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Targeting of the CREB gene leads to up-regulation of a novel CREB mRNA isoform

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To define the role of cAMP signaling in gene control, we have generated mice with a mutation in the cAMP response element binding protein (CREB) gene. Mice carrying this mutation are viable but show an impairment in memory consolidation. In the further analysis of these mice, we have found an up-regulation of a CREB isoform that has not been described previously. The new isoform, termed CREBβ, has nearly the same transactivation potential as the other CREB isoforms and is expressed ubiquitiously. The up-regulation appears to be due to an increase in alternative splicing or mRNA stability, but not to an increase in transcriptional rate. Due to the relatively low levels of expression in all tissues, the role of this isoform is likely to be minor in the wild-type mouse. However, its dramatic up-regulation in the mutant mouse, together with the specific deficiencies recently observed in these mice, suggest that it has a very specific role in compensating for CREBα and Δ in some, but not all, areas where CREB function has been implicated. Together with the up-regulation of the cAMP response element modulator protein (CREM) mRNA and protein levels demonstrated previously in CREB mutant mice, we suggest that the up-regulation of CREBβ may also contribute to compensation within the CREB/ATF family of transcription factors, when CREBΔ and CREBα are absent.

Keywords: CREB/CREM/mRNA/up-regulation

Introduction

A variety of extracellular signals, including hormones and neurotransmitters, stimulate gene transcription by increasing intracellular cAMP, resulting in activation of protein kinase A (PKA) and the subsequent phosphorylation of target proteins. The cAMP response element binding protein, CREB, is one of the best characterized nuclear target proteins for PKA phosphorylation (Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989) and has been identified as a substrate for other kinases such as CaM kinase II and IV (Dash *et al.*, 1991; Matthews *et al.*, 1994), CREB kinase (Ginty *et al.*, 1994) and glycogen synthase kinase-3 (GSK-3; Fiol *et al.*, 1994). While the role of progressive phosphorylation by the kinases CKII and GSK3 at other serine residues remains undefined, it is clear that phosphorylation of CREB at Ser133 is required for increased DNA binding and transactivation (Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989) which is mediated through cAMP-responsive elements (CREs; reviewed in Ziff, 1990). The CRE is recognized by a family of DNA binding proteins, the CREB/ATF family, consisting of a dozen or so different proteins. Within this group, CREB heterodimerizes with activating transcription factor 1 (ATF-1) (Hurst *et al.*, 1991) and CRE modulator protein (CREM) (Foulkes *et al.*, 1991) *in vitro* (for review, see Hurst, 1995). CREB, CREM and ATF-1 are strongly related in sequence and are all involved in cAMP-mediated signaling to the nucleus (Brindle and Montminy, 1992; Lee and Masson, 1993). Together, these data indicate that CREB, CREM and ATF-1 form a subgroup within the CREB/ATF family of transcription factors.

In order to assess the role of CREB in development and in physiological and pathological processes in the adult, our laboratory has generated mice with a disruption of the CREB gene by homologous recombination (Hummler *et al.*, 1994). A null mutation of the gene was intended by the interruption of exon 2 of the CREB gene, which is part of the multiple mRNAs generated by alternative splicing identified thus far (Ruppert *et al.*, 1992). The most abundant mouse cDNA isoform is the homolog of the human and rat CREBΔ cDNA clones (Hoeffler *et al.*, 1988; Gonzalez and Montminy, 1989; Yamamoto *et al.*, 1990) and is referred to as CREBΔ. All other cDNAs cloned have insertions or deletions with respect to the CREBΔ isoform; however, all contain exon 2, which encodes the ATG translation initiation codon. It was for this reason that the neomycin phosphotransferase cassette in the targeting vector for the CREB mutation was inserted into exon 2, assuming that all isoforms containing this exon would be disrupted.

Mice which were genotyped as CREB mutants appeared healthy and exhibited no impairment of growth or development. These findings were puzzling for two reasons. First, activation of gluconeogenic enzymes, which is crucial for survival in the perinatal period, had been shown to be mediated partially by CREs and was thus expected to be impaired in CREB mutant mice (Pilkis and Granner, 1992; Schmid *et al.*, 1993). Secondly, mice carrying a transgene expressing a dominant-negative mutant of CREB directed to the pituitary were severely growth retarded (Struthers *et al.*, 1991).

Similarly, surprising results were obtained in experiments utilizing the CREB mutant mice in the analysis of various processes in the central nervous system (CNS) in which CREB had been implicated as having an important role. In three paradigms examined (memory consolidation, migroglial proliferation and opiate withdrawal response; see Discussion) mice homozygous for the CREB mutation

were severely impaired (Bourtchuladze *et al.*, 1994; Gehrmann *et al.*, in preparation; Maldonado *et al.*, in preparation). However, in two studies (seizure induction and kindling response) no differences between wild-type and mutant animals were observed (Blendy *et al.*, 1995; Butler and MacNamara, personal communication). This variety of responses in CREB$^-$ mice may be explained by the possibility that these mice carry a CREB mutant gene that can be considered a hypomorphic allele. Here we describe the isolation and characterization of a novel isoform of CREB, CREBβ, which is strongly up-regulated in the CREB mutant mice and which contributes to the partial compensation of CREB function observed in the CREB mutant mice.

Results

Presence of CREB immunostaining in CREB mutant mice

CREB is relatively abundant in the brain and has been implicated recently as an important component in transduction of a variety of neuronal signaling paradigms (Ginty *et al.*, 1993; for review, see Montminy *et al.*, 1990; Ghosh and Greenberg, 1995). Several studies using the CREB mutant mice generated by gene targeting addressed the role of CREB in CNS function. These experiments had led to conflicting conclusions. Although CREB function was implicated in all aspects addressed, only in some processess were distinct phenotypes observed (Bourtchuladze *et al.*, 1994; Gehrmann *et al.*, in preparation; Maldonodo *et al.*, in preparation), while in others the mutant animals were not distinguishable from wild-type controls (Blendy *et al.*, 1995). We hypothesized that the CREB mutation might be a hypomorphic allele and tried to identify new, CREB-related proteins in the mutant mice.

Initially, brains from wild-type and CREB mutant animals were analyzed for the presence of CREB and CREM proteins by immunohistochemistry. Figure 1A illustrates that CREM immunoreactivity, as detected by an antibody recognizing all isoforms of the CREM gene, is distributed ubiquitously throughout the brain and is up-regulated in CREB mutant mice in accordance with previous RNA analysis results (Hummler *et al.*, 1994), in particular in areas of the cerebral cortex and hippocampal formation. Surprisingly, however, specific immunostaining with a CREB-specific antibody was seen in CREB mutant mice as well, although to a lesser extent than observed in wild-type controls (Figure 1B).

In order to investigate the molecular nature of this CREB-like protein in the CREB mutant mice, we performed Western blot analysis on brains from wild-type and CREB mutant mice with antisera raised against CREB and phosphorylated CREB. With both antisera, a protein band of ~40 kDa is detected in both wild-type and CREB mutant samples (Figure 2). This band has ~30% of the intensity of the CREB band in wild-type mice.

Cloning and characterization of CREB1β

Previously, no CREB mRNA had been detected in RNA samples from CREB mutant animals with a probe covering parts of exons 1 and 2 of the mouse CREB gene (Hummler *et al.*, 1994). As it seemed likely that the new 40 kDa band detected with the anti-CREB antibodies represented a novel splice variant of the CREB gene, we used a probe derived from a CREBΔ cDNA (Ruppert *et al.*, 1992) which contains sequences from exons 1, 2, 4 and 7 of the CREB gene (see Figure 3) to probe RNA from wild-type and mutant mice in RNase protection experiments. As is shown in Figure 4, the bands corresponding to CREBΔ (exons 1, 2, 4 and 7) and CREBγ (exons 1, 2 and 4) are present only in wild-type RNA, as is expected since exon 2 was interrupted by the neomycin resistance cassette in the CREB –/– mice. A novel mRNA, which we interpret to be the result of splicing of exons 1, 4 and 7, is present both in wild-type and in CREB mutant mice. This novel mRNA, which we have termed CREBβ, is up-regulated ~6-fold in brain and 4-fold in liver (Figure 4). In order to prove that the band at 236 nucleotides in the RNase protection is in fact a new splice variant, we cloned CREB cDNAs from CREB –/– mRNA by 5′ RACE-PCR. Of the 24 clones analyzed by sequencing, all corresponded to CREBβ, and the complete sequence of the cDNA is depicted in Figure 5.

The CREBβ cDNA encodes a protein with a calculated molecular weight of 39 456 Da, which is in close agreement with the size of the up-regulated CREB immunoreactive band observed in Western blots (see Figure 2). CREBβ is identical to CREBΔ, with the exception of lacking 40 amino acids at the N-terminus, where 40% of the Q-domain is deleted. An alternate translation initiation codon is just inside the fourth exon of the CREB gene, and is surrounded by bases that match six out of 10 bases of the Kozak consensus sequence (Kozak, 1987). The fact that this initiation codon is not as close to the consensus as that of CREBΔ and CREBγ (seven out of 10 bases) might explain why there seems to be less CREB protein than would have been expected from the relative levels of mRNA (compare Figures 2 and 4). This notion is also supported by *in vitro* translation experiments, in which CREBβ mRNA consistently is only about half as efficiently translated as that of CREBΔ (data not shown).

Direct cloning of 5′ RACE-PCR products has been utilized previously to identify the start site of transcription for RNA polymerase II transcripts. This is based on the fact that nascent RNA polymerase II transcripts are post-transcriptionally capped with a modified guanosine, which can be reverse transcribed (Hirzmann *et al.*, 1993). The presence of additional Gs at the 5′ end of the cloned PCR product, which are not found in the genomic sequence, indicate the transcription initiation site. Such additional guanosines were found in the CREBβ clones and are indicated in Figure 5. These transcription initiation sites are in very close proximity to those found for CREBα and Δ (Cole *et al.*, 1992).

Tissue distribution of CREBβ

As CREBΔ has been shown to be expressed ubiquitously in adult tissues (Ruppert *et al.*, 1992), we were interested in examining the tissue distribution pattern of CREBβ in wild-type mice. As shown in Figure 6A, CREBβ is present in all tissues examined and makes up ~12–22% of the total translated CREB isoforms. Although some CREB isoforms (ψ, Ω) could not be differentiated with the probe used, these isoforms do not encode functional proteins (Ruppert *et al.*, 1992).

The up-regulation of CREBβ has been observed in

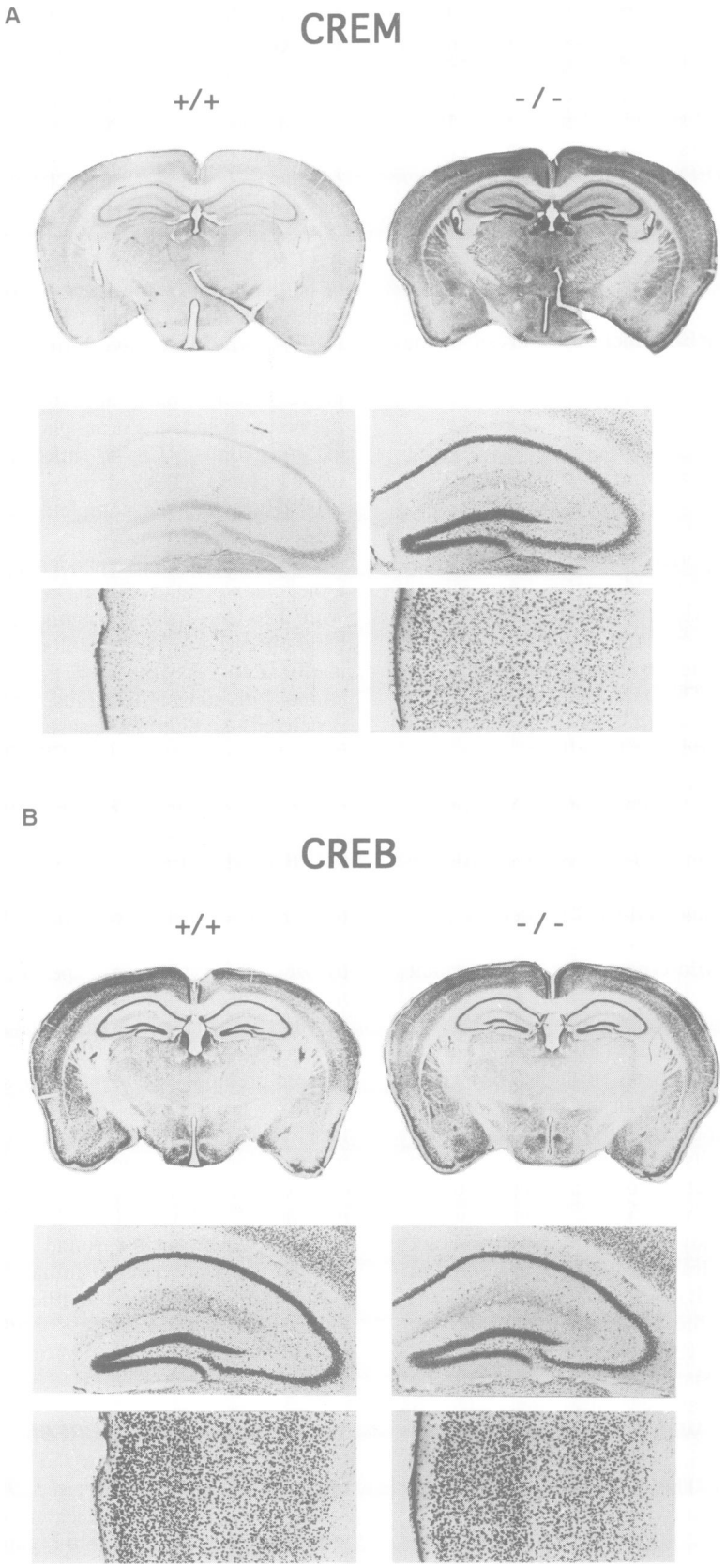

Fig. 1. Expression analysis of CREM and CREB in brains of wild-type and CREB mutant mice. (**A**) Coronal sections through adult mouse brains stained with an antibody specific for CREM. +/+, wild-type animals; –/– CREB mutant animals. (**B**) Adjacent sections of the same brains stained with an antibody specific for CREB. Note the specific, though weaker, CREB signal in the CREB mutant brains (–/–).

a number of tissues, most notably in brain. This up-regulation in brain, however, does not seem to be region specific, as indicated in Figure 6B. CREBβ is up-regulated both in liver (Figure 4) and in testis (data not shown), but not to the same extent as in brain.

Comparison of transactivation potentials of CREBΔ and CREBβ

In order to assess the functional significance of CREBβ, we investigated its transactivation potential in a transient co-transfection assay (Gonzalez and Montminy, 1989). We compared the CREBβ and CREBΔ isoforms, differing only by exon 2, in the same vector background. Full-length mouse cDNAs for CREBβ and CREBΔ, cloned into the eukaryotic expression plasmid pHD (Müller *et al.*, 1988) were co-transfected into F9 teratocarcinoma cells together with the somatostatin promoter–CAT plasmid [Δ(–71)SOM–CAT; Montminy *et al.*, 1986] as a reporter and an expression plasmid encoding the catalytic subunit of PKA (Uhler and McKnight, 1987). In the presence of the CREBΔ expression plasmid, we observed an 8-fold increase of transactivation when compared with basal levels (Figure 7). A lower, but significant increase of transactivation is observed in the presence of CREBβ. The combination of both Δ and β does not appear to have any additive effects, as the level of transactivation is not different from that of Δ alone. No activaton of the reporter plasmid lacking the CRE was observed with any of the CREB expression plasmids (data not shown).

Analysis of the mechanism of up-regulation of CREBβ and CREM

In an attempt to address the mechanism of up-regulation of CREBβ as well as CREM in CREB mutant animals, we

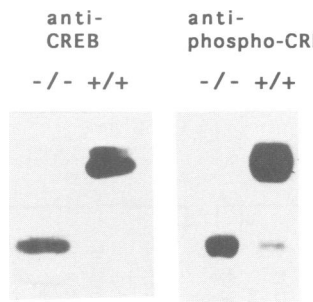

Fig. 2. Brains from CREB mutant mice are enriched in a novel CREB immunoreactive protein. Westen blot analysis of CREB protein in brain nuclear extracts from wild-type (+/+) and CREB mutant (–/–) mice using anti-peptide antibodies specific for CREB or phospho-CREB as indicated. Arrows identify the bands for CREBα/Δ and β.

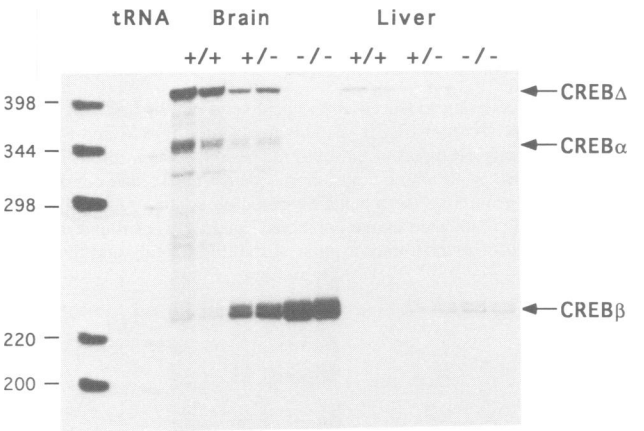

Fig. 4. CREBβ is up-regulated in CREB mutant mice. RNase protection analysis of total RNA (20 μg in each lane) from brains or livers of CREB mutant (–/–), heterozygous (+/–) or wild-type (+/+) animals with the *Eco*RI–*Stu*I 418 probe indicated in Figure 3. The protected fragments of CREBΔ, α or β are indicated by arrows.

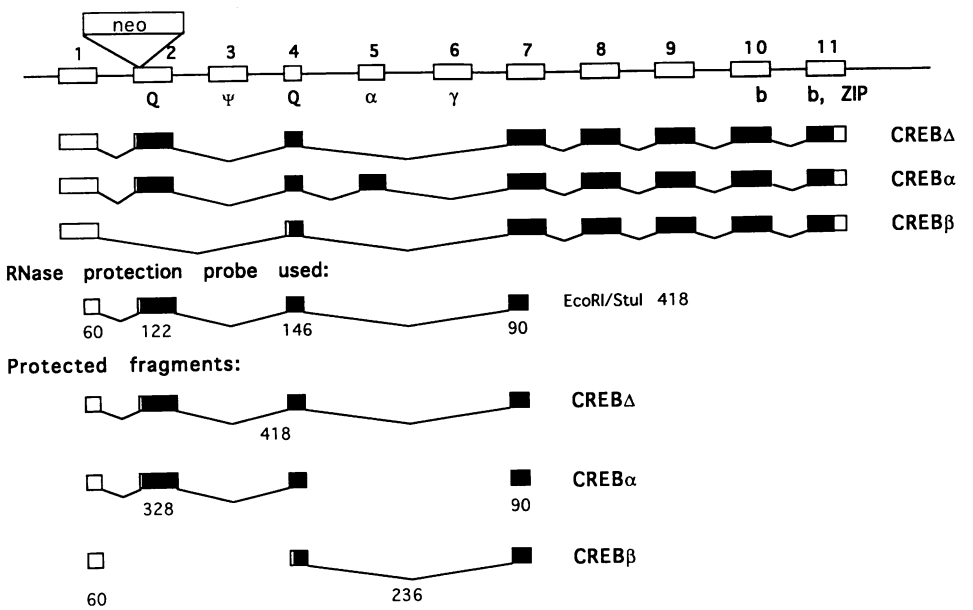

Fig. 3. Structure of the mouse CREB gene and the major CREB cDNAs. The top line indicates the 11 exons (open boxes) of the mouse CREB gene (Ruppert *et al.*, 1992) and the position of the neomycin resistance cassette (neo) in the CREB mutant mice (Hummler *et al.*, 1994). The introns (thin lines) are not drawn to scale. The next three lines show the three major CREB isoforms Δ, α and β. The other CREB isoforms described in Ruppert *et al.* (1992) were omitted, as their translation does not result in functional CREB proteins. The bottom half of the figure explains the probe used in the RNase protection analysis and the protected fragments resulting from hybridization of the probe with either CREBΔ, α or β.

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        G           G
  1 GCTCCCTGGCTGCGGCTCCTCAGTCGGCGGCGGCTGCTGCTGCCTGTGGCCCGGGCGGCTGGGAGAAGCGGAGTGTTGGTGAGTGACGCGGCGGAGGTGTAGTTTGACGCGGTGTGTTAC  120

121 GTGGGGGAGAGAATAAAACTCCAGCGAGATCCGGGCCGCGAACGAAAGCAGTGACGGAGGAGCTTGTACCACCGGTATCCATGCCAGCAGCTCATGCAACATCATCTGCTCCCACTGTAA  240
                                                                                                    M  P  A  A  H  A  T  S  S  A  P  T  V  T
        oligo B                         oligo A
241 CCTTAGTGCAGCTGCCCAATGGGCAGACAGTCCAGGTCCATGGCGTTATCCAGGCGGCCCAGCCATCAGTTATCCAGTCTCCACAAGTCCAAACAGTTCAGATTTCAACTATTGCAGAAA  360
      L  V  Q  L  P  N  G  Q  T  V  Q  V  H  G  V  I  Q  A  A  Q  P  S  V  I  Q  S  P  Q  V  Q  T  V  Q  I  S  T  I  A  E  S

361 GTGAAGATTCACAGGAGTCTGTGGATAGTGTAACTGATTCCCAAAAACGAAGGGAAATCCTTTCAAGGAGGCCTTCCTACAGGAAAATTTTGAATGACTTATCTTCTGATGCACCAGGGG  480
      E  D  S  Q  E  S  V  D  S  V  T  D  S  Q  K  R  R  E  I  L  S  R  R  P  S  Y  R  K  I  L  N  D  L  S  S  D  A  P  G  V

481 TGCCAAGGATTGAAGAAGAAAAGTCAGAAGAGGAGACTTCAGCCCCTGCCATCACCACTGTAACAGTGCCAACCCCCATTTACCAAACTAGCAGTGGGCAGTACATTGCCATTACCCAGG  600
      P  R  I  E  E  E  K  S  E  E  E  T  S  A  P  A  I  T  T  V  T  V  P  T  P  I  Y  Q  T  S  S  G  Q  Y  I  A  I  T  Q  G

601 GAGGAGCAATACAGCTGGCTAACAATGGTACGGATGGGGTACAGGGCCTGCAGACATTAACCATGACCAATGCAGCTGCCACTCAGCCGGGTACTACCATTCTACAGTATGCACAGACCA  720
      G  A  I  Q  L  A  N  N  G  T  D  G  V  Q  G  L  Q  T  L  T  M  T  N  A  A  A  T  Q  P  G  T  T  I  L  Q  Y  A  Q  T  T

721 CTGATGGACAGCAGATTCTAGTGCCCAGCAACCAAGTTGTTGTTCAAGCTGCCTCAGGCGATGTACAAACATACCAGATCCGCACAGCACCCACGAGCACCATTGCCCCTGGAGTTGTTA  840
      D  G  Q  Q  I  L  V  P  S  N  Q  V  V  V  Q  A  A  S  G  D  V  Q  T  Y  Q  I  R  T  A  P  T  S  T  I  A  P  G  V  V  M

841 TGGCGTCCTCCCCAGCACTTCCTACACAGCCTGCTGAAGAAGCAGCACGGAAGAGAGAGGTCCGTCTAATGAAGAACAGGGAGGCAGCAAGAGAATGTCGTAGAAAGAAGAAAGAATATG  960
      A  S  S  P  A  L  P  T  Q  P  A  E  E  A  A  R  K  R  E  V  R  L  M  K  N  R  E  A  A  R  E  C  R  R  K  K  K  E  Y  V

961 TGAAATGTTTAGAGAACAGAGTGGCAGTGCTTGAAAACCAAAACAAAACATTGATTGAGGAGCTAAAAGCACTTAAGGACCTTTACTGCCACAAATCAGATTAATTTGGGATTTAAGTTC 1080
      K  C  L  E  N  R  V  A  V  L  E  N  Q  N  K  T  L  I  E  E  L  K  A  L  K  D  L  Y  C  H  K  S  D  *

1081 TCTCCTGTACGGTGGAGAATGGACTGGCTTGGCACAACCAGAAAGACAAGTAAACATTTATTTTCTAAACATTTCTTTTTT  1161
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Fig. 5. Nucleotide and deduced amino acid sequence of CREBβ. The sequence is derived from the cDNA fragments obtained by 5′ RACE-PCR from brain mRNA from CREB –/– animals using oligos A and B (see Materials and methods for details) and the previously published mouse CREBΔ sequence (Ruppert *et al.*, 1992). The first ATG in the CREBβ mRNA is located just inside exon 4 and is in-frame with the rest of the protein. CREBβ is 40 amino acids shorter than CREBΔ and contains only nine out of the 15 glutamine residues in the so-called Q-domain. The nucleotides surrounding the translation initiation codon in exon 4 which match the Kozak consensus sequence (Kozak, 1987) are underlined. The guanosine bases indicated above positions 7 and 21 were found in the 5′ RACE-PCR products and are not encoded in the CREB gene. These Gs were the result of reverse transcription of the mRNA cap, and thus represent two transcriptional start sites of the CREB gene.

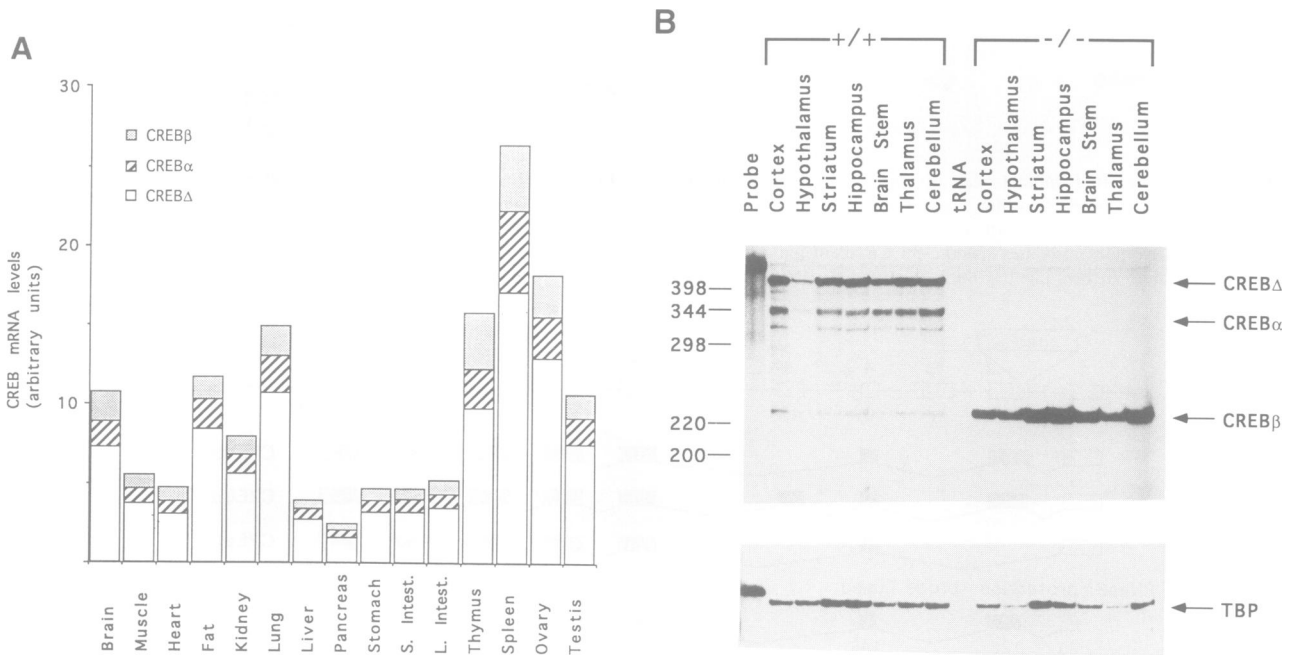

Fig. 6. Tissue distribution of the major CREB isoforms. (**A**) Total RNA (20 μg in each lane) from the adult mouse tissues indicated was analyzed for CREB expression with the *Eco*RI–*Stu*I 418 probe indicated in Figure 3 and for TBP as the control. The bands obtained were quantified using a PhosphorImager and normalized for the number of ^{32}P-labeled uridine bases contained in each protected fragment, and thus represent an accurate representation of the contributions of the isoforms. CREBβ constitutes between 12 and 22% of the three isoforms in all tissues. (**B**) CREBβ is up-regulated in all brain tissues. Total RNA was prepared from the brain tissues indicated from wild-type (+/+) or CREB mutant mice (–/–) and RNase protection analysis performed as in (A). The protected fragments of CREBΔ, α or β and TBP are indicated by arrows.

performed nuclear run-off transcription assays. Newly transcribed RNAs were measured in nuclei prepared from the brains of wild-type or CREB mutant animals. For the CREB gene, two different DNA fragments were used to examine the transcriptional rate 5′ as well as 3′ of the gene targeting insert. As shown in Figure 8, the transcription rate of CREB in wild-type animals is roughly equivalent to that in CREB mutant animals in both areas of the locus. Similarly, the transcriptional rate of CREM is not altered dramatically in CREB mutant animals when compared with wild-type controls. Thus, the up-regulation of CREM as well as CREBβ in CREB mutant mice occurs post-transcriptionally.

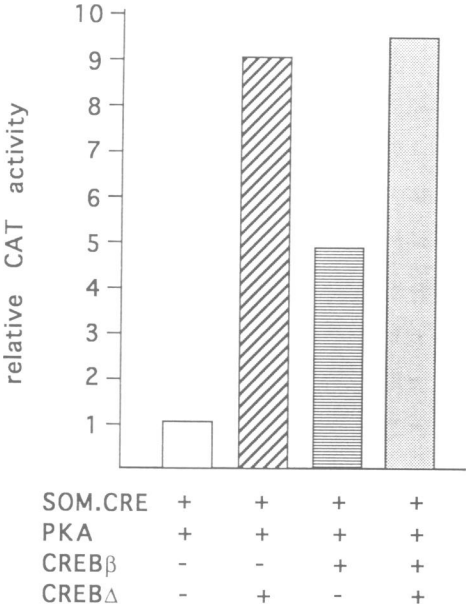

Fig. 7. CREBβ can transactivate promotors containing CREs. Expression plasmids harboring the full-lenghth cDNAs for CREBβ or Δ were co-transfected with a somatostatin promotor construct [Δ(−71)SOM–CAT; Montminy *et al.*, 1986] as a reporter, together with a plasmid directing the expression of the catalytic subunit of PKA into undifferentiated F9 teratocarcinoma cells. CAT activities are corrected for β-galactosidase activity of an internal reference and calculated as fold stimulation, setting the control without CREB expression plasmid to 1.

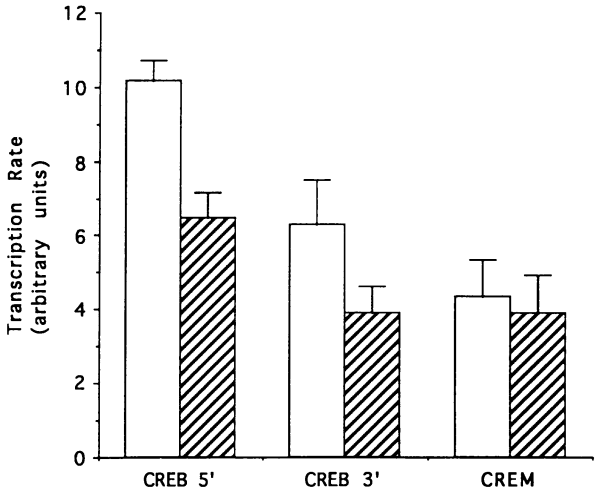

Fig. 8. CREB and CREM gene transcription is not up-regulated in CREB mutant mice. Nuclear run-on assays were performed on brains from wild-type (solid bars) or mutant (hatched bars) CREB mice. Data presented were normalized for the transcriptional activity of a housekeeping gene and represent the mean ± the standard error of three animals per group.

Discussion

Mutation in CREB gene generated by gene targeting is not a null allele

The CREB gene has been implicated as a key regulator in the transcriptional control of many genes. Surprisingly, mice with a targeted mutation of this gene develop normally and appear healthy when examined for a variety of physiological functions. CREB-deficient male mice are fertile, despite previous expression data suggesting that CREB has a role in development of primary spermatocytes (Ruppert *et al.*, 1992). CREB mutant mice exhibit normal pre- and post-natal growth compared with their littermates. This finding is interesting in light of the fact that transgenic mice generated with a mutation in the PKA phosphoacceptor site of the CREB protein and under the control of the growth hormone promoter display a dwarf phenotype, atrophic pituitary and absence of somatotroph cells (Struthers *et al.*, 1991). Lastly, gluconeogenic enzymes such as tyrosine aminotransferase (TAT), in which a CRE was identified as an essential element for the activation of these genes, were not affected by the targeted mutation of the CREB gene.

All the results indicated above suggested functional redundancy within the CREB/ATF family of transcription factors, particularly as one member of this family, CREM, is up-regulated in these mutant mice. However, as more complex analyses were performed using the CREB mutants as a model system, a number of differences in responses were observed in the mutant mouse when compared with wild-type controls. In the case of learning and memory studies, previous data had suggested an important role for CREB-like proteins in memory storage in *Aplysia* (Schacher *et al.*, 1988; Ghirardi *et al.*, 1992) and a central role for cAMP signaling in learning in *Drosophila* (Tully *et al.*, 1994; Yin *et al.*, 1995). Thus, it seemed likely that CREB function is important for memory in higher organisms. This was demonstrated to be true in studies with fear conditioning, which showed that mice with a targeted disruption of the CREB gene are profoundly deficient in long-term memory (Bourtchuladze *et al.*, 1994). Similarly, CREB is thought to be crucial for activation of microglia following nerve transection (Herdegen *et al.*, 1992). Compared with wild-type mice, CREB mutant mice showed drastically reduced microglial proliferation and activation following nerve transection (Gehrmann *et al.*, in preparation). Lastly, changes in cAMP signaling had been implicated in the mechanisms of opiate dependence and withdrawal. In particular, it has been shown that alterations in the state of phosphorylation of CREB are pronounced following acute and chronic morphine administration (Guitart *et al.*, 1992). Recent experiments in our laboratory indicate that CREB mutant mice show a marked decrease in withdrawal symptoms following chronic morphine administration (Maldonado *et al.*, in preparation).

However, not all aspects of CREB-dependent brain function are equally affected. While CREB mutant mice have deficits in learning and memory, microglial proliferation and behavioral responses to opiate withdrawal as detailed above, several other paradigms of neuronal signaling are not altered. For example, CREB has been implicated as a key regulator in the transcriptional control of immediate early genes (IEGs) (e.g. c-*fos*; Sassone-Corsi *et al.*, 1988; Sheng and Greenberg, 1990); this control is not apparent in CREB mutant mice following limbic seizure activity. In this study, a number of IEGs were measured following pharmacologically induced seizures and found to be induced equally in wild-type and CREB mutant animals (Blendy *et al.*, 1995). Furthermore, these mice were studied using the kindling model, which is one means of assessing fleeting changes of neuronal

activity which lead to lasting changes in neuronal structure and function in the mature nervous system. However, the CREB mutants show no differences in their kindling response when compared with wild-type controls (L.Butler and J.MacNamara, personal communication). Hence, an interesting paradox is emerging with respect to the variety of effects seen in CREB mutant mice. These divergent results may be explained partially by compensation among other members of the CREB/ATF family of transcription factors, as was suggested previously (Hummler *et al.*, 1994). An additional explanation includes the presence of residual CREB activity reported here in these mutant mice.

Using immunological detection methods and RNA analysis, we have identified a novel CREB mRNA, termed CREBβ. This isoform is generated by alternative splicing of the primary gene transcript, and leads to a product which differs from other CREB transcripts by the absence of exon 2 and the use of an ATG start codon in exon 4. Previously, six different CREB mRNAs, which differ in their coding information, have been identified in our laboratory (Ruppert *et al.*, 1992). These isoforms are generated by alternative splicing, resulting in the inclusion or exclusion of different exon sequences, and were termed Δ, ψ, α, γ and Ω. CREBΔ and CREBα are the most abundant CREB isoforms and, along with CREB Ω, were the only isoforms identified which contain the basic region and the leucine zipper dimerization domain which are critical for DNA binding and subsequent transactivation of target genes. Furthermore, these isoforms contain the kinase-inducible domain which when phosphorylated leads to an enhancement of these activities (Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989). The novel isoform of the mouse CREB family, CREBβ, described here, includes all of these domains.

Western blot and immunohistochemistry results show that the CREBβ isoform is recognized by both CREB- and phospho-CREB-specific antibodies, indicating that the CREBβ mRNA is translated *in vivo*. Furthermore, analysis of this novel CREB isoform in a transactivation assay indicates its ability to transactivate a CRE-containing reporter construct in undifferentiated F9 cells, as has been shown, and repeated in these studies, for CREBΔ. CREBβ differs from CREBΔ and α by the exclusion of exon 2, which encodes ~40% of the first glutamine-rich domain. It is likely that the reduced stimulation of the CRE-containing reporter construct in the co-transfection experiment by CREBβ is due to this reduced transactivation (Q) domain, although it cannot be ruled out that less efficient tanslation of the CREBβ mRNA plays a role as well.

Distribution analysis and up-regulation of CREBβ

The presence of CREBβ in wild-type tissues indicates that this is a naturally occurring isoform. It has most likely gone undetected in previous screens due, in part, to its low abundance. A thorough tissue distribution analysis indicates that this isoform is present in all tissues to about the same extent as CREBα. Together both α and β make up ~1/3 of the total CREB transcripts present in all tissues examined, with CREBΔ being the predominant isoform.

The dramatic up-regulation of CREBβ seen in CREB mutant mice has made possible the initial cloning and characterization of this isoform. The mechanism underlying this up-regulation is an increase in alternative splicing or stability of the CREBβ mRNA, rather than an increase of the transcriptional activity of the CREB locus. Nuclear run-on analyses indicate that, despite the presence of several CRE sites in the promoter of CREB (Meyer *et al.*, 1993), the lack of CREBα and Δ does not result in a dramatic change in the activity of the CREB promoter.

The CREB mutation is a hypomorphic allele

In summary, the CREB mutant mice described previously represent a partial loss-of-function mutation of the CREB gene. This is not the first example where gene targeting has resulted in hypomorphic alleles. In the case of the N-*myc* locus, the *neo*^{r} gene was insterted into the first intron of N-*myc* in such a way that alternative splicing around this insertion could result in the generation of a normal N-*myc* transcript in addition to a mutant transcript (Moens *et al.*, 1992). In addition, the targeted mutation of the β-amyloid precursor protein did not completely abolish expression of this gene in the mouse, because the cassette designed to terminate transcription was only partially effective and the disrupted exon was skipped during splicing of the readthrough transcript, resulting in the formation of a reduced amount of β-amyloid precursor protein (Müller *et al.*, 1994). In both cases, interesting phenotypes were observed with these hypomorphic alleles.

To date, mice carrying the targeted allele of the CREB gene have been used for at least five independent investigations. Three out of the five paradigms tested (memory consolidation, microglial proliferation and opiate withdrawal response) show abnormalities in the CREB mutant mice, whereas the other paradigms tested (seizure induction and kindling) show no difference between wild-type and mutant mice. It seems likely that the up-regulation of CREBβ, rather than CREM, is responsible for the relatively mild phenotype observed in the CREB mutant mice, as the majority of the CREM isoforms in the brain have been described as negative regulators of transcription (Mellström *et al.*, 1993), while CREBα and Δ are transcriptional activators, as is CREBβ. However, as detailed above, even CREBβ is not sufficient in some cases to overcome the deficiencies observed in the CREB mutant mice.

Mice carrying this hypomorphic allele of CREB have already been proven to be a useful model system to test certain aspects of neuronal signaling. The importance of CREBβ and the usefulness of a hypomorphic mutation in this gene are underscored by recent findings in our laboratory with mice carrying a null mutation in the CREB gene. Preliminary data sugggest that this null mutation leads to perinatal death, as no surviving mutants were found at 4 weeks of age (Rudolf and Schütz, unpublished observation). Hence the importance of generating multiple alleles at a given locus to elucidate the various functions of a gene should not be underestimated.

Materials and methods

Animals

CREB mutant mice were maintained in a mixed (129Sv×C57Bl6) background and genotyped as described previously (Hummler *et al.*, 1994).

Western blot and immunohistochemical analysis

Nuclear extracts were prepared from whole brains of wild-type (+/+) or CREB mutant mice (−/−) as described (Gorski *et al.*, 1986). Western blot analysis was carrried out as described (Hummler *et al.*, 1994) using peptide antibodies specific for CREB or phospho-CREB (Ginty *et al.*, 1993; kindly provided by M.Greenberg, Harvard Medical School, Boston, MA).

For immunocytochemistry, animals were sacrificed by transcardiac perfusion with 4% (w/v) paraformaldehyde. Brains were post-fixed overnight in the same fixative prior to vibratome sectioning. Expression of CREB or CREM was examined in coronal 50 μm vibratome sections at the level of the dorsal hippocampus. All antibodies were affinity purified. The specificity of the antibodies was proven in immunoprecipitation and Western blot experiments as described elsewhere (Herdegen *et al.*, 1991). Sections were incubated in normal goat serum [2% in phosphate-buffered saline (PBS) and 0.2% Triton X-100] for 1 h, followed by the primary antisera for 36 h at 4°C. The primary antisera were diluted 1:3000 to 1:20 000. Immunoreactivity was visualized by the avidin–biotin complex method (Vectastain, Vector Laboratories, USA), as described previously (Gass *et al.*, 1992). Sections were developed in 0.02% diaminobenzidine with 0.02% hydrogen peroxide and a subset counterstained with hemalum. No immunoreactivity was observed following incubation with pre-immune serum (data not shown).

RNA isolation and RNase protection analysis

Total RNA from a variety of mouse tissues was isolated using guanidinium thiocyanate (Chomczynski and Sacchi, 1987). The quality of the RNA preparations was controlled by ethidium bromide staining of the 18S and 28S rRNAs after electrophoretic separation of the RNA in denaturing agarose gels. RNase protection analysis was performed as described previously (Kaestner *et al.*, 1989) using [α-^{32}P]UTP-labeled antisense RNA probes derived from Bluescript (Stratagene) subclones of the CREBΔ cDNA (Ruppert *et al.*, 1992) indicated in Figure 3. A 150 bp subclone of the mouse TBP gene (Tamura *et al.*, 1991) was used as an internal control, as the mRNA of this gene is expressed in all tissues at roughly equal levels, with the exception of ovary and testis. The antisense probes were hybridized overnight against total RNA at 54°C in 80% formamide. Excess probes were removed by digestion with RNases A and T1 and the protected probe fragments analyzed on denaturing 6% polyacrylamide gels. In some cases, the signals obtained were quantified using a PhosphorImager (Molecular Dynamics) and normalized over those of TBP.

PCR amplification of the 5′ end of the CREBβ mRNA

The 5′ end of the CREBβ cDNA was cloned using the Amplifinder Kit (Clontech) according to the manufacturer's instructions. The poly(A)$^+$ RNA fraction from total brain RNA of CREB −/− animals was isolated using oligo(dT)–Dynabeads (Dynal). The cDNA synthesis was primed with primer A (indicated in Figure 5), the resulting cDNA ligated to the anchor oligo provided, and amplified by 30 cycles of PCR (annealing temperature 68°C) using the anchor primer oligo and primer A. Further amplification was achieved by 25 cycles of PCR with the anchor primer and oligo B (see Figure 5). The PCR products were ethanol precipitated, digested with *Eco*RI and *Sma*I, subcloned into Bluescript II (Stratgene) and sequenced. All 24 independent subclones analyzed corresponded to CREBβ.

Cell culture and co-transfection assays

For co-transfection assays, the full-length CREBβ cDNA was subcloned into the expression vector pHD (Müller *et al.*, 1988), the CREBΔ cDNA in the same vector (pHDCREBΔ, Ruppert *et al.*, 1992) was used for comparison, and the CREBΔ cDNA in antisense orientation (pHDBERC) as the negative control. Mouse F9 teratocarcinoma cells were cultured and transfected as described previously (Ruppert *et al.*, 1992). Briefly, 1.2×10^6 cells were plated onto 60 mm culture dishes and transfected 2–3 h after plating with calcium phosphate–DNA co-precipitates. All dishes received 1 μg of Δ(−71)somatostatin–CAT as the reporter (Montminy *et al.*, 1986), 1 μg of CαEV (mouse catalytic subunit Cα of PKA; Uhler and McKnight, 1987) to phosphorylate fully CREB and 2.5–5 μg of the appropriate CREB expression plasmid. After a medium change 16 h after transfection, the cells were harvested after 45 h of incubation. CAT extracts were prepared and analyzed as described previously (Neumann *et al.*, 1987).

Nuclear run-off transcription assays

Newly transcribed RNAs were measured as described by Marzluff and Huang (1985). Nuclei were prepared from the brains of three wild-type or CREB mutant animals (littermates). Nascent transcripts of 2×10^7 nuclei each were extended in the presence of [^{32}P]UTP. Labeled RNA was isolated and hybridized against dot-blots containing 1 μg of gel-purified DNA fragments. Fragments used were: a 4.3 kb *Eco*RI fragment containing exon 1 of the mouse CREB gene (CREB 5′, Ruppert *et al.*, 1992), a 7.0 kb *Hin*dIII fragment containing exon 10 of the mouse CREB gene (CREB 3′, Ruppert *et al.*, 1992), a 8.3 kb *Eco*RI fragment of the mouse CREM gene, a 0.8 kb *Xba*I–*Pst*I fragment of the rat glyceraldehyde phosphate dehydrogenase gene (for normalization, Fort *et al.*, 1985), and pBluescript (Stratagene) to subtract background hybridization. The filters were washed at 65°C in 0.1× SSC, 0.1% SDS and the signals analyzed on a Molecular Dynamics PhosphorImager.

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