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Functionally Distinct Isoforms of the CRE-BP DNA-Binding Protein Mediate Activity of a T-Cell-Specific Enhancer

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Expression of the CD3δ gene of the T-cell receptor (TCR) complex is regulated by a T-cell-specific enhancer. A highly conserved 40-bp motif (element δA) within the CD3δ enhancer is responsible for mediating its activity and specificity. Element δA exhibits sequence similarities to the cyclic AMP response element (CRE) but does not respond to changes in the level of cyclic AMP. Using the δA element as a probe, we have isolated three cDNA clones encoding three distinct protein isoforms, products of differential splicing and alternate promoter usage of the CRE-BP gene. These isoforms share the DNA binding and dimerization domains at the C terminus of the protein but differ at their N termini. In transfection assays, their activities as transcription regulators differ: CRE-BP2 is a potent activator, CRE-BP3 is a weak activator, and CRE-BP1 is transcriptionally inert. Mutations in the basic region of the CRE-BP1 protein which abrogate its ability to bind DNA render this protein a dominant repressor of the δA enhancer. Antibodies to the CRE-BP protein interact specifically with the ubiquitous and predominantly T-cell-restricted nuclear complexes that bind to the δA element and suggest the presence of this protein in homo- and heterodimeric complexes. Since the δA motif is also present in the enhancer and promoter of the TCR α and β genes, the CRE-BP isoforms may mediate expression of other members of the CD3/TCR complex during T-cell development.

Differentiation of a precursor stem cell into a given cell lineage is characterized by the novel expression of genes that are identified as lineage-specific markers. Expression of the invariant δ, γ, and ε polypeptides of the CD3/T-cell receptor (TCR) complex (5, 13) is detected in the early prethymocyte and may occur upon or soon after T-cell commitment. Immature thymocytes and T-cell mutants contain low levels of CD3δ mRNA. The higher levels expressed in mature T cells can be further increased upon T-cell activation (27, 34).

In a search for the regulatory elements that mediate expression of CD3δ, a tissue-specific enhancer was identified at the 3′ of the gene (8). This enhancer was dissected into two *cis*-acting elements, δA and δB (7). Element δA can function as a strong enhancer when reiterated but requires the presence of δB for high levels of activity when present as a single copy in the context of the CD3δ enhancer. The sequence of element δA shows strong similarity to the cyclic AMP (cAMP) response element (CRE) (4, 9, 14, 22), but its activity is not modulated by an increase in the level of cAMP. Element δB does not function independently, even when reiterated, but stimulates the activity of δA element by threefold in cells of the T lineage (7). The activity of element δA in various T-cell lines correlates with that of the CD3δ enhancer and with the expression levels of the endogenous CD3δ gene. Study of the nuclear factors that interact with elements δA and δB in T and non-T cells revealed a specific nuclear complex whose presence correlates with the δA enhancer activity (7).

As a first step in understanding how the nuclear factors that interact with the CD3δ enhancer elements mediate expression of the CD3δ gene during T-cell development, we have cloned three cDNAs encoding proteins that bind to element δA. Here we report that these cDNAs are products of differential splicing and alternate promoter usage of the CRE-BP gene (25). The three protein isoforms encoded by these cDNAs interact with element δA with high affinity but differ in the ability to modulate the activity of this enhancer element. The three CRE-BP isoforms range from strong activators to transcriptionally inert DNA-binding proteins. To test the role of the CRE-BP gene in mediating the activity of the T-cell-specific δA element, we have generated CRE-BP mutants which will not bind DNA but which will dimerize with both mutant and wild-type molecules. These CRE-BP mutants strongly repress the activity of the δA element when expressed in mature T cells. Furthermore, antibodies raised to the CRE-BP isoforms interact specifically with the nuclear complexes that bind to the δA element in T cells.

MATERIALS AND METHODS

Cloning of three distinct isoforms of the CRE-BP gene which bind specifically to the δA element of the CD3δ enhancer. An oligo(dT)-primed cDNA library from the T-cell line EL4 was made in the λ ZAP expression vector by using standard protocols (Stratagene, San Diego, Calif.). One million recombinant phage were screened with a pentameric copy of element δA according to the protocol of Singh et al. (28) as modified by Vinson et al. (33). Eight specific clones were isolated, which were characterized by restriction analysis and allocated into three groups (Fig. 1). Each group was further analyzed by sequencing. Analysis of the DNA and protein data was done by using the University of Wisconsin ALIGN, FASTA, and PEPPLOT programs.

Mutagenesis in the basic DNA binding domain of the CRE-BP protein. Nonconservative amino acid substitutions were introduced into a conserved region of the DNA binding domain of the CRE-BP protein, using the polymerase chain reaction (PCR). The amino acid sequence GAGSAAA was substituted for the wild-type RCRQKRK sequence. These changes are expected to disrupt the α-helical secondary

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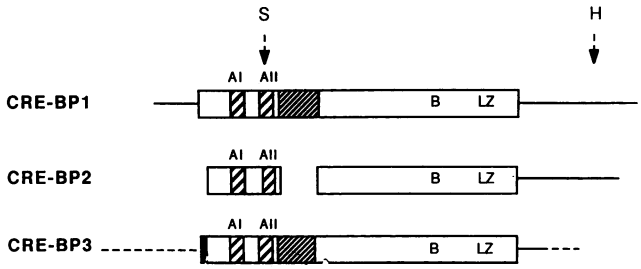

FIG. 1. Schematic representation of the CRE-BP1, -BP2, and -BP3 cDNAs. The coding regions are shown as open rectangles. The alternatively spliced region present in CRE-BP1 and -BP3 but absent from CRE-BP2 is shown as a hatched box. The difference in the coding region between CRE-BP3 and CRE-BP1 and -BP2 cDNAs is shown as a black box. Lines indicate 5′ and 3′ untranslated regions. The broken line in CRE-BP3 indicates differences in the untranslated regions of this cDNA which differ from the respective regions in the CRE-BP1 cDNA. AI and AII designate putative activation domains. LZ and B designate the leucine zipper and basic regions. Arrows labeled S and H show the locations of unique *Sal*I and *Hin*dIII sites used for subsequent construction of full-length CRE-BP2 (CRE-BP2.1) in the expression vector CDM8 (Fig. 5).

structure of this region. The nucleotide sequences of the CRE-BP mutants were determined. Truncated CRE-BP molecules with deletions through the DNA binding and the leucine zipper dimerization domains were also generated. In the CRE-BP LZ5 mutant, the N terminus including the basic region was deleted (nucleotides [nt] 1 to 1170), leaving intact the leucine zipper dimerization domain. In the CRE-BP LZ4 mutant, the deletion was extended into the dimerization domain, and one of the five leucines was deleted (nt 1 to 1192). In the CRE-BP LZ3 mutant, two of the five leucines of the zipper dimerization domain were removed (nt 1 to 1212). A peptide encoding a nuclear localization signal was introduced at the N terminus of each of these truncated CRE-BP proteins to allow for their efficient translocation to the nucleus. To control for the specificity of the repression mediated by the CRE-BP DNA-binding mutant molecules, the leucine zipper dimerization domain of CREB (nt 980 to 1220) was used in parallel experiments (4, 9, 14).

Expression of the CRE-BP isoforms and mutant variants. The CRE-BP1, -BP2, and -BP3 cDNAs were subcloned into the CDM8 expression vector (1). The tkCAT reporter gene driven by five or three copies of the δA element was cotransfected with recombinant CDM8 expression vectors. Different concentrations of the reporter gene and expression vector were tested, and a ratio within the linear range for activation of the reporter gene by recombinant CDM8 plasmids was used for further experiments. The recombinant plasmids (CDM8/CRE-BP2.1, -BP2, and -BP3 [see Fig. 5; 2.5 μg each]) were cotransfected with 10 μg of the 5δAtkCAT reporter plasmids into the mature T-cell line EL4, the immature thymoma cell line BW5147, and the fibroblast cell line NIH 3T3 as previously described (7). To control for transfection efficiency, the RSVGH and SL3GH plasmids were included in the transfections of fibroblast and lymphoid cells, respectively. Transfections were performed in triplicate for each recombinant expression vector, and chloramphenicol acetyltransferase (CAT) activity did not vary more than 15%. The level of expression from the CDM8 vector in the different cell types was tested by using the CDM8-GH gene.

An oligonucleotide was introduced at the 5′ of the CRE-BP1 and -BP2.1 clones which allows for efficient translation of sequences upstream of the first initiation codon. These sequences encode a putative finger domain which in the human CRE-BP1 homolog appears to be involved in transactivation by the adenovirus E1a protein (23). The activity of the finger plus CRE-BP isoforms were tested in a transfection assay as described above.

In a similar fashion, CRE-BP mutants in DNA binding were tested for the ability to alter expression of the tkCAT reporter gene driven by three copies of the δA element when cotransfected in the mature T-cell line EL4. The effect of expression of a CREB DNA-binding mutant was studied in parallel.

Finally, the effects of expression of CRE-BP wild-type and DNA-binding mutants were tested on the activity of the tkCAT, 5δAtkCAT, and tkCATSL3 reporter genes.

Specificity and affinity of binding of the CRE-BP isoforms for the δA sequence motif. Bacteria harboring the recombinant phagemids were grown to A_{600} = 0.3 and then induced with isopropylthiogalactopyranoside (IPTG) to produce the recombinant protein. Bacterial lysates and nuclear extracts made from the T-cell line EL4 were assayed in parallel by gel retardation and footprinting assays as previously described (7). The 40-bp GGAGAAGTTTCCATGACATCATGAATGGGGTGGCACTCGA double-stranded oligonucleotide containing the δA element (underlined) was used as a probe. δA oligonucleotides with nucleotide substitutions in the 8-bp hyphenated palindrome (double underlined) were used in competition assays. These substitutions were TGACGTCA, TGATATCA, and TAAGATCA.

In vitro transcription of the CRE-BP2.1 wild-type and CRE-BP LZ5 mutant was performed from the T7 promoter of the CDM8 recombinant vectors. Wild-type and mutant molecules were either cotranslated or mixed after translation (for 30 min at 37°C), and the protein products were assayed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and gel retardation as described previously (12).

Antibodies to the CRE-BP protein interact with the T-cell-specific complexes and the in vitro-translated CRE-BP2.1 that bind to the δA sequence motif. CRE-BP fragments from nt 315 to 1560 and 481 to 783 of the coding region (covering the 98 amino acids spliced out in the CRE-BP2 isoform) were fused in frame with the glutathione transferase gene in the pGEX1 vector (29). Fusion proteins were generated, purified over a glutathione column, and injected in rabbits. After three additional boosts, rabbit serum was collected and immunoglobulin G antibodies were purified over a protein A column. Dilutions of the purified antibodies (1:5 and 1:25) were assayed in a gel retardation assay for the ability to interact with the δA nuclear complexes and with in vitro-translated CRE-BP2.1.

Northern (RNA) and PCR analysis of the CRE-BP1, -BP2, and -BP3 transcripts. RNA was generated from the T-cell lines EL4, BW5147, and SL12.1, from NIH 3T3 fibroblast cells, from the A20 myeloma cell line, and from spleen, thymus, brain, liver, lung, kidney, and heart tissues. Total RNA (30 μg) was separated on a formaldehyde-agarose gel, transferred to a membrane, and hybridized sequentially with CRE-BP1, CRE-BP3, and actin probes at 65°C in the presence of 5% SDS.

For PCR analysis, 30 μg of total RNA from different tissues was hybridized to the appropriate specific primer and reverse transcribed. The reverse transcription reaction was subjected to 15 cycles of amplification with a second specific primer, and the products were analyzed by gel electropho-

resis and by Southern transfer. Amplified products of CRE-BP1 and -BP2 cDNAs were proportional to input cDNA under these conditions.

RESULTS

Cloning of the DNA-binding proteins that interact with the δA enhancer element of the CD3δ gene. To isolate and characterize the DNA-binding proteins which mediate transcription of the CD3δ gene during T-cell development, we generated a cDNA expression library from the mature T-cell line EL4. This T-cell line has the highest levels of the transcriptionally active factor(s) that binds to element δA, as assayed by transient transfection and gel retardation assays. One million recombinant λ phage clones were screened by using a reiterated δA element probe. Eight clones encoded proteins that bound the δA probe but failed to bind similarly sized pentamers of unrelated sequence (data not shown). Restriction analysis allocated these clones to three groups (Fig. 1).

The CRE-BP1 cDNA clone is 3.8 kb in size and encodes a 49-kDa protein. CRE-BP2 overlaps the CRE-BP1 clone but is shorter at the 5′ and 3′ ends by 394 and 300 bp, respectively. Furthermore, a 294-bp sequence in the coding region of CRE-BP1 is absent in CRE-BP2. We assume that differential splicing in this region accounts for these protein isoforms. The CRE-BP1 and -BP3 clones share most of the coding region and differ only by 15 amino acids at the N terminus (Fig. 2). The 5′ and 3′ untranslated regions of the CRE-BP3 cDNA differ from those of CRE-BP1 (Fig. 2). The CRE-BP3 cDNA includes a long 5′ untranslated region (465 bp) containing multiple initiation sites followed by stop codons. This is reminiscent of the structure of the 5′ untranslated region of several cDNAs subject to translational control (19). PCR analysis (see below) confirmed that the cDNA sequences represent bona fide RNAs and are not artifacts of the library construction (data not shown for CRE-BP3).

The human CRE-BP1 clone isolated by Maekawa et al. (25) diverges upstream of the translation initiation site of the mouse CRE-BP1 clone isolated here into a novel open reading frame and gives rise to a larger protein. The two proteins also differ at the last 10 C-terminal amino acids. Diversification of the human and mouse species or alternate splicing may account for these changes. Hai et al. (11) and Kara et al. (17) have also isolated partial human and mouse CRE-BP clones from osteosarcoma, B-cell, and spleen cell libraries. These clones start at positions 535, 661, and 314 of the CRE-BP1 clone. All of these previously identified CRE-BP clones were isolated by using the CRE as a probe for expression screening.

Structure of the CRE-BP1, -BP2, and -BP3 DNA-binding proteins. Sequence analysis revealed that the common region of these proteins has the characteristics of a leucine zipper DNA-binding protein, namely, a region of basic amino acids followed by a series of five leucines separated by six amino acids in a sequence capable of forming an amphipathic helix (Fig. 2) (20, 21). The putative DNA binding and dimerization domains of the CRE-BP isoforms show extensive homology to the c-Jun, c-Fos, and CREB members of the leucine zipper family (25).

CRE-BP2 differs from CRE-BP1 by the removal of 98 amino acids at position 100 of this protein. This stretch of 98 amino acids is relatively hydrophobic and serine rich and has a propensity for forming β-sheet structure. CRE-BP3 replaces the first 15 amino acids of the CRE-BP1 protein with a hydrophobic, 8-amino-acid peptide but is otherwise identical throughout the coding region.

Differential expression of CRE-BP1, -BP2, and -BP3 in the mouse. The expression of CRE-BP1, -BP2, and -BP3 was studied in different mouse cell lines and tissues, using RNA hybridization with clone-specific probes. A probe common to all CRE-BP isoforms hybridized predominantly with a 4.2-kb transcript(s), but hybridization with faster-migrating transcripts was also evident (Fig. 3A). A CRE-BP3-specific probe composed of the first 500 bp identified the faster-migrating species as CRE-BP3 related (Fig. 3B). An actin-specific probe was used to control for variability in the RNA levels in different samples (Fig. 3B). The 4.2-kb transcript(s) was strongly expressed in all tissues tested. In contrast, the CRE-BP3 isoform was strongly expressed only in an embryonic blastoderm cell line; its level of expression was very low in all other tissues tested (Fig. 3A and B).

PCR analysis was used to determine the relative levels of expression of CRE-BP1 and -BP2 isoforms, using oligonucleotides flanking the alternate exon (Fig. 3C). Amplification of control mixtures of the two cDNAs gave the expected 270- and 570-nt products in proportion to the ratio of starting cDNAs under the conditions used (data not shown). The CRE-BP1-dependent band was observed in all tissues tested at similar levels. The CRE-BP2-dependent band was most prevalent in T cells and in brain tissue, in which it was fivefold less abundant than CRE-BP1. Restriction digests and hybridization to internal sequences confirmed the identity of these PCR products and revealed low levels of CRE-BP2 in the SL12.1 T-cell mutant, in NIH 3T3 fibroblasts, and in thymic tissue (Fig. 3C). The low level of CRE-BP2 expression detected in the thymus is presumably due to the relatively small proportion of mature T cells in this tissue. Parallel expression patterns of the CRE-BP isoforms in T cells and in the brain suggest shared regulatory mechanisms between these two tissues. A number of genes (e.g., Thy1 and CD4 [19]) have been previously reported to be specifically expressed in these two tissues.

Three additional CRE-BP1/2-related PCR products were detected upon Southern hybridization and may represent incomplete or additional splicing products from this region of the CRE-BP molecule (Fig. 3C). Of these PCR products, one appears to be T-cell restricted and may represent yet another T-cell-restricted CRE-BP splicing variant.

Affinity of the CRE-BP1, -BP2, and -BP3 isoforms for the δA element. The affinity and specificity of the CRE-BP1, -BP2, and -BP3 isoforms for binding to the δA element were tested together with nuclear extracts from the mature T-cell line EL4. A fivefold molar excess of cold δA oligonucleotide competed effectively for binding of each of the three bacterially produced isoforms (Fig. 4A). The three CRE-BP isoforms protected the same DNA sequence in the δA binding site from DNase I digestion (Fig. 4B). All three CRE-BP isoforms appear to bind element δA with the same affinity and specificity.

Modulation of δA enhancer activity by expression of the CRE-BP isoforms in T and non-T cells. Our previous studies have shown that the δA element is a strong enhancer in T-cell lines with a mature phenotype (EL4) but is a very weak enhancer in mutant T cells (BW5147, SL12.1, and SAK8) and fibroblasts (NIH 3T3). Several nuclear complexes which interact specifically with element δA were identified. One of these complexes, found predominantly in mature T cells, correlates with activity levels of the δA enhancer and expression of the CD3δ gene (7).

The ability of the three CRE-BP isoforms to modulate the

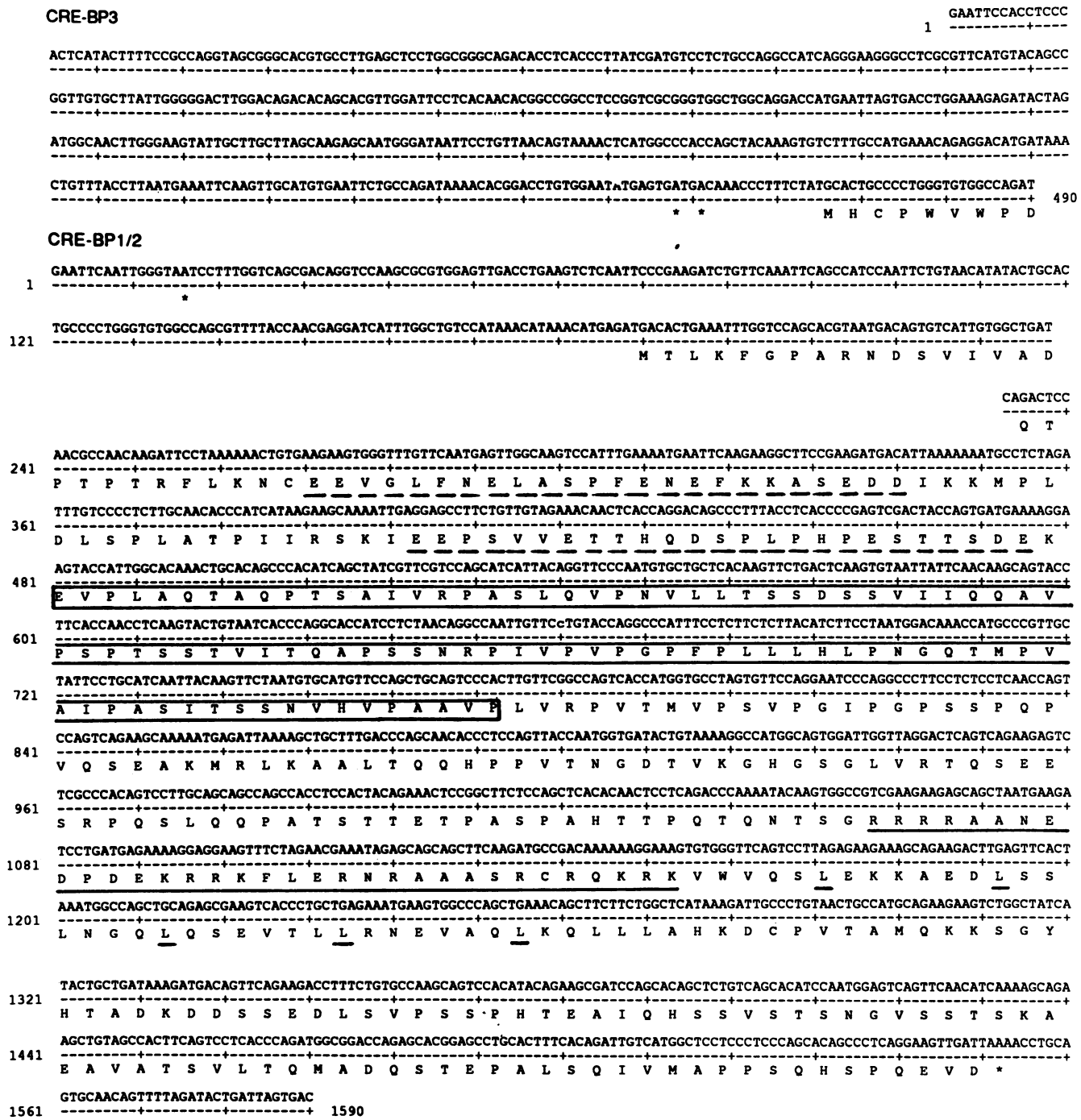

FIG. 2. Nucleotide and deduced amino acid sequences of CRE-BP1, -BP2, and -BP3 cDNAs. The boxed sequence indicates the region removed by splicing in CRE-BP2. Stars indicate stop codons. A stop codon is located 15 bp in from the 5′ end of this clone, preventing fusion of the CRE-BP1 protein with β-galactosidase. The translation initiation site is located 170 bp downstream of this stop codon. Leucine residues participating in the formation of the zipper motif are underlined. The basic region involved in DNA binding is underlined. Putative acidic activation domains are indicated with a broken line under the amino acid sequence.

activity of element δA were assayed in these cells. To compare the degree of activation from the CRE-BP1 and -BP2 isoforms, we constructed a hybrid CRE-BP2.1 molecule that differs from CRE-BP1 only by the 294-bp internal deletion. The structures of the CRE-BP cDNAs inserted into the expression vector CDM8 are represented schematically in Fig. 1. A reporter tkCAT plasmid containing five copies of the δA element (5δAtkCAT) was cotransfected with expression vectors carrying the different CRE-BP cDNAs. The level of expression of the reporter gene mediated by endogenous factors present in each cell line was estimated upon cotransfection with the CDM8 vector alone. To determine whether the effect of expression of the CRE-BP isoforms was specific for the δA element, we tested their ability to

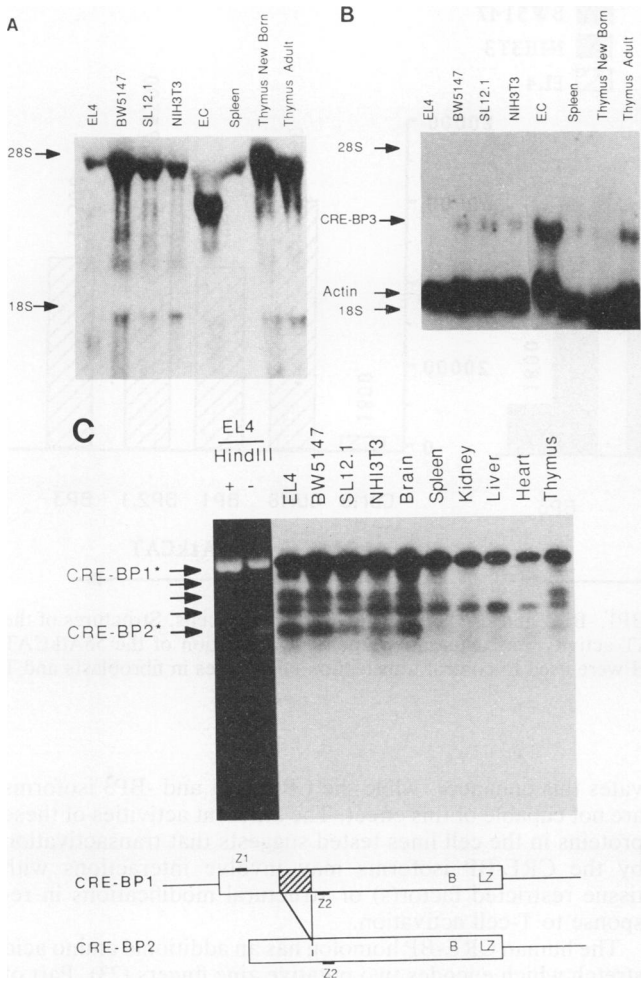

FIG. 3. Tissue distribution of the CRE-BP1, -BP2, and -BP3 cDNAs determined by Northern hybridization and by PCR analysis. (A and B) Northern hybridization of RNAs from the T-cell lines EL4, BW5147, and SL12.1, NIH 3T3 fibroblasts, the embryonic blastoderm line E.C, and tissues of brain, spleen, newborn, and adult thymus. CRE-BP (shared fragment between all isoforms) (A) and CRE-BP3 (500-bp fragment derived from the 5′ end of this cDNA) and actin-specific probes (B) were used to hybridize the same RNA filter sequentially. (C) PCR analysis of RNAs from the T-cell lines EL4, BW5147, and SL12.1, the B-cell myeloma A20, the fibroblast line NIH 3T3, and brain, spleen, lung, liver, heart, and thymus. Z1 and Z2 oligonucleotides were used for analysis of CRE-BP1 and -BP2 transcripts. Arrows indicate specifically amplified bands. The CRE-BP1 and -BP2 related bands are labelled accordingly. The presence of a unique *Hin*dIII site at the splicing junction which generates the CRE-BP2 isoform was used to confirm the identity of the CRE-BP2-related band in the PCR-amplified products in EL4 cells. A cDNA probe shared by all isoforms was used to determine the specificity of the CRE-BP1 and -BP2 PCR products.

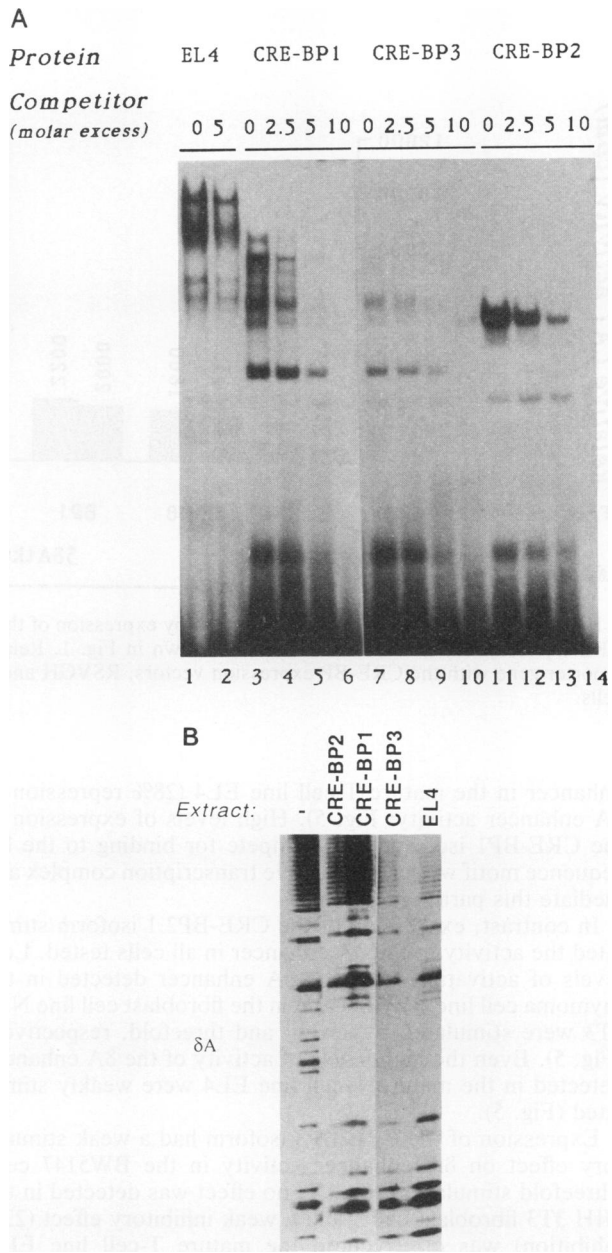

FIG. 4. DNA binding specificity of the CRE-BP1, -BP2, and -BP3 proteins. (A) Relative affinities of bacterially expressed CRE-BP1, -BP2, and -BP3 proteins for the δA element. The CRE-BP1, -BP2, and -BP3 bacterial lysates (2 μg each) were used in a binding assay together with 50,000 cpm of end-labelled 37-bp δA oligonucleotide and 1 μg of poly(dI-dC) as a nonspecific competitor. An unlabelled δA (double-stranded 37-mer) oligonucleotide was used at the indicated molar excesses in binding assays to determine the relative affinities of the different CRE-BP isoforms for the δA enhancer. Binding assays with nuclear extracts from EL4 T cells were run in comparison. (B) Binding specificity of the CRE-BP isoforms relative to the EL4 nuclear extract as determined by DNase I footprinting. δA denotes the protected δA sequence.

transactivate the reporter tkCAT without δA elements, the 5δAmutkCAT reporter with mutated δA elements (δA mutant TAAGATCA; the mutation abrogates CRE-BP binding and transcriptional activity of the δA element), and the reporter driven by a heterologous enhancer, the SL3 long terminal repeat, which does not contain any δA binding sites. Cotransfection of the CRE-BP isoforms elicited no changes in activity of these reporter genes which do not contain a δA binding site (data not shown).

Expression of the CRE-BP1 isoform had no significant effect on the low level of activity of the δA enhancer in the thymoma BW5147 and fibroblast NIH 3T3 cell lines (Fig. 5). However, CRE-BP1 exhibited a small but specific and reproducible inhibitory effect on the strong activity of the δA

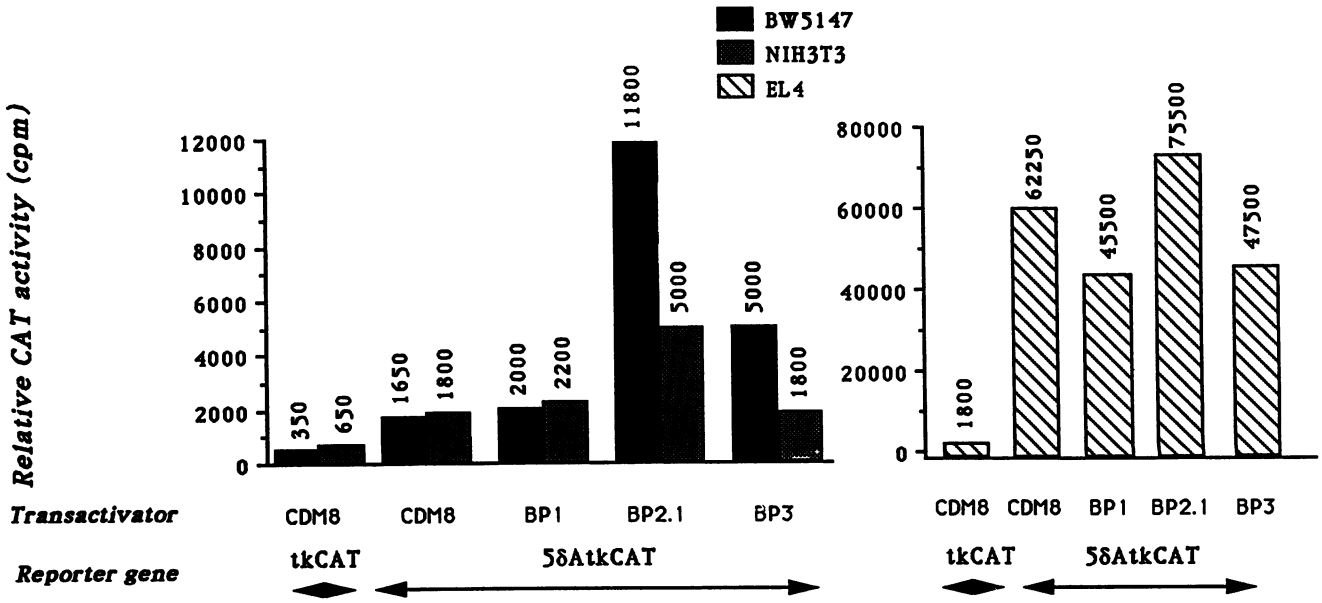

FIG. 5. Transactivation of the δA element by expression of the CRE-BP1, -BP2, and -BP3 cDNAs in T and non-T cells. Structures of the CRE-BP cDNAs used for expression are shown in Fig. 1. Relative CAT activity was determined upon cotransfection of the 5δAtkCAT reporter gene with the CRE-BP expression vectors. RSVGH and SL3GH were used to control transfection efficiencies in fibroblasts and T cells.

enhancer in the mature T-cell line EL4 (28% repression of δA enhancer activity; Fig. 5). High levels of expression of the CRE-BP1 isoform may compete for binding to the δA sequence motif with a more active transcription complex and mediate this partial inhibition.

In contrast, expression of the CRE-BP2.1 isoform stimulated the activity of the δA enhancer in all cells tested. Low levels of activation from the δA enhancer detected in the thymoma cell line BW5147 and in the fibroblast cell line NIH 3T3 were stimulated by seven- and threefold, respectively (Fig. 5). Even the high levels of activity of the δA enhancer detected in the mature T-cell line EL4 were weakly stimulated (Fig. 5).

Expression of the CRE-BP3 isoform had a weak stimulatory effect on δA enhancer activity in the BW5147 cells (threefold stimulation; Fig. 5), no effect was detected in the NIH 3T3 fibroblast cells, and a weak inhibitory effect (23% inhibition) was observed in the mature T-cell line EL4. Again, high levels of the CRE-BP3 isoform may compete for binding to the δA site with a more potent activating complex (e.g., a complex that contains the CRE-BP2.1 isoform) and thus lower the activity of this enhancer.

The level of stimulation of the δA enhancer by a given CRE-BP isoform varies depending on the isoform and the cell type. This variable effect is not due to differential expression from the CDM8 vector, since transfections with a CDMhGH control plasmid resulted in comparable levels of expression of the growth hormone reporter gene in all the cell lines tested (data not shown). Although it is possible that differential stability of the CRE-BP isoforms accounts for the inability of CRE-BP1 and -BP3 to stimulate the activity of the δA enhancer, the small but specific repression of enhancer activity detected upon their expression in T cells argues strongly that all of these isoforms are stably produced. Therefore, it appears that these distinct products of the CRE-BP gene differentially modulate activity of a T-cell-specific enhancer: the CRE-BP2 isoform strongly transactivates this enhancer, while the CRE-BP1 and -BP3 isoforms are not capable of this effect. The different activities of these proteins in the cell lines tested suggests that transactivation by the CRE-BP isoforms may involve interactions with tissue restricted factor(s) or structural modifications in response to T-cell activation.

The human CRE-BP homolog has an additional amino acid stretch which encodes two putative zinc fingers (23). Part of this amino acid stretch is also present in CRE-BP1 isoform but remains untranslated because of the absence of an initiation codon. Since it is possible that a splicing variant exists that allows for translation of this sequence, we have introduced an initiation codon upstream of this region in CDM8 in order to assess its role in transcriptional activity of the CRE-BP1 and -BP2.1 isoforms. No difference in activity was detected between CRE-BP1 and -BP2.1 isoforms with and without the putative finger motifs upon cotransfection in the T-cell line BW5147 (data not shown). It is possible that additional factors (e.g., an E1a-like activity) are required to manifest the role of this putative domain in transcriptional activity of the CRE-BP DNA-binding protein.

Mutations in the DNA binding domain of the CRE-BP protein render the molecule a dominant repressor of the δA enhancer. Since the CRE-BP protein is a member of the leucine zipper family of DNA-binding proteins, it is possible to generate mutant molecules to directly test their role as transcriptional enhancers. The DNA binding of this family of proteins is mediated by a basic region in the protein that is oriented with respect to the homologous region of another molecule by the leucine zipper domain which mediates their dimerization (20). Nonconservative substitutions in the basic region abrogate DNA binding without interfering with dimerization (6, 21, 32). Such molecules may heterodimerize with wild-type proteins to form complexes incapable of DNA binding and therefore act as specific dominant suppressors of enhancer activity.

Seven highly conserved amino acids in the basic region of

the CRE-BP were substituted for an amino acid sequence which contains α-helix breakers (glycine residues), expected to disrupt the secondary structure of this domain and DNA binding. When expressed in mature T cells, this CRE-BP mutant decreases the expression of the tkCAT reporter gene driven by three copies of the δA enhancer by fourfold (Fig. 6A), presumably by substituting for endogenous CRE-BP in an inactive heterodimer. The 3δAtkCAT reporter gene was tested in parallel with the 5δAtk CAT reporter gene in transfection assays and was transactivated by the CRE-BP isoforms in a similar fashion (data not shown).

We tested a series of truncated CRE-BP molecules in which either the basic domain or part of the leucine zipper was deleted for the ability to repress δA enhancer function. A peptide carrying the nuclear localization signal of simian virus 40 large T antigen was introduced at the N terminus of each of these truncated molecules to ensure their efficient transfer to the nucleus. Expression of a truncated CRE-BP molecule with a deleted basic region but an intact dimerization domain was the most potent repressor for the δA enhancer (CRE-BP LZ5 inhibited enhancer activity by 5.75-fold). Deletion past the first leucine of the dimerization domain decreased repression by twofold, and deletion past the second leucine alleviated repression altogether (Fig. 6A).

The specificity of the repression mediated by the CRE-BP DNA-binding mutants on the activity of the δA element was tested by cotransfection assays with a reporter gene driven by a heterologous enhancer (the viral SL3 long terminal repeat) which does not have sequence similarities to the δA element. The level of expression of the tkCAT reporter gene driven by this heterologous enhancer was not affected by coexpression of these mutants (Fig. 6A).

Overexpression of a leucine zipper motif can lead to the formation of zipper heterodimers which do not occur naturally and which can interfere with the activity of the regulatory elements that each zipper partner binds to. We have chosen to overexpress the zipper motif of CREB, a leucine zipper which can bind the same DNA sequence as CRE-BP but which is not involved in regulating the δA enhancer element (7). Regulatory elements (CREs) are transactivated by CREB in response to cAMP agonists, in contrast to the δA element, which is constitutively active.

We have generated a truncated CREB which is composed of a small part of the basic region, not sufficient to bind DNA but appropriate for nuclear localization, and the leucine zipper dimerization domain. CRE-BP and CREB DNA-binding mutants were cotransfected in parallel with the reporter tkCAT gene driven by the δA element in the mature T-cell line EL4. The level of expression of the reporter gene was not affected by expression of the CREB leucine zipper motif but was strongly diminished by expression of the CRE-BP DNA-binding mutant (CRE-BP LZ5) (Fig. 6A).

The ability of the CRE-BP LZ5 to dimerize and inhibit binding of the wild-type CRE-BP2.1 isoform to the δA element was tested in vitro in a gel retardation assay. Upon in vitro translation of CRE-BP2.1 (Fig. 6B), a novel retarded band was detected, which supershifted in the presence of anti-CRE-BP antibodies and was abolished by specific competitor DNA (Fig. 6C, Ab1 and S. competitors). Anti-CRE antibodies raised to an epitope not present in the CRE-BP2.1 isoform did not interact with this retarded complex (Fig. 6C, Ab2). Cotranslation of CRE-BP2.1 with the CRE-BP LZ5 mutant or mixing of their translated products diminished expression of the CRE-BP2.1 retarded complex (Fig. 6B, CRE-BP2.1+LZ5).

Since down-regulation of the δA enhancer in mature T cells by mutant CRE-BP molecules is not dependent on DNA binding, it cannot be due to competition with some active factor for the δA binding site. The dependence of this inhibition on the ability of these mutants molecules to dimerize with themselves or endogenous factors leads us to postulate that endogenous CRE-BP factors mediate the activity of the δA enhancer in T cells.

Antibodies to CRE-BP interact with T-cell-restricted and ubiquitously expressed complexes. The δA element is an 8-bp hyphenated palindrome that differs from the CRE perfect palindrome by a 1-bp substitution. This element is conserved between the mouse and human CD3δ enhancers (7a). At least two nuclear complexes have been identified, one that appears to be T-cell restricted and another that appears to be ubiquitously expressed.

We have investigated the composition of the δA nuclear complexes by using antibodies (Ab1 and Ab2) to the CRE-BP protein (Fig. 7). Ab1 recognizes the N-terminal regions shared by CRE-BP1, -BP2, and -BP3, while Ab2 is specific for the internal 98-amino-acid region present in CRE-BP1 and -BP3 but absent in CRE-BP2. Both antibodies interact specifically with the T-cell-restricted (δA-c1) and the ubiquitously expressed (δA-c2) complexes which bind to the δA element. The δA-c1 and -c2 complexes appeared to supershift when specific anti-CRE-BP antibodies were added to the binding reaction, but no change in their mobility was detected in the presence of preimmune serum (Fig. 7, lanes 2 to 6). Since these antibodies interact with specific parts of the molecule which are not conserved in other members of the leucine zipper family, it is highly improbable that this interaction is due to cross-reactivity between specific CRE-BP antibodies and a distinct DNA-binding protein member of the leucine zipper family. Antibodies to the c-Jun and c-Fos proteins were also tested, but no interaction with the δA nuclear complexes was detected (data not shown).

These data strongly suggest that CRE-BP protein isoforms are present in both the T-cell-restricted and ubiquitous complexes. The presence of the CRE-BP protein(s) in the more T-cell-restricted complex (δA-c1) which correlates with enhancer activity suggests the formation of a T-cell-restricted CRE-BP heterodimer. This heterodimer may be composed of two CRE-BP isoforms, e.g., CRE-BP1 and the more T-cell-restricted CRE-BP2, or it may consist of a CRE-BP isoform and a more T-cell-restricted transcriptional activator. Formation of such a heterodimer may also impart activity to the transcriptionally inert CRE-BP1 isoform or further modulate the activity of the CRE-BP2 and -BP3 isoforms.

DISCUSSION

In search of the transcriptional regulators that mediate activity and specificity of the δA element of the CD3δ enhancer, we have isolated three mouse cDNAs derived from the CRE-BP gene by differential splicing and alternate promoter usage.

The CRE-BP gene has been cloned previously from several tissues on the basis of its affinity for the CRE (11, 17, 25) and is a member of the leucine zipper family of DNA-binding proteins. Strong amino acid similarities were found between the CRE-BP and the c-Jun, c-Fos, and CREB proteins over the basic and leucine zipper motifs (25). A heptad array of leucines is involved in dimerization which is required for DNA binding (20) mediated by a basic amino acid region. Different members of this family can form heterodimers with novel binding characteristics (11, 12, 16, 18, 26).

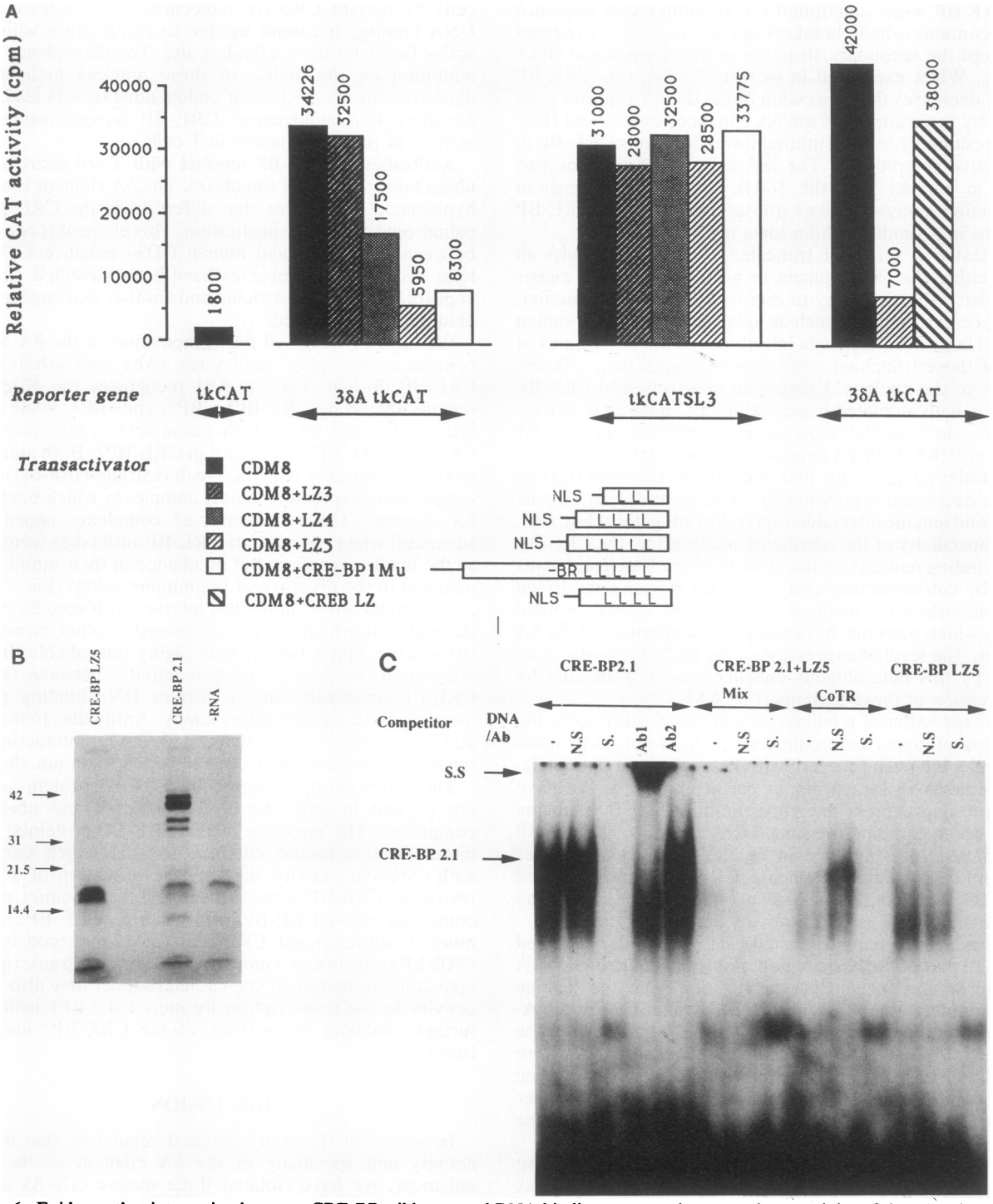

FIG. 6. Evidence that interaction between CRE-BP wild-type and DNA-binding mutants down-regulates activity of the δA enhancer in T cells. (A) Relative CAT activity determined upon cotransfection of 3δAtkCAT and tkCATSL3 reporter genes with the following CRE-BP and CREB mutants: CRE-BP LZ3 (nt 1212 to 1561 of the coding region; three of the five leucines of the zipper dimerization domain are retained), CRE-BP LZ4 (nt 1192 to 1561; four of the five leucines of the zipper dimerization domain are retained), CRE-BP LZ5 (nt 1170 to 1561; all five leucines of the zipper dimerization domain are retained), and CRE-BP Mu carrying a seven-amino-acid substitution in its basic region which abrogates its ability to bind DNA and CREB (nt 980 to 1220 of basic region and intact zipper dimerization domain). The 3δAtkCAT and 5δAtkCAT gene reporter genes were tested and found to be transactivated by the CRE-BP isoforms in a similar fashion. NLS, nuclear localization signal; L, leucine; BR, basic region. Data represent averages of at least three experiments with each transfection point performed in duplicate or triplicate. Expression vectors and reporter genes were cotransfected with SL3GH. (B) In vitro translation of CRE-BP2.1 and CRE-BP LZ5. CRE-BP2.1 and CRE-BP LZ5 RNAs were transcribed in vitro from the T7 promoter of the CDM8 vector and used to program rabbit reticulocyte lysates. The products of in vitro translation were analyzed by SDS-PAGE on a 12% acrylamide gel. Arrows indicate the positions (in kilodaltons) travelled by the molecular weight markers. (C) Evidence that DNA-binding mutants of CRE-BP inhibit binding of wild-type CRE-BP2.1 to the δA site. CRE-BP LZ5, composed of the leucine zipper dimerization domain, was cotranslated or mixed after

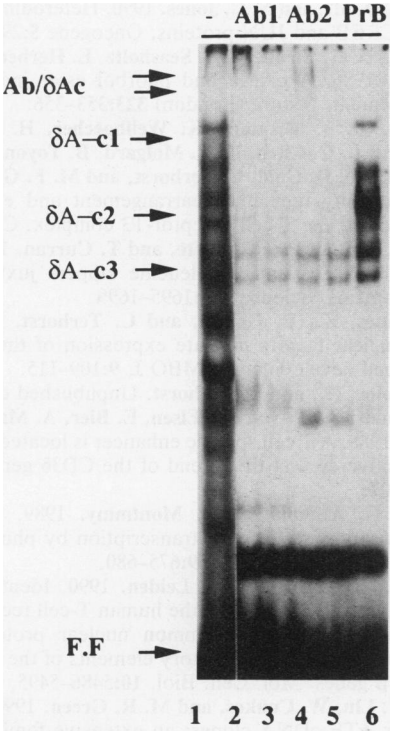

FIG. 7. Recognition of the δA nuclear complexes by antibodies (Ab) to the CRE-BP protein. DNA binding assays were performed with T-cell nuclear extract (EL4; 2 μg) and 50,000 cpm of end-labelled δA oligonucleotide in the presence of 1 μl of 1× preimmune serum (lane 6), 1:5 and 1:25 dilutions of CRE-BP Ab1 (lanes 2 and 3), and 1:5 and 1:25 dilutions of CRE-BP Ab2 (lanes 4 and 5) and in the absence of any serum (lane 1). δA-c1, δA-c2, δA-c3 are T-cell nuclear complexes binding the δA element.

The CRE-BP isoforms reported here are identical over the DNA binding and dimerization motifs but differ at the N-terminal region of the protein. Expression of two of these isoforms, CRE-BP2 and -BP3, appears to be regulated at the level of differential splicing and alternate promoter usage (Fig. 3). To assess the role of the CRE-BP isoforms for the δA enhancer, each isoform was tested for its ability to transactivate the δA enhancer in a variety of cells.

Expression of the CRE-BP2 isoform stimulated the low levels of δA enhancer activity in both immature T cells and fibroblasts and was capable of stimulating further the high levels of enhancer activity detected in mature T cells (Fig. 5). The effect of expression of this isoform on the low levels of the δA enhancer was more dramatic in T cells than in fibroblasts, suggesting that a second mechanism may be involved in mediating high levels of enhancer activity in T cells. This may entail either interaction with a T-cell-restricted factor or tissue-specific posttranslational modification of this isoform.

Expression of CRE-BP1 and -BP3 isoforms had no effect on the low levels of δA enhancer activity detected in fibroblast cells, and only the CRE-BP3 isoform could weakly transactivated this enhancer in the immature thymoma cell line BW5147 (Fig. 5). The strong activity of the δA enhancer element detected in the mature T-cell line EL4 was weakly repressed upon expression of the CRE-BP1 and -BP3 isoforms. This small but specific repression may be due to competition for binding to the δA site between overexpressed CRE-BP1 and -BP3 homodimers which are transcriptionally inert and a transcriptionally more active nuclear complex (e.g., a heterodimer between CRE-BP1/3 and CRE-BP2 or a T-cell-restricted factor) present in these cells.

The CRE-BP2 isoform differs from CRE-BP1 by an internal deletion of 98 amino acids near the N terminus of the molecule. The deleted domain is hydrophobic in nature, is serine rich, and exhibits a high propensity for β-sheet structure. Two stretches of acidic amino acids lie upstream of the deleted region (Fig. 2). Acidic domains have been shown to be important for the activation properties of several transcription factors (15, 24), and this hydrophobic region may be involved in regulating their accessibility. Removal of this amino acid domain from CRE-BP1 may either directly affect its conformation or prevent the molecule from interacting with a repressor protein. Either of these changes could uncover the putative activation domains present in this molecule and convert it from an inert DNA-binding protein (CRE-BP1) to a strong activator of transcription (CRE-BP2). The transcriptional activities of the yeast heat shock factor (30) and of the proto-oncogene c-*jun* (2) also appear to be regulated by the presence of analogous repressive sequences. Deletion of an amino acid domain from the N terminus of c-Jun leads to its permanent activation and deregulation of its oncogenic potential. Liu and Green (23) have reported that CRE-BP1 (ATF-2) is transcriptionally inert but becomes a potent transactivator when coexpressed with the adenovirus E1a. They have postulated that E1a interacts directly with the DNA-bound CRE-BP1 to provide activation domains necessary for the transcriptional stimulation mediated by this complex. However, the results presented here raise the possibility that interaction with E1a might also unveil the activation domains of CRE-BP1.

To address the role of the CRE-BP protein in mediating δA enhancer activity in T cells, we have taken advantage of the modular nature of the DNA binding and dimerization domains of this family of nuclear proteins. Studies with other members of the leucine zipper family have shown that mutations in the basic region of the molecule disrupt its structure and abrogate DNA binding but do not affect dimerization of the molecule (6, 32). Expression of such mutants is expected to interfere with the DNA binding and enhancer function of wild-type endogenous factors by sequestering them in heterodimers incapable of DNA binding. CRE-BP mutants which lack the ability to bind DNA but retain the ability to dimerize had a strong inhibitory effect on the high levels of δA enhancer activity detected in mature T cells (Fig. 6). In contrast, CREB DNA-binding mutants had no effect on activity of the δA element. CRE-BP mutants unable to dimerize had no such effect either. Together, these observations confirm the direct involvement of this family of

translation with the CRE-BP2.1 isoform. The in vitro-translated homo- and heterodimeric complexes were assayed for the ability to bind to the δA element. Tenfold excesses of cold specific (S.; TGACATCA) and nonspecific (N.S.; TAAGATCA) competitors were added to the reaction mixture to determine the specificity of interaction. Antibodies (Ab) raised against a shared CRE-BP peptide (Ab1) and against a peptide spliced out in CRE-BP2.1 (Ab2) were added to the DNA binding reaction mixture to confirm the identities of the shifted bands. Arrows indicate the CRE-BP2.1 complex and the CRE-BP2.1-antibody supershifted (S.S) complex.

isoforms in generating δA enhancer activity. Since δA like elements have been reported in the promoter and enhancers of the TCR α and β chains (10, 31), the CRE-BP family of transcription factors may be involved in regulating the expression of other members of the TCR/CD3 complex. It will be important to determine whether stable introduction of the dominant suppressor CRE-BP mutants can induce phenotypic changes in the mature T cell.

We have examined the composition of the T-cell-restricted complex (δA-c1) and the ubiquitously expressed nuclear complexes (δA-c2 and -c3) that bind to the δA element, using antibodies specific for CRE-BP isoforms. Two of these nuclear complexes, the T-cell-restricted δA-c1 and the δA-c2 complexes, were specifically disrupted by the anti-CRE-BP antibodies (Fig. 7). Interaction of these two distinct complexes with the same antibody raises the possibility that CRE-BP forms homo- and heterodimeric complexes over the δA site. Limiting amounts of a tissue-specific partner may account for the inhibitory effect, detected upon overexpression of the inert CRE-BP1 and -3 isoforms, on the δA enhancer activity (Fig. 5). Lack of expression of this T-cell-restricted partner from immature T cells and fibroblasts may also account for the inability of CRE-BP1 to stimulate transcription and for the lower levels of activation by the active CRE-BP2 isoform in fibroblasts. The conserved asymmetry of the δA binding site also supports the binding of a heterodimer on this element. Recent reports have demonstrated the ability of CRE-BP1 to form heterodimers with c-Jun in vitro (3, 11, 16). In our studies, c-Jun or c-Fos was not found to transactivate the δA enhancer element or to be present in the δA nuclear complexes (unpublished data). This raises the possibility of heterodimer formation with an unidentified T-cell-specific partner as a mechanism of activation of the δA enhancer. This partner may be either a T-cell-restricted CRE-BP isoform or a tissue-restricted product of a novel gene, and its identification and characterization will be pursued further.

In conclusion, the induction of the δA enhancer activity during T-cell development involves several layers of complexity. A ubiquitously expressed DNA-binding protein, CRE-BP, appears to be crucial for the activity of this T-cell-specific enhancer. Alternate promoter usage and differential splicing of the CRE-BP gene can generate at least three functionally distinct isoforms. Expression of the active isoforms appears to be regulated in different tissues at the level of splicing. Finally, formation of heterodimeric complexes between transcriptionally inert CRE-BP isoforms and an active T-cell-restricted factor(s) may further modulate the activity of the δA enhancer in T cells.

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