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A *Drosophila* CREB/CREM Homolog Encodes Multiple Isoforms, Including a Cyclic AMP-Dependent Protein Kinase-Responsive Transcriptional Activator and Antagonist

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We have characterized a *Drosophila* gene that is a highly conserved homolog of the mammalian cyclic AMP (cAMP)-responsive transcription factors CREB and CREM. Uniquely among *Drosophila* genes characterized to date, it codes for a cAMP-responsive transcriptional activator. An alternatively spliced product of the same gene is a specific antagonist of cAMP-inducible transcription. Analysis of the splicing pattern of the gene suggests that the gene may be the predecessor of the mammalian *CREB* and *CREM* genes.

Activation of the cyclic AMP (cAMP) signal transduction pathway can have long-lasting global consequences through its influence on the expression of specific genes. In mammalian systems, many of the known cAMP-responsive genes serve important neural and endocrine roles (38, 54, 63, 70).

Some members of the CREB family of transcription factors in mammals are known to participate in the control of gene expression by cAMP (13, 25, 32, 36). Proteins of this family consist of two major domains. The carboxyl-terminal portion contains a basic region-leucine zipper (bZIP) domain, involved in sequence-specific DNA binding and protein dimerization (9, 33, 40, 74, 79). The remainder of the protein comprises the activation domain. This contains regions that can presumably interact with other components of the transcription machinery and with signal transduction pathways to influence gene expression (10, 25, 27, 43). CREB proteins can bind as dimers to a conserved palindromic DNA sequence, the cAMP-responsive element (CRE; consensus sequence, 5′ TGACGTCA 3′). CRE sequences have been characterized from upstream control regions of cAMP-responsive genes (15, 39, 55, 63, 80) and have been identified as the *cis*-acting component of the CREB-mediated transcriptional response to cAMP.

cAMP-responsive transcriptional activation from CREs appears to require the presence of cAMP-dependent protein kinase (PKA) in the nucleus (50). Persistent elevation of cAMP levels can lead to the migration of free catalytic subunits of PKA to the nucleus (5, 22, 32, 34, 56). Experimentally, elevation of PKA levels or injection of PKA into the nucleus can substitute for all earlier cytoplasmic steps in this signal transduction pathway (7, 32, 51, 60, 61). In the nucleus, PKA is believed to phosphorylate and activate nuclear substrates that include a subset of CREB family proteins (3, 50). Phosphorylation of a particular serine residue by PKA is obligatory for cAMP-dependent transcriptional activation by the best characterized of the cAMP-responsive activator proteins, CREB (28, 43).

Only two other genes in the CREB family, besides *CREB* itself, are known to code for PKA-responsive activators: *CREM* (23, 25) and *ATF-1* (46, 59). Other CREB family members that have been tested do not appear to respond to PKA (47, 67). The *CREM* gene, besides producing PKA-responsive transcriptional activators, gives rise to isoforms that explicitly antagonize cAMP-dependent transcription (23, 24).

We undertook the cloning of *Drosophila* CREB/ATF family members with the specific aim of obtaining tools for examining the involvement of cAMP-dependent gene expression in *Drosophila* long-term memory. We have isolated and characterized a *Drosophila* CREB family gene that produces a number of alternatively spliced isoforms. This gene, which we call *dCREB2*, is the same gene previously reported as *dCREB-B* and partially characterized by Usui et al. (73) on the basis of one cloned cDNA isoform. That isoform (corresponding to the dCREB2-c isoform illustrated in Fig. 5) did not encode a PKA-responsive transcriptional activator. Among the isoforms that we have characterized is one that is a PKA-responsive transcriptional activator (dCREB2-a) and another that is an antagonist of PKA-responsive transcriptional activation (dCREB2-b). In our communications, we have used the nomenclature dCREB2-x to differentiate the various alternatively spliced forms, where x is a letter code signifying the particular isoform (see Fig. 5). Furthermore, our analysis of the genomic organization of *dCREB2* identified additional coding exons, requiring a numbering scheme different from that in reference 73. For these reasons, in this report we will use our own nomenclature for the gene.

dCREB2 appears to be closely related to, and perhaps an ancestral form of, both of the mammalian genes *CREB* and *CREM*. The presence of a PKA-responsive transcriptional activator from the CREB family in *Drosophila melanogaster* suggests that this organism may share mechanisms for the control cAMP-dependent gene expression with the CREB-mediated system of mammals. The characterization of the various gene products from this gene will provide useful biological tools for examining the involvement of cAMP-dependent gene expression in long-term memory (76, 77) and in other areas of *Drosophila* biology where cAMP is an important second messenger.

MATERIALS AND METHODS

Expression cloning of *dCREB2*. Standard protocols for expression cloning by DNA-binding activity (4, 66) were followed except as noted. A double-stranded 3xCRE oligonucleotide based on an adenovirus E4 CRE (45) was synthesized

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and cloned in between the *Xba*I and *Kpn*I sites of pGEM7Zf+ (Promega). The sequence of one strand of the oligonucleotide was 5′ CGTCTAGATC TATGACGTAATATGACGTAATATGACGTAATGGTACCAGATCTGGCC 3′ (CREs underlined). The oligonucleotide was excised as a *Bgl*II-*Hin*dIII fragment and labeled by filling in the overhanging ends with Klenow fragment in the presence of [α-^{32}P]dGTP, [α-^{32}P]dCTP, and unlabeled dATP and dTTP (4). Just prior to use, the labeled fragment was preabsorbed to blank nitrocellulose filters to reduce background binding. All other steps were as described previously (4). After secondary and tertiary lifts, positive clones were subcloned into Bluescript pKS$^+$ (Stratagene) and sequenced. Standard hybridization and screening methods (4) were used in the oligonucleotide-based screen of a *Drosophila* head cDNA library.

Gel shift analysis. Gel mobility shift assays were performed as described previously (4), with the following modifications. The 4% polyacrylamide gel (cross-linking ratio, 80:1) was cast and run in 5× Tris-glycine buffer supplemented with 3 mM $MgCl_2$. The oligonucleotides used as the DNA probes were boiled and slowly cooled to room temperature at a concentration of 50 μg/ml in 0.1 M NaCl. Fifty nanograms of double-stranded probe was end labeled by using polynucleotide kinase in the presence of 100 μCi of [γ-^{32}P]ATP. The double-stranded oligonucleotides were purified on a native polyacrylamide gel and used in a mobility shift assay at about 0.5 ng per reaction.

A *dCREB2-b* cDNA was subcloned and subjected to site-directed mutagenesis to introduce restriction sites immediately 5′ and 3′ of the open reading frame (ORF). This ORF was subcloned into the pET11A expression vector (Novagen) and used to induce expression of the protein in bacteria. The cells containing this vector were grown at 30°C to an approximate density of 2×10^8/ml and heat induced at 37°C for 2 h. The cells were collected by centrifugation and lysed as described previously (8). The crude extract was clarified by centrifugation and loaded onto a DEAE column that had been previously equilibrated with 50 mM Tris-HCl (pH 8.0)–10% sucrose–100 mM KCl. dCREB2-b protein was eluted by steps with increasing concentrations of KCl in the same buffer. Fractions were evaluated by gel mobility shift assay. The peak fraction was dialyzed against the loading buffer and used in the binding experiment. The specific competitor that was used was the wild-type CRE oligonucleotide. The sequence of one strand of each of the double-stranded oligonucleotides used in the gel shift analysis is listed below. For the first two oligonucleotides, wild-type and mutant CREs are underlined. The sequences are as follows: wild-type 3xCRE, 5′ AAATGACGT AACGGAAATGACGTAACGGAAATGACGTAACG 3′; mutant 3xmCRE, 5′ AAATGAATTAACGGAAATGAATTAACGGAAATGAATTAACGG 3′; nonspecific competitor 1, 5′ TCGACGGGTTTTCGACGTTCACTGGTAGTG TCTGATGAGGCCGAAAGGCCGAAACGCGATGCCCATAACCACCACG CTCAG 3′; nonspecific competitor 2, 5′ TCGACCCACAGTTTCGGGTTTT CGAGCAAGTCTGCTAGTGTCTGATGAGGCCGAAAGGCCGAAACGC-GAAGCCGTATTGCACCACGCTCATCGAGAAGGC 3′; nonspecific competitor 3, 5′ CTAGAGCTTGCAAGCATGCTTGCAAGCAAGCATGCTTGC AAGCATGCTTGCAAGC 3′; and nonspecific competitor 4, 5′ CTCTAGA GCGTACGCAAGCGTACGCAAGCGTACG 3′.

Northern (RNA) blots. Total head and body RNA was isolated from adult flies as described previously (16). Total RNA from other developmental stages was a gift from Eric Schaeffer. All RNA samples were selected twice on oligo(dT) columns (5 Prime-3 Prime) to isolate poly(A)$^+$ RNA. Aliquots containing 2 μg of poly(A)$^+$ RNA were fractionated on 1.2% formaldehyde-formamide agarose gels and transferred to nitrocellulose, and the filters were probed with a uniformly labeled, strand-specific antisense RNA probe. The template for the synthesis of the probe was pJY199, which contains a partial *dCREB2* cDNA coding for the carboxyl-terminal 86 amino acids of the dCREB2-b protein plus about 585 bp of 3′ untranslated mRNA, subcloned into Bluescript pKS$^+$ (Stratagene). All Northern blots were washed at high stringency (0.1% sodium dodecyl sulfate, 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 65°C).

In situ hybridization to tissue sections. Frozen frontal sections of *Drosophila* heads were cut and processed under RNase-free conditions essentially as described previously (57), with modifications for riboprobes as noted here. Digoxigenin-labeled riboprobes were transcribed from the pJY199 template, using a Genius kit (Boehringer Mannheim). One microgram of *Xba*-linearized template in a T3 RNA polymerase reaction was used to make the antisense probe. One microgram of *Hin*dIII-linearized template was transcribed by T7 RNA polymerase to generate the control sense probe. Alkaline hydrolysis in 1 M calcium carbonate solution (pH 10) for 30 min at 60°C was used to reduce the average probe fragment size to about 200 bases. The hydrolyzed probe was diluted 1:250 in hybridization solution (57), boiled, quickly cooled on ice, added to the slides, and hybridized at 42°C overnight. The slides were then treated with RNase A (20 mg of RNase A per ml in 0.5 M NaCl–10 mM Tris [pH 8.0] for 1 h at 37°C) prior to two 50°C washes. Digoxigenin detection was done as described previously (57).

RT-PCR analysis of dCREB2 and identification of alternatively spliced exons. The template for reverse transcription-mediated PCR (RT-PCR) was total RNA or poly(A)$^+$ RNA isolated from *Drosophila* heads as described previously (16). Total RNA used was exhaustively digested with RNase-free DNase I (50 μg of RNA digested with 50 U of DNase I for 60 to 90 min at 37°C and then subjected to phenol extraction, phenol-chloroform extraction, and ethanol precipitation) prior to use. Results from separate experiments (75a) indicate that this DNase treatment effectively eliminates the possibility of PCR products derived from any contaminating genomic DNA. Two rounds of selection using commercial oligo(dT) columns (5 Prime-3 Prime) were used to isolate poly(A)$^+$ RNA from total RNA. The template for an individual reaction was either 100 to 200 ng of total RNA or 10 to 20 ng of poly(A)$^+$ RNA.

The RT-PCRs were performed as specified by the supplier (Perkin-Elmer) with a hot-start modification (Perkin-Elmer RT-PCR kit instructions). All components of the reverse transcription reaction, except the r*Tth* enzyme, were assembled at 75°C, and the reaction was initiated by adding the enzyme and lowering the temperature to 70°C. At the end of 15 min, the PCR components (including trace amounts of [α-^{32}P]dCTP), preheated to 75°C, were added quickly, the reaction tubes were put into a preheated thermocycler, and PCR amplification was begun. Cycling parameters for reactions (100-μl total volume) in a Perkin-Elmer 480 thermocycler were 94°C for 60 s and 70°C for 90 s. For 50-μl reactions in an MJ Minicycler (MJ Research), the cycle parameters were 94°C for 45 s and 70°C for 90 s.

All primers used in these procedures were designed to have 26 nucleotides complementary to their target sequences. Some primers had additional nucleotides for restriction sites at their 5′ ends to facilitate subsequent cloning of the products. Primers were designed to have about 50% GC content, with a G or C nucleotide at their 3′-most ends and with no G/C runs longer than three. For RT-PCRs with a given pair of primers, the Mg^{2+} concentration was optimized by running a series of pilot reactions at Mg^{2+} concentrations ranging from 0.6 to 3.0 mM. Reaction products were analyzed on urea-polyacrylamide denaturing gels by autoradiography. Any product that appeared larger than the band predicted from the cDNA sequence was purified from a preparative native gel, reamplified by using the same primers, gel purified, subcloned, and sequenced.

To verify that a given RT-PCR product was actually derived from RNA, we ran control reactions to show that the appearance of the product was eliminated by RNase A treatment of the template RNA. Also, products generated from reactions using total RNA as the template were reisolated from reactions by using twice-selected poly(A)$^+$ RNA as the template.

Plasmids. Construction of the plasmid *RSV-dCREB2-a* entailed a number of cloning steps, and details are available upon request. In brief, the *dCREB-a* ORF was first reconstructed in the plasmid Bluescript pKS$^+$ by sequentially adding each of the three exons (exons 2, 4, and 6) to *dCREB2-b* cDNA which had been subcloned from phage into pKS$^+$. Site-directed mutagenesis was used to introduce unique restriction enzyme sites both 5′ and 3′ of the *dCREB2-b* ORF to facilitate the subcloning process and allow removal of 5′ and 3′ untranslated sequences. Once the activator was assembled, the resulting ORF was checked by sequencing and moved into a modified RSV-SG vector, RSV-0, that contains a polylinker located between the Rous sarcoma virus (RSV) long terminal repeat promoter and the simian virus 40 polyadenylation sequences. *RSV-dCREB2-b* was made by moving the *dCREB2-b* cDNA, which had been subcloned into pKS$^+$, into RSV-0.

Other constructs used in transfection experiments were pCaEV (51), which contains the cDNA for mouse PKA catalytic subunit cloned under the control of the mouse metallothionein 1 promoter, RSV-βgal (21), which expresses the *lacZ* gene under the control of the RSV long terminal repeat promoter (30), RSV-CREB (29), which is a CREB cDNA fragment containing the complete 341-amino-acid ORF under the control of the RSV long terminal repeat promoter in RSV-SG, and the Δ(−71) CAT reporter (55), which is a fusion of a CRE-containing fragment of the rat somatostatin promoter and the bacterial chloramphenicol acetyltransferase (CAT) coding region.

F9 cell culture and transfection. Undifferentiated F9 cells were maintained and transfected by the calcium phosphate method as described previously (11) except that chloroquine was added to cultures to a final concentration of 100 μM immediately before transfection. Precipitates were washed off 10 h after transfection, at which time the dishes received fresh, chloroquine-free medium. Amounts of DNA in transfections were made equivalent by adding RSV-0 when required. Cells were harvested 30 h after transfection. Extracts were made by three cycles of freeze-thawing, with brief vortexing between cycles. Particulate matter was cleared from extracts by 10 min of centrifugation in the cold. β-Galactosidase activity assays were performed as described previously (52). CAT activity assays were performed as described previously (65), using aliquots of extract which were heat treated at 65°C for 10 min and then centrifuged for 10 min to remove debris. Results reported are from three experiments run on different days with at least three dishes per condition within each experiment. Error bars represent standard errors of the means, with error propagation taken into account (31).

RESULTS

Expression cloning of *dCREB2*. Numerous cDNAs from *dCREB2* were obtained in a DNA-binding screen of an expression library of *Drosophila* head cDNAs, using a radiolabeled duplex oligonucleotide probe, 3xCRE, containing three copies of a functional, noncanonical CRE from the adenovirus type 5 E4 promoter (37). Sequencing of the *dCREB2* clones revealed two alternatively spliced forms, *dCREB2-b* and *dCREB2-c*. As illustrated in Fig. 4, these forms differ in the presence or

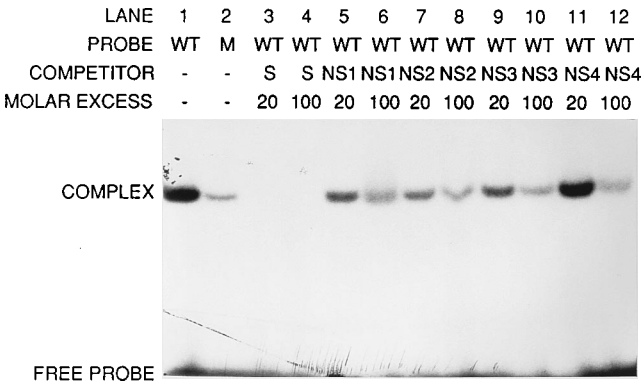

FIG. 1. Gel mobility shift assays of bacterially expressed dCREB2. All reactions contained labeled wild-type (WT) 3xCRE DNA probes except the reaction in lane 2, which contained labeled mutant (M) 3xmCRE probe. Competitors were added at two different molar ratios. Reactions in lanes 3 and 4 contained specific (S; 3xCRE) competitor oligonucleotides, while the reactions in lanes 5 to 12 contained nonspecific competitors (NS1 to NS4).

absence of exon 4. They also had differences in their 5′ and 3′ untranslated sequences.

Chromosomal in situ hybridization using a *dCREB2* probe resulted in diffuse labeling centered at 17A2 on the X chromosome, in agreement with the results of Usui et al. (73). This region contains several lethal complementation groups (18).

DNA-binding properties of *dCREB2-b*. The DNA-binding activity of dCREB2-b was tested in a gel mobility shift assay (Fig. 1). Bacterial extracts expressing the dCREB2-b protein retarded the migration of a 3xCRE probe. The protein had lower but detectable affinity for a mutated 3xCRE oligonucleotide (compare lanes 1 and 2). Competition experiments using unlabeled competitor oligonucleotides showed that the binding of dCREB2-b to 3xCRE was specific (lanes 3 to 12), with higher affinity for CRE sites than to nonspecific DNA. Together with the conserved amino acid sequence, this functional similarity suggested that *dCREB2* was a CREB family member.

Expression pattern of *dCREB2*. Northern blot analysis of poly(A)$^+$ RNA from larval stages and heads and bodies of adult flies (Fig. 2A) showed a complex pattern of bands, with at least 12 different-size transcripts apparent. Two bands of approximately 0.8 and 3.5 kb were common to all of the stages. The adult head contained transcripts of at least six sizes (0.8, 1.2, 1.6, 1.9, 2.3, and 3.5 kb). In situ hybridization to RNA in *Drosophila* head tissue sections, using a riboprobe with antisense sequences for the dCREB2 bZIP region, resulted in signal in most or all cells. In the brain, cell bodies, but not neuropil, were stained (Fig. 2B).

***dCREB2* has alternatively spliced forms.** Our initial transfection experiments showed that the dCREB2-c isoform was not a PKA-responsive transcriptional activator (data not shown), a result confirming preliminary observations (73). The complex developmental expression pattern of transcripts from *dCREB2*, plus information from the mammalian *CREM* gene that alternative splicing was required to generate PKA-responsive activators (23, 25, 42), suggested that additional domains might be required for activating isoforms from *dCREB2*.

RT-PCR was used to identify new coding exons. Comparison of *dCREB2* genomic DNA sequence with that of cDNAs indicated the general exon/intron organization and assisted in the search for additional exons. Sense and antisense primers spaced across the *dCREB2-b* cDNA were synthesized and used pairwise in RT-PCRs primed with *Drosophila* head mRNA to look for these new coding regions. Positions of the PCR primers used in this analysis are shown in Fig. 3. Reactions with

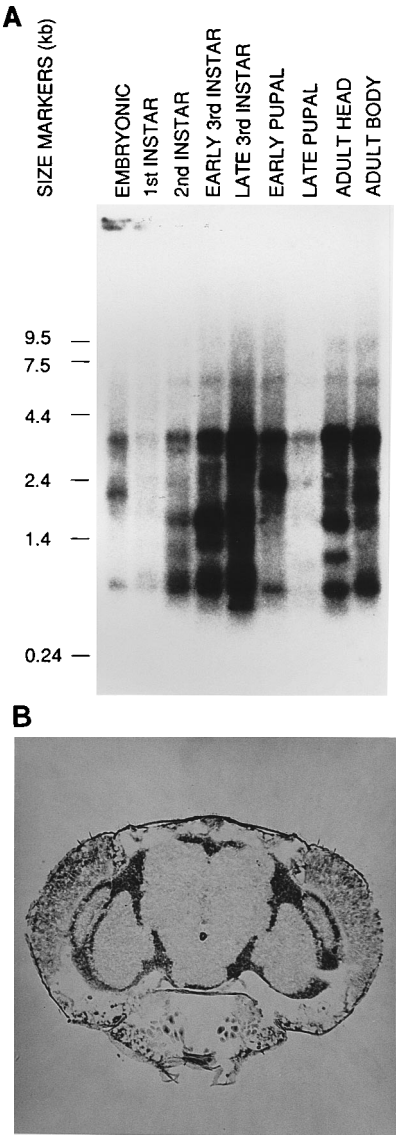

FIG. 2. (A) Northern blot analysis of *dCREB2* transcripts. Poly(A)$^+$ RNA samples from different developmental stages were separated and probed with an antisense *dCREB2* probe. (B) *dCREB2* RNA in a representative (medial) tissue section from *Drosophila* head. An antisense riboprobe to *dCREB2* was hybridized to serial frontal sections of wild-type (Can-S) flies. Sections hybridized with the corresponding sense riboprobe showed no signal.

primer pair A and B, hybridizing in exons 5 and 7, generated two products, one of the size predicted by our cDNA clones and one that was larger. Cloning and sequencing of the larger product suggested the presence of exon 6. A primer within exon 6 (primer C) was synthesized, end labeled, and used to screen a *Drosophila* head cDNA library. Two clones were isolated, sequenced, and found to be identical. This splicing isoform, *dCREB2-d*, confirmed the splice junctions and exon sequence inferred from the RT-PCR products.

Initial attempts to isolate exon 2 proved difficult. We conceptually translated the genomic sequence that separated exons 1 and 3 in all frames and identified one relatively long ORF. Of three antisense primers (D, E, and F) synthesized on the basis of the genomic sequence, only one (primer D) fell within the ORF. Three sets of RT-PCR each using a different one of the three antisense primers paired with primer G, a

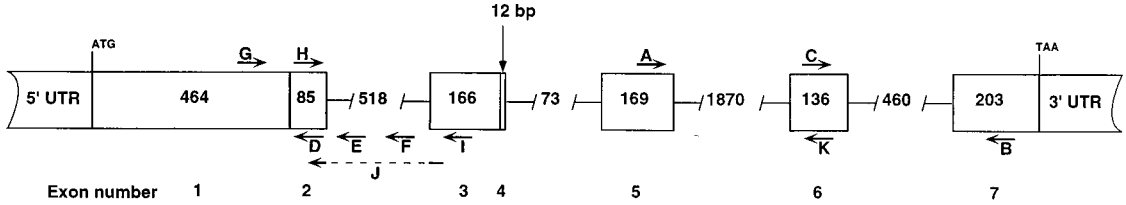

FIG. 3. Schematized *dCREB2* genomic region showing PCR primer locations. Boxes represent exons containing known coding sequences. Enclosed numbers indicate lengths of coding regions in base pairs. Lines between boxes indicate introns and approximate lengths in base pairs. Horizontal arrows represent PCR primers discussed in the text. UTR, untranslated region.

sense primer in exon 1, were run in parallel. Only the reaction that used primer D produced a PCR product. The sequence of this product matched a continuous stretch of nucleotides from the genomic sequence, extending 3′ from exon 1 past the splice junction in the *dCREB2-b* cDNA to the location of primer D. This fragment suggested that exon 1 might be extended in some mRNAs by use of an alternative 5′ splice site located 3′ to the site used to make *dCREB2-b*. On the basis of the newly identified exon sequences, we made primer H, which when used in a PCR with primer I in exon 3 generated a new product whose sequence established the location of the alternative 5′ splice site. The sequence added to exon 1 by alternative 5′ splice site selection is designated exon 2. The exon 2 sequence also showed that the same 3′ splice site was used both in the cDNAs that we originally isolated and in those used for the RT-PCR product. To independently verify this alternative splicing pattern, we carried out RT-PCR using primer J, which spans the 3′ splice junction of exon 2, in combination with primer G in exon 1. The sequence of the product corroborated the splice junctions of exon 2 shown in Fig. 3.

To determine if exons 2 and 6 could be coordinately spliced into the same molecule, we carried out an RT-PCR with primers H and K, located in exons 2 and 6, respectively. The reaction produced a product of the size predicted for a fragment containing exons 2, 3, 4, 5, and 6, and the identity of this product was confirmed by extensive restriction analysis.

***dCREB2* is a *Drosophila CREB* gene.** Figure 4 shows the DNA sequence and inferred amino acid sequence of *dCREB2-a*, the ORF which results from combining all of the identified *dCREB2* exons. The translation start site indicated for *dCREB2-a* is likely to be authentic because (i) stop codons occur upstream from this ATG in all reading frames in our *dCREB2* cDNAs (sequences not shown), (ii) this ATG was selected by computerized analysis (68) as the best ribosome binding site in the DNA sequence that contains the ORF, and (iii) use of the next ATG in the ORF (480 nucleotides downstream) would not predict a protein that would be a PKA-dependent activator (see below). This does not exclude the possibility that internal translation initiation sites are used in this transcript, as happens with the *CREM* gene's S-CREM isoform (14).

The *dCREB2-a* ORF predicts a protein of 360 amino acids. A computerized amino acid sequence homology search (71) with the predicted dCREB2-a protein sequence identifies CREB, CREM, and ATF-1 gene products as the closest matches to dCREB2-a. As noted previously (73), amino acid homology is particularly striking between dCREB2 and these three mammalian CREBs in the carboxyl-terminal bZIP domain (Fig. 4B). Amino acid homology is much less strong, but still present, in the activation domain. The predicted dCREB2-a product has a region of amino acids containing consensus phosphorylation sites (58) for PKA, calcium/calmodulin-dependent kinase II, and protein kinase C, analogous to the more extensive P-box or kinase-inducible domains defined

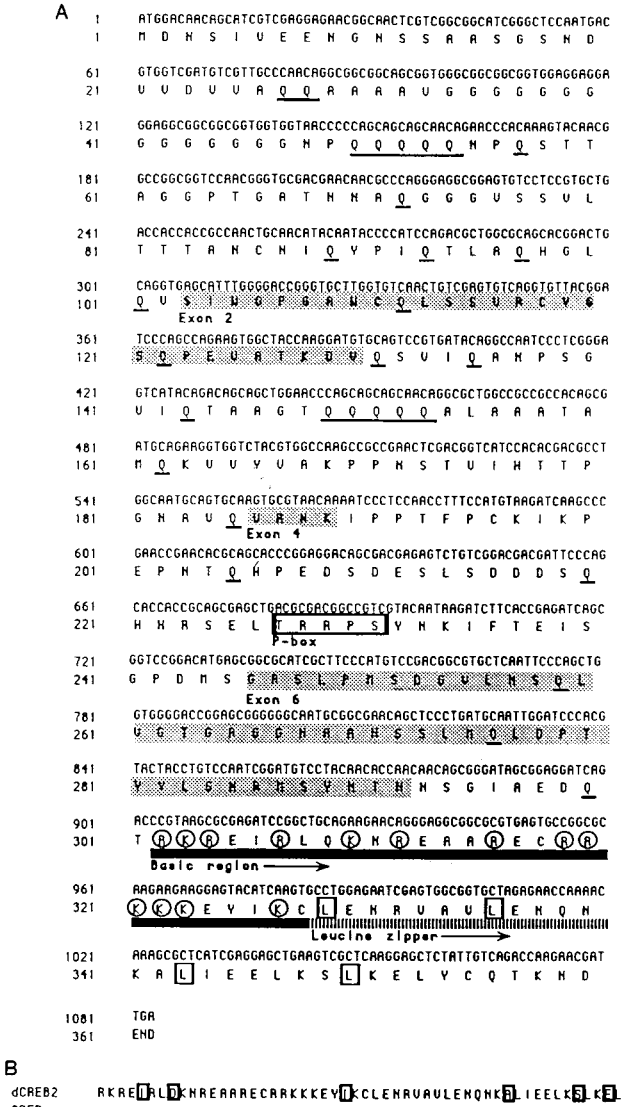

FIG. 4. (A) DNA sequence and predicted amino acid sequence of the *dCREB2-a* coding region. The basic region and leucine zipper domains are indicated by solid and broken bold underlining, respectively. Positively charged residues in the basic region are circled; periodic leucines in the zipper motif are boxed; glutamines in the activation domain are underlined. The short amino acid motif with target sites for kinases, starting at residue 227, is indicated by a bold outline. Sequences specified by alternatively spliced exons 2, 4, and 6 are shaded. (B) Amino acid sequence comparison of bZIP domains of dCREB2, mammalian CREB, CREM, and ATF-1. Differences between dCREB2 and CREB are boxed.

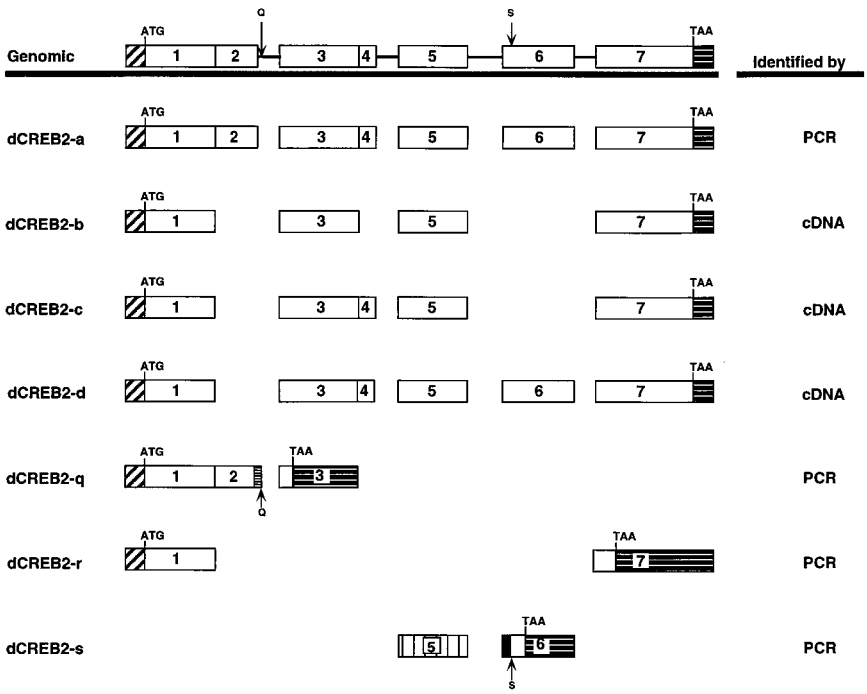

FIG. 5. Diagram of *dCREB2* isoforms. Exon boundaries are defined with respect to *dCREB2-a*. Diagram is not drawn to scale.

in CREB, CREM, and ATF-1. Like the P boxes in CREB and CREM-τ, the dCREB2-a P box is located carboxyl terminal to a glutamine-rich region. As in CREM τ, the dCREB2-a P box lies between a pair of alternatively spliced domains implicated in transcriptional activation (42).

Figure 5 shows the exons present in each of the *dCREB2* alternative splice forms that we have detected, both as cDNAs and by RT-PCR. The splice products of *dCREB2* fall into two broad categories. One class of transcripts (*dCREB2-a*, *-b*, *-c*, and *-d*) employs alternative splicing of exons 2, 4, and 6 to produce isoforms whose predicted protein products all have the bZIP domains attached to different versions of the activation domain. Members of the second class of transcripts (*dCREB2-q*, *-r*, and *-s*) all use alternative splice site selection. Splice forms q and s are generated from alternative 5′ and 3′ splice sites respectively, while form r is a direct splice from exon 1 to exon 7. These splicing variations change the reading frame and result in translation termination at various positions 5′ of the bZIP domain. Thus, they predict a set of truncated activation domains lacking the DNA-binding or dimerization activity that the basic region and leucine zipper provide.

Two different dCREB2 isoforms, dCREB2-a and dCREB2-b, have opposite roles in PKA-responsive transcription. The ability of dCREB2 isoforms to mediate PKA-responsive transcription was tested in F9 cells. These cells have been used extensively to study CREB-dependent activation because their endogenous cAMP-responsive transcription system is inactive (28, 49). In this system, expression constructs for candidate cAMP-responsive transcription factors are transiently transfected with and without a construct expressing the PKA catalytic subunit. CREB-dependent changes in gene expression are measured by using a cotransfected reporter construct that has a CRE-containing promoter fused to coding sequences of the bacterial CAT gene.

In this assay, dCREB2-a is a PKA-dependent activator (Fig. 6). Transfection of expression constructs for PKA or dCREB2-a alone gave only modest activation of the CRE reporter above baseline values. Cotransfection of these constructs together, however, gave levels of activation 5.4-fold greater than the activation seen with PKA alone. This level of PKA-dependent activation was only slightly less than that obtained with mammalian CREB-341 in parallel experiments (data not shown).

dCREB2-b did not function as a PKA-dependent activator in this assay. It failed to stimulate CRE reporter activity in the presence or absence of PKA (data not shown). Instead, dCREB2-b worked as a direct antagonist of PKA-dependent activation by dCREB2-a (Fig. 7). Cotransfection of equimolar amounts of the *dCREB2-a* and *dCREB2-b* expression constructs, along with a construct expressing PKA, resulted in a

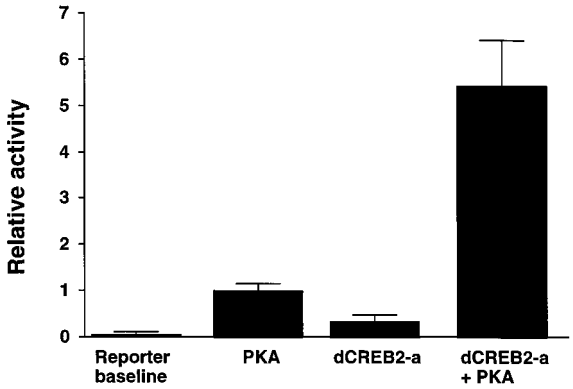

FIG. 6. PKA-responsive transcriptional activation by dCREB2-a. F9 cells were transiently transfected with 10 μg of Δ(−71) CAT plasmid as a CRE-directed reporter; 5 μg of RSV-βgal reporter was included in each dish as a normalization control for transfection efficiency. Different groups received 8 μg of dCREB2-a expression vector and 4 μg of PKA expression vector, separately or in combination, as indicated. All results are expressed as CAT/β-galactosidase enzyme activity ratios, standardized to values obtained with PKA-transfected dishes.

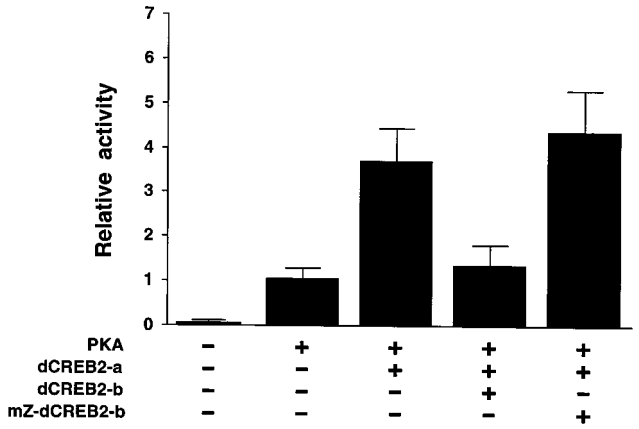

FIG. 7. Transcriptional effect of dCREB2-b and a mutant variant on PKA-responsive activation by dCREB2-a. F9 cells were transiently cotransfected with 10 μg of Δ(−71) CAT along with the indicated combinations of the following expression constructs: RSV-*dCREB2-a* (5 μg), pMtC (2 μg), RSV-*dCREB2-b* (5 μg), and RSV-*mLZ-dCREB2-b*, which expresses a leucine zipper mutant of *dCREB2-b* (5 μg). The DNA mass for each dish was made up to 27 μg with RSV-0. Other experimental conditions are as described in the legend to Fig. 6.

nearly complete block of the PKA-dependent activation produced by dCREB2-a.

Near identity between the predicted leucine zippers of dCREB2 and mammalian CREB suggested that the effects of mutations in CREB in this region could serve as a guide for making mutations in dCREB2. A DNA coding for a mutant dCREB2 molecule, *mLZ-dCREB2-b*, was made by introducing two single-base changes that convert the middle two leucines of the predicted leucine zipper to valines. An identical mutation in CREB abolishes homodimerization in vitro (17). In cotransfection experiments, expression of a construct for mLZ-dCREB2-b failed to block PKA-dependent activation by dCREB2-a (Fig. 7).

DISCUSSION

We have investigated the *dCREB2* gene and found that it encodes the first known PKA-responsive CREB transcriptional activator in *D. melanogaster*. Previously, the mammalian *CREB*, *CREM*, and *ATF-1* genes were the only CREB family members known to express PKA-responsive transcriptional activators. These three mammalian genes form a CREB subfamily defined by this shared function and by amino acid homology, which is especially strong in the bZIP region. A protein database homology search indicated that dCREB2-a is most similar to CREB, CREM, and ATF-1 proteins, especially in the bZIP region, where up to 90% amino acid identity was found. For these reasons, we propose that *dCREB2* is a member of this subgroup of CREB family genes that produce PKA-responsive transcriptional activators and thus might play roles in *D. melanogaster* analogous to those served by the mammalian genes in this group.

The *dCREB2* transcript undergoes alternative splicing. *dCREB2-a*, *-b*, *-c*, and *-d* are splice forms that predict variants of the activation domain attached to a common bZIP region. These alternative splice forms result in seemingly minor changes in the size and spacing of parts of the activation domain. Nevertheless, alternative splicing of the activation domain has profound effects on the functional properties of *dCREB2* products. dCREB2-a is a PKA-responsive transcriptional activator in cell culture, whereas dCREB2-b, lacking exons 2 and 6, produces a specific antagonist. Preliminary data (not shown) suggest that dCREB2-c and dCREB2-d are not activators, suggesting that the activator form requires both exons 2 and 6. Although we have not detected a cDNA which corresponds to dCREB2-a (which is a composite splice form inferred from our cDNA and RT-PCR analysis), we have shown that this molecule functions both in cell culture (Fig. 6) and in transgenic flies (76) as a PKA-responsive activator. This *dCREB2* splicing pattern (and its functional consequences) is virtually identical to that seen in the *CREM* gene. In *CREM*, alternative splicing of exons flanking the P box determines whether a particular isoform is an activator or an antagonist (23, 25, 42). Activator forms contain the alternatively spliced exons, while blocking forms are missing the exons.

In contrast to the *dCREB2* splicing variants that encode isoforms with a bZIP domain, the *dCREB2-q*, *-r*, and *-s* splice forms incorporate in-frame stop codons, resulting in predicted proteins which are truncated amino terminal to the bZIP region. Isoforms of this type have been identified among the products of the *CREB* gene (13, 64) but not among those of the *CREM* gene. The function of these truncated molecules is not known, but at least one such *CREB* mRNA is cyclically regulated in rat spermatogenesis (75).

Other CREBs have been identified in *D. melanogaster*, but dCREB2-a is, so far, the only cAMP-responsive *Drosophila* CREB transcription factor. These other *Drosophila* proteins, BBF-2/dCREB-A (1, 67) and dCREB1 (76a), have substantially less amino acid homology to mammalian CREB, CREM, and ATF-1 than does dCREB2. It may be that in *D. melanogaster*, *dCREB2* is the only representative of this family of genes.

Protein homology and structural gene similarity between the mammalian *CREB* and *CREM* genes has led some to suggest that these genes may be the result of a gene duplication (48, 64). The *Drosophila dCREB2* gene has mRNA splicing isoforms similar to exclusive products of *CREB* and *CREM*. In combination with amino acid sequence homology and the functional similarity between the predicted proteins from *dCREB2* and those from *CREB*, *CREM*, and *ATF-1*, the variety of *dCREB2* splice products suggests that *dCREB2* may be an ancestral form of the mammalian family of PKA-responsive *CREB* genes.

We believe that the net level of gene activation by CREB family members in response to activation of the cAMP pathway depends on the ratio of the amounts and activities of activator isoforms to blocking isoforms, a proportion that we call the A/B ratio (76). In mammals, three genes (*CREB*, *CREM*, and *ATF-1*) can possibly contribute to this ratio, thus making it difficult to evaluate the function of any single gene in a particular process. For instance, *CREB* knockout mice appear normal, but at the molecular level show increased levels of *CREM* expression (37), showing that compensation can occur among genes of this group. In other experiments, expression of a CREB blocker transgene under control of the somatostatin promoter in mice results in dwarfism (70), while the knockout mice are normal in size.

If dCREB2 is the only PKA-responsive CREB in *D. melanogaster*, experiments aimed at disrupting CREB-mediated cAMP-responsive transcription may be easier to perform and interpret in this organism than in the multigene mammalian systems. Although *D. melanogaster* apparently uses only a single gene, the *dCREB2* gene retains many of the subtleties of its mammalian counterpart, including the production of both activators and blockers. By combining the well-developed molecular genetic techniques available for *D. melanogaster* with the ability to make inducible transgenic flies and isolate mutations,

dCREB2 could provide insights not currently available elsewhere into the role of cAMP-responsive transcription in a variety of biological processes.

One such process is the possible involvement of CREB in long-term memory formation. Results from studies with *Aplysia californica* pointed to a requirement for a CREB factor in a cellular model for long-term memory (2, 12). Recently we have performed experiments with *D. melanogaster* to test the effects of blocker or activator *dCREB2* isoforms on long-term memory in the intact, behaving animal. Using a conditionally expressed transgene, we have shown that induced *dCREB2-b* expression specifically abolishes long-term memory (77). Most recently, we have obtained a complementary result in *dCREB2-a* transgenic flies, in which induction of the activator actually potentiates formation of long-term memory (76). We believe that long-term memory depends on PKA-responsive gene activation via CREBs and that this transcriptional switch is a function of the A/B ratio in relevant brain cells during and after behavioral training.

Discovery of a cAMP-responsive transcriptional control system in *D. melanogaster* can also be rapidly integrated into the framework of ongoing molecular genetic investigations. Two examples of where this is occurring are biological rhythms and development. Rhythmic phosphorylation of CREB and cyclic expression of a blocking CREM isoform, ICER, is correlated with a phase of a biological clock in mammals (26, 69, 72). Mutations directly affecting cAMP metabolism are reported to alter behavioral rhythms in *D. melanogaster* (44), in which a clock gene has been cloned (20, 78). Experiments evaluating the effects of inducing *dCREB2* transgenes on behavior or expression of the clock protein may prove informative.

Lane and Kalderon (41) showed the general involvement of the cAMP second-messenger system in *Drosophila* development by using mutants in the catalytic subunit of PKA. Recently, the *hedgehog* developmental pathway has become the subject of intense interest in both *Drosophila* and mammalian systems (6, 19, 35, 53, 62), and results of studies with *D. melanogaster* suggest a role for cAMP in this system (10a, 37a, 63a, 69a). The possible involvement of CREB-mediated transcription in these processes now can be tested by using reverse genetic strategies.

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