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Targeted mutation of the CREB gene: Compensation within the CREB/ATF family of transcription factors

(CREM/gene targeting/homologous recombination)

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ABSTRACT **The cAMP response element binding protein (CREB) has been implicated as a key regulator in the transcriptional control of many genes. To assess the functional importance of CREB *in vivo* and its role in development, we used gene targeting to generate mice with a disruption of the CREB gene. Homozygous mutant mice appeared healthy and exhibited no impairment of growth or development. In this report we demonstrate that CREB and two other members of the CREB/ATF family, cAMP response element modulation protein (CREM) and activating transcription factor 1 (ATF1), appear to form a unique subgroup within this extensive class of transcription factors. Examination of CREM mRNA and protein levels in CREB mutant mice demonstrated overexpression of CREM in all tissues examined, but no change in ATF1 levels. These data demonstrate that CREB is not the sole mediator of cAMP-dependent transcriptional regulation and probably acts in concert with a specific subset of cAMP response element-binding proteins to transduce the cAMP signal and, in its absence, these same proteins can compensate for CREB function *in vivo*.**

Transcription of many genes is affected by changes in cAMP levels in response to a variety of external signals and is mediated via a cAMP response element (CRE). This DNA sequence is recognized by a diverse family of DNA binding proteins (1–5), of which the CRE-binding protein (CREB) has been best characterized (6–12). Activation of the protein kinase A (PKA) pathway leads to phosphorylation of CREB at Ser^{133}, which is required for CREB to initiate transcription of target genes (6, 13). Since the cloning of CREB, a large number of CRE-binding proteins have been identified. They all contain a leucine-zipper DNA binding motif and for some members the potential for heterodimerization has been demonstrated *in vitro* (14). Transcription factor CREB heterodimerizes with activating transcription factor 1 (ATF1) (15) and CRE modulator protein (CREM) (16) *in vitro*. CREM, ATF1, and CREB are strongly related in sequence and appear to be involved in cAMP signaling to the nucleus (1, 2).

The importance of these proteins for cAMP-mediated transcriptional regulation has been clearly demonstrated but not much is known about the specific physiological roles of these proteins. The block of CREB function in transgenic mice by a nonphosphorylatable transdominant-negative CREB mutant prevented proliferation of somatotrophic cells resulting in pituitary atrophy and dwarfism (17). To address the role of CREB in development and in physiological processes in the adult, we generated mice with a disruption of the CREB gene by homologous recombination in mouse embryonic stem (ES) cells.

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MATERIALS AND METHODS

Gene Targeting. The targeting vector consisted of 7.1 kb of mouse CREB genomic DNA and a promoterless neomycin-resistance (Neo) gene inserted in-frame in exon 2 of the mouse CREB gene via a unique *Nco* I site (ref. 18; Fig. 1*A*). The construct (20 μg) was linearized with *Not* I and used to electroporate 1×10^7 D3 ES cells derived from 129Sv/J mice, which were cultured on mitomycin-treated embryonic fibroblast feeder layers (19). DNA from clones surviving G418 selection (200 μg/ml) were individually analyzed on Southern blots and hybridized with probes located either 5′ or 3′ of the genomic sequence contained in the construct. Depending on the probe used blots were prepared by digestion with *Pvu* II (3′ probe) or *Nco* I (5′ probe).

Generation of CREB −/− Mice. ES cells from two clones were used for injection into blastocysts derived from C57BL/6J mice. Blastocysts were transferred to pseudopregnant NMRI/Han females and chimeric offspring were detected by the presence of agouti hairs (genotype A^w) on a nonagouti (*a*) background. Chimeric males were mated to females to produce ES-cell-derived offspring that were then analyzed on Southern blots containing DNA isolated from mouse tails (20). Mice heterozygous for the gene-targeting event were then used to generate homozygous mutant CREB −/− mice.

RNA Analysis. Total RNA was isolated and prepared from tissues of wild-type (+/+), heterozygous (+/−), or homozygous (−/−) CREB mutant adult mice as described (21). A ^{32}P-labeled antisense CREB RNA probe was generated from a 166-bp *Nco* I–*Hin*cII fragment and hybridized with 10 μg of total RNA. Protected fragments were treated and analyzed on 6% polyacrylamide gels as described (22). As a control, a glucocorticoid receptor RNA probe was transcribed from a 320-bp *Ava* II–*Hin*dII fragment of exon 2 of the mouse glucocorticoid receptor gene (23). A CREM-specific RNA probe was a 105-bp fragment (nt 10–114) of the mouse CREMτ cDNA (24), and the ATF1-specific RNA probe was a 226-bp *Bam*HI–*Pst* I fragment (nt 167–393) of the mouse ATF1 cDNA (25). The CREM-isoform-specific RNA probe was a 231-bp fragment from nt 10 to nt 240 of the mouse CREMτcDNA (24). Loading in each RNA sample was controlled by analysis of expression of the cytochrome oxidase gene.

Abbreviations: CRE, cAMP response element; PKA, protein kinase A; Neo, neomycin resistance; ES, embryonic stem.

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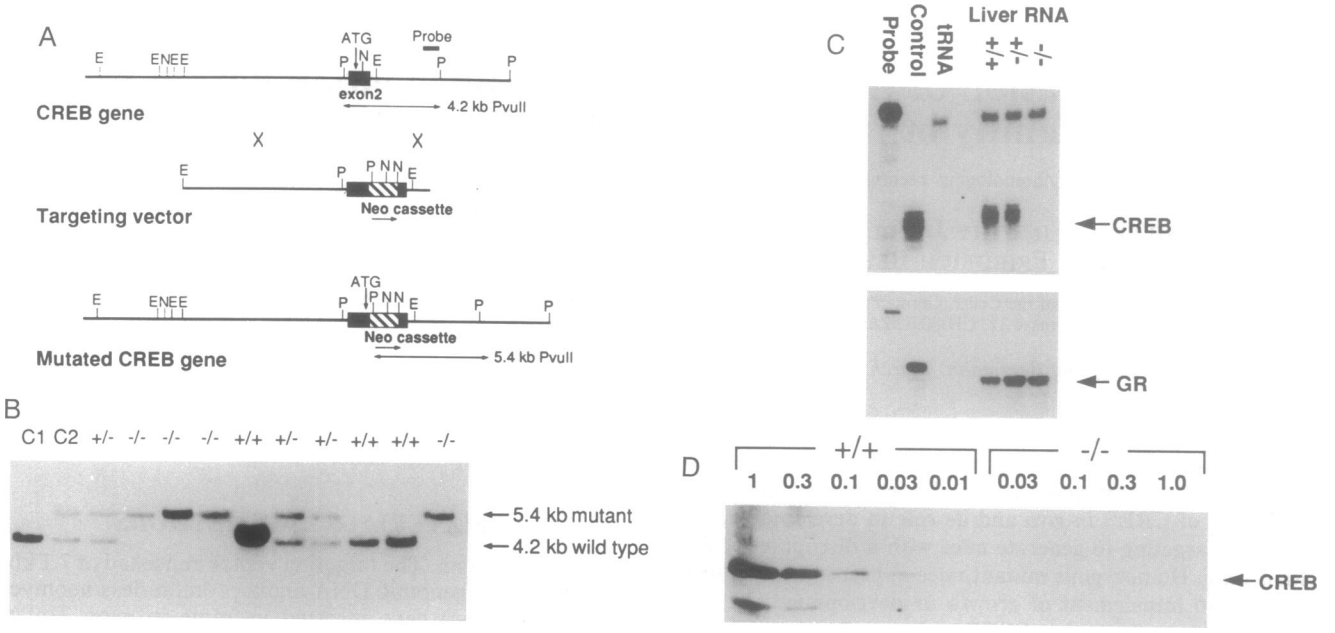

FIG. 1. Inactivation of the mouse CREB gene. (*A*) Structure of the wild-type CREB gene, the targeting vector, and the predicted structure of the targeted CREB locus. Insertion of a promoterless Neo gene cassette in the second exon of the CREB gene is indicated by a hatched box. Expected fragment sizes of the wild-type and mutant CREB allele are indicated after digestion with *Pvu* II and hybridization with a 3′ flanking probe (solid bar). Targeted clones were later confirmed with a 5′ flanking probe (data not shown). E, *Eco*RI; N, *Nco* I; P, *Pvu* II. (*B*) Southern blot analysis of offspring derived from heterozygote intercrosses. Tail DNAs were digested with *Pvu* II and hybridized with the 3′ flanking probe. The CREB genotype is indicated above each lane. (*C*) RNase protection analysis of total RNA (10 μg) from livers of wild-type (+/+), heterozygote (+/−), and homozygote mutant CREB −/− mice by using RNA probes (lane 1) specific for CREB and the glucocorticoid receptor (GR). Controls were *in vitro*-transcribed CREB or GR sense RNA (lane 2) and 10 μg of yeast tRNA (lane 3). (*D*) Western blot analysis of CREB protein in liver nuclear extracts from wild-type (+/+) and mutant CREB −/− mice by using an anti-CREB peptide antibody.

Protein Analysis. Liver nuclear extracts were prepared from wild-type CREB (+/+) and mutant CREB −/− mice as described (26). Extracts were diluted serially 1:3.3 starting with 20 mg of protein and analyzed for immunostaining with a peptide antibody specific for CREB as described (9). For immunohistochemical analysis, pituitary glands were fixed in buffered 4% (wt/vol) paraformaldehyde, paraffin-embedded, and cut in 4-μm sections. Immunohistochemistry was performed using commercial rabbit antisera (Dako) and the avidin–biotin complex detection system.

Immunoprecipitation and "Far-Western" Blot Analysis. Nuclear extracts were prepared from hepatoma cells and from testis and brain of wild-type (+/+) mice as described (26) and phosphorylated with [γ-^{32}P]ATP by the PKA catalytic subunit. Extracts were incubated with a phosphopeptide antibody (kindly provided by M. Greenberg, Harvard Medical School, Boston), raised against a 14-amino acid phosphorylated peptide corresponding to amino acids 123–136 of the CREB PKA domain (27) or a CREB antibody. Immunoprecipitates were analyzed by SDS/PAGE and autoradiography. Protein markers and nuclear extracts from liver, brain, and testis were separated by SDS/PAGE, transferred to a nylon membrane, and probed with ^{32}P-labeled bacterially expressed CREB (10^8 dpm/mg) as described (28). Protein markers of CREB and ATF1 were synthesized by *in vitro* transcription and translation. Bacterially expressed CREMτ was a generous gift from P. Sassone-Corsi (Institut National de la Santé et de la Recherche Médicale, Strasbourg).

RESULTS AND DISCUSSION

Targeted Mutation of the CREB Gene by Homologous Recombination. The mouse CREB gene was disrupted in ES cells by a replacement targeting vector as illustrated in Fig. 1*A*. As CREB is expressed in ES cells, a promoterless Neo gene was inserted in-frame into the second exon, as this is a common exon to all known mRNA isoforms. Twelve correctly homologously recombined clones were identified, representing a frequency of 1 targeted clone per 25 stably transformed cells. After blastocyst injection of two clones, we obtained 14 male and 11 female chimeras. Several chimeras transmitted the mutated allele to their offspring. Heterozygous males and females were mated to generate homozygous mutant CREB −/− mice (Fig. 1*B*). Among 311 adult mice analyzed, only 15% of the mice were genotyped as −/− mutants (+/+, 31%; +/−, 54%), which clearly deviates from the expected Mendelian frequency. Backcrossing of heterozygote offspring to wild-type mice indicated no transmission distortion of the mutated CREB allele (data not shown). This suggests that homozygous mutant CREB −/− mice may have a disadvantage in surviving to adulthood. Those mice that do survive, however, exhibit an apparently normal phenotype, showing no histological or morphological defects. RNase protection analysis of total RNA from liver revealed no CREB expression in CREB −/− mice (Fig. 1*C*), and the lack of CREB protein in liver was confirmed by Western blot analysis (Fig. 1*D*).

Absence of CREB Does Not Affect cAMP Signaling in the Pituitary. Previous studies have shown that cAMP serves as a mitogenic signal for anterior pituitary somatotrophic cells (29). In a study using transgenic mice, a transdominant negative mutant of CREB was expressed under the control of the growth hormone gene promoter (17). The mutated CREB protein, which cannot be phosphorylated at Ser^{133} but is still able to form dimers and bind DNA, leads to pituitary hypoplasia and dwarfism. We therefore investigated whether the proliferation of these cells was affected in homozygous CREB −/− mice. Histological examination of pituitaries of all three CREB genotypes showed normal anterior, intermediate, and posterior lobes. Immunohistochemical analysis revealed normal staining patterns for growth hormone (Fig. 2 *A* and *B*), corticotropin (Fig. 2 *C* and *D*), follicle stimulating

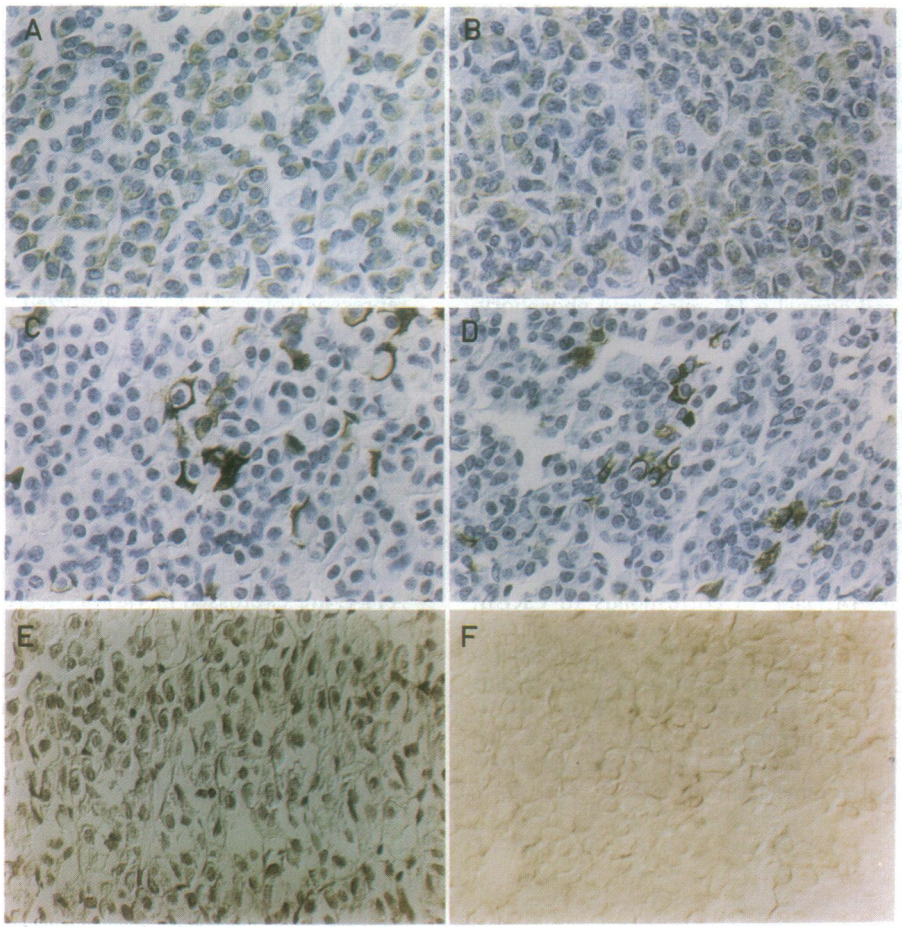

FIG. 2. Immunohistochemical analysis of the anterior pituitary lobe in CREB +/+ (*A*, *C*, and *E*) and CREB −/− (*B*, *D*, and *F*) mice. (*A* and *B*) Growth hormone. (*C* and *D*) Corticotropin. (*E* and *F*) CREB. No immunoreactivity was detected in CREB −/− mice (*F*).

hormone, luteinizing hormone, and prolactin (data not shown). CREB immunoreactivity in wild-type mice was largely confined to the nucleus and was not detected in CREB −/− mice (Fig. 2 *E* and *F*). The difference in pituitary

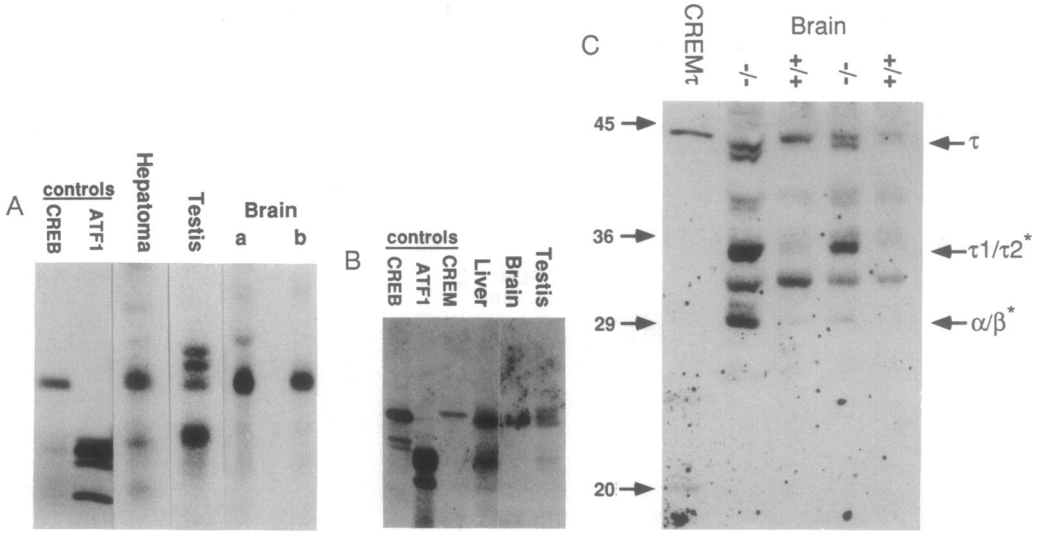

FIG. 3. CREB, CREM, and ATF1 are targets for phosphorylation by PKA and can heterodimerize. (*A*) Immunoprecipitation from hepatoma cell line (FTO2B), testis, and brain extracts, after phosphorylation *in vitro* by PKA, using a phosphopeptide antibody. Immunoprecipitation from brain extracts was performed using both the phosphopeptide antibody (lane a) and a CREB-specific antibody (lane b). Labeled markers for CREB and ATF1 were synthesized by *in vitro* transcription/translation. (*B*) Analysis of proteins dimerizing with CREB by far-Western blot analysis (28). Labeled markers of CREB, ATF1, and CREMτ served as controls. (*C*) Western blot analysis of CREM protein in brain nuclear extracts from wild-type (+/+) and mutant CREB −/− mice using an affinity-purified CREM antibody directed against the CREM γ-peptide (16). Bacterially expressed CREMτ was used as a control (lane 1). Molecular mass markers (indicated by arrows) were ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20 kDa). The position of the CREM τ1, τ2, α, and β isoforms (indicated by *) is designated based on their relative sizes as calculated from their amino acid sequences (16, 24, 30).

phenotypes between the transcriptionally inactive CREB mutant (acting as a dominant negative mutant) and the CREB −/− mutation suggests that other CRE-binding proteins functionally compensate for CREB in CREB −/− mice.

Functional Compensation Within a Subset of the CREB/ATF Gene Family. We investigated the potential of other members of the CREB/ATF family to substitute for CREB function in mutant mice. To identify family members that may be activated by the PKA pathway, we performed *in vitro* PKA phosphorylation assays with nuclear extracts from wild-type mice. After phosphorylation, protein was immunoprecipitated with an antibody raised against a 14-amino acid phosphorylated peptide of the CREB PKA domain (Fig. 3*A*). From extracts of the FTO hepatoma cell line, two major bands were immunoprecipitated that corresponded to CREB and ATF1, another member of the CREB/ATF family (3). In testis extracts, in addition to CREB and ATF1, two other bands with slightly higher molecular weights were detected. These two bands probably represent CREM, due to size and the abundance of CREM in testis (16, 24, 30). In nuclear extracts from brain, however, the predominant protein immunoprecipitated by the phosphopeptide antibody (Fig. 3*A*, lane a) and the CREB antibody (Fig. 3*A*, lane b) corresponds to CREB. Far-Western blot protein analysis was performed to identify potential dimerization partners of CREB (Fig. 3*B*). By using a labeled CREB probe, the only proteins identified in extracts from liver, testis, or brain corresponded to CREB, CREM, and ATF1. Thus, these experiments strongly suggest that CREB, CREM, and ATF1 are the only targets for PKA phosphorylation that also dimerize with CREB. Therefore, these three proteins form a structurally and functionally related subset of the CREB/ATF family of transcription factors.

Is the absence of a major phenotypic change in CREB −/− mice due to functional compensation by CREM and/or ATF1? Examination of RNA expression in wild-type mice by RNase protection analysis (Fig. 4*A*) showed that all three genes were expressed in all tissues and cell lines tested. Furthermore, analysis of RNA from CREB −/− mice revealed no overall change in the levels of ATF1 transcripts, but a 2- to 3-fold increase in the expression of CREM in liver, kidney, and brain (Fig. 4*B*). As CREM exists in activator and repressor isoforms (16), we analyzed which of these isoforms is up-regulated in CREB −/− mice. Both the activator CREMτ isoform RNA and the repressor CREMα/β isoform RNA, which lacks an activator-specific exon, are up-regulated in RNA isolated from brain (Fig. 4*C*), liver, and kidney (data not shown). Analysis of protein extracts from brain of CREB −/− mice equally demonstrates increased expression of the respective CREM proteins (τ, α, and β; Fig. 3*C*).

Our data indicate that the three functionally related mediators of cAMP signaling in the nucleus, CREB, CREM, and ATF1, are expressed in various degrees in all tissues examined. In addition, this gene subfamily is characterized by a common dimerization domain and a conserved kinase-inducible domain that is a target for phosphorylation by PKA. In CREB −/− mice, CREM (which is up-regulated) and ATF1 seem to compensate for the lack of CREB protein, thus resulting in a normal phenotype. This type of functional redundancy is not unique to this subgroup of transcription

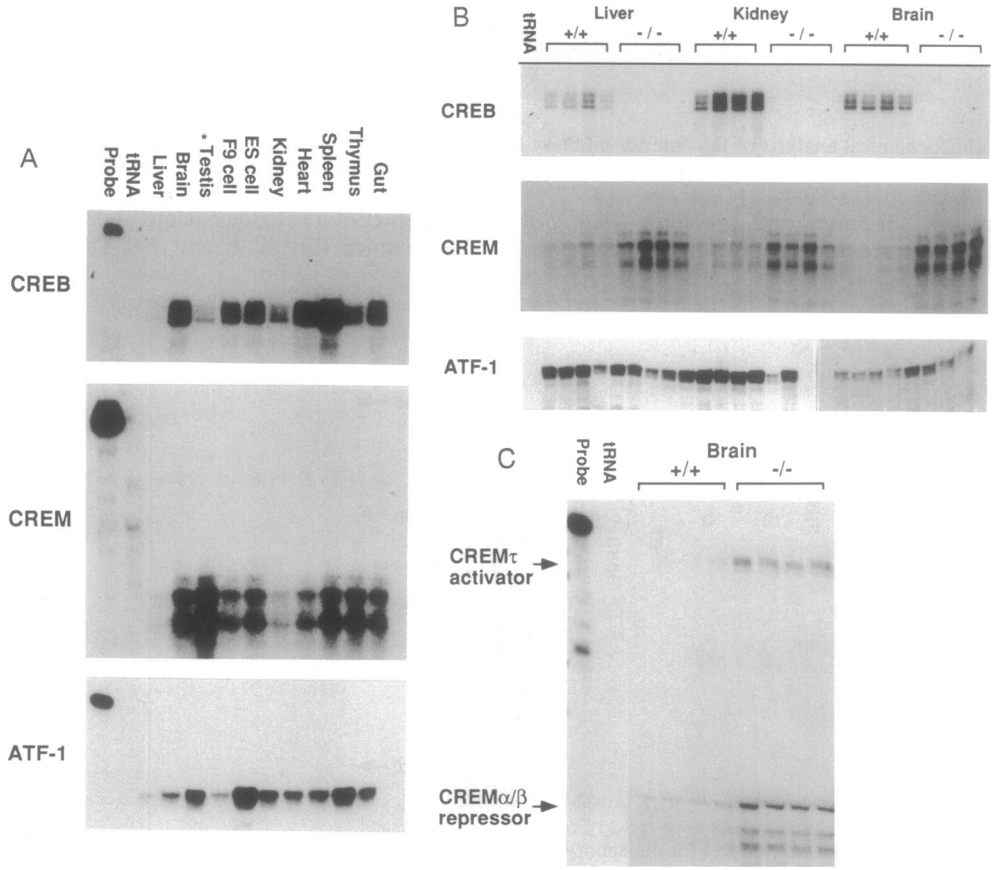

FIG. 4. Expression analysis of CREB, CREM, and ATF1 in wild-type and CREB −/− mice. (*A*) RNase protection analysis of various mouse tissues and F9 and D3ES cells using CREB-, CREM-, and ATF1-specific RNA probes. All RNA samples were 50 μg of total RNA except testis (indicated by *), which was 5 μg. (*B*) Expression of CREB, CREM, and ATF1 RNA by RNase protection for four individual wild-type and CREB −/− mice in liver (50 μg), kidney (25 μg), and brain (10 μg). (*C*) Expression of CREMτ-activator and CREMα/β-repressor isoform RNAs in brain by RNase protection analysis in four individual wild-type and CREB −/− mice. CREMτ transcripts give complete RNase protection (231 bp) and CREMα/β transcripts, which lack an activator-specific exon, protect a 105-bp fragment.

factors as it has also been observed for Myf5 in MyoD-deficient mice (31). These data also provide evidence that in wild-type mice CREB may directly or indirectly negatively regulate CREM expression. The relatively high abundance of CREB protein in brain (Fig. 3*A*) and recent data implicating cAMP-inducible genes in long-term potentiation (32, 33) and the maintenance of circadian rhythms (27) make the CREB −/− mouse a useful model to study selective effects on neuronal function.

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1. Brindle, P. K. & Montminy, M. R. (1993) *Curr. Opin. Genet. Dev.* **2**, 199–204.
2. Lee, K. A. W. & Masson, N. (1993) *Biochim. Biophys. Acta* **1174**, 221–233.
3. Hai, T., Liu, F., Coukos, W. J. & Green, M. R. (1989) *Genes Dev.* **3**, 2083–2090.
4. Flint, K. J. & Jones, N. C. (1991) *Oncogene* **6**, 2019–2026.
5. Lee, K. A. W. (1991) *Curr. Opin. Cell Biol.* **3**, 953–959.
6. Gonzalez, G. A. & Montminy, M. R. (1989) *Cell* **59**, 675–680.
7. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S. & Montminy, M. (1992) *Cell* **70**, 105–113.
8. Boshart, M., Weih, F., Nichols, M. & Schütz, G. (1991) *Cell* **66**, 849–859.
9. Nichols, M., Weih, F., Schmid, W., DeVack, C., Kowenz-Leutz, E., Luckow, B., Boshart, M. & Schütz, G. (1992) *EMBO J.* **11**, 3337–3346.
10. Ruppert, S., Cole, T. J., Boshart, M., Schmid, E. & Schütz, G. (1992) *EMBO J.* **11**, 1503–1512.
11. Lee, C. Q., Yun, Y., Hoeffler, J. P. & Habener, J. F. (1990) *EMBO J.* **9**, 4455–4465.
12. Waeber, G. & Habener, J. F. (1991) *Mol. Endocrinol.* **5**, 1431–1438.
13. Sheng, M. E., Thompson, M. A. & Greenberg, M. E. (1991) *Science* **252**, 1427–1430.
14. Meyer, T. E. & Habener, J. F. (1993) *Endocrinol. Rev.* **14**, 411–414.
15. Hurst, H. C., Totty, N. F. & Jones, N. C. (1991) *Nucleic Acids Res.* **19**, 4601–4609.
16. Foulkes, N. S. & Sassone-Corsi, P. (1992) *Cell* **68**, 411–414.
17. Struthers, R. S., Vale, W. W., Arias, C., Sawchenko, P. E. & Montminy, M. R. (1991) *Nature (London)* **350**, 622–624.
18. Cole, T. J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Schütz, G. & Ruppert, S. (1992) *Genomics* **13**, 974–982.
19. Doetschman, T. C., Eistetter, H., Katz, M., Schmid, W. & Kemler, R. J. (1985) *Embryol. Exp. Morphol.* **87**, 27–45.
20. Beermann, F., Hummler, E., Schmid, E. & Schütz, G. (1993) *Mech. Dev.* **42**, 59–65.
21. LeMeur, M., Glanville, N., Mandel, J. C., Gerlinger, P., Palmiter, R. & Chambon, P. (1981) *Cell* **23**, 561–573.
22. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1992) *Current Protocols in Molecular Biology* (Greene and Wiley, New York).
23. Strähle, U., Schmidt, A., Kelsey, G., Stewart, A. F., Cole, T. J., Schmid, W. & Schütz, G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6731–6735.
24. Foulkes, N. S., Mellström, B., Benusiglio, E. & Sassone-Corsi, P. (1992) *Nature (London)* **355**, 80–84.
25. Lee, M.-R., Chung, C.-S., Liou, M.-L., Wu, M., Li, W.-F., Hsueh, Y.-P. & Lai, M.-Z. (1992) *J. Immunol.* **148**, 1906–1912.
26. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
27. Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S. & Greenberg, M. E. (1993) *Science* **260**, 238–291.
28. Masson, N., Hurst, H. C. & Lee, K. A. W. (1993) *Nucleic Acids Res.* **21**, 1163–1169.
29. Billestrup, N., Swanson, L. M. & Vale, W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6854–6857.
30. Foulkes, N. S., Borelli, E. & Sassone-Corsi, P. (1991) *Cell* **64**, 739–749.
31. Rudnicki, M. A., Braun, T., Hinuma, S. & Jaenisch, R. (1992) *Cell* **71**, 383–390.
32. Schacher, S., Castellucci, V. F. & Kandel, E. R. (1988) *Science* **240**, 1667–1669.
33. Dask, K. P., Hochner, B. & Kandel, E. R. (1990) *Nature (London)* **345**, 718–721.