THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 13, Issue of March 26, pp. 12117–12123, 2004

Printed in U.S.A.

Phosphorylation of Conserved Casein Kinase Sites Regulates cAMP-response Element-binding Protein DNA Binding in *Drosophila**

Received for publication, December 17, 2002, and in revised form, December 12, 2003
Published, JBC Papers in Press, December 15, 2003, DOI 10.1074/jbc.M212839200

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The *Drosophila* homolog of cAMP-response element-binding protein (CREB), dCREB2, exists with serine 231, equivalent to mammalian serine 133, in a predominantly phosphorylated state. Thus, unlike the mammalian protein, the primary regulation of dCREB2 may occur at a different step from serine 231 phosphorylation. Although bacterially expressed dCREB2 bound cAMP-response element sites, protein from *Drosophila* extracts was unable to do so unless treated with phosphatase. Phosphorylation of recombinant protein by casein kinase (CK) I or II, but not calcium-calmodulin kinase II or protein kinase A, inhibited DNA binding. Up to four conserved CK sites likely to be phosphorylated *in vivo* were responsible for this effect, and these sites were phosphorylated by a kinase present in *Drosophila* cell extracts that biochemically resembles CKII. We propose that the relative importance of different signaling pathways in regulating CREB activity may differ between *Drosophila* and mammals. In *Drosophila*, the dephosphorylation of CK sites appears to be the major regulatory step, while phosphorylation of serine 231 is necessary but secondary.

The cAMP-response element-binding protein (CREB)[1] has been implicated in a large number of cellular and physiological processes (1). It is a transcription factor that binds to CRE sites and is involved in adaptive responses to a wide variety of stimuli, including neuronal activity, memory formation, drugs of abuse, growth factors, hormones, and peptide signaling. These responses contribute to its transcriptional role in circadian biology, the stress response, neuritogenesis, developmental plasticity, and memory formation (2–5).

In mammals, the CREB family consists of three different genes: CREB, CREM, and ATF-1 (1). Although their protein products are very similar, minor sequence differences exist, and the physiological distinctions between them are not entirely clear. Genetic knock-out studies indicate that these genes probably have similar functions since loss-of-function mutations in individual genes result in compensatory changes in expression of the others (6, 7).

Sequence alignment of CREB genes from many species identifies two regions of the protein that are highly conserved (1), a basic region-leucine zipper structural motif involved in dimerization and DNA binding and a 60-amino acid kinase-inducible domain (KID) (Fig. 1). CREB activity is regulated by phosphorylation of sites within the KID. Notably phosphorylation of a protein kinase A (PKA) site at serine 133 (Ser-133) allows CREB to interact with the co-activator CREB-binding protein, which in turn activates transcription by recruiting RNA polymerase II complexes to CRE-containing promoters and by acetylating histones (8–12). A variety of other kinases, including the extracellular signal-regulated kinase/mitogen-activated protein kinase/ribosomal S6 kinase cascade, protein kinase C, and calcium/calmodulin-dependent kinases (CaMKs), have also been shown to target Ser-133 (13).

Other regulatory sites within the KID include Ser-129 and Ser-142 (14). Ser-129 is believed to be a substrate for glycogen synthase kinase 3. Phosphorylation of this site occurs only after phosphorylation of Ser-133 and can function to reduce DNA binding of CREB (15, 16). Ser-142 can be phosphorylated by CaMKII and is implicated in either inhibiting CREB activity (17) or stimulating CREB-binding protein-independent CREB activation (18). In addition, serine residues thought to be targets for casein kinases (CKs) are found at residues 108, 111, 114, 117, and 121.

The *Drosophila* CREB homolog, dCREB2, is of particular interest since it is a critical molecule in long term memory formation in flies. Studying its regulation may yield insights to the regulation of memory formation. Similar to CREB from other species, dCREB2 has a conserved KID domain (Fig. 1). Ser-231, which is homologous to the critical mammalian Ser-133 residue, can be phosphorylated by PKA, and a serine to alanine mutation at this site completely abolishes activity (19). Mammalian Ser-129 and Ser-142 are also conserved as Thr-227 and Ser-240, respectively. In addition, the CK sites are positionally conserved N-terminal to Ser-133/231 and are similarly surrounded by acidic amino acids (20).

CKII is a ubiquitous, highly conserved serine/threonine kinase (21). Currently its regulation and precise biological roles are poorly understood, but it is essential for viability of eukaryotes. It has been reported to interact with the basic-leucine zipper domains of several transcription factors, including ATF-1 and CREB (22). In some cases, phosphorylation by CKII has been reported to regulate binding to DNA. It is proposed that CKII inhibits DNA binding of the c-Jun transcription factor (23) and enhances the DNA binding properties of CREM (24). The CK sites of CREB are phosphorylated during early S phase in a cell cycle-dependent manner, although the role of

* This work was supported in part by Grant NS 35575 (to J. C. P. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CREB, cAMP-response element-binding protein; CK, casein kinase; CRE, cAMP-response element; CREM, cAMP-response element modulator; KID, kinase-inducible domain; PKA, protein kinase A; CaMK, calcium/calmodulin-dependent kinase; ATF-1, activating transcription factor-1; EMSA, electrophoretic mobility shift assay; S2, Schneider-2; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

this phosphorylation has not been elucidated (25). In *Drosophila*, CKII interacts with and phosphorylates basic-helix-loop-helix proteins (26).

In the current study, we showed that a large proportion of dCREB2 Ser-231 exists in a phosphorylated state. This suggested to us that, in flies, an alternate regulatory mechanism might be preferentially utilized. We propose that the CK sites of dCREB2 may provide this mechanism by regulating DNA binding. In support of this model, we demonstrated that phosphorylation of the CK sites prevents DNA binding, while dephosphorylation allows dCREB2 to bind. Furthermore we showed that phosphorylation of these sites is likely to occur *in vivo* and that a kinase that shares similarities to CKII is present in *Drosophila* extracts and phosphorylates these sites. Regulation of DNA binding through phosphorylation/dephosphorylation of these sites may be a novel mechanism of controlling CREB activity separate from Ser-133 phosphorylation.

MATERIALS AND METHODS

Extract Preparation—Drosophila head extracts were made by freezing flies in liquid nitrogen, isolating heads, and crushing them with a pestle. For Western analysis, heads were crushed directly in SDS loading buffer (27) and used at a concentration of 5 μl/head. For extracts used in electrophoretic mobility shift assays (EMSAs), crushed heads were extracted by addition of 3 μl/head cold homogenization buffer (15 mM Hepes, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Extracts were centrifuged twice at 14,000 $\times$ *g* to remove debris. An equal volume of 2$\times$ HEMG (200 mM KCl, 40 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) was added, and protein concentrations were determined using the Bradford assay. Extracts containing 100 mM EDTA were made as above except that the EDTA concentration was raised to 100 mM in all solutions used. For extracts fractionated using phosphocellulose, the 2$\times$ HEMG was supplemented with 1 M NaCl to make a final extract concentration of 500 mM NaCl.

Schneider-2 (S2) cells were grown and passaged as described previously (28). Extracts were made by resuspending cells in 3 times their packed cell volume of homogenization buffer and lysing by freeze/thawing in liquid nitrogen three times in rapid succession. Debris were removed from extracts by centrifugation, and an equal volume of 2$\times$ HEMG was added. Protein concentrations were determined using the Bradford assay.

For phosphocellulose fractionation of extracts, cellulose phosphate P-11 was prepared as recommended by the manufacturer (Whatman BioSystems Ltd.) and equilibrated in 1$\times$ HEMG with 500 mM NaCl. Head extracts were added to the phosphocellulose at a ratio of 100 μl of extract to 50 μl of phosphocellulose and incubated at 4 °C for 1 h on a rotator. Mixtures were centrifuged at 1000 $\times$ *g* for 10 min to separate a supernatant (flow-through) fraction from the pellet fraction. After washing with HEMG containing 500 mM NaCl, the pellet fraction was mixed with 0.5 times the original extract volume of HEMG with 1.5 M NaCl. Mixtures were rotated at 4 °C for 1 h, and then the 1.5 M NaCl solution was collected (elution fraction). Both flow-through and elution fractions were dialyzed against 1$\times$ HEMG.

Mutagenesis, Plasmid Construction, Purification of Recombinant dCREB2 Protein, and Generation of Transgenic Flies—In vitro oligonucleotide-directed mutagenesis was performed on pJY386 (20), a cDNA of the dCREB2-b isoform originally isolated from a *Drosophila* head cDNA library and subcloned into Bluescript pKS+ (Stratagene). The mutations that were made were: **S231A** (TCG codon changed to GCG), **(CK1–4)A**, S210A (AGC changed to GCC), S213A (AGT changed to GCT), S215A (TCG changed to GCC), S219A (TCC changed to GCC), and **S240A** (AGC changed to GCC). All derivatives were sequenced on both strands. These were subcloned into pET30C to express protein in bacteria and into CaSpeR-HS to make transgenic flies expressing the open reading frames under the control of the heat shock promoter. Plasmid names are as follows: pJY1048 = S231A in pBluescript, pJY1060 = S231A in pET30C, pJY1056 = S231A in CaSpeR-HS, pJY1049 = (CK1–4)A in pBluescript, pJY1061 = (CK1–4)A in pET30C, pJY1057 = (CK1–4)A in CaSpeR-HS, pJY1051 = S240A in pBluescript, pJY1062 = S240A in pET30C, and pJY1058 = S240A in CaSpeR-HS. Growth of bacteria, induction, and purification of proteins on nickel-nitrilotriacetic acid-agarose columns was done according to the manufacturer's recommendations (Qiagen). Transgenic flies were produced using standard techniques. Transgenes were induced using a 30-min temperature shift from 25 °C to 37 °C followed by 3 h at 25 °C prior to extract preparation.

Kinase and Phosphatase Treatment—CKI, CKII, PKA, CaMKII, and λ protein phosphatase were purchased from New England Biolabs. Phosphorylation and phosphatase reactions were performed as recommended by the manufacturer. For gel shift phosphatase treatments, 10 μg of extract or 6 ng of recombinant dCREB2 were dephosphorylated in a total volume of 10 μl using 200 units of phosphatase. Recombinant dCREB2 was phosphorylated using S2 extract by mixing 10 ng of recombinant protein with 20 μg of extract in a total volume of 10 μl of 20 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, and 100 μM ATP. Mixtures were incubated at room temperature for 1 h. For 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibition experiments, DRB was added at the indicated concentrations.

EMSAs—Sequences of the 3xCRE double-stranded oligonucleotide probe and the mutated 3xCRE oligonucleotide have been described previously (20). 200 ng of probe were radiolabeled using polynucleotide kinase in the presence of 100 μCi of [γ-^{32}P]ATP as recommended by the manufacturer (New England Biolabs). Probe (4–5 $\times$ 10^5 cpm) was incubated with 5 μg of *Drosophila* extract, 4 μg of mouse extract, or 3 ng of recombinant dCREB2 for each gel shift experiment. Incubations were carried out in a volume of 10 μl at room temperature for 1 h in 12 mM Hepes, pH 7.9, 4 mM Tris-Cl, pH 7.9, 1 mM EDTA, 12% glycerol, 1 mM dithiothreitol, 5 mM $MgCl_2$, 60 mM KCl, 1 mg/ml bovine serum albumin, and 0.4 mg/ml poly(dI-dC). Mixtures were then run on a 4% acrylamide (acryl:bis = 80:1), Tris-glycine (380 mM glycine, 50 mM Tris base, 2 mM EDTA, 3.6 mM $MgCl_2$, and 1% glycerol) gel, transferred to Whatman No. 3MM paper, and exposed to film. For antibody supershift experiments, 0.5 μl of anti-mouse CREB antibody (New England Biolabs) or anti-dCREB2 antibody (3) were added to each binding reaction.

Peptide Analysis—Recombinant dCREB2 was phosphorylated with S2 extract as described above except that the recombinant protein used was between 50 ng and 1 μg, and the ATP concentration was 60 pM cold ATP and 50 μCi of [γ-^{32}P]ATP. Phosphorylation reactions were separated on a 10% SDS-polyacrylamide gel. The radiolabeled CREB band, identified by Coomassie staining and exposing the wet gel to film, was excised, and gel slices were extensively washed with 50% methanol, 10% acetic acid and then with 50% methanol before being lyophilized. Slices were then treated with 1 ml of trypsin (75 mg/ml in 50 mM NH_4CO_3H, pH 8.0) for 20 h at 37 °C. Supernatants from the trypsin digestion were lyophilized, resuspended in 200 μl of H_2O, lyophilized, resuspended in 100 μl of 10% acetic acid, 1% pyridine, lyophilized, and finally resuspended in 10 μl of 25% acetonitrile, 7.5% acetic acid, 0.75% pyridine. Samples were applied with phenol red tracking dye to TLC plates and electrophoresed at 400 V at pH 3.5 until the phenol red migrated halfway to the anode. Following electrophoresis, plates were dried and subjected to thin layer chromatography in a solvent of 25% 1-butanol, 7.5% acetic acid, and 37.5% pyridine until the phenol red migrated close to the top of the plate. Plates were dried and exposed to film at −80 °C with intensifying screens or analyzed by PhosphorImager.

Denaturing Non-SDS-PAGE—Denaturing non-SDS-polyacrylamide gels were similar to standard SDS gels (27) except that SDS was omitted and replaced with 8 M urea in the resolving and stacking gels and sample loading buffer. The concentration of the reducing agent dithiothreitol in sample loading buffer was 100 mM.

Ser-231-phosphospecific Antibody—Two peptides were synthesized, one with the sequence H-Cys-Glu-Leu-Thr-Arg-Arg-Pro-Ser-Tyr-Asn-Lys-Ile-Phe-Thr-NH_2 and another with the identical sequence but with the serine phosphorylated. The phosphorylated peptide was conjugated to keyhole limpet hemocyanin using an Imject Immunogen EDC conjugation kit (Pierce) as recommended by the manufacturer and injected into rabbits using standard techniques (29). To purify rabbit polyclonal serum, both phosphorylated and unphosphorylated peptides were reduced (Reduce-Imm reducing kit, Pierce) and cross-linked to agarose beads in a column (AminoLink immobilization kit, Pierce). Columns were run sequentially, and purified sera consisted of the flow-through of the column containing unphosphorylated peptide and the elution of the column containing the phosphorylated peptide.

RESULTS

Most dCREB2 Ser-231 Exists in a Phosphorylated Form—In mammals, CREB activity has been correlated with Ser-133 phosphorylation, and Ser-133-phosphospecific antibody has been used widely to identify tissues or areas where CREB has been activated (30, 31). We wanted to quantify the total amount of dCREB2 present in fly heads and determine the

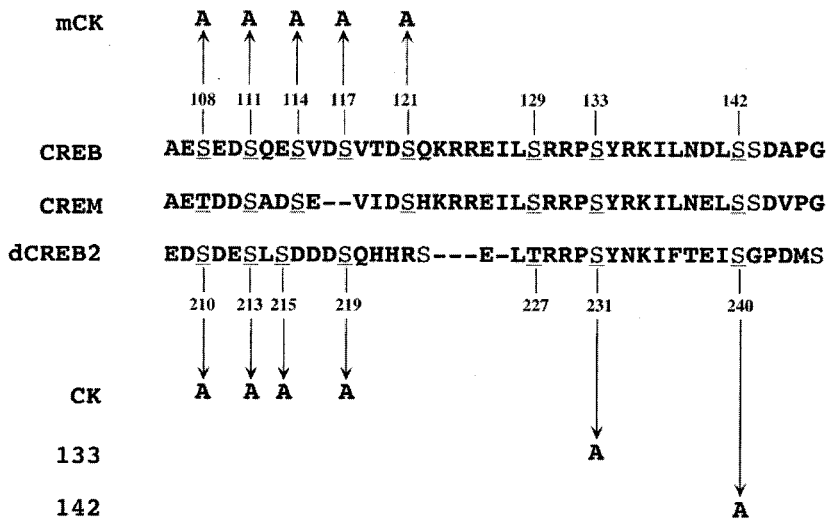

FIG. 1. **Sequence comparisons within the kinase-inducible domains of mammalian CREB, CREM, and *Drosophila* CREB2.** Ser-133/231, Ser-142/240, Ser-129/Thr-227, and the CK sites are *underlined* and *numbered*. Mutations generated and used in this study are indicated. "*mCK*" refers to mutations created in rat CREB and used in the tissue culture transfection experiments. "*CK*" shows the Ser to Ala mutations present in rec-dCREB2-CK and expressed in hs-CREB-CK flies, "*133*" shows the mutation present in rec-dCREB2-133 and expressed in hs-CREB-133 flies, and "*142*" shows the mutation present in rec-dCREB2-142 and expressed in hs-CREB-142.

percentage of this protein that was phosphorylated at Ser-231, the equivalent site to mammalian Ser-133 (Fig. 1). Recombinant dCREB2-b protein (an inhibitory isoform) was purified from bacteria and is referred to as rec-dCREB2-wt. Various dilutions of the rec-dCREB2-wt protein and varying amounts of *Drosophila* head extracts were analyzed on Western blots using dCREB2-specific monoclonal and Ser-231-phosphospecific antibodies. Fig. 2 shows that our monoclonal antibody had similar affinity for non-phosphorylated or phosphorylated protein and results in linearity of band intensity with protein levels in the range tested. Using this antibody, we estimated that a single *Drosophila* head contains ~0.67 ng of dCREB2 protein (based on three to five heads). An equivalent blot, probed with the Ser-231-phosphospecific antibody, demonstrates the specificity of the antibody since 30 ng of non-phosphorylated protein gave no signal, while 2 ng of PKA-phosphorylated protein produced a clear band. Similar to the monoclonal antibody, the band intensity produced by the phosphospecific antibody varied proportionally with rec-dCREB2-wt levels (Fig. 2, *lower panel*, *lanes 1–6*). Comparison of band intensities between head extracts and recombinant protein led to an estimate of ~0.67–0.8 ng of Ser-231-phosphorylated dCREB2/head (based on three to five heads). This result indicates that a large proportion, if not all, of the dCREB2 present in *Drosophila* heads exists in a Ser-231-phosphorylated state. This is a surprising result since the majority of mammalian CREB seems to exist in a non-phosphorylated and inactive form. Activation of mammalian CREB critically depends upon stimulation of pathways that lead to phosphorylation of Ser-133.

Phosphatase Treatment Unmasks dCREB2 DNA Binding Activity—We next wanted to determine whether our identified dCREB2 was able to bind DNA. When extracts were tested for CRE DNA binding using EMSAs, one shifted band was detected (Fig. 3*A*, *lane 2*). If the extracts were treated with λ phosphatase prior to the binding reactions, a second, slower migrating band was observed (*lane 3*). The proteins in both of these complexes bound specifically to CRE sites since excess cold CRE, but not mutated CRE, oligonucleotides competed for binding (*lanes 5* and *6*). To determine which of these two complexes contains dCREB2, supershift studies were performed using dCREB2 monoclonal antibody. As shown in Fig. 3*A*, *lane 4*, antibody was able to supershift only the phosphatase-dependent band. The phosphatase-independent band seemed to be unrelated to dCREB2 and was not studied further. Thus, in *Drosophila* head extracts, there are at least two CRE-binding protein complexes, but only the phosphatase-dependent one

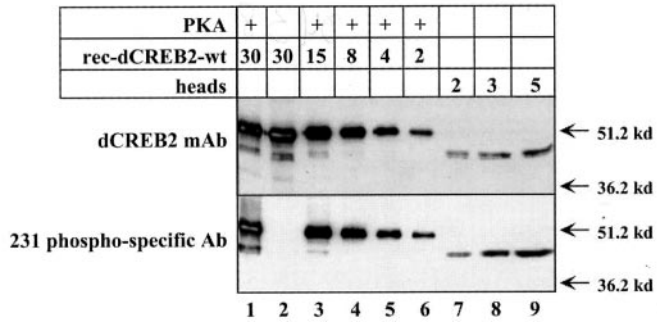

FIG. 2. **Comparison of amounts of total dCREB2 and phospho-Ser-231 dCREB2 in *Drosophila* heads.** Varying amounts of recombinant dCREB2 and head extracts were subjected to PAGE and Western analysis. In the row marked "*rec-dCREB2-wt*," *numbers* refer to the amount (in ng) of recombinant dCREB2 loaded on the gel. In the row marked "*heads*," *numbers* refer to the number of head equivalents loaded. Recombinant protein was either treated (+) or untreated with PKA as indicated. Western blots were probed with either monoclonal dCREB2 antibody (*dCREB2 mAb*), which does not differentiate between phosphorylation states, or Ser-231-phosphospecific antibody (*Ab*). Recombinant dCREB2 runs at a higher molecular weight compared with endogenous dCREB2. Part of the reason for this aberrant mobility is that recombinant dCREB2 contains extra residues encoded in pET30C, including a His_6 tag used for purification, fused onto the C terminus of the protein.

contains dCREB2 protein.

The phosphatase dependence of dCREB2 binding was not limited to head extracts since dCREB2 from *Drosophila* S2 cell extracts behaved identically (Fig. 3*B*). These data suggest that, in *Drosophila*, the dCREB2 protein exists in a phosphorylated state that is unable to bind DNA and that binding requires the dephosphorylation of one or more residues.

Casein Kinase Treatment Inhibits Recombinant dCREB2 DNA Binding Activity—If *Drosophila* contain a kinase that phosphorylates and inactivates dCREB2 binding activity, then bacterially expressed dCREB2 should be free from this phosphorylation and able to bind DNA. As expected, rec-dCREB2-wt was able to bind CRE sites as assayed using EMSA (Fig. 4). Phosphorylation of the protein using CKI or CKII severely inhibited binding (Fig. 4, *left panel*), while phosphorylation using PKA or CaMKII had only slight or negligible effects. In all cases, the incorporation of phosphate into dCREB2 was verified in parallel experiments using [γ-^{32}P]ATP as a phosphate source (Fig. 7 and data not shown).

One trivial explanation for the disappearance of DNA bind-

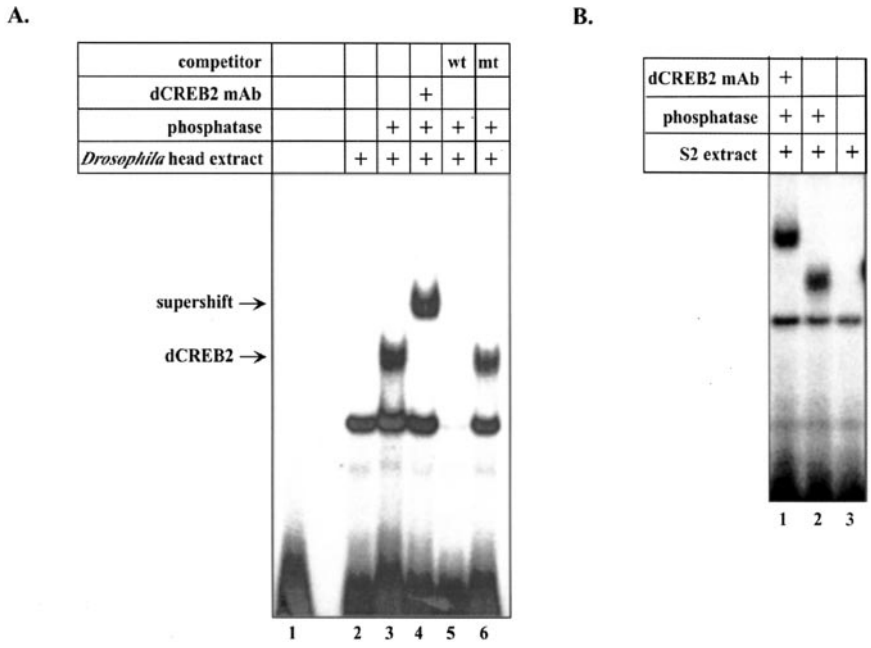

FIG. 3. *A*, CRE-binding proteins in *Drosophila* head extracts. Head extracts from wild type *Drosophila* were made as described under "Materials and Methods." 5 μg of extract were either left untreated (*lane 2*) or treated (*lanes 3–6*) with λ phosphatase prior to being mixed with radiolabeled CRE DNA probe and subjected to EMSA. Free probe alone is shown in *lane 1*, supershift using dCREB2 antibody is shown in *lane 4*, and oligonucleotide competition experiments with wild type (*wt*) and mutated (*mt*) CRE elements, respectively, are shown in *lanes 5* and *6*. *B*, CRE-binding proteins in Schneider-2 cell extracts. 5 μg of S2 extract were either treated (*lanes 1* and *2*) with λ phosphatase or left untreated (*lane 3*), mixed with CRE probe, and subjected to EMSA. Supershift with dCREB2 antibody is shown in *lane 1*. *mAb*, monoclonal antibody.

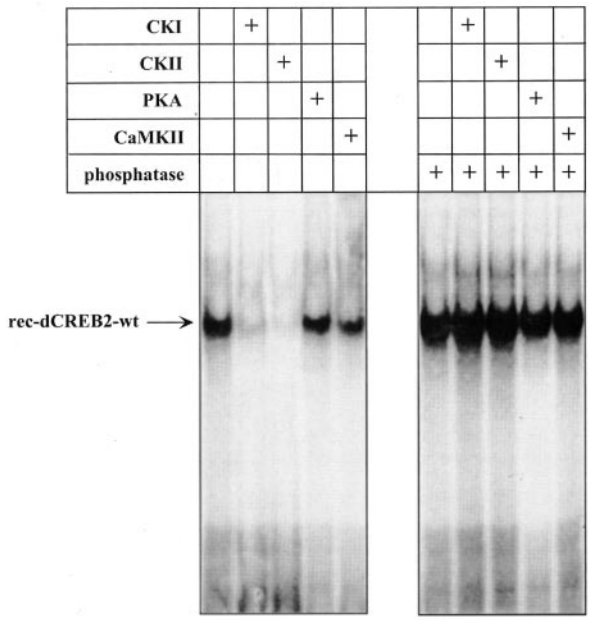

FIG. 4. **Binding to CRE sites of recombinant dCREB2 phosphorylated *in vitro*.** 3 ng of purified rec-dCREB2-wt were either left unphosphorylated (*lanes 1* and *6*) or phosphorylated with the indicated kinases (*lanes 2–5* and *7–10*) before being mixed with CRE radiolabeled probe and subjected to EMSA. Samples in *lanes 6–10* were treated with excess λ phosphatase after kinase treatment and prior to EMSA. The position of recombinant dCREB2 is indicated.

ing subsequent to the addition of CKI or CKII would be proteolysis of rec-dCREB2-wt due to the presence of contaminating proteases in these samples. We demonstrated that this was not the case since addition of excess phosphatase after the kinase reactions restored binding activity (Fig. 4, *right panel*). This reversal of kinase-mediated inhibition suggests that CKI and CKII exert their effects through phosphorylation. Furthermore the inhibition of binding was dependent on the presence of ATP as a phosphate source during incubation with the kinase (data not shown). Thus we propose that CKI, CKII, and/or another kinase with similar site specificity is present in *Drosophila* and prevents dCREB2 from binding to CRE sites.

Mutating Serines 210, 213, 215, and 219 Prevents Casein Kinase II-mediated Inhibition of DNA Binding—We hypothesized that the conserved CK sites in the KID of dCREB2 might be involved in regulating binding. Thus we mutated a number of different sites on the dCREB2-b isoform, including the CK sites, and tested mutant recombinant protein for DNA binding activity. rec-dCREB2-133 contains a mutation of Ser-231 to Ala. rec-dCREB2-CK contains Ser to Ala substitutions at the four putative CK sites located at residues 210, 213, 215, and 219. rec-dCREB2-142 is dCREB2 with residue 240 mutated to Ala. As shown in Fig. 5, all recombinant proteins bound similarly. However, only the rec-dCREB2-CK protein bound DNA after treatment with CKII. Phosphorylation of serines corresponding to mammalian residues 133 and 142 appear not to be involved in regulating DNA binding. Treatment with excess phosphatase after kinase treatment led to a recovery of DNA binding.

Schneider Cell Extracts Contain a Kinase That Inhibits Binding—Incubation of bacterially expressed dCREB2 protein in an S2 extract inhibited DNA binding (Fig. 6, *lane 3*). However, when rec-dCREB2-CK was incubated, it was still able to bind DNA (*lane 4*). Subsequent phosphatase treatment restored DNA binding of dCREB2-wt (*lanes 5* and *6*). These observations indicate that there is an active kinase present in S2 extracts that phosphorylates dCREB2 at the CK sites and inhibits DNA binding.

The bands present in Fig. 6, *lanes 5* and *6*, are somewhat darker and broader than the bands present in *lanes 1* and *2* (recombinant proteins alone). This broadening is likely due to two separate factors. Phosphatase treatment allows not only recombinant rec-dCREB2-wt to bind but also unmasks binding of endogenous dCREB2 proteins present in S2 extracts (see Fig. 3*B*). Gel shift species containing recombinant dCREB2 migrated at a position slightly higher than those containing endogenous dCREB2 (see Figs. 2 and 8*A*), resulting in a darkening and broadening of the shifted band, which now included recombinant dCREB2, endogenous dCREB2, and possibly heterodimers of the two. In addition, phosphatase treatment may have slightly enhanced binding of recombinant dCREB2 (see Fig. 3), suggesting that the recombinant protein may be phosphorylated at unknown sites when expressed in bacteria.

Peptide Mapping Confirms That the Inhibitory Kinases Phosphorylate Casein Kinase Sites—The preceding results all indicate that CKI, CKII, and S2 extracts all phosphorylate

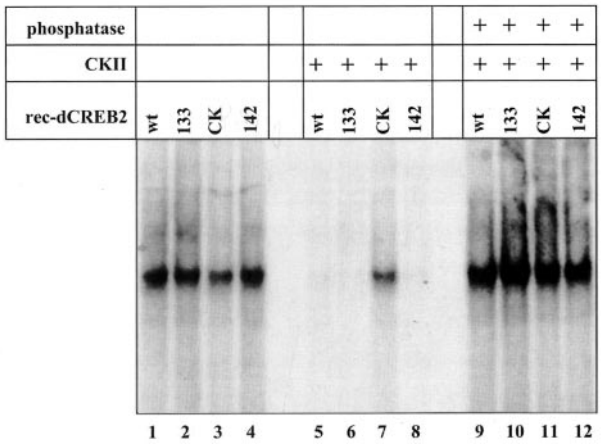

FIG. 5. **Effects of CKII phosphorylation on CRE binding of wild type and mutated recombinant dCREB2.** *Lanes 1–4* show EMSA of 3 ng of unphosphorylated recombinant dCREB2 proteins. *wt* refers to wild type, *133* indicates a mutation converting Ser-231 to Ala, *CK* indicates Ser to Ala mutations at the four CK sites, and *142* indicates a Ser-240 to Ala mutation. In *lanes 5–8*, the recombinant proteins were first phosphorylated using CKII prior to EMSA. In *lanes 9–12*, proteins were first phosphorylated and then dephosphorylated using λ phosphatase prior to EMSA.

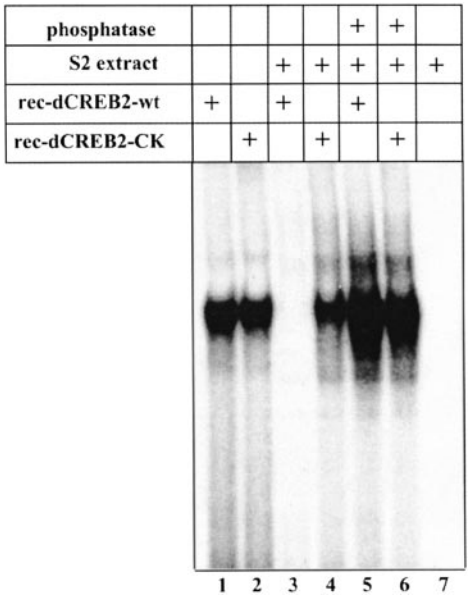

FIG. 6. **EMSA of recombinant dCREB2 treated with Schneider-2 cell extract.** EMSA was performed on rec-dCREB2-wt and rec-dCREB2-CK. *Lanes 1* and *2* show gel shifts of 3 ng of the recombinant proteins alone. *Lanes 3* and *4* show gel shifts of the proteins after incubating them with S2 extract in the presence of ATP. In *lanes 5* and *6*, proteins were first incubated with S2 extract and ATP and then subsequently dephosphorylated with excess λ phosphatase before being subjected to EMSA. Note that phosphatase treatment allows both recombinant protein and dCREB2 from extracts to bind. *Lane 7* shows EMSA of untreated S2 extract.

dCREB2 and prevent DNA binding. Furthermore they suggest that the putative CK sites play important roles in this regulation of binding. To verify the similarities between the three kinases and their relation to the CK sites, we performed peptide mapping experiments.

rec-dCREB2-wt protein was phosphorylated *in vitro* using PKA, CaMKII, CKI, CKII, and S2 extracts in the presence of [γ-^{32}P]ATP (Fig. 7*A* and data not shown). Phosphorylated products were subjected to PAGE, and the radiolabeled protein band was purified and digested with trypsin. Resultant peptides were separated in two dimensions using isoelectric focusing and thin layer chromatography. As shown in Fig. 7*B*, CKI, CKII, and the S2 extract all phosphorylated a common peptide or peptides (labeled *B*), while PKA and CaMKII phosphorylated a different peptide (labeled *A*), which presumably contains Ser-133 and Ser-142.

To confirm the relationship between peptide B and the putative CK sites, rec-dCREB2-CK as well as rec-dCREB2-wt was used as a substrate for the kinase in S2 extracts. As seen in Fig. 7*C*, peptide B was phosphorylated when the substrate was rec-dCREB2-wt but remained unphosphorylated when the substrate was rec-dCREB2-CK. Taken together, these results provide strong supporting evidence that the kinases that inhibit dCREB2 binding function by phosphorylating the sites that are mutated in CREB-CK.

The Kinase in Schneider-2 Cell Extracts Shares Some Properties with Casein Kinase II—We were interested in characterizing the kinase in S2 extracts to determine whether it was more similar to CKI or to CKII. CKII is known to bind to phosphocellulose at high ionic strengths (>0.5 M NaCl) (21). When *Drosophila* S2 extracts were adjusted to 0.5 M NaCl and then batch bound to phosphocellulose, more than 99% of the proteins were in the unbound flow-through fraction as measured using a Bradford assay. Bound proteins were eluted at 1.5 M NaCl and constituted less than 1% of total proteins (data not shown). Western analysis of the flow-through *versus* elution fraction demonstrated that all measurable amounts of dCREB2 and CKI were in the flow-through fraction (data not shown). CKII was one of the few proteins present in the bound and eluted fraction. If the S2 kinase resembles CKI, it should be in the flow-through fraction, whereas if it resembles CKII, it should be in the elution fraction.

Fig. 8*A* demonstrates that the S2 kinase is present in the elution fraction. *Lane 1* shows the migration of rec-dCREB2-wt when assayed by EMSA. Mixing recombinant dCREB2 with the phosphocellulose flow-through fraction in the presence of ATP did not inhibit DNA binding (*lane 4*), while mixing with elution fraction completely abolished binding (*lane 7*). This effect of the elution fraction on binding was reversed by phosphatase treatment (*lane 8*). Endogenous dCREB2 migrated at a position slightly lower than recombinant dCREB2 and was present in the flow-through fraction (*lane 3*). Although the kinase was not active in the flow-through fraction, endogenous dCREB2 remained phosphorylated and unable to bind DNA unless treated with phosphatase (*lanes 2* and *3*). When the flow-through fraction was mixed with rec-dCREB2-wt and subsequently treated with phosphatase, both endogenous and recombinant dCREB2 were able to bind resulting in a darker, broader gel shift. This broader gel species contained both recombinant and endogenous proteins and possibly species due to heterodimer-mediated binding.

The observation that the S2 kinase is in the eluted fraction suggests that it may be related to CKII. To further test this idea, we determined whether this kinase could use GTP as a phosphate source. CKI has been shown to strictly require ATP as a phosphate donor, while CKII can use either ATP or GTP. In the *left panel* of Fig. 8*B*, rec-dCREB2-wt was phosphorylated using an S2 extract in the presence of either [γ-^{32}P]ATP or [γ-^{32}P]GTP, separated by PAGE, and analyzed using autoradiography. The S2 kinase activity was able to use either nucleotide triphosphate as a phosphate source. As further characterization, we determined whether the S2 kinase was inhibited by DRB, a potent and specific inhibitor of CKII (6 μM DRB reduces CKII activity 50%) (32, 33). The *right panel* of Fig. 8*B* shows the incorporation of radioactive phosphate into rec-dCREB2-wt using an S2 extract in the presence of varying

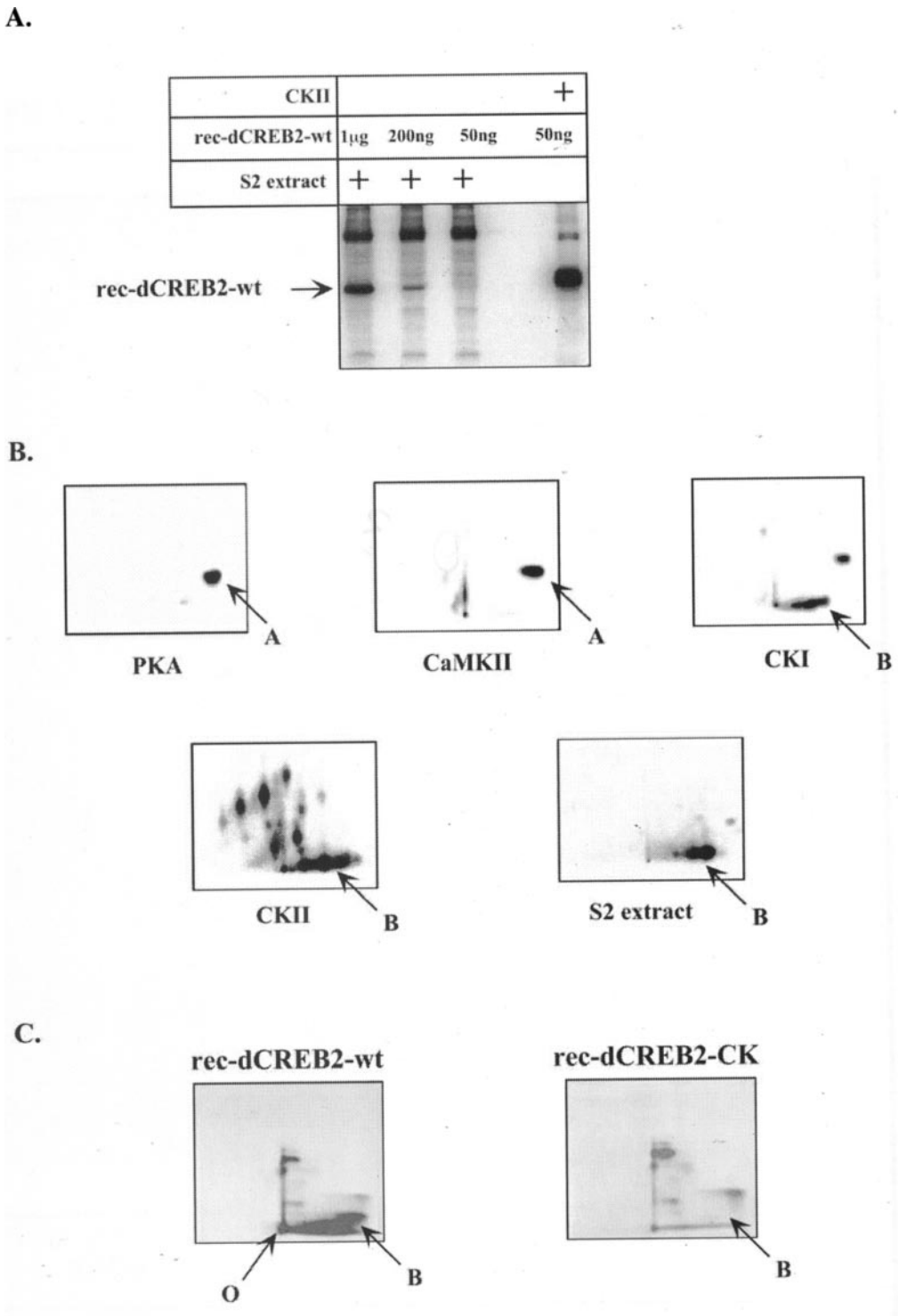

FIG. 7. **Peptide analysis of phosphorylated sites on dCREB2.** *A*, the indicated amounts of rec-dCREB2-wt were phosphorylated using S2 extract or CKII in the presence of [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography. The position of dCREB2 is marked. *B*, rec-dCREB2-wt phosphorylated with [γ-^{32}P]ATP using the indicated kinases or S2 extract was subjected to SDS-PAGE and autoradiography as in *A*. Gel slices containing phosphorylated rec-dCREB2-wt were excised and trypsinized. Resulting peptides were eluted, separated in two dimensions by electrophoresis and chromatography, and analyzed by autoradiography. A peptide phosphorylated by PKA and CaMKII is labeled "*A*," and a second peptide/peptides phosphorylated by CKI, CKII, and S2 extract is labeled "*B*." *C*, rec-dCREB2-wt and rec-dCREB2-CK were phosphorylated using S2 extract and subjected to tryptic peptide mapping as in *B*. The position of peptide "*B*" is marked. In *B* and *C*, all blots were aligned such that electrophoresis proceeded in the horizontal direction with the cathode to the right. Chromatography was performed in the vertical direction proceeding upward. Samples were loaded in an equivalent position on each blot, labeled with an "*O*" on one blot.

concentrations of DRB. Proteins were incubated in the presence or absence of drug, separated using PAGE, and visualized using autoradiography. As seen, 6 μM DRB severely inhibited incorporation, and higher concentrations led to complete inhibition of incorporation. Taken together, these results suggest that the kinase active in S2 extracts that prevents DNA binding is CKII.

Transgenic dCREB2-CK Binds CRE Sites in the Absence of Phosphatase Treatment—All previous experiments demonstrating the importance of the CK sites for dCREB2 DNA

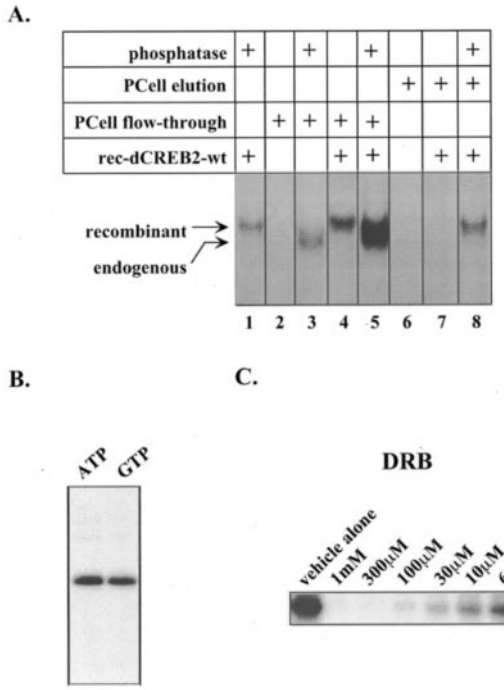

FIG. 8. **Analysis of the kinase active in Schneider-2 cell extracts.** *A*, S2 extracts were separated, using phosphocellulose, into two fractions, a PCell flow-through, which does not bind to phosphocellulose at 0.5 M NaCl, and a PCell elution, which binds at 0.5 M and elutes at 1.5 M NaCl. To identify which fraction contains the S2 kinase, fractions were mixed with rec-dCREB2-wt and assayed by EMSA. *Lanes 1*, *2*, and *6* show the gel shifts of unmixed rec-dCREB2-wt, PCell flow-through, and PCell elution, respectively. *Lanes 4* and *7* show gel shifts of dCREB2-wt after being mixed with PCell flow-through and PCell elution, respectively. *Lanes 5* and *8* show shifts after mixing and subsequent phosphatase treatment. *Lane 3* identifies the gel shift of endogenous dCREB2, which is in the PCell flow-through and adds background bands to *lane 5*. In this panel, all lanes are from the same autoradiogram, and irrelevant lanes were removed for clarity. *B*, rec-dCREB2-wt was phosphorylated *in vitro* by mixing with S2 extract in the presence of either [γ-^{32}P]ATP or [γ-^{32}P]GTP. Phosphorylation reactions were subjected to SDS-PAGE, and phosphate incorporation into rec-dCREB2-wt was analyzed by autoradiography. *C*, rec-dCREB2-wt was phosphorylated using S2 extract in the presence of [γ-^{32}P]ATP and various concentrations of the CKII inhibitor DRB. Incorporation of phosphate into CREB-wt was analyzed by SDS-PAGE followed by autoradiography.

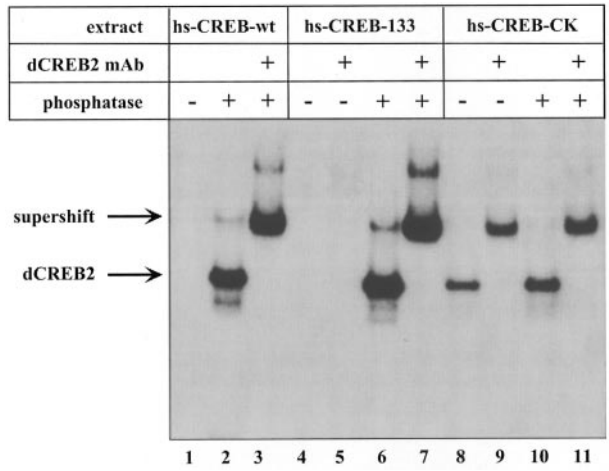

FIG. 9. **CRE binding of transgenic dCREB2.** Transgenic flies that overexpress wild type and mutated forms of dCREB2 under control of a heat shock promoter were generated as described under "Materials and Methods." hs-CREB-wt expresses a wild type repressor isoform of dCREB2, hs-CREB-133 expresses a Ser-231 to Ala mutated form, and hs-CREB-CK expresses a dCREB2 with the four CK sites mutated to Ala. Transgenes were induced by heat shocking at 37 °C for 30 min. 3 h after heat shock, flies were harvested, and head extracts were made. EMSA was performed on 5 μg of extract. Assays were performed either in the absence or presence of λ phosphatase as indicated. Supershifts using dCREB2 monoclonal antibody are also indicated. *mAb*, monoclonal antibody.

binding involved phosphorylation reactions *in vitro*. However, *in vitro* phosphorylation varies considerably depending on how much kinase is added, and it is difficult to determine the appropriate ratio of substrate to kinase to match *in vivo* conditions. To partially address this problem, we made transgenic flies carrying mutations in various phosphorylation sites. hs-CREB-wt refers to a transgenic line expressing dCREB2-b, an isoform nearly identical to rec-dCREB2-wt used in our *in vitro* analysis. hs-CREB-133 expresses the same isoform with a Ser-231 to Ala mutation, and hs-CREB-CK expresses this isoform with the CK sites mutated, similar to recombinant rec-dCREB2-CK. Overexpressed dCREB2 behaved similarly to endogenous protein with respect to DNA binding (Fig. 9, *lanes 1–3*). Extracts from hs-CREB-wt flies did not show CRE binding activity, but phosphatase treatment prior to EMSA unmasked binding. Extracts from hs-CREB-133 flies behaved identically to hs-CREB-wt fly extracts (*lanes 4–7*) supporting the idea that phosphorylation of Ser-231 does not have a major effect on DNA binding. On the other hand, dCREB2 from extracts made from hs-CREB-CK flies was able to bind CRE sites in the absence of phosphatase treatment, and there was no significant increase in binding activity upon treatment (*lanes 8–11*). These results further support the idea that the four CK sites play a major role in the regulation of DNA binding by dCREB2. They also suggest, but do not prove, that phosphorylation of these sites may be occurring *in vivo*.

dCREB2 Is Likely to Be Phosphorylated at the Casein Kinase Sites in Vivo—Since we demonstrated that there was an active kinase in our extracts, it is possible that the phosphorylation occurred exclusively *in vitro* after extracts had been made rather than *in vivo*. To address this issue, we made *Drosophila* head extracts in the presence of 100 mM EDTA, a concentration that abolishes CKI, CKII, and S2 kinase activity in extracts (data not shown). These were used in two experiments. In the first, extracts were adjusted to 0.5 M NaCl and then fractionated using phosphocellulose. Phosphocellulose can be used to separate dCREB2 from S2 kinase since dCREB2 is found in the flow-through fraction and the kinase is found in the elution fraction (Fig. 6*A*). The flow-through fraction, which no longer contains kinase, was subsequently dialyzed to physiological salt and EDTA concentrations and tested for DNA binding. Indistinguishable from previous experiments, dCREB2 did not bind in the absence of phosphatase (data not shown), indicating that phosphorylation of the CK sites most likely occurs in *Drosophila* prior to extract preparation.

In a second experiment, high EDTA extracts were compared with low EDTA extracts that had been either treated or untreated with phosphatase in a denaturing non-SDS-PAGE system. SDS-PAGE cannot be used to distinguish between phosphorylated and dephosphorylated dCREB2 (data not shown). Since this is likely due to the excess SDS-mediated negative charge masking any small charge differences due to phosphorylation, we replaced SDS with 8 M urea as the denaturant in our gels and assayed for dCREB2 by Western blotting. As shown in Fig. 10, when low EDTA extracts from the hs-CREB-wt line were run on this gel system, a clear difference was seen in dCREB2 mobility between untreated and phosphatase-treated samples (*lanes 1* and *2*). When hs-CREB-wt extract was made in high EDTA conditions, dCREB2 migrated at the same position as non-phosphatase-treated extracts (*lane 3*), suggesting that dCREB2 exists in a phosphorylated state

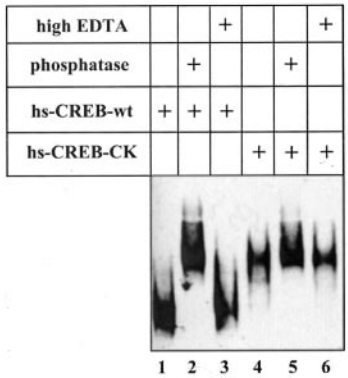

FIG. 10. **Analysis of transgenic dCREB2 using a denaturing non-SDS-PAGE system.** Transgenic dCREB2 protein was induced in hs-CREB-wt and hs-CREB-CK flies as described in Fig. 9. Head extracts were made in buffer containing either 0.1 mM EDTA or 100 mM EDTA (*high EDTA*). Extracts made in low EDTA were either treated with λ phosphatase or left untreated. Samples were separated on a PAGE system lacking SDS and containing 8 M urea to observe mobility differences between different phosphorylated forms of dCREB2. dCREB2 was identified by Western blotting.

prior to extract preparation. To determine whether this *in vivo* phosphorylation occurs at the CK sites, extracts were made from hs-CREB-CK lines, and migration of dCREB2 was compared with that of wild type. As shown in *lanes 4–6*, dCREB2 mutated at the CK sites migrated close to the same position as dephosphorylated dCREB2 and separate from non-phosphatase-treated dCREB2, indicating that the faster migrating form of dCREB2 is due in large part to CK site phosphorylation. We attribute the slight difference in mobility between CREB-CK and dephosphorylated dCREB2 or CREB-CK (the bands in *lanes 4* and *6* migrate slightly farther than those in *lanes 2* and *5*) to phosphorylation occurring at Ser-231. These results indicate that dCREB2 is likely to exist in *Drosophila* in a form where the CK sites are phosphorylated.

DISCUSSION

The importance of Ser-133 phosphorylation in CREB activation has been well characterized and is a conserved requirement between mammalian and *Drosophila* CREB proteins (1, 12, 19). However, Ser-133 phosphorylation alone may not be sufficient for CREB activation (34). In a number of cases, stimuli that induce Ser-133 phosphorylation do not completely parallel CREB-dependent transcription (35), and the preponderance of conserved phosphorylation sites between CREB proteins from different species suggests that other sites may play important roles in regulation. Recent reports have begun to elucidate the role of these sites.

However, it has been difficult in mammalian systems to unambiguously define a regulatory role for these other sites. Ser-129 has been reported to decrease CREB DNA binding when phosphorylated (16), but phosphorylation of Ser-142 has been reported both to increase activity and decrease activity (17, 18, 36). In addition, phosphorylation of the CK sites has been reported to increase DNA binding of CREM (24) but have no effect on binding for mammalian CREB (15). We have utilized *Drosophila* CREB to try to clarify some of these discrepancies.

Similarities between *Drosophila* and mammalian CREB are indicated by the conservation of Ser-133/231 and other sites within the KID (1, 20). However, there seem to be differences in the relative importance and usage of different regulatory pathways in regulating CREB activity. In mammals, most CREB exists with Ser-133 in a dephosphorylated state, and phosphorylation of this site is a crucial step in CREB activation (37). In *Drosophila*, we showed that a large proportion of dCREB2 was already phosphorylated at Ser-231, indicating that regulation by increasing Ser-231 phosphorylation may be less important than regulation by other means. In a second, Ser-231-independent regulatory step that we present here, we demonstrated that dCREB2 was completely unable to bind CRE sites unless first dephosphorylated at sites other than Ser-231. Preliminary results in mammalian extracts suggests that this step is conserved on mammalian CREB for at least a fraction of the detectable protein (data not shown). These results suggest a conserved mechanism of CREB regulation, although in this case the pathway is more tightly regulated in *Drosophila* than in mammals. The tight on/off type regulation of dCREB2 binding allowed us to unambiguously identify the CK sites as important for this effect. In addition, we showed that *Drosophila* CKII was likely to phosphorylate these sites and these sites were likely to exist in a phosphorylated state *in vivo*.

Although we identified four CK sites on dCREB2 that play significant roles in altering DNA binding, it is not absolutely certain that phosphorylation of all four sites is necessary to inhibit binding. Possibly phosphorylation of only a subset of these sites is adequate. Indeed, in our peptide mapping experiments, peptide B, which was phosphorylated by CKI, CKII and the S2 kinase, seems to consist of several closely spaced spots that may reflect differentially phosphorylated species. In addition, although the four sites are clearly important, we cannot dismiss the possibility that there is another site or sites that also contribute to regulating DNA binding. In our peptide mapping experiments, the phosphopeptide pattern for CREB phosphorylated by CKII included many peptides unrelated to the one containing the CK sites. However, purified recombinant CKII is an extremely active enzyme that phosphorylates many sites *in vitro* that are not necessarily phosphorylated *in vivo*. Upon dilution of recombinant CKII, we obtained phosphopeptide patterns that resembled the pattern obtained when CREB was phosphorylated using CKI or S2 kinase. The excess enzymatic activity of recombinant CKII can also be used to explain a possible slight reduction of binding of CREB-CK upon CKII treatment seen in Figs. 5 and 6. We suspect that we identified the relevant sites for the following reasons. In our peptide mapping experiments, peptide B seemed to be the major species phosphorylated by the S2 kinase. Also, in hs-CREB-CK flies, dCREB2 bound extremely well in the absence of phosphatase, and the addition of phosphatase did not significantly increase binding. Finally, in our urea PAGE experiments, CREB-CK ran at a position very similar to dephosphorylated CREB. A slight mobility change between CREB-CK and dephosphorylated CREB is likely to be due to phosphorylation of Ser-133/231, which is normally phosphorylated in *Drosophila*.

Our data on *Drosophila* CREB showed an inhibitory effect of phosphorylation on binding. However, previously published reports on the modulation of CREB DNA binding are inconsistent with our results. Bullock and Habener (15) have shown that phosphorylation of the casein kinase sites has little effect on CREB binding affinity, although they found that having acidic amino acids next to these sites affects CREB binding affinity. De Groot *et al.* (24) report an increase in DNA binding upon phosphorylation of these sites on CREM. It is unclear what factors, such as species differences or experimental conditions, are responsible for these differences. In our studies, we have focused on binding to high affinity symmetric CRE sites originally found in the somatostatin promoter region (12). Binding of CREB to lower affinity non-symmetric sites may be regulated differently (38, 39). Additionally alterations in CKII activity due to cell division cycle and due to redox state may further complicate matters (40, 41).

Our results demonstrated that the CK sites were phospho-

rylated in extracts and are likely to be phosphorylated *in vivo*. The development of phospho- and dephosphospecific antibodies will be needed to demonstrate this unequivocally. Since CREB activity requires both DNA binding and transcriptional activation, it is likely that the active form of CREB is dephosphorylated, or otherwise modified, at the CK sites and phosphorylated at Ser-231. We should note, however, that we have not yet identified this species of CREB in wild type extracts, and it will be of great interest to determine how this form is generated.

Many groups have observed that stimuli able to produce CRE-dependent gene expression consist of only those able to induce a particularly long lasting Ser-133 phosphorylation (34, 35, 42). It is possible that the complex regulation of CREB may explain this requirement for sustained phosphorylation, and this requirement has interesting implications about CREB in memory formation. In *Drosophila*, long term memory formation, which is dCREB2-dependent, requires multiple training trials with rest intervals between the individual trials (spaced training) (43, 44). Equal numbers of massed trials, where there are no interspersed rest intervals, do not produce long term memory. Our current results suggest several intriguing possibilities regarding these temporal requirements for CREB activation. We propose that inactive dCREB2 exists in flies in a state where both the CK sites and Ser-231 are phosphorylated. In one model, activation of dCREB2 may occur through an induction of a phosphatase that dephosphorylates the CK sites specifically. This would result in a relatively simple one-step activation of dCREB2. However, sequence-specific selectivity in serine phosphatases has not been demonstrated in general, suggesting a second possibility. In this second model, activation of dCREB2 results from induction of a phosphatase that dephosphorylates both Ser-231 and the CK sites. Dephosphorylated dCREB2 is able to bind DNA but requires the subsequent activity of a Ser-231 kinase to become fully active. Following activation, CKII or a related kinase can inactivate dCREB2 by phosphorylating the CK sites. Thus, dCREB2 activation would require two sequential steps, a nonspecific dephosphorylation step followed by at least one specific phosphorylation step. The duration of dCREB2 activation would depend on the longevity of Ser-231 phosphorylation and the kinetics of CK site phosphorylation. Thus it is possible that dCREB2 activation would require inhibition of CKII as well as activation of a phosphatase.

This model predicts that continued activation of dCREB2 may be partially counterproductive since induction of the phosphatase may dephosphorylate Ser-231 and other residues whose phosphorylation are required for transcriptional activity. This suggests that maximal induction of dCREB2 may require phasic induction, which might explain the efficacy of spaced training. Studies on the role of the signal transduction pathways that regulate casein kinase activity or the activity of a putative endogenous phosphatase that would dephosphorylate the CK sites will be of great interest in the future.

Acknowledgments—We thank Atsuko Horiuchi for generous help with peptide mapping experiments. We also thank Robert Filipkowski for helpful comments on the manuscript.

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