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Two distinct forms of active transcription factor CREB (cAMP response element binding protein)

(signal transduction/DNA-binding proteins/alternative splicing/c-fos promoter)

LAURA A. BERKOWITZ AND MICHAEL Z. GILMAN*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724

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ABSTRACT **Mammalian cells express two distinct forms of transcription factor CREB (cAMP response element binding protein) that are apparently the products of alternative splicing of the CREB gene transcript. The two proteins differ by a 14-amino acid serine-rich insertion present in one of the CREB isoforms. We show that both CREB isoforms are expressed in many cell types and mammalian species. Both encode proteins that bind specifically to a cAMP response element *in vitro*. As expected for proteins of this class, the CREB proteins bind DNA as dimers. Both proteins impart cAMP-regulated transcriptional activity to a heterologous DNA-binding domain, showing that cAMP directly modulates the transcriptional stimulatory activity of CREB. The presence of multiple CREB isoforms with identical DNA-binding specificities but differences in the presumed regulatory domain raises the possibility that CREB proteins may be able to integrate distinct regulatory signals at the level of gene transcription.**

cAMP is an example of a "second messenger" that relays extracellular signals to intracellular targets. Elevation of intracellular cAMP concentrations leads to activation of cAMP-dependent protein kinase (PKA) and subsequently to changes in the pattern of cellular gene transcription (1). Activation of PKA is both necessary and sufficient for the transcriptional response to cAMP (2–5).

Most genes induced by cAMP share a conserved DNA sequence that constitutes a cAMP-responsive element (CRE) (for review, see ref. 6). Mammalian cells contain multiple proteins that bind this sequence (see, for example, refs. 7–12). One of these proteins, CREB (cAMP response element binding protein) (7, 8), is a 43-kDa phosphoprotein that is a substrate for PKA *in vitro* (8). Mutation of the serine residue phosphorylated by PKA prevents activation of CREB by PKA *in vitro* (13). Two cDNAs encoding CREB have recently been isolated—one from a human placenta library (11) and a second from a rat brain library (12). The two clones encode highly related proteins that differ primarily by a 14-amino acid region found only in the rat clone. This difference could be due to species or tissue specificity, and its functional significance is not known. We report here on the isolation of CREB cDNA clones from a human T-cell cDNA library.† We establish that both CREB isoforms are expressed ubiquitously and describe the properties of the proteins encoded by the two cDNAs.

MATERIALS AND METHODS

cDNA Isolation. CREB cDNAs were amplified by polymerase chain reaction from a human peripheral blood T-cell cDNA library in λgt10 (ref. 14; extracted phage DNA kindly provided by C. Nicolet, Cold Spring Harbor Laboratory), using two oligonucleotides that carried sequence complementary to the first and last 20 nucleotides of the CREB open reading frame, respectively (11). Amplification reaction mixtures (100 μl) contained 70 ng of library DNA, 100 pmol of each phosphorylated oligonucleotide, and 2.5 units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin–Elmer/Cetus). The amplification cycle was 1 min at 94°C, 1 min at 65°C, 5 min at 74°C, and it was repeated for a total of 35 cycles. The 1-kilobase CREB cDNA fragment was the major product of the reaction. The CREB cDNA fragment was cloned in pBSM13+ (Stratagene). Eight independent clones carrying CREB inserts were fully sequenced by using a set of oligonucleotide primers based on a published CREB sequence (11). All eight clones were fully sequenced on one strand, while one clone of each class was also fully sequenced on the opposite strand.

Other Constructions. To construct the probe plasmid for RNase protection assays of CREB mRNA, a 176-base-pair (bp) *Hae* III fragment of the CREB-B cDNA was cloned into the *Hinc*II site of pBSM13+.

Mutagenesis of the c-fos promoter to convert its natural CRE to a GAL4 binding site was performed by site-directed mutagenesis (15). The parental plasmid was a c-fos–chloramphenicol acetyltransferase fusion plasmid carrying c-fos sequences from −71 to +109 and six tandem repeats of a synthetic simian virus 40 enhancer element (16). The GAL4 sequence used (see Fig. 5) was based on Webster *et al.* (17).

To construct the GAL4–CREB fusion protein expression plasmids, we placed the entire CREB open reading frame downstream of the yeast GAL4 DNA-binding domain [GAL4, 1–147; originally derived from the plasmid pMA424 (18)]. The fusion protein was expressed under the control of the cytomegalovirus promoter and 5′-untranslated sequences from the herpes simplex virus thymidine kinase gene. The structure of this plasmid is shown in Fig. 5; details of this construction are available upon request.

RNA Analysis. Methods for transient expression assays and RNA analysis have been described (16). RNA from mouse tissues was a gift from M. Steinhelper, L. Field, and J. Skowronski (Cold Spring Harbor Laboratory).

***In Vitro* Translation Assays.** Templates for *in vitro* transcription of CREB cDNAs were prepared by polymerase chain reaction amplification of single-stranded phagemid DNA. Amplification yielded a double-stranded fragment carrying the CREB cDNA linked to adjacent bacteriophage promoters. For preparing truncated CREB proteins, we prepared oligonucleotides that hybridized to CREB DNA at the desired locations (see Fig. 4) and carried at the 5′ end a T7 RNA polymerase promoter. These were used as primers

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Abbreviations: PKA, cAMP-dependent protein kinase A; CRE, cAMP response element; CREB, cAMP response element binding protein; IBMX, isobutylmethylxanthine.
*To whom reprint requests should be addressed.
†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34356).

in polymerase chain reactions in conjunction with a primer that hybridized to the 3′ end of the CREB coding sequence. Approximately 1 μg of each amplified fragment was transcribed *in vitro* with T7 RNA polymerase. The resulting RNAs were translated *in vitro* by using a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. Mobility-shift assays were carried out as described (16).

RESULTS

Two Distinct CREB cDNAs. To study the role of CREB in the control of c-fos transcription of cAMP (16), we wished to obtain a full-length CREB cDNA. We designed oligonucleotides corresponding to the first seven and last seven codons of the human CREB sequence obtained by Hoeffler *et al.* (11) and used these oligonucleotides in a polymerase chain reaction to amplify CREB cDNAs from a human peripheral blood T-cell cDNA. The amplified DNA was cloned into a plasmid vector and eight isolates were completely sequenced. The eight clones fell into two distinct groups that differed by a 42-bp insertion present in three of the eight clones. In addition, we noted 13 nucleotide substitutions that were unique to individual clones, which we presume are due to misincorporation by *Taq* polymerase during amplification. Because each substitution was found in only a single isolate, consensus nucleotides at each position were easily established.

Fig. 1 shows the consensus nucleotide sequences of the two CREB cDNAs. The cDNA termed CREB-A was identical in sequence to that reported by Hoeffler *et al.* (11) except for a region spanning amino acids 57–64, where our sequence showed five substitutions and one inserted amino acid relative to the reported sequence. In each case, the amino acids encoded at these positions in our clones matched those found in the rat clone (12). The second cDNA, termed CREB-B, contained a 42-bp insertion, encoding 14 additional amino acids beginning at amino acid 88 and resuming CREB-A sequence at amino acid 102. This precise 14-amino acid stretch is found in an identical position in the rat cDNA. Except for this insertion, the two human cDNAs were identical in nucleotide sequence. Thus, two distinct forms of human CREB are apparently encoded by alternate transcripts of a single gene. The discrepancy between the two previously reported sequences (11, 12) is not due to species differences.

Both CREB mRNAs Are Ubiquitously Expressed. To study the pattern of expression of the two CREB mRNAs, we prepared an RNase protection probe that spanned the CREB-B insert. CREB-B mRNA can hybridize contiguously to the probe, leading to protection of the entire CREB sequence in the probe. Hybridization to CREB-A mRNA loops out the insert region of the probe, which is cleaved by RNase T1 to generate two smaller probe fragments, only the larger of which is seen in the figures. Thus, probe fragments protected by the two mRNAs were easily resolved by gel electrophoresis. Fig. 2*A* shows that both mRNAs were observed in several human cell lines, including H9 and Jurkat T lymphoblasts, WI-38 fibroblasts, HeLa, and HepG2 hepatoma cells. In addition, full protection of identical probe fragments was observed in RNA samples from several rodent cell lines, including rat 208F fibroblasts, BALB/c 3T3 fibroblasts, and mouse F9 embryonal carcinoma stem cells and differentiated cultures. Thus, both CREB mRNAs were expressed simultaneously in all these cell lines. In general, CREB-A mRNA was expressed at severalfold higher levels than CREB-B mRNA.

Because we could obtain protection of the full human CREB probe with mouse RNA, we also surveyed a variety of mouse tissues. Again, both CREB mRNAs were expressed in all tissues examined, with CREB-A mRNA being more abundant (Fig. 2*B*). CREB-B mRNA appeared to be especially abundant in brain. The main conclusion from this analysis is that both CREB mRNAs are widely expressed and that there is no striking tissue specificity to their expression.

Both cDNAs Encode Proteins That Bind a CRE *in Vitro*. To determine whether both cDNAs encode CREBs, we transcribed both cDNAs into mRNA with T7 RNA polymerase and translated the mRNAs in a rabbit reticulocyte lysate. Fig. 3*A* shows that both mRNAs encode proteins with apparent molecular masses of ≈45 kDa, consistent with the reported size of the purified rat protein (7). Fig. 3*B* shows a mobility-shift assay of the proteins translated *in vitro*. The probe carries a CRE from the mouse c-fos promoter (16). Translation products from both mRNAs yield strong CRE binding activities not detectable in control lysates (compare lanes 2–7 with lanes 8–10). These complexes closely comigrated with the major complex observed with nuclear extracts from H9 T lymphoblasts (lane 1). The complexes are sequence specific, because they did not form with a similar probe carrying four nucleotide substitutions that inactivate the CRE (16) (lanes 11 and 12). Thus, both CREB mRNAs encode proteins that are functional in DNA-binding assays *in vitro*.

CREB belongs to the "leucine zipper" family of DNA-binding proteins (for review, see ref. 19). These proteins bind to DNA as dimers, and DNA binding requires a region rich

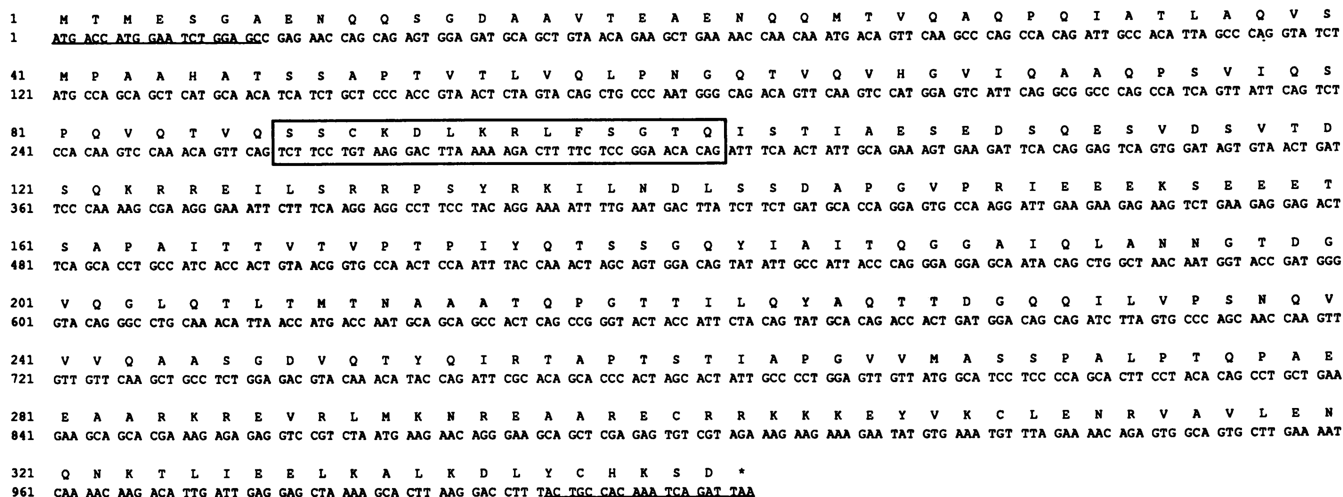

FIG. 1. Sequence of CREB cDNA clones. The sequence shown represents the consensus nucleotides at each position in eight independently isolated clones (see text). The underlined sequences at the 5′ and 3′ ends were encoded in the oligonucleotide primers and therefore were not independently determined. The boxed region indicates sequence present only in the CREB-B cDNA.

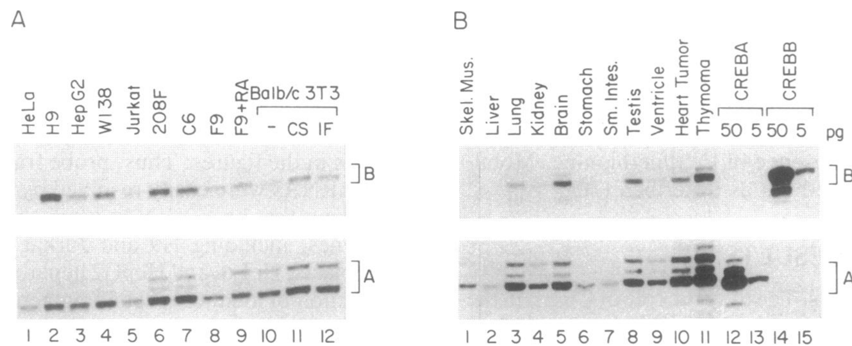

FIG. 2. *In vivo* expression pattern of CREB mRNAs. (*A*) RNase protection analysis of CREB mRNAs from human and rodent cell lines (10 μg of total cytoplasmic RNA was assayed in each lane). Lanes: 1, HeLa human epithelioid carcinoma; 2, H9 human T lymphoblast; 3, HepG2 human hepatoma; 4, WI-38 human fetal fibroblast; 5, Jurkat human T lymphoblast; 6, 208F rat fibroblast; 7, C6 rat glioma; 8, F9 mouse embryonal carcinoma; 9, F9 culture treated with retinoic acid to induce differentiation; 10–12, quiescent BALB/c 3T3 mouse fibroblasts (lane 10) treated for 60 min with 10% calf serum (lane 11) or IBMX and forskolin (lane 12). The probe fragments protected by CREB-A (A) and CREB-B (B) mRNAs are indicated on the right. (*B*) RNase protection analysis of CREB mRNAs in mouse tissues. The tissues examined are shown above the gel (lanes 1–11); 10 μg of RNA was assayed. Lanes 12–15, control hybridizations performed with *in vitro*-transcribed CREB-A and CREB-B mRNAs. Each panel is derived from a single gel from which empty space was removed to conserve space.

in basic amino acids N terminal to the leucine zipper. To determine whether CREB proteins behaved similarly, we prepared a series of N-terminal truncations by *in vitro* transcription and translation of polymerase chain reaction-amplified templates. Fig. 4 shows the mobility-shift complexes obtained with full-length CREB-A (lane 2) and CREB-B (lane 11) proteins. Lanes 3–6 show the complexes obtained with N-terminally truncated CREB-B proteins. A truncation beginning at the insert had no detectable DNA-binding activity (lane 3), although significant levels of the 256-amino acid protein were synthesized (data not shown). Whether this represents a specific regulatory effect of the insert or a nonspecific folding problem is not yet clear. A shorter protein carrying the C-terminal 202 amino acids was fully active, yielding a faster migrating complex (lane 4). A 69-residue protein beginning just N terminal to the basic region retained specific DNA-binding activity (lane 6). A protein lacking the basic region but retaining the leucine zipper (45 amino acids) was not active for DNA binding (lane 6). To determine whether the CREB proteins formed dimers, we cotranslated full-length CREB-A mRNA with truncated CREB-B mRNAs. In two cases, complexes of intermediate mobility were observed (lanes 8 and 10, arrows), showing that the active binding species is a dimer. When CREB-A mRNA was cotranslated with the two CREB-B truncations that were not active as homodimers, no heterodimeric protein–DNA complexes were observed (lanes 7 and 10). However, in the case of the 256-amino acid CREB-B truncation, the yield of CREB-A homodimer complex was substantially reduced, suggesting that the truncated CREB-B protein may still be competent for dimerization, yielding heterodimers with no DNA-binding activity. Thus, these experiments show that CREB binds DNA as a dimer. They further suggest that CREB-A:CREB-B heterodimers may form, but we have not demonstrated this directly with full-length proteins. Similar studies were recently reported by Dwarki *et al.* (26).

Both CREB mRNAs Encode Proteins That Impart cAMP-Responsiveness to a Heterologous DNA-Binding Domain *in Vivo*. *In vivo* assay of the activities of these proteins encoded by these cDNAs is complicated by the presence of CREB and related activities in all of the cell lines we examined. Moreover, we wished to separate cAMP effects on CREB transcriptional activity from any possible effects on DNA binding. Therefore, we reprogrammed the DNA-binding specificity of the CREB proteins by fusing the DNA-binding domain of the yeast transcriptional activator GAL4 (18) to the N terminus of the CREB proteins. The fusion proteins contained the first 147 amino acids of the GAL4 protein,

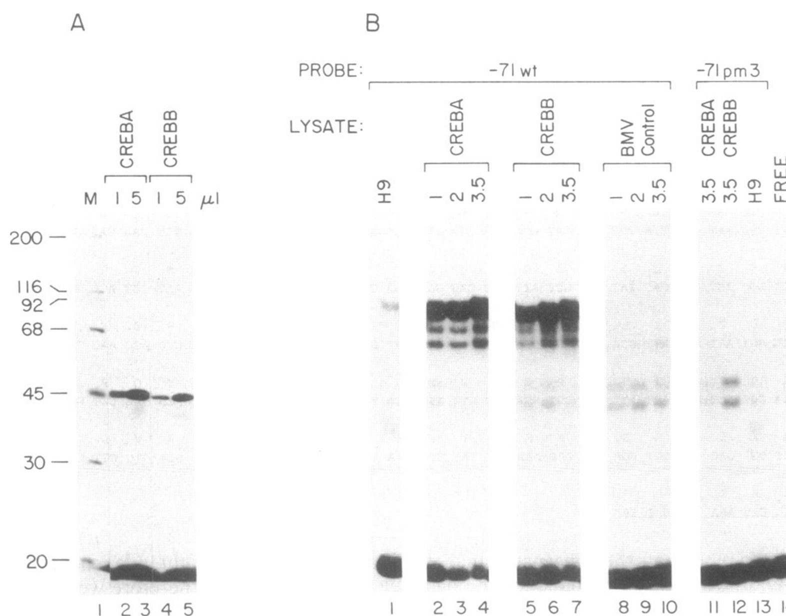

FIG. 3. *In vitro* translation of CREB proteins. (*A*) SDS/PAGE analysis of *in vitro* translated proteins. Lane 1, molecular mass markers (kDa); lanes 2 and 3, 1 and 5 μl of translation lysate programmed by CREB-A mRNA; lanes 4 and 5, 1 and 5 μl of translation lysate programmed by CREB-B mRNA. (*B*) Mobility-shift assay of *in vitro* translated CREB proteins. The probe was a 50-bp restriction fragment carrying the wild-type c-fos CRE (lanes 1–10 and 14) or an inactive mutant (pm3; lanes 11–13) (18). Lanes: 1 and 13, H9 T-lymphoblast cell extract; 2–4, and 11, the indicated volumes (in μl) of translation lysate programmed by CREB-A mRNA; 5–7, and 12, the indicated volumes (in μl) of translation lysate programmed by CREB-B mRNA; 8–10, the indicated volumes (in μl) of translation lysate programmed by brome mosaic virus mRNA.

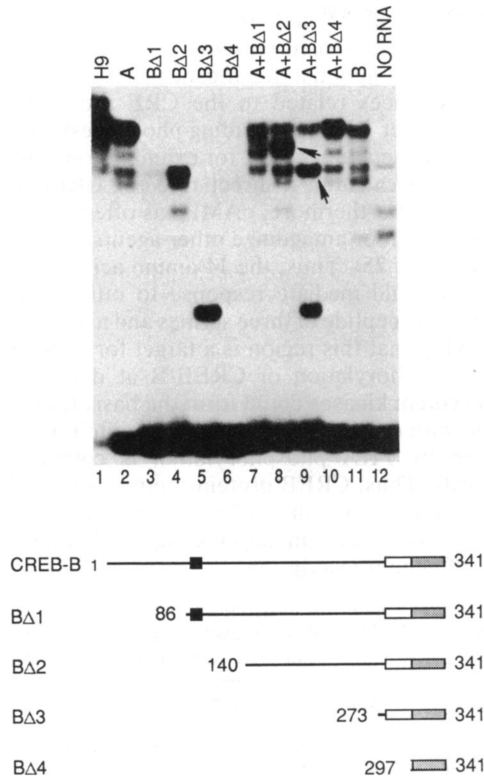

FIG. 4. Mobility-shift assay of truncated CREBs. The drawings represent the N-terminally truncated CREB-B prepared by *in vitro* translation. Solid box indicates the insert unique to CREB-B; open box represents the basic region; stippled box represents the leucine zipper. Lanes: 1, H9 cell extract; 2, full-length CREB-A; 3–6, the indicated truncated CREB-B; 7–10, cotranslated CREB-A and CREB-B derivatives; 11, full-length CREB-B; 12, unprogrammed translation lysate. Arrows point to complexes in cotranslations (lanes 8 and 9) with mobilities intermediate between those obtained with the two proteins translated individually; these complexes represent binding by heterodimers of full-length CREB-A and truncated CREB-B.

followed by 7 linker amino acids that included several potential helix breakers, and then the entire CREB proteins, including the DNA-binding domain and leucine zipper. To assay the transcriptional activities of these proteins *in vivo*, we used site-directed mutagenesis to convert the CRE in a c-fos promoter derivative to a GAL4 binding site (Fig. 5). We transfected this test plasmid into BALB/c 3T3 cells together with plasmids that expressed the GAL4–CREB fusion proteins from a viral promoter. Also included in the transfections was a plasmid encoding the human α-globin gene, which served as an internal control for transfection efficiency and RNA recovery. The transfected cells were starved for serum for 48 hr and subsequently treated for 1 hr with isobutylmethylxanthine (IBMX) (a phosphodiesterase inhibitor) and forskolin (an activator of adenylate cyclase). Total cytoplasmic RNA was harvested and assayed for correctly initiated c-fos and globin transcripts by RNase protection.

Fig. 5 shows that whereas the wild-type Δ-71 c-fos promoter was strongly induced by IBMX and forskolin (lanes 1 and 2), the construct in which the c-fos CRE was substituted with a GAL4 site was not [lanes 3 and 4; the small induction seen in this and other control lanes is likely due to the influence of CRE-like sequences present in vector DNA upstream of the c-fos promoter (16)]. When this substituted c-fos promoter was cotransfected with a plasmid expressing full-length yeast GAL4 protein, its activity was augmented in the presence and absence of stimuli (lanes 5 and 6), showing that the promoter carries an active GAL4 binding site and can respond to a transcription factor bound there. When cotransfected with plasmids expressing either of the full-length GAL4–CREB fusion proteins, the substituted c-fos promoter became strongly cAMP-responsive (lanes 7–10). Thus, the presence of these fusion proteins converted the GAL4 binding site into a CRE. To show that this effect required the expression of fusion proteins in trans, we prepared a small insertion by filling in a *Bst*XI site near the 5′ end of the CREB cDNA, shifting the reading frame. This plasmid carries all of the DNA sequence present in the active plasmid, but it

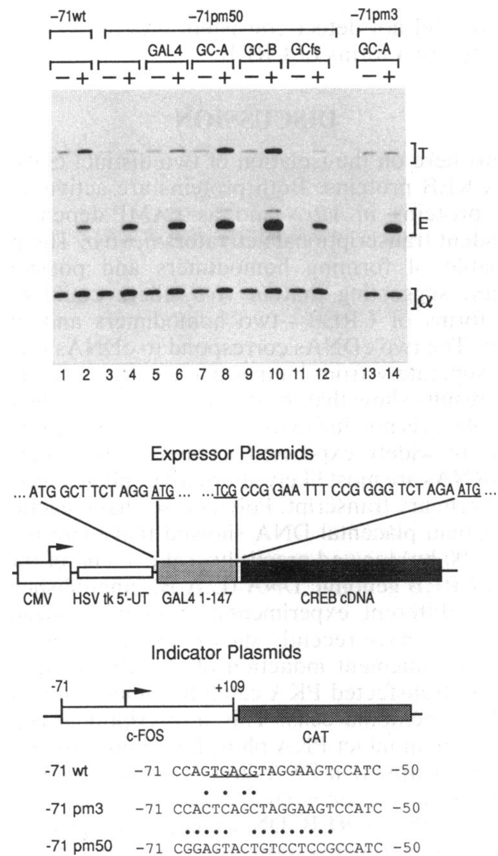

FIG. 5. Functional assay of CREBs *in vivo*. Indicated combinations of indicator and expressor plasmids were transiently transfected into duplicate cultures of BALB/c 3T3 cells. Transfections contained 10 μg of indicator plasmid, 100 ng of expressor plasmid, and 2 μg of α-globin internal control plasmid. Two days after transfection, one culture from each pair was treated with IBMX and forskolin (lanes +). Indicator plasmids carried either a wild-type CRE (lanes 1 and 2), an inactive mutant CRE (lanes 13 and 14), or a GAL4 binding site in place of the CRE (lanes 3–12). Expressor plasmids expressed full-length yeast GAL4 protein (lanes 5 and 6). GAL4–CREB-A fusion (lanes 7, 8, 13, and 14), GAL4–CREB-B fusion (lanes 9 and 10), GAL4–CREB-B with a frameshift mutation (lanes 11 and 12). Equal amounts of RNA from transfected cells were assayed by RNase protection as described (18, 20). Half of the hybridization reactions were analyzed to verify that endogenous c-fos induction was equivalent in all samples. Subsequently, the remaining portions of the hybridization were run on a second gel (shown here) with the amounts adjusted to equalize the signals from the internal control. T, E, and α indicate the probe fragments protected by transcripts of the transfected indicator plasmid, endogenous c-fos, and α-globin internal control plasmid, respectively. Shown below are schematic representations of the expressor and indicator plasmids. For the expressor plasmids, junction sequences between segments are shown; underlined nucleotides are the first and last codons in each unit. In the promoter sequences of the indicator plasmids, dots above each sequence mark positions where each plasmid differs from wild type. CMV, cytomegalovirus; HSV, herpes simplex virus; tk, thymidine kinase; UT, untranslated region.

expresses a protein in which only 15 amino acids of CREB are fused to the GAL4 DNA-binding domain. Cotransfection of this plasmid did not lead to cAMP induction of the substituted c-fos promoter (lanes 11 and 12). To show that the effect of the fusion proteins required a specific binding site in cis, we cotransfected an active GAL4–CREB plasmid with a c-fos promoter in which the CRE was substituted with sequences that inactivated the site without creating a GAL4 binding site. This promoter did not respond to cAMP (lanes 13 and 14). We conclude that both CREB cDNAs encode cAMP-responsive transcription factors that can act in trans through a heterologous DNA-binding domain. Under our experimental conditions, we did not detect consistent differences in activity between the two forms of CREB.

DISCUSSION

We report here on the isolation of two distinct cDNAs that encode CREB proteins. Both proteins are active as DNA-binding proteins *in vitro* and as cAMP-dependent and -independent transcriptional activators *in vivo*. The proteins are capable of forming homodimers and possibly heterodimers, suggesting that *in vivo*, there could be three distinct forms of CREB—two homodimers and one heterodimer. The two cDNAs correspond to cDNAs previously isolated separately from human placenta and rat brain (11, 12). Our results show that the difference between the original CREB isolates is not due to tissue or species specificity. Both mRNAs are widely expressed in human and rodent cells. These mRNAs are most likely alternative splicing products of the same primary transcript. Polymerase chain reaction analysis of human placental DNA showed that there is a small intron (≈500 bp) located precisely at the 5′ end of the insert region in CREB genomic DNA (L.A.B., unpublished data).

Using a different experimental strategy, Gonzalez and Montminy (13) have recently shown that the rat homolog of CREB-B can augment induction of a CRE-containing promoter by cotransfected PKA catalytic subunit in murine F9 embryonal carcinoma cells. This transcriptional activity is dependent on an intact PKA phosphorylation site in CREB, strongly suggesting that PKA directly regulates CREB function by phosphorylation *in vivo*. This activation could reflect changes in either CREB DNA-binding or transcriptional activity. Our experiments show that both forms of CREB can confer cAMP-regulated transcriptional activation to a heterologous DNA-binding domain. Thus, increased concentrations of cAMP, presumably acting through PKA, must influence the transcriptional stimulatory properties of CREB.

After this manuscript was submitted, Yamamoto *et al.* (21) reported characterization of the rat homologues of two CREB isoforms described here. In contrast to our results, they observed a large difference in the transcriptional activities of the two proteins *in vivo*. The discrepancy probably reflects the rather significant differences in the assay systems used. One possible explanation is that, as we discuss below, the two proteins could respond differentially to an intracellular signal expressed at different levels in F9 and BALB/c 3T3 cells; for example F9 cells could contain high levels of a CREB-B-specific protein kinase absent from fibroblasts. Another potential explanation stems from the relative concentrations of endogenous and exogenous CREB proteins in the transfected cells. If the concentration of exogenous CREB is low, it may form dimers predominantly with endogenous proteins, distorting measurement of the activity intrinsic to the protein encoded by the transfected cDNA. Our GAL4–CREB fusion proteins, like intact CREB, could also act in part by recruiting endogenous CREB via their free leucine zippers. However, mutation of the serine residue phosphorylated by PKA substantially reduces the activity of the GAL4–CREB proteins *in vivo* (L.A.B., unpublished data), so most of the activity we are measuring must be intrinsic to the introduced proteins.

What is the functional significance of the two forms of CREB? Sequences related to the CRE are implicated in response to other stimuli, including phorbol esters, calcium, and viral trans-activators (see, for example, refs. 20, 22–24). In the case of calcium, a direct role for CREB has been proposed (24). Furthermore, cAMP has often been reported to synergize with or antagonize other agents (see, for example, refs. 22 and 25). Thus, the 14-amino acid peptide found in CREB-B could mediate response to other stimuli. The presence in this peptide of three serines and a threonine raises the possibility that this region is a target for cellular protein kinases. Phosphorylation of CREB-B at distinct sites by different protein kinases could form the basis for synergistic activation with cAMP or could provide a site through which stimulation by PKA phosphorylation is counteracted by other signals. Thus, CREB proteins could serve as a molecular focus for "crosstalk" often observed among signal transduction pathways, integrating signaling information at the level of gene transcription.

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