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# Identification and Functional Analysis of Novel cAMP Response Element Binding Protein Splice Variants Lacking the Basic/Leucine Zipper Domain

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## ABSTRACT

Two novel cAMP response element binding protein (CREB) splice variants were found by reverse transcription-polymerase chain reaction cloning by using mouse brain RNA as a template. One splice variant, named Δ-14, lacks 14 nucleotides at the beginning of exon 9 of the CREBΔ isoform. The other, named Δ-35, lacks 35 nucleotides at the beginning of exon 8 of CREBΔ. These nucleotide deletions cause frame shifts for codon usage, producing proteins which conserve the major phosphorylation site ($Ser^{133}$) but lack the basic/leucine zipper domain, which is essential for binding to DNA and to other transcription factors. Both variants are widely expressed in peripheral tissues, but are enriched in brain, thymus, and testis. CREBΔ-14 and Δ-35 variant proteins were expressed by using an in vitro translation system and by transfecting into human embryonic kidney 293 cells. Both variants were detected by a CREB antibody that recognizes the CREBΔ amino terminus, but not by an antibody which recognizes the CREBΔ carboxy terminus, as would be predicted based on the frame shift. Activation of the cAMP pathway increased phospho-CREB immunoreactivity, indicating that these variants are substrates of cAMP-dependent protein kinase. In addition, immunocytochemical analysis demonstrated that CREBΔ-14 and Δ-35 are primarily cytosolic, whereas CREBα is predominantly in the nucleus. Finally, expression of CREBΔ-14 or Δ-35 decreased cAMP responsive element-chloramphenicol acetyltransferase reporter activity, demonstrating that both can function as repressors of endogenous CREB.

Various extracellular signals such as neurotransmitters and hormones influence cellular function by increasing the intracellular cAMP cascade. This second messenger cascade includes activation of cAMP-dependent protein kinase (PKA) and regulation of gene transcription via cAMP response elements (CRE; Ziff, 1990) in the promoter regions of target genes. The CRE binding protein (CREB) mediates the action of the cAMP cascade on gene expression (Hoeffler et al., 1988; Yamamoto et al., 1988; Gonzalez and Montminy, 1989). In addition to CREB, there are several other CRE binding proteins that form the CREB/activating transcription factor (ATF) family (Maekawa et al., 1989; Hai and Curran, 1991).

Phosphorylation of CREB at $Ser^{133}$ by PKA initiates CREB transactivation. Phosphorylated CREB binds to a coactivator protein, CREB binding protein (Chrivia et al., 1993), which then binds to a transcription factor/RNA polymerase II complex, which directly transactivates the target gene (Kwok et al., 1994). The $Ser^{133}$ residue can be phosphorylated not only by PKA, but also by other kinases, including CaM kinase II and IV (Dash et al., 1991; Matthews et al., 1994), indicating that intracellular elevation of calcium can also transactivate target genes via CREB. In the CREB/ATF family the basic/leucine zipper (bZIP) motif in the C terminus region is highly conserved. The bZIP motif is essential for DNA binding and heterodimerization of the CREB/ATF proteins (Johnson and McKnight, 1989). In addition, the bZIP domain is necessary for translocation of CREB into the nucleus (Waeber and Habener, 1991).

A study of the mouse CREB gene has revealed that it consists of 11 exons and multiple variants produced by alternative splicing (Cole et al., 1992; Ruppert et al., 1992). Following that report, additional splice variants of mammalian CREB have been identified, some of which exhibit tissue specific expression (Waeber and Habener, 1992; Ellis et al., 1995; Blendy et al., 1996; Girardet et al., 1996; Walker et al., 1996; Youg et al., 1997). The major identified CREB isoforms, α, β, and Δ (Ruppert et al., 1992; Blendy et al., 1996) have all of the three domains which are essential for the transactivation function of CREB, including a glutamine rich domain (Q domain), a kinase inducible domain (KID domain), and a bZIP domain.

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**ABBREVIATIONS:** PKA, cAMP-dependent protein kinase; CRE, cAMP response elements; CREB, cAMP responsive-element binding protein; HEK293, Human embryonic kidney 293 cells; KID, kinase-inducible domain; ATF, activating transcription factor; CMV, cytomegalovirus.

In contrast, some members of the CREB/ATF family have the bZIP domain but lack the glutamine rich Q and kinase-inducible domains (KIDs), and act as repressors of CREB transactivation (Walker et al., 1996). Examples of these are the I-CREBs and ICER, an isoform of cAMP response element modulator. In addition, CREB variants that lack the bZIP domain also have been reported (Ruppert et al., 1992; Waeber and Habener, 1992; Ellis et al., 1995; Youg et al., 1997; Bartsch et al., 1998), although the functional significance of these proteins is still controversial. These variants are localized primarily to the cytosol due to their lack of a bZIP domain (Waeber et al., 1991; Bartsch et al., 1998).

CREB plays an important role in numerous cellular functions (Lalli and Sassone-Corsi, 1994). In the brain, CREB is reported to play a role in circadian rhythm and formation of learning and memory (Ginty et al., 1993; Milner et al., 1998). Furthermore, the function and expression of CREB in specific brain regions has been implicated in the neuronal adaptations or plasticity due to chronic psychotropic drug treatments (Nibuya et al., 1996); Duman et al., 1997; Nestler and Aghajanian, 1997). To further characterize the expression of CREB variants in the brain, reverse transcriptase-polymerase chain reaction (RT-PCR) cloning of CREB variants was performed. Two novel splice variants, which are predicted to lack the bZIP domain, but conserve the KID domain, were identified.

## Materials and Methods

**RT-PCR Cloning of CREB Variants.** Total RNA was extracted from mouse brain (striatum) using the RNAquous kit (Ambion, Austin, TX) according to the manufacture's standard protocol. A sense primer (designated primer 8; Fig. 1) and an antisense primer (designated primer 9; Fig. 1), which recognize exons 4 and 9 of the mouse CREB, respectively, were designed for RT-PCR. The sequence of primer 8 was 5′-CAGTCTCCACAAGTCCAAACAGTT-3′, and that for primer 9 was 5′-GTAGAATGGTAGTACCCGGCTGA-3′. RT-PCR was carried out with the striatal RNA as template by using the Access RT-PCR system (Promega, Madison, WI), according to the manufacture's recommended method. RT-PCR products of the primer 8–9 set were subjected to agarose gel separation and visualized by ethidium bromide staining. Several RT-PCR products of the predicted size [434 base pairs (bp) and 392 bp] of known variants (CREB$\alpha$ and CREBΔ) were observed. In addition, several products of unpredicted size were also observed (data not shown). These RT-PCR products were isolated by using a gel extraction kit (Qiagen, Chatsworth, CA), subcloned into pGEM-T Easy vector (Promega) and verified by sequencing. Two distinct plasmids, encoding novel splice variants of the CREBΔ isoform, were isolated and designated CREBΔ-14/8–9 and CREBΔ-35/8–9 (Fig. 1 and Results).

**RNase Protection Assay (RPA) of Splice Variants.** RNase protection analysis was performed by RPAII kit (Ambion) according to the manufacture's standard protocol, with total RNA extracts from various mouse tissues as templates. The riboprobes were generated by linearizing CREBΔ-14 or Δ-35 plasmids with *Nco*I and $^{32}$P-labeled using SP6 RNA polymerase (Boehringer Manheim, Indianapolis, IN). Protected fragments were loaded onto an 8% Acrylamide-TBE gel and the separated bands were detected by autoradiography. Measurement and quantification of protected band density were carried out by using the Macintosh-based NIH image analysis program (version 1.52).

**Subcloning of CREB Variants into Expression Plasmids for Mammalian Cells.** To obtain the cDNAs encoding CREBΔ-14 or Δ-35 with initiation sites of CREBΔ isoform, another RT-PCR was performed using primer set 6–7 (Fig. 1). The sense (6) and antisense (7) primers were 5′-CTAAATGACCATGGAATCTGGAGCA-3′ and 5′-AGTTACACTATCCACAGACTCCTG-3′, respectively. RT-PCR products encoding CREBΔ isoforms were identified based on their predicted size and subcloned into a pGEM-T Easy vector (designated as CREBΔ/6–7). To subclone CREBΔ14 or CREBΔ35 cDNA with the CREBΔ initiation site, ExoRI/PleI fragments of CREBΔ/6–7 and PleI/*Eco*RI fragments of CREBΔ14/8–9 or CREBΔ−35/8–9, respectively, were ligated into the ExoRI sites of the pCI plasmid, an expression plasmid for mammalian cells with a CMV promoter. To subclone CREBΔ-14 and Δ-35 cDNA containing the CREBΔ initiation site into pCI (Promega), an expression plasmid for mammalian cells with a CMV promoter, *Eco*RI/PleI fragments of CREBΔ/6-7 and PleI/*Eco*RI fragments of CREBΔ-14/8-9 and CREBΔ-35/8-9, respectively, were ligated into the *Eco*RI site of pCI plasmids.These plasmids were designated as CREBΔ-14 pCI and CREBΔ-35 pCI, respectively.

To express FLAG-tagged CREBΔ-14 or CREBΔ-35 protein in mammalian cells, PCR was performed using CREBΔ-14 pCI or CREBΔ-35 pCI as templates. The sense primer used was 5′-TTGAATT CATGACCATGGAATCTGGAGCA-3′. The antisense primers used for CREBΔ-14 was 5′-TTGAATTCTTAGCCAGCTGTATTGCTCCT-3′ and for CREBΔ-35 was 5′-TTGAATTCTCAATCCTTGGCACCCCTGTA-3′. These PCR fragments, which contain the coding region of CREBΔ-14 or CREB Δ-35 isoforms, were digested with *Eco*RI and subcloned into the *Eco*RI site of pTB701 FL, an expression plasmid used to fuse the FLAG peptide to the N terminus of the target protein (Kuroda et al., 1996). The plasmids obtained were designated CREBΔ-14 FL and CREBΔ-35 FL, respectively. All PCR products used were verified by sequencing after the subcloning into pGEM-T Easy.

**Cell Transfection and CAT Assay.** Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle medium containing 25 mM glucose, which was buffered with 44 mM NaHCO3 and supplemented with 10% fetal bovine serum, in a humidified atmosphere containing 5% $CO_2$ at 37°C. Transfection of plasmids was performed by lipofection with TransIT-LT2 reagent (Panvera, Madison, WI), into subconfluent 293 cells (approximately $6 \times 10^6$ cells/10 cm dishes). For Western blotting, cells were reseeded into two or three 10-cm dishes 16 h after the transfection and were harvested 48 h later. For CAT assays, 5 $\mu$g of CRE-CAT constructs (Montominy et al., 1986) plus 15 $\mu$g of either CREB pCI variant or pCI vector alone plus 1 $\mu$g pCMV-$\beta$gal (Promega) were cotransfected into 293 cells. Cells were reseeded into six-well dishes (3.0 cm in diameter) 24 h after the transfection. After the transfection, cells were allowed to settle on the dishes (approximately 8 h after reseeding), then treated with or without forskolin (5 $\mu$M; Sigma Chemical Co., St Louis, MO) for 16 h, and then the CAT assay was carried out by CAT enzyme assay system (Promega) as described previously (Widnell et al., 1996a). $\beta$-galactosidase activity was simultaneously measured by the $\beta$-galactosidase enzyme assay system (Promega) and CRE-CAT activity was normalized to the $\beta$-gal activity.

**Western Blotting, Immunocytochemistry and In Vitro Transcription/Translation.** Transfected or untransfected cells were washed and harvested with isotonic homogenate buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 50 mM Tris/HCl, 200 $\mu$g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4), then lysed with RIPA buffer (10 mM Tris-HCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 200 $\mu$g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at 19,000 $\times$ *g* for 15 min. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis. Western blotting was carried out as described previously (Takahashi et al., 1999). For phospho-CREB immunoblotting, cells were harvested after treatment with 50 $\mu$M forskolin for 10 min. Phosphatase inhibitors (1 mM $Na_3VO_4$, 1 mM NaF and 100 nM calyculin A) were added to homogenates and RIPA buffer. Immunoblotting for N- and C-terminal CREB was performed using polyclonal CREB (Upstate Biotechnology Incorporated, Lake Placid, NY; 1:500 dilution) and monoclonal CREB (X-12; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 di-

lution) antibodies, respectively. Immunoblotting for phospho-CREB was performed using polyclonal phospho-CREB antibody (New England Biolabs, Beverly, MA; 1:500 dilution).

For FLAG immunocytochemistry, transfected cells were reseeded into a Lab-Tek chamber slide (Nalge Nunc International, Naperville, IL). FLAG immunoreactive cells were detected with monoclonal anti-FLAG antibody (M5; Sigma; 1:1000 dilution) as a primary antibody and Cy3-conjugated anti-mouse IgG (Amersham, Buckinghamshire, UK) as a secondary antibody, according to methods described previously (Tolbert and Lameh, 1996). Fluorescence of FLAG-immunoreactivity was observed by fluorescence microscopy.

In vitro transcription/translation was carried out by the TNT quick coupled transcription/translation system (Promega) using CREBΔ-14 pCI and CREBΔ-35 pCI as templates. [$^{35}$S]Methionine-labeled proteins were subjected to SDS-polyacrylamide gel electrophoresis and were detected by autoradiography.

## Results

**Sequence Analysis and Predicted Structure of Novel CREB Splicing Variants.** By using RT-PCR and total RNA extracted from mouse striatum as a template, we have isolated two cDNAs which encode novel CREB splice variants. The primers used for RT-PCR were derived from the nucleotide sequence of exons 4 and 9 of the mouse CREB gene (Fig. 1). Both variants lack exon 5, suggesting that they are most closely related to the CREBΔ isoform, which also lacks this exon. One splice variant, referred to as CREBΔ-14, also lacks 14 nucleotides at the beginning of exon 9, around the boundary of intron 8 and exon 9 (Fig. 1). Deletion of 14 nucleotides would result in a change of codon usage, including termination of translation in exon 9. Based on this analysis and the initiation site in exon 2, which is the same as that used for CREB$\alpha$ and Δ isoforms, CREBΔ-14 is predicted to have 175 amino acids.

The other splice variant, referred to as CREBΔ minus 35 (CREBΔ-35), lacks 35 nucleotides at the beginning of exon 8, around the boundary of intron 7 and exon 8 (Fig. 1). This deletion in CREBΔ-35 would also cause a frame shift resulting in termination of protein translation in exon 8. The predicted number of amino acids in CREBΔ-35 is 122, based on the use of the same translation initiation site in exon 2 discussed for CREBΔ-14 and the CREB$\alpha$ and Δ isoforms.

The splicing rule for acceptor and donor sites is kept in both CREBΔ-14 and Δ-35 (i.e., the end of the deleted nucleotide sequence in each case is "AG"; Fig. 1). In addition, both CREBΔ-14 and Δ-35 translated proteins are predicted to lack the bZIP domain, which is encoded in exon 11, but would retain the conserved PKA phosphorylation site at Ser$^{133}$ in exon 7.

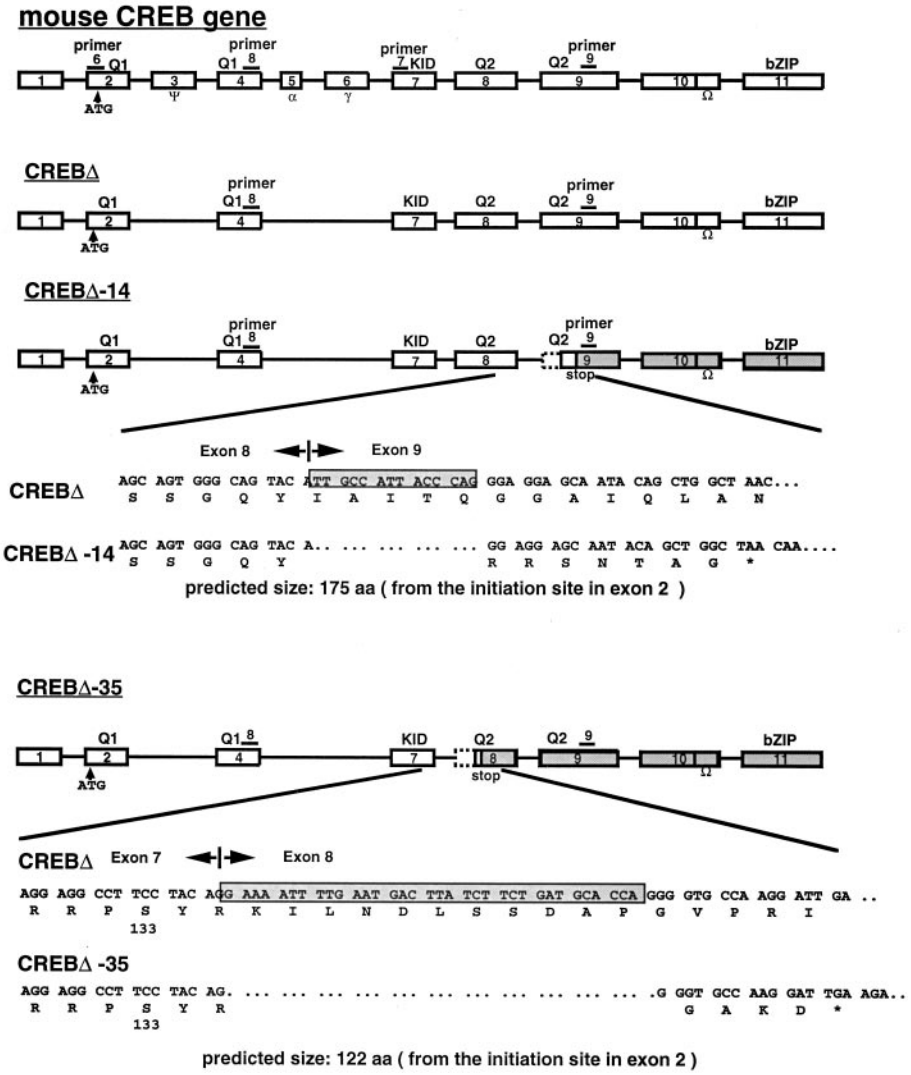


**Fig. 1.** Identification, sequence analysis, and predicted structure of the CREBΔ-14 and CREBΔ-35 variants. A schematic of the mouse CREB gene is shown at the top with the exons depicted as the boxed areas. Below are diagrams of CREBΔ, as well as the novel splice variants CREBΔ-14 and CREBΔ-35. In the CREBΔ-14 variant, 14 nucleotides are deleted at the beginning of exon 9 around the boundary of intron 8 and exon 9. For the CREBΔ-35, variant 35 nucleotides are deleted at the beginning of exon 8 around the boundary of intron 7 and exon 8. In both variants, new translation termination codons are predicted near the deleted region that would result in CREB variants lacking the bZIP domain. The shaded boxes indicate the coding regions that variants would lack. Boxed and shaded nucleotides in CREBΔ isoform are the nucleotides that are deleted in either CREBΔ-14 or CREBΔ-35. The nomenclature used here is for the mouse CREB gene according to Ruppert et al. (1992). Note that in each case, the deletion is followed by standard acceptor and donor sites. Q1, glutamine rich region 1; Q2, glutamine rich region 2; KID, kinase-inducible domain; bZIP, basic/leucine zipper domain

**Tissue Distribution of CREBΔ-14 and CREBΔ-35 Splice Variants.** To characterize CREBΔ-14 and Δ-35 expression, we analyzed levels of mRNA for each variant in different tissues. Expression was determined by RPA with antisense riboprobes derived from CREBΔ-14 or Δ-35 cDNAs, referred to as CREBΔ-14/8-9 and CREBΔ-35/8-9, respectively. The predicted fragment size for each CREB isoform in the RPA is summarized in Fig. 2A. As shown in Fig. 2B, CREBΔ-14 and Δ-35 are expressed in all tissues examined, although at different levels, providing evidence for widespread distribution of both variants. CREBΔ-14 and Δ-35 were found to be enriched in most brain regions, particularly cerebellum, as well as in thymus and testis, relative to other peripheral tissues; the CREBΔ and $\alpha$ isoforms are also expressed at relatively high levels in these tissues. Both variants are expressed at lower levels in the other tissues examined, including lung, heart, liver, spleen, and kidney. Note that larger amounts of total RNA were used for these latter tissues, due to lower levels of expression, to observe clear expression of the variants.

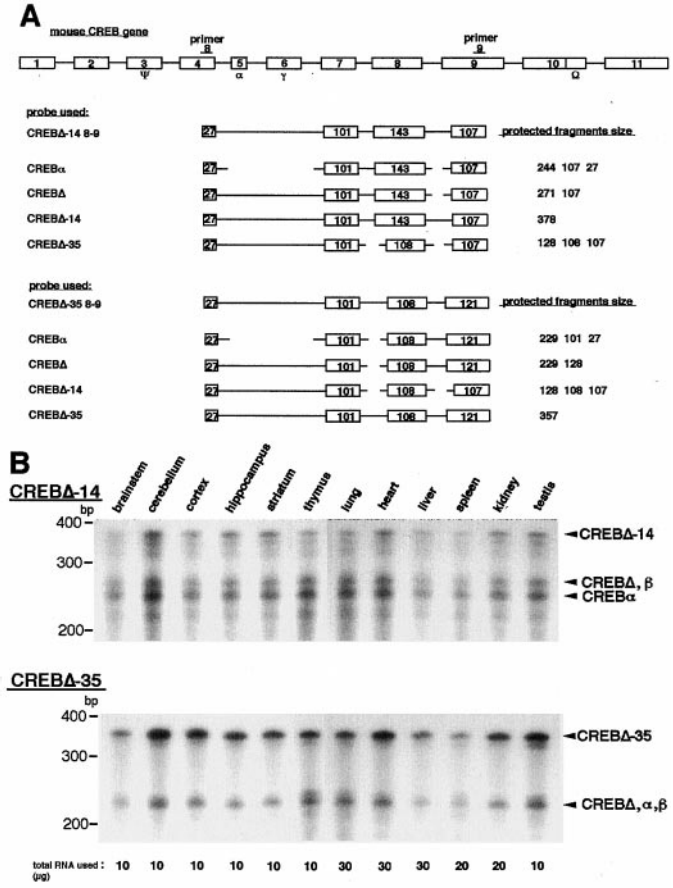


**Fig. 2.** Schematic of the CREB gene and riboprobes used for analysis of CREBΔ-14 and CREBΔ-35 expression in brain and peripheral tissues. A, structure of the CREB gene is shown at the top and that for the CREBΔ-14/8-9 and CREBΔ-35/8-9 riboprobes is shown below. The protected RNA hybrids for CREB$\alpha$, CREBΔ, CREBΔ-14, and CREBΔ-35 that are predicted to result from RPA with the CREBΔ-14/8-9 and CREBΔ-35/8-9 riboprobes are shown. The sizes (bp) of the protected hybrids are shown on the right. The predicted size of CREBΔ-14 is 378 bp and that for CREBΔ-35 is 357 bp when using the respective riboprobes for each of variant. B, tissue distribution of CREBΔ-14 and CREBΔ-35 mRNA. Expression of CREBΔ-14 and CREBΔ-35 mRNA is enriched in brain, including the cerebellum, cerebral cortex, hippocampus, striatum, and brainstem. Levels of these variants are also enriched in thymus and testis, and are widely expressed in the other tissues examined. Note that larger amounts of total RNA were used for some of the peripheral tissues, including lung, heart, liver, spleen, and kidney, so that a significant signal could be observed.

**Expression of CREBΔ-14 and CREBΔ-35 Protein.** To characterize the structural and functional properties of the CREB variants, expression vectors for CREBΔ-14 and Δ-35 were prepared. As described in *Materials and Methods*, RT-PCR was used to isolate the 5′ coding region of the CREBΔ isoform, including the translation initiation start site, which was then subcloned separately with each of the original RT-PCR products into a pCI expression plasmid. Sequence analysis confirmed that the expression plasmids contained the predicted 5′ coding region, as well as the coding region for each variant. CREBΔ-14 and Δ-35 were first expressed by using an in vitro transcription/translation system under the control of T7 promoter. The CREBΔ-14 and Δ-35 proteins have molecular masses of approximately 27 and 22 kDa, respectively (Fig. 3A).

As shown in Fig. 1, the predicted sequence of the CREBΔ-14 and Δ-35 proteins lack the bZIP domain at the C terminus. To determine whether this is the case, antibodies specific to the amino and carboxy terminus of CREB were used for immunoblot analysis of CREBΔ-14 and Δ-35 after transfection into HEK293 cells. As shown in Fig. 3B, CREBΔ-14 and Δ-35 proteins were detected by the CREB antibody, which recognizes the amino terminus, but not by the antibody which recognizes the carboxy terminus. The molecular masses of the CREBΔ-14 and Δ-35 were 27 and 21.5 kDa, respectively, similar to the sizes observed for the products of the in vitro transcription/translation system.

One more prediction based on sequence analysis is that the $Ser^{133}$ phosphorylation site is conserved in both CREBΔ-14 and Δ-35 (see Fig. 1). To determine whether this is the case, a phospho-CREB specific antibody was used for immunoblot analysis of the CREBΔ-14 and Δ-35. After transfection, HEK293 cells were treated with forskolin to activate the cAMP-PKA signaling cascade, and levels of phospho-CREB were determined. The phospho-CREB antibody is directed against the $Ser^{133}$ phosphorylation site found in CREB and CREB related transcription factors, ATF1 and cAMP response element modulator. As shown in Fig. 3C, forskolin treatment increased levels of phospho-CREB immunoreactivity for bands of 27 and 21.5 kDa in cells transfected with CREBΔ-14 and Δ-35, but not in cells transfected with vector plasmid alone. Forskolin treatment also increased several immunoreactive bands of larger molecular weight that correspond to the size of endogenous CREB and ATF-1 isoforms. These phosphorylated bands were observed in both the CREBΔ-14 and Δ-35 transfected, as well as nontransfected, cells as expected.

To examine whether these two variants are expressed in native tissues at the protein level, immunoblot analysis was conducted by using the antibody that recognizes the N-terminus of CREB. As shown in Fig. 3D, an immunoreactive band that comigrates with recombinant CREB Δ-14 or Δ-35 is seen in every brain region examined, as well as in two peripheral tissues, demonstrating the presence of CREBΔ-14- and Δ-35-like proteins.

**Localization of CREBΔ-14 and Δ-35 in Transfected Cells.** To examine the localization of the CREB variants in transfected HEK293 cells, we constructed plasmids for expression of FLAG-tagged versions of the CREB variants (re-

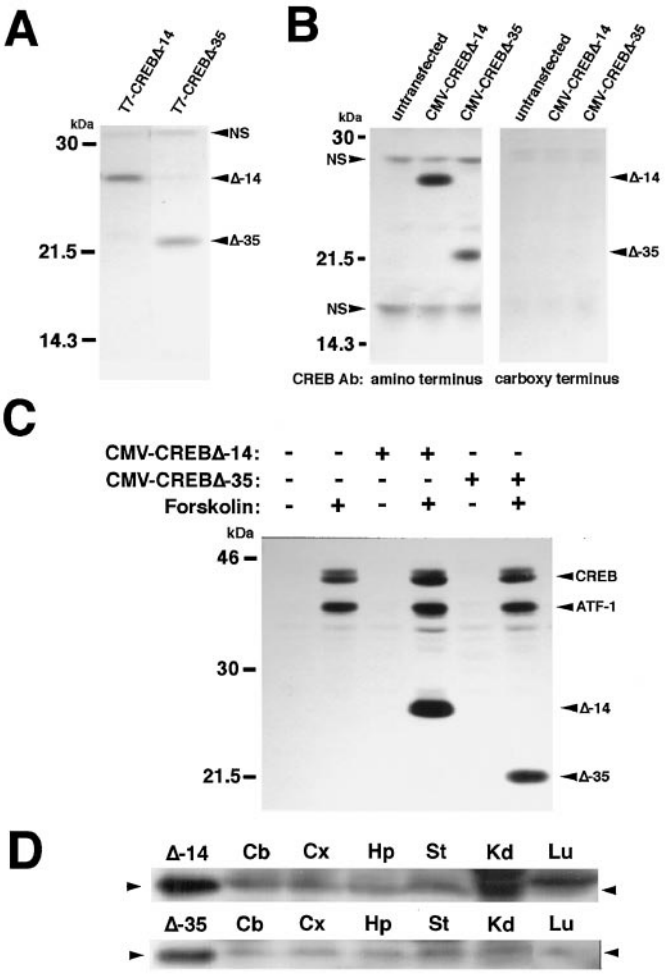


**Fig. 3.** Expression of recombinant CREBΔ-14 and CREBΔ-35 proteins. A. Expression of CREBΔ−14 and CREBΔ-35 protein using an in vitro translation/transcription system and the expression vectors CREBΔ-14 pCI and CREBΔ-35 pCI. In these vectors, expression is under the control of the CMV promoter. Immunoblot analysis with a CREB amino terminus antibody demonstrates that CREBΔ-14 and CREBΔ-35 have molecular masses of approximately 27 and 22 kDa, respectively. B, expression of CREBΔ-14 and CREBΔ-35 proteins in mammalian cells. The CREBΔ-14 pCI and CREBΔ-35 pCI expression vectors were transfected into HEK293 cells, and immunoblot analysis was conducted with CREB antibodies selective for either the amino- or carboxy terminus. The new stop codon resulting from the nucleotide deletions predict that the CREBΔ-14 and Δ-35 variants will have a truncation of the carboxy terminus and would not be reactive to the antibody selective for this portion of CREB. This is confirmed by the presence of immunolabeled bands of the appropriate size detected with the antibody selective for the amino, but not the carboxy terminus of CREB. C, CREB Δ-14 and CREBΔ-35 are phosphorylated by activation of PKA. The variants retain the consensus phosphorylation site ($Ser^{133}$). To determine whether these sites undergo phosphorylation, CREBΔ-14- or CREBΔ-35-transfected cells were treated with or without 50 $\mu$M forskolin for 10 min and levels of phospho-CREB immunoreactivity were determined. The results demonstrate the presence of phospho-CREB immunoreactive bands at the appropriate molecular weight in response to forskolin treatment, but not under basal conditions. Higher-molecular-weight bands, which most likely represent CREB and ATF-1, also were observed in the presence of forskolin. D, the expression of CREBΔ-14 and Δ-35 protein in native tissues was determined by immunoblot analysis with the antibody selective for the amino terminus of CREB. Shown are representative immuonoblots of recombinant CREBΔ-14 or Δ-35 protein and lysates from various mouse brain regions (100 $\mu$g of protein) and peripheral tissues (150 $\mu$g of protein). The regions examined included: Cb, cerebellum; Cx, cortex; Hp, hippocampus; St, striatum; Kd, kidney; and Lu, lung. Immunoreactive bands which comigrate with either recombinant CREBΔ-14 or Δ-35 protein were detected.

ferred to as CREBΔ-14FL and CREBΔ-35FL; see *Materials and Methods*). We also transfected FLAG-tagged CREB$\alpha$ cDNA (a gift from Dr. M.Greenberg, Harvard Medical School, Cambridge, MA) as a control. FLAG immunocytochemistry was performed in cells transfected with FLAG-CREBΔ-14, FLAG-CREBΔ-35 and FLAG-CREB$\alpha$. In cells expressing either FLAG-CREBΔ-14 or FLAG-CREBΔ-35, FLAG-immunoreactivity was seen predominately in the cytosol and rarely in the nucleus (Fig. 4). These observations are consistent with the prediction that the CREB variants are not translocated to the nucleus because they lack the bZIP domain. In contrast, in cells transfected with FLAG-CREB$\alpha$, FLAG immunoreactivity was observed largely in the nucleus, although immunoreactivity in the cytosol was also observed (Fig. 4).

**Effects of CREBΔ-14 and CREBΔ-35 on CRE-Reporter activity.** To study the functional characteristics of the CREB variants, we cotransfected each with a CRE-CAT reporter construct into HEK293 cells. Basal and forskolin-stimulated CRE-CAT activity was determined in the cotransfected cells. Forskolin incubation (5 $\mu$M) increased levels of CRE-CAT activity by more than 4-fold, relative to basal reporter activity (Fig. 5). Overexpression of either CREBΔ-14 or Δ-35 repressed CRE-CAT activity (Fig. 5). The inhibitory effects of the CREB variants were more prominent on basal CRE-CAT than on forskolin-stimulated activity (basal CRE activity: 100 ± 5.7; Δ-14, 43 ± 3.3; Δ-35, 31 ± 3.4; forskolin-stimulated CRE-CAT activity: control, 444 ± 21; Δ-14, 341 ± 20; Δ−35, 319 ± 12; mean ± S.E.M. percentage of control, basal CRE-CAT activity) (Fig. 5).

To futher examine the effect of the CREB variants on the

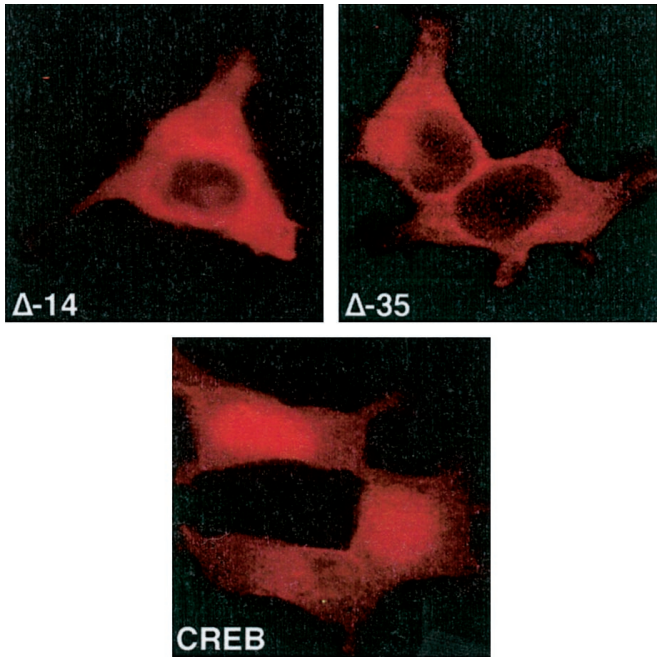


**Fig. 4.** Cellular localization of CREBΔ-14 and CREBΔ-35 variants in transfected HEK293 cells. FLAG-tagged CREBΔ-14 and CREBΔ-35, as well as FLAG-tagged CREB$\alpha$, were transfected into HEK293 cells. The FLAG-tagged CREB variants were visualized by FLAG-immunoreactivity by using Cy3-conjugated secondary antibody and fluorescence microscopy. The nucleus (n) of each labeled cell is indicated. The results demonstrate that CREBΔ-14 and CREBΔ-35 are localized primarily in the cytosol. In contrast, CREB$\alpha$ is localized predominately in the cell nucleus .

function of CREB, and the influence of the CREB variants on CRE-CAT activity was determined in the presence of of recombinant CREB. The CREB construct was cotransfected with different amounts of either CREBΔ-14 or Δ-35 and the CRE-CAT constructs into HEK 293 cells (Fig. 5B). Both CREBΔ-14 and Δ-35 dose-dependently repressed basal CRE-CAT activity, but not forskolin-stimulated CRE-CAT activity.

**CREB Variants Are Regulated in CREB-Overexpressing Transgenic Mice.** The CREB gene contains a CRE, and previous studies have demonstrated that activation of the cAMP system regulates the expression of CREB isoforms (Widnell et al., 1994). To determine whether CREBΔ-14 and Δ-35 are also regulated by the cAMP system, we examined the expression of these variants in CREBα-overexpressing mice. We recently have developed inducible CREBα-overexpressing transgenic mice by using the tetracycline-regulated system (Chen et al., 1998). In these mice, the tetracycline transactivator is under the control of the neuron-specific enolase promoter and expression of CREBα is under the control of the tetracycline-responsive promoter. Addition of tetracycline induces a conformational change in tetracycline transactivator and thereby blocks its ability to activate tetracycline-responsive promoter-CREBα expression (tetracycline-off system). For these studies, we used one of the lines (6-B) that exhibits high levels of CREBα overexpression in the striatum and cerebellum (Chen et al., 1998).

In a preliminary study with riboprobes that distinguish the major CREB variants, we found that overexpression of CREBα up-regulates endogenous CREBΔ and β isoforms (not shown). In the present study, the riboprobes used for analysis of CREBΔ-14 and Δ-35 do not distinguish between these variants, but a clear increase of more than 400% in the combined levels of CREBα, Δ, and β isoforms was observed in the transgenic mice (Fig. 6A). This is most likely largely due to overexpressed CREBα, as well as up-regulation of CREBβ and Δ. Levels of CREBΔ-14 mRNA in the CREBα transgenic mice were reduced to 81 ± 5% (mean ± S.E.M) of that in control mice. In contrast, CREBΔ-35 mRNA was up-regulated by 277 ± 13% (mean ± S.E.M.) compared with control mice. These findings demonstrate that CREBΔ-14 and Δ-35 are differentially regulated by CREBα, and raise the possi-

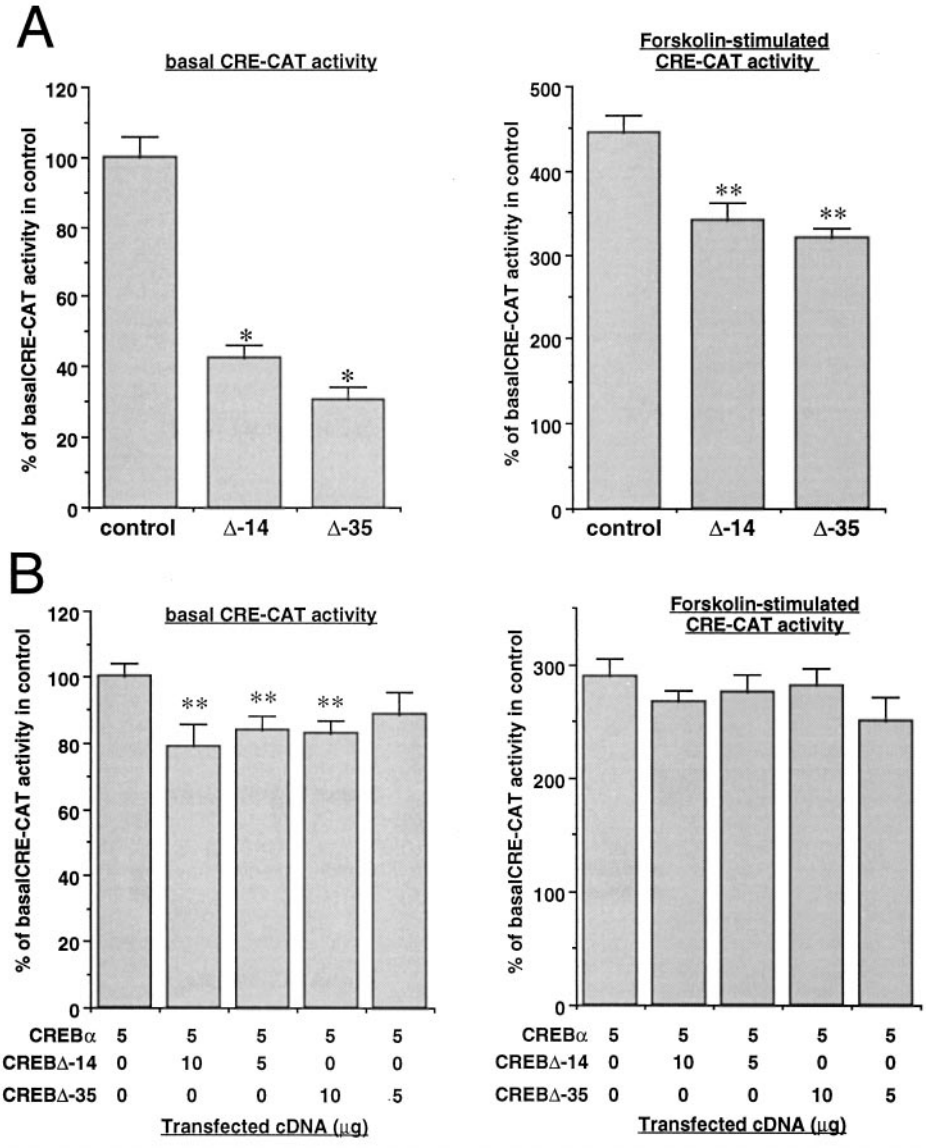


**Fig. 5.** Effects of CREBΔ-14 and CREBΔ-35 expression on CRE-CAT activity. A, HEK293 cells were transfected with either CREBΔ-14 pCI or CREB-35 pCI plus the CRE-CAT reporter construct. Cells were also transfected with pSV-βgal as a marker for transfection efficiency. For the control, the empty pCI vector was transfected instead of constructs containing the CREB variants. Either basal or forskolin (5 μM) stimulated CRE-CAT activity was measured in transfected cells. Expression of either CREBΔ-14 or CREBΔ-35 suppressed CRE-CAT activity under both basal and forskolin-stimulated conditions. Data were normalized to the level of β-gal activity. B, the influence of the CREB variants on the regulation of CRE-CAT activity in response to recombinant CREB was also determined. Expression constructs for CREBα and CREBΔ-14 or Δ-35 were cotransfected into HEK293 cells as indicated. The cells also were transfected with CRE-CAT, different amounts of the empty pCI vector to serve as a control and to maintain equivalent amounts of DNA under each condition, and with pSV-βgal as a marker for transfection efficacy. Either basal or forskolin (5 μM)-stimulated CRE-CAT activity was measured in transfected cells. The results are the mean ± S.E.M.; $n = 6$ per group. $^{*}P < .01$ or $^{**}P < .05$ compared with control (Student's $t$ test).

bility that expression of these variants can be regulated by the cAMP system under physiological conditions.

## Discussion

In the present study, we have identified two novel CREB splice variants, CREBΔ-14 and CREBΔ-35. Previous studies have demonstrated the presence of several CREB isoforms as a result of alternative splicing (Waeber et al., 1991; Ruppert et al., 1992; Ellis et al., 1995; Blendy et al., 1996; Girardet et al., 1996; Walker et al., 1996; Youg et al., 1997; Bartsch et al., 1998). In most of these previous reports, the CREB variants were generated by splicing out an entire exon (Ruppert et al., 1992; Youg et al., 1997) or the insertion of a novel exon (Waeber et al., 1991; Ruppert et al., 1992; Girardet et al., 1996; Bartsch et al., 1998). The CREBΔ-14 and CREBΔ-35 variants described in this study have a deletion of exon 5, similar to that in the CREBΔ variant. In addition, CREBΔ-14 and Δ-35 also lack several nucleotides at the beginning of exon 9 and 8, respectively, indicating that they are novel CREB splice variants. This indicates that CREBΔ-14 and Δ-35 are generated by recognition of alternative intron-exon boundaries within these regions. In this regard, CREBΔ-14 and Δ-35 are similar to the βCREB−1 variant (Ellis et al., 1995), in which an insertion of several nucleotides was found at the beginning of exon 7.

The sequence deletions in CREBΔ-14 and Δ-35 suggest an alternative codon usage that would result in an early stop codon. This would result in the formation of proteins with reduced molecular weight relative to CREBΔ, and loss of the carboxy bZIP domain that is required for dimerization and DNA binding. Expression by either a transcription/translation system or transfection of HEK293 cells demonstrates that CREBΔ-14 and Δ-35 have molecular masses of approximately 27 and 22 kDa, respectively. In addition, deletion of the bZIP domain in CREBΔ-14 and Δ-35 was directly demonstrated by immunoblot analysis by using antibodies that selectively recognize the amino or carboxy terminus of CREB. In cells overexpressing the CREB variants, immunoreactivity against the amino, but not the carboxy, terminus was observed, consistent with the sequence prediction. In addition, immunocytochemical analysis of cells expressing FLAG-tagged recombinant CREBΔ-14 and Δ-35 demonstrated that the variants were localized primarily to the cytosol, with very low levels in the nucleus, as would be predicted from the loss of nuclear localization signals and the DNA binding domain.

In contrast to these deletions, both variants contain the kinase-inducible domain located in exon 7, indicating that CREBΔ-14 and Δ-35 are substrates for phosphorylation by CREB kinases, including PKA and CaMKII and IV. This was examined by immunoblot analysis by using phospho-CREB specific antibodies. In the basal or untreated cells, levels of phospho-CREB immunoreactivity were undetectable. Stimulation of the cAMP cascade by incubation of cells with forskolin resulted in high levels of a phospho-CREB bands of the appropriate size in cells expressing either CREBΔ-14 or Δ-35 (i.e., 27 or 22 kDa, respectively). These findings confirm that

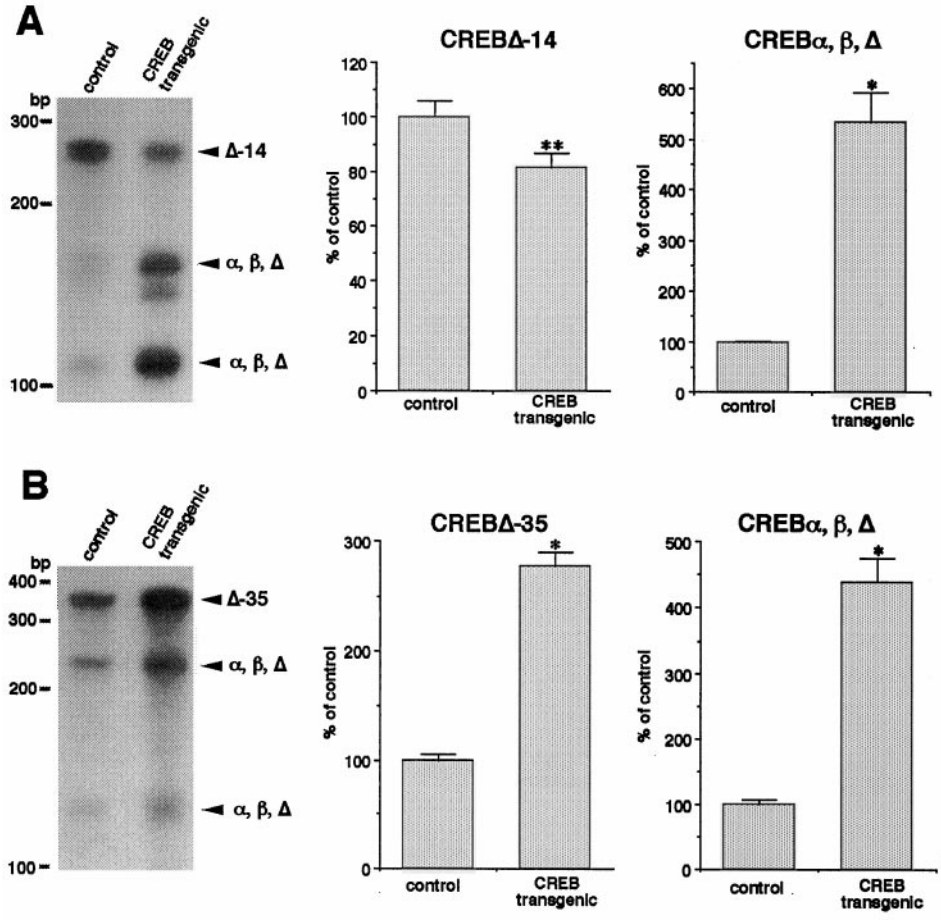


**Fig. 6.** Regulation of CREBΔ-14 or CREBΔ−35 expression in CREB transgenic mice. Levels of CREBΔ-14 (top) and Δ-35 (bottom) mRNA were examined by RPA in the striatum of mice overexpressing CREBα. Representative autoradiograms are shown on the left and quantitation of the results are shown on the right. The results demonstrate that there is a small but significant down-regulation of CREBΔ-14 in the CREBα-overexpressing mice. In contrast, levels of CREBΔ-35 were up-regulated in the CREBα transgenic mice. In both cases, levels of the CREBα, Δ, and β isoforms were significantly increased in the CREB transgenic mice, as a result of overexpression of the CREBα transgene and up-regulation of CREBΔ and β (see text for discussion). The results are expressed as mean ± S.E.M.; $n$ = 10 per group. $^{*}P < .01$ or $^{**}P < .05$ compared with control mice (nontransgenic mice; Student's $t$ test).

CREBΔ-14 and Δ-35 are substrates of PKA, and suggest that they are likely to be substrates for other CREB kinases.

The functional characteristics of CREBΔ-14 and Δ-35 were also examined by using a CRE-CAT reporter assay. Expression of either CREBΔ-14 or CREBΔ-35 repressed both basal and forskolin-stimulated CRE-CAT activity in transfected cells. In addition, the function of recombinant CREB was also repressed by expression of the CREB variants. Previous studies have reported two other CREB variants that, like CREBΔ-14 or CREBΔ-35, lack the bZIP domain but retain the kinase inducible, phosphorylation domain. These are the CREBw isoform in rat (Waeber et al., 1991) and the CREB1c isoform in Aplysia (Bartsch et al., 1998). The cellular localization of these isoforms in the cytosol is similar to that found in the present study for CREBΔ-14 and Δ-35. However, one of these variants (CREBw) but not the other (CREB1c) was reported to repress CRE-reporter activity (Girardet et al., 1996; Walker et al., 1996; Bartsch et al., 1998). This discrepancy could be explained by different experimental conditions or expression efficiency.

The mechanism by which CREBΔ-14, CREBΔ-35, and CREBw inhibit endogenous CREB function is not clear. Because these variants lack the bZIP domain they can not dimerize or bind to DNA to inhibit CREB function. The most likely mechanism for the inhibition is that these variants may act as pseudosubstrates of PKA in the cytosol and thereby decrease the phosphorylation of endogenous CREB in the nucleus. The question remains as to whether endogenous expression of these isoforms is at a level that is sufficient to repress CREB function under physiological conditions. CREBΔ-14 and CREBΔ-35 are widely expressed in all tissues that were examined, and were particularly enriched in brain, thymus, and testes. Moreover, CREBΔ-35 mRNA appears to be abundant and is expressed at high levels relative to the other major CREB isoforms, CREB $\alpha$, $\beta$, and Δ. Expression of immunoreactive bands of the appropriate molecular weight were also observed, indicating that CREBΔ-14 and Δ-35 are expressed at the protein levels. In addition, the finding that CREBΔ-35 is up-regulated in CREB transgenic mice suggests that expression of this isoform may serve a negative feedback role for the cAMP system. These findings raise the possibility that CREBΔ-35, as well as CREBΔ-14, could influence CREB function in vivo. Alternatively, these isoforms may have other, unidentified functions in the cytosol that are regulated by phosphorylation. This possibility is supported by studies demonstrating that CREB$\alpha$ is present in developing dendrites (Crino et al., 1998) and in mitochondria (Cammarota et al., 1999).

Yet another possibility is that CREBΔ-14 and CREBΔ-35 may be aberrant splice variants that are repressed under physiological conditions. This has been demonstrated for other aberrant splice variants that are associated with human disease (Nakai and Sakamoto, 1994). For example, the pathogenesis of sporadic amyotrophic lateral sclerosis recently was determined to result, in part, from aberrant RNA processing of a glutamate transporter (EAAT2; Lin et al., 1998). In this regard, the CREBΔ-14 and CREBΔ-35 variants could be involved in the regulation of the cAMP-CREB signaling pathway under pathological conditions. Further examination will be needed to determine the function of the CREBΔ-14 and Δ-35 variants in normal physiological signaling, and possibly in aberrant conditions.

**References**


Bai G and Lipton, SA (1998) Aberrant RNA splicing in sporadic amyotrophic lateral sclerosis. *Neuron* **20:**363–366

Bartsch D, Casadio A, Karl KD, Serodio P and Kandel ER (1998) CREB1 encodes a nuclear activator, a repressor and a cytoplasmic modulator that form a regulatory unit critical for long-term facilitation. *Cell* **95:**211–223

Blendy JA, Kaestner KH, Schmid W, Gass P and Schutz, G (1996) Targeting of the CREB gene leads to up-regulation of a novel CREB mRNA isoform. *EMBO J* **15:**1098–1106

Cammarota M, Paratcha G, Bevilaqua LRM, Levi de Stein M, Lopez M, Pellegrino de Iraldi A, Izquierdo I and Medina JH (1999) cAMP-response binding protein in brain mitochondria. *J Neurochem* **72:**2272–2277.

Chen J, Kelz, MB, Zeng, G, Sakai, N, Steffen, C, Shckett, PE, Picciotto, MR, Duman, RS and Nestler, EJ (1998) Transgenic animals with inducible, targeted gene expression in brain. *Mol Pharmacol* **54:**495–503

Chrivia JC, Kwok, RP Lamb N, Hagiwara M, Montminy MR and Goodman RH (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature (Lond)***365:**855–859

Cole TJ, Copeland NG, Gilbert DJ, Jenkins NA, Schutz G and Ruppert S (1992) The mouse CREB (cAMP responsive element binding protein) gene: Structure, promoter analysis, and choromosomal localization. *Genomics* **13:**974–982

Crino P, Khodakhah K, Becker K, Ginsberg S, Hemby S and Eberwine J (1998) Presence and phosphorylation of transcription factors in developing dendrites. *Proc Natl Acad Sci USA* **95:**2313–2318

Dash PK, Karl KA, Colicos MA, Prywes R and Kandel ER (1991) cAMP respose element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* **88:**5061–5065

Duman RS, Heninger GR and Nestler EJ (1997) A molecular and cellular theory of depression. *Arch Gen Psychiatry* **54:**597–606

Ellis MJ, Hurst HC and Goodbourn S (1995) A novel cyclic AMP response element-binding protein-1 (CREB-1) splice product may down-regulate CREB-1 activity. *J Mol Endocrinol* **14:**191–198

Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS and Greenberg ME (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science (Wash DC)* **260:**238–241

Girardet C, Walker WH and Habener JF (1996) An alternatively spliced polycistronic mRNA encoding cyclic adenosine3′ 5′ monophosphate (cAMP)-responsive transcription factor CREB (cAMP response element-binding protein) in human testis extinguishes expression of an internally translated inhibitor CREB isoform. *Mol Endocrinol* **10:**879–891

Gonzalez GA and Montminy, MR (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59:**675–680

Hai T and Curran T (1991) Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci USA* **88:**3720–3724

Hoeffler JP, Meyer TE, Yun Y, Jameson JL and Habener JF (1988) Cyclic AMP-responsive DNA-binding proteins: structure based on a cloned placental cDNA. *Science (Wash DC)* **242:**1430–433

Johnson PF and McKnight SL (1989) Eukaryotic transcriptional regulatory proteins. *Annu Rev Biochem* **58:**799–839

Kuroda S, Tokunaga T, Kiyohara Y, Higuchi O, Konishi H, Mizuno K, Gill GN and Kikkawa U (1996) Protein-protein interaction of zinc finger LIM domains with protein kinase C. *J Biol Chem* **271:**31029–31032

Kwok PP, Lundblad JR, Chirivia JC, Richards JP, Bachinger HP, Brennan RG, Roberts SG, Green MR and Goodman R (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature (Lond)* **370:**223–226

Lalli E and Sassone-Corsi P (1994) Signal transduction and gene regulation: The nuclear response to cAMP. *J Bio Chem* **269:**17359–17362

Lin C-LG, Bristol LA, Jin L, Dykes-Hoberg M, Crawford T, Clawson L and Rothstein JD (1998) Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* **20:**589–602

Maekawa T, Sakura H, Kanei-Ishii C, Sudo T, Yoshimura T, Fujisawa J, Yoshida M and Ishii S (1989) Luecine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO J* **8:**2023–2028

Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR and McKnight GS (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol Cell Biol* **14:**6107–6116

Milner B, Squire LR and Kandel ER (1998) Cognitive neuroscience and the study of memory. *Neuron* **20:**445–468

Montominy MR, Sevarino KA, Wagner JA, Mandel G and Goodman RH (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc Natl Acad Sci USA* **83:**6682–6686

Nakai K and Sakamoto H (1994) Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene* **141:**171–177

Nestler EJ and Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science (Wash DC)* **278:**58–63

Nibuya M, Nestler EJ and Duman RS (1996) Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J Neurosci* **16:**2365–2372

Ruppert S, Cole TJ, Boshart M, Schmid E and Schutz G (1992) Multiple mRNA isoforms of the transcription activator protein CREB: Generation by alternative splicing and specific expression in primary spermatocytes. *EMBO J* **11:**1503–1512

Takahashi M, Terwillinger R, Lane C, Mezes PS, Conti M and Duman RS (1999) Chronic antidepressant administration increses the expression of cAMP-specific phosphodiesterase 4A and 4B isoforms. *J Neurosci* **19:**610–618

Tolbert LM and Lameh J (1996) Human muscarinic cholinergic receptor Hml internalizes via clathrin-coated vesicles. *J Biol Chem* **271:**17335–17342
Waeber G and Habener JF (1991) Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3′, 5′-monophosphate response element-binding protein CREB. *Mol Endocrinol* **5:**1431–1438
Waeber G and Habener JF (1992) Novel testis germ cell-specific transcript of the CREB gene contains an alternatively spliced exon with multiple in-frame stop codons. *Endocrinol* **131:**2010–2015
Waeber G, Meyer TE, LeSieur M, Hermann HL, Gerard N and Habener JF (1991) Developmental stage-specific expression of cyclic adenosine 3′, 5′-monophosphate response element-binding protein CREB during spermatogenesis involves alternative exon splicing. *Mol Endocrinol* **5:**1418–1430.
Walker WH, Girardet C and Habener JF (1996) Alternative exon splicing controls a translational switch from activator to repressor isoforms of transcription factor CREB during spermatogenesis. *J Biol Chem* **271:**20145–21050.
Widnell KL, Chen J, Iredale P, Walker WH, Duman RS, Habener JF and Nestler EJ (1996a) Transcriptional regulation of CREB (cyclic AMP response element-binding protein) expression in CATH.a cells. *J Neurochem* **66:**1770–1773.
Widnell KL, Russell, D and Nestler, EJ (1994) Regulation of cAMP response element binding protein in the locus coeruleus in vivo and in a locus coeruleus-like (CATHa) cell line in vitro. *Proc Natl Acad Sci USA* **91:**10974–10951.
Widnell KL, Self DW, Lane SB, Russell DS, Vaidya V, Miserendino MJD, Rubin CS, Duman RS and Nestler EJ (1996b) Regulation of CREB expression: In vivo evidence for a functional role in morphine action in the nucleus accumbens. *J Pharmacol Exp Ther* **276:**306–315
Yamamoto KK, Gonzalez GA, Biggs WHI and Montminy MR (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature (Lond)* **334:**494–498
Youg L, Lanier ER and Kraig E (1997) Identification of a novel, spliced variant of CREB that is preferentially expressed in the thymus. *J Immunol* **158:**2522–2525
Ziff EB (1990) Transcription factors: A new family gathers at the cAMP response site. *Trends Genet* **6:**69–72

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