and C and data not shown) or by the consensus AP-1 oligonucleotide (Fig. 2B). Thus, the 6A2 cDNA encodes a fusion protein that binds specifically to the CREs in both the TCRα enhancer and the somatostatin promoter. We have designated this cDNA CREB-2.

CREB-2 Contains Leucine-Zipper and Basic Domains but Lacks PKA and Protein Kinase C (PKC) Phosphorylation Sites. The 6A2 cDNA was used as a hybridization probe to identify a 1.4-kb CDNA clone approximating the size of the CREB-2 mRNA (1.5 kb) (see Fig. 4). This full-length CREB-2 cDNA contains a single long open reading frame of 1053 nucleotides and encodes a 351-amino acid protein of predicted molecular mass = 38.5 kDa (Fig. 3A). The COOH-
terminal end of the predicted CREB-2 protein contains a leucine-zipper motif and an adjacent basic domain that have been shown to be involved in the dimerization and DNA binding, respectively, of a number of other transcriptional regulatory proteins including c-fos, c-jun, several jun- and fos-related proteins—CEBP (30-33), CREB (7, 11), CRE-BP1 (also called HB16 and ATF-2) (6, 9, 10), and ATFs 1 to 6 (6). Of note, the amino acid sequence of the COOH-terminal region of CREB-2 is identical to that predicted from the partial-length cDNA, ATF-4 (6). However, because only the amino acid sequence of a partial-length ATF-4 cDNA has been reported, it is impossible to conclude definitively that these two cDNAs are identical. Unlike CREB and CRE-BP1, the NH2-terminal region of CREB-2 lacks potential PKA and PKC phosphorylation sites.

CREB-2 is Expressed Ubiquitously in Tumor Cell Lines and Mouse Organs. Northern blot analyses using RNA derived from a variety of human tumor cell lines and normal mouse organs demonstrated that a single 1.5-kb CREB-2 transcript was expressed at high levels in all of the tumor cell lines studied (Fig. 4A). In addition, the Epstein-Barr virus-transformed B-cell line clone 13 contained a 1.2-kb CREB-2 mRNA. The structure of this second transcript remains unclear. CREB-2 mRNA was also detected in all of the mouse organs assayed (Fig. 4B).

Overexpression of CREB-2 Represses CRE-Dependent Transcription. To better understand the transcriptional activity of the CREB-2 protein, we constructed eukaryotic expression vectors containing the CREB-2, and PKA catalytic subunit cDNAs (Fig. 5A). These vectors were used to overexpress the relevant proteins after transfection into cAMP-responsive monkey CV-1 cells. The pENKAT-12 plasmid in which CAT transcription is under the control of the CRE-containing human enkephalin promoter (Fig. 5A) was included in each transfection as a reporter for CRE-dependent transcription. Indirect immunofluorescence analyses of cells transfected with CREB and CREB-2 vectors demonstrated that (i) both CREB and CREB-2 are expressed at relatively low levels in the nuclei of untransfected CV-1 cells, (ii) CREB-2 is expressed in a more restricted pattern, and (iii) overexpression of CREB-2 represses CREB-dependent transcription.

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Identification and characterization of the 6A2 CREB-2 cDNA. A Jurkat T-cell Agt11 cDNA library was screened with a radiolabeled multimerized Tα1 oligonucleotide probe. One clone, 6A2, was identified that bound to multimerized Tα1 and somatostatin CRE (SOM) probes but not to multimerized mutant Tα1 oligonucleotide (mTα1) or mutant somatostatin oligonucleotide (mSOM) probes. EMSAs of 6A2 CREB-2 lysogen extracts. Extracts prepared from Escherichia coli Y1089 lysogenized with either wild-type Agt11 (gtll) or the 6A2 CREB-2 cDNA clone were used in EMSAs with radiolabeled Tα1 (A and B) or somatostatin (SOM) CRE (C) probes. Unlabeled competitor oligonucleotides were added to some binding reaction mixtures as shown. The Tα3 oligonucleotide (AGAGATAGCATCGCCCCAGGCCACGTGCCGAGGG) from the human TCRα enhancer (20) was used as a negative control in the competition experiments.

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Structure of CREB-2. A schematic diagram of the CREB-2 cDNA and protein is shown at the top. The 5' untranslated region is shown as a solid box, the open reading frame is shown as an open box, and the 3' untranslated region is shown as a lightly shaded box. The leucine-zipper and basic domains are labeled. The deduced amino acid sequences of the leucine-zipper and basic domains of CREB-2 are also shown.

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Northern blot analysis of CREB-2 gene expression. (A) CREB-2 expression in human tumor cell lines. Poly(A)+ RNA (0.5 µg) from Jurkat and PEER human T cells, K562 chronic myelogenous leukemia cells, clone 13 Epstein-Barr virus-transformed B cells, and HeLa cells was subjected to Northern blot analysis using a radiolabeled 6A2 CREB-2 cDNA probe. (B) CREB-2 gene expression in mouse organs. Size markers in kb are shown to the left of each autoradiogram.
cells and (ii) transfection with either the pCDM7/CREB or pCDM7/CREB-2 expression vectors resulted in high-level nuclear expression of the appropriate protein in 1-2% of the cells in each culture (data not shown).

A set of transient transfections was performed to determine the effects of overexpression of CREB and CREB-2 on CRE-dependent transcription in these cells. Transfection of the pENKAT-12 reporter plasmid alone resulted in low-level CAT activity that was normalized to a value of 1 (Fig. 5B). Overexpression of PKA increased CAT activity by 39-fold. This finding, which apparently reflects the activation of endogenous CREB proteins by PKA, was consistent with previous experiments that have demonstrated that the enkephalin promoter is cAMP-responsive and that this responsiveness is mediated by the activation of PKA (14). Overexpression of CREB in conjunction with PKA caused a further 4-fold increase in CRE-dependent transcription (Fig. 5B). This increase was specific for the enkephalin promoter and not simply the result of a generalized increase in transcription because overexpression of CREB had no effect on transcription from the cotransfected pMVS/βgal and pCH110 β-galactosidase reference plasmids. These results were in accord with previous studies that have demonstrated that CREB is a transcriptional activator that requires phosphorylation by PKA for its activity (12).

In contrast to the results observed after overexpression of CREB, overexpression of CREB-2 in conjunction with PKA suppressed CAT transcription back to baseline levels (i.e., to levels similar to those produced by transfection with pENKAT-12 alone; Fig. 5B), suggesting that, unlike CREB, CREB-2 functions as a transcriptional repressor in this system. Several control experiments were performed to demonstrate the specificity of this transcriptional repressor effect. (i) The level of transcriptional repression was shown to be proportional to the amount of CREB-2 expression vector transfected (Fig. 5C). (ii) The CREB-2-mediated transcriptional repression was shown not to be the result of feedback of CREB-2 on the pCMVCA PKA expression vector, because overexpression of CREB-2 also repressed CRE-dependent transcription in transfections utilizing a PKA expression vector containing the metallothionein promoter and in transfections lacking a PKA expression vector (data not shown). In addition, this transcriptional repression was not the result of competition between the cytomegalovirus promoters contained on the pCDM7/CREB-2 and pCMVCA plasmids, because the total amount of DNA in the transfections was held constant by the addition of the appropriate amount of the pCDM7 expression plasmid that contains the cytomegalovirus promoter but lacks a cDNA insert. Most importantly, the transcriptional repressor effect of CREB-2 was specific for the enkephalin promoter and not the result of generalized toxicity of CREB-2 or pCDM7 because the activity of the pCH110 β-galactosidase reference plasmid, which was included in each transfection, was not significantly decreased by the overexpression of CREB-2 (data not shown). Thus, the overexpression of CREB-2 results in the specific repression of CRE-dependent transcription in CV-1 cells.

**Localization of the Transcriptional Repressor Domain of CREB-2.** To better localize the transcriptional repressor domain of the CREB-2 protein, a PCR was used to prepare a CREB-2 deletion mutant that contained only the COOH-terminal 102 amino acids including the entire leucine-zipper and basic domains. Overexpression of the truncated version of CREB-2 (CREB-2a) resulted in the complete repression of CRE-mediated transcription in CV-1 cells (Fig. 5B).

**DISCUSSION**

The studies described in this report have identified and characterized an ATF/CREB family member, CREB-2. CREB-2 is related to, but distinct from, the previously described CREB and CRE-BP1 proteins but may be identical to ATF-4. Although all of the ATF/CREB proteins including CREB-2 share highly related basic and leucine-zipper domains, CREB-2 lacks the PKA and PKC phosphorylation sites and the α-helical transcriptional activator domain found in the NH₂ terminus of CREB. Unlike CREB, which activates transcription from CRE-containing promoters, CREB-2 functions as a specific repressor of CRE-dependent transcription. Deletional analyses demonstrated that the transcriptional repressor activity of CREB-2 is contained within a 102-amino acid COOH-terminal region of the molecule (amino acids 249–351).

Several distinct molecular mechanisms could be responsible for the transcriptional repressor effects of CREB-2, including the possibility that CREB-2 monomers or heterodimers can bind to CRE sites thereby displacing CREB activator proteins, and the possibility that CREB-2 can form heterodimers with other ATF/CREB activator proteins thereby preventing DNA binding and/or transcriptional activation (squelching). Both of these models would be consistent with the deletional analyses that demonstrated that the
transcriptional repressor function of CREB-2 is retained by a truncated protein that contains the leucine-zipper and basic domains of the molecule that have been implicated in the dimerization and DNA binding, respectively, of a number of related transcriptional regulatory proteins. Of note, an EMSA using in vitro-translated proteins has demonstrated that CREB-2 cannot dimerize with CREB, CRE-BP1, c-jun, or c-fos (data not shown).

What is the physiological role of CREB-2 in regulating CRE-dependent transcription? Because it is expressed ubiquitously, it may serve the general function of preventing “leaky” transcription from CRE-containing promoters until appropriate levels of phosphorylated CREB (or other CREB activator proteins) have been generated in response to elevations of intracellular cAMP. In addition, because CREs can modulate basal levels of transcription in a cAMP-independent fashion, CREB-2 might represent a negative regulator of cAMP-independent CRE-mediated transcription, similar to the recently described CREM transcription factor (34). The availability of structurally and functionally characterized full-length ATF/CREB cDNA clones should facilitate future studies aimed at better understanding the interaction of these proteins and their roles in regulating cAMP-dependent and -independent gene expression.

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