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A Monomeric Derivative of the Cellular Transcription Factor CREB Functions as a Constitutive Activator

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The mammalian transcriptional activator CREB binds as a dimer to a broad spectrum of inducible promoters. CREB activity is modulated by several signalling agents (protein kinase A [PKA], Ca^{2+}, and transforming growth factor β) and via functional interactions with cell-specific transcription factors. In addition, CREB can activate transcription constitutively and repress the activity of several other transcriptional activators. The mechanisms that allow CREB to act in such a malleable manner and the role that CREB dimerization might play in this are poorly understood. To probe the latter issue, we have created monomeric forms of CREB by fusing CREB to the DNA-binding domain of a protein (B-cell specific activator protein [BSAP]) that binds to DNA as a monomer. Remarkably, monomeric CREB acts as a potent, constitutive activator under conditions in which native CREB is inducible by PKA. Thus, CREB contains constitutive activation regions that are unable to function in native CREB. Two glutamine-rich domains that are important for native, PKA-inducible CREB activity are required for the constitutive activity of monomeric CREB. In contrast, two elements within the kinase-inducible domain of CREB are dispensable for constitutive activity. We discuss our results in relation to inducible and constitutive CREB activity and the potential modes of action of other activators that directly interact with CREB.

The bZIP family of transcription factors bind to DNA as dimers and activate or repress transcription of a variety of mammalian promoters. The bZIP domain is responsible for dimerization and DNA binding and consists of a leucine zipper structure (ZIP) that mediates dimerization and an adjacent basic region (b) that directly contacts DNA (2, 6, 54). The cyclic AMP (cAMP) response element-binding protein (CREB) is one of the best-characterized bZIP proteins. CREB is implicated in a host of biological functions, including neuronal excitation (10), setting of circadian rhythms (20), pituitary proliferation (49), gluconeogenesis (4), and opiate tolerance (23). Accordingly, CREB is activated via phosphorylation induced by several distinct signals (protein kinase A [PKA], Ca^{2+}, and transforming growth factor β) (12, 22, 30, 48) and through interactions with cell-specific transcription factors (4, 40). In addition to acting as an inducible activator, CREB can function as a constitutive activator (36) or as a potent inhibitor of a diverse class of transcriptional activators (35). The molecular mechanisms that allow CREB to act in such a malleable manner are poorly understood.

The role of CREB in cAMP signalling has provided the main focus for studying CREB (34). PKA activates CREB by direct phosphorylation of a single serine (22). In addition to the PKA phosphoacceptor site (Ser-119), several other regions of CREB are required for transcriptional activation (see Fig. 1). The PKA site is part of the kinase-inducible domain (KID) (21, 32), which contains an element termed β (21, 32), an α-helical region (α2), and a protein kinase C (PKC) site (21). In addition to the KID, two glutamine-rich regions (Q1 and Q2) that are similar to constitutive activation domains of other transcription factors (11, 16, 19, 51, 52) are both required for PKA-inducible CREB activity (5, 21).

The mechanism by which phosphorylation activates CREB is not well characterized. The KID itself is able to mediate PKA-dependent transcriptional activation when linked to a heterologous DNA-binding domain (5, 32), although the level of activation is low. Phosphorylation of CREB by PKA is, however, required for CREB to interact with a coactivator protein termed CBP (9), and it is therefore likely that CBP functions (at least in some cases) to allow CREB to interact with other components in the transcription complex. Phosphorylation of the KID also changes the conformation of CREB (21), and this change is accompanied by an increase in DNA-binding activity (43). Whether allosteric effects of phosphorylation increase CREB transcriptional activity remains to be determined.

While the role for dimerization in DNA binding of bZIP proteins is established (3, 29, 33), the effect of dimerization on transcriptional activity per se has not been addressed. We have probed this by fusing CREB to a monomeric DNA-binding protein (B-cell specific activator protein [BSAP]) and determining the transcriptional properties of monomeric CREB. Remarkably, BSAP-CREB acts as a constitutive activator under conditions in which native CREB is inducible by PKA. In addition, two CREB monomers are necessary for synergistic activation but can cooperate only when close together at the promoter. Our results indicate that dimerization may directly influence CREB activity and have implications for the different modes of action of CREB.

MATERIALS AND METHODS

Plasmids and constructions. Reporter plasmids used are as follows. pG1E4TCAT contains a single GAL4 binding site upstream of the adenovirus E4 TATA box as previously described (15). All BSAP reporter constructs were obtained by inserting double-stranded oligonucleotides into the *Sac*I site of pΔERSVCAT (53). The BSAP binding site (GAGAATGGGG CACTGAGGCGTGACCACCGC) corresponds to the high-affinity site in the human CD19 promoter (1). pBS1CAT, pBS2CAT, and pBS4CAT contain one, two, and four BSAP sites, respectively. Single-site reporters have the promoter-proximal (pNMCAT) and promoter-distal (pMNCAT) BSAP

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binding sites mutated. The mutated sequence (TCTCCGTAT TACAGTCTTATGTGAAGGTCT) simply replaces the wild-type sequence in pBS2CAT, thereby maintaining the spacing between the single BSAP site and the TATA box. pBS2+5CAT and pBS2+10CAT have 5 and 10 bp, respectively, inserted between the two BSAP binding sites in pBS2CAT. pBS2DCAT has 10 bp inserted between the proximal BSAP binding site and the TATA box. pGem3/BSAP contains a single BSAP binding site cloned into the *Sac*I site of the pGem3 polylinker.

Activator plasmids and all PCR-derived constructs are as follows. pZ1 (32) expresses GAL4-CREB and contains the GAL4 DNA-binding domain (amino acids 1 to 147) linked to the amino terminus of ΔCREB (amino acids 1 to 261) under control of the simian virus 40 early promoter. All other activators are expressed from the same vector. pBSAP expresses the BSAP DNA-binding domain alone (amino acids 1 to 163). pBSAP/CREB expresses the BSAP DNA-binding domain linked to the amino terminus of ΔCREB (amino acids 1 to 261) lacking the bZIP domain. pBSAP/CREB was obtained by inserting a PCR product (encoding amino acids 1 to 163 of BSAP) between the *Hin*dIII and *Eco*RI sites of pZ1 (thereby replacing the GAL4 DNA-binding domain with BSAP). Nomenclature for mutations within the DLSSD motif is as previously described (21). KID mutants (pD140N, pD140/144N, pϕ, and pS119A) were constructed by site-directed mutagenesis using PCR as follows. Oligonucleotides containing the desired mutations were used to amplify sequences between the *Stu*I site in CREB (amino acid 117) and the *Sac*I site at the C terminus of CREB in pZ1 by PCR. PCR products were then inserted between the *Stu*I and *Sac*I sites of pZ1, and mutations were confirmed by DNA sequencing. Q2 deletions were obtained as follows. pΔ140-182 was constructed by inserting an oligonucleotide encoding CREB amino acids 117 to 139 into *Stu*I-*Acc* 651-digested pZ1. pΔ140-248 was derived from pΔ140-182 by digestion with *Acc* 651 and *Xba*I, filling in of the ends, and religation. pΔ140-200 and pΔ140-221 were obtained by inserting PCR products encoding the required CREB residues between the *Acc* 651 and *Sac*I sites of pΔ140-182. pBSAP/CREM was obtained by three-way ligation of *Eco*RI-*Sac*I-digested pBSAP/CREB, an *Eco*RI-*Afl*III-ended oligonucleotide encoding cAMP response element modulation protein (CREM) amino acids 1 to 14, and an *Afl*III fragment from pCREMα encoding CREM amino acids 15 to 140. pMtC (41) expresses the catalytic subunit of PKA.

Transfections and chloramphenicol acetyltransferase (CAT) assays. Transfection of JEG3 cells was done by calcium phosphate coprecipitation with 5 μg of reporter plasmid, 5 μg of activator plasmid, 5 μg of pMtC where indicated, and pGem3, to total 20 μg of DNA. One-third of the precipitate was added to JEG3 cells at ~50% confluence in 60-mm-diameter dishes. All experiments were performed at least twice with different preparations of expression and reporter plasmids. Quantitation was achieved by removing radioactive spots from the thin-layer chromatography plate and counting in a scintillation counter.

Preparation of nuclear extracts. JEG3 cells were transfected with pBSAP/CREB as described for CAT assays, and extracts were prepared as follows. Cell pellets were resuspended in ~100 μl of lysis buffer consisting of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0), 20 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, pepstatin, chymostatin, trasylol, and TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone]), left on ice for 5 min, and centrifuged for 1 min at top speed in a Microfuge at room temperature. The crude nuclear pellet was extracted with 20 μl of buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, and protease inhibitors) for 15 min, resuspended again, and extracted for a further 15 min. Nuclear debris was removed by centrifugation for 1 min in a Microfuge, and the supernatant (high-salt nuclear extract) was quick-frozen in liquid nitrogen.

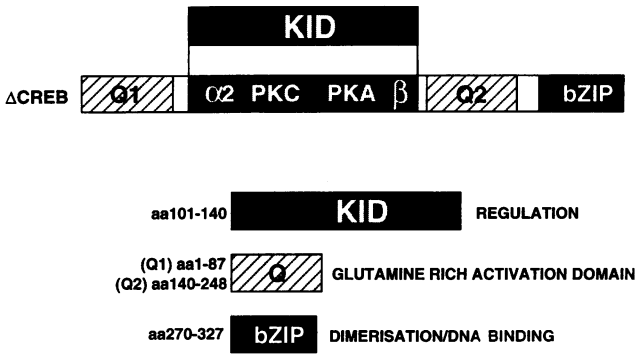

FIG. 1. Functional regions of CREB. The known functional regions of ΔCREB (34) are shown. The carboxy-terminal bZIP domain is necessary and sufficient for dimerization and DNA binding. Several distinct elements (α2, PKC, PKA, and β) constitute the KID and are defined by discrete deletions (21, 22, 32, 34). PKA represents a single PKA phosphoacceptor site (Ser-119), and β contains a critical 5-bp motif, DLSSD (21). The PKA site and β are stringently required for CREB activity. α2 and PKC form part of a region that contains phosphoacceptor sites for several protein kinases, including PKC. This region has only a modest effect on activity (21). Q1 and Q2 represent the two glutamine-rich activation domains of CREB, both of which contribute to transcriptional activity (5, 21). aa, amino acids.

Gel mobility shift assays. Incubation mixtures (15 μl) contained 0.1 to 1 μl of nuclear extract, 1 ng of ^{32}P-labelled DNA probe, 500 ng of poly(dI-dC), and buffer containing 10 mM Tris (pH 7.5), 60 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. Gels were fixed in 50% methanol, dried, and autoradiographed. DNA probe was prepared by reverse transcriptase end labelling of gel-purified *Hin*dIII-*Pvu*II fragment from pGem3/BSAP containing a BSAP binding site cloned into the *Sac*I site of the pGem3 polylinker.

RESULTS

To date, CREB has been functionally analyzed by introducing native dimeric CREB into F9 embryonal carcinoma cells (which lack an endogenous PKA pathway [22]) or by fusing CREB to the DNA-binding domain of the dimeric yeast activator GAL4 (21, 27, 32, 46). The above studies have defined several functional elements that are required for PKA-inducible transcription (Fig. 1; see also Fig. 4A). Moreover, the requirements for activation by GAL4-CREB fusion proteins and native CREB are very similar (21, 32), thus establishing GAL4-CREB as an appropriate model system.

To assay monomeric CREB, we fused CREB to the DNA-binding domain of BSAP, which binds DNA as a monomer (1), and tested the ability of BSAP-CREB to activate reporters containing BSAP binding sites (Fig. 2). Human JEG3 cells were used since CREB is highly responsive to PKA in this cell type (14, 18, 32). A reporter containing a single BSAP binding site (BS1CAT) is not efficiently activated by BSAP-CREB in either the presence or the absence of PKA. In contrast, reporters containing two (BS2CAT) or four (BS4CAT) sites are strongly activated, and two sites are sufficient for maximal

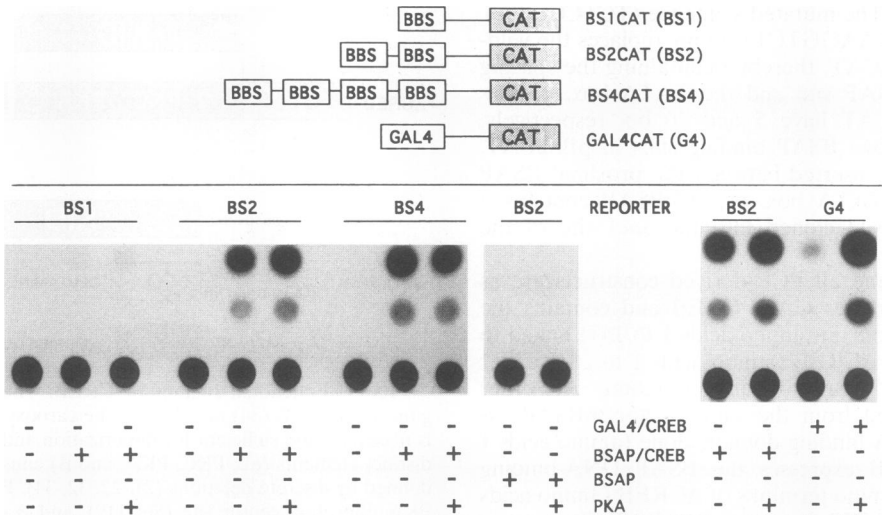

FIG. 2. Activation of transcription by BSAP-CREB fusion proteins in vivo. Vectors expressing BSAP-CREB, GAL4-CREB, and the catalytic subunit of PKA and reporter constructs containing BSAP binding sites linked to CAT were cotransfected into PKA-responsive JEG3 cells, and CAT activity was assayed 40 h later. Representative autoradiograms are shown. pBS1CAT (BS1), pBS2CAT (BS2), and pBS4CAT (BS4) contain the indicated numbers of BSAP binding sites (BBS), respectively, upstream of the Rous sarcoma virus TATA box fused to CAT. The spacing between each BBS in BS2 is 30 bp, placing each site on the same side of the DNA helix. GAL4CAT (G4) contains a single GAL4 binding site upstream of the adenovirus E4 TATA box in pG1E4TCAT as previously described (15). pBSAP expresses the BSAP DNA-binding domain alone (amino acids 1 to 163). pBSAP/CREB expresses the BSAP DNA-binding domain linked to the amino terminus of ΔCREB (amino acids 1 to 261) lacking the bZIP domain. GAL4-CREB is expressed from pZ1 (32) and contains the GAL4 DNA-binding domain (amino acids 1 to 147) linked to the amino terminus of ΔCREB (amino acids 1 to 261). The catalytic subunit of PKA is expressed from pMtC (41).

activity. The BSAP DNA-binding domain alone is unable to activate BS2CAT, demonstrating that activation is dependent on CREB. Remarkably, activation by BSAP-CREB is not stimulated by cotransfection with PKA under conditions in which the ability of GAL4-CREB to activate a reporter containing a single GAL4 binding site is stimulated 40-fold (Fig. 2). In addition, the level of constitutive activation achieved by two BSAP-CREB monomers is comparable to the PKA-induced level achieved by a single GAL4-CREB dimer (Fig. 2). We therefore conclude that CREB contains activation regions that are capable of potent, PKA-independent transcriptional activation and that these regions are functionally repressed in native CREB.

To determine more precisely the promoter requirements for activation by monomeric BSAP-CREB, we tested a series of mutant promoters (Fig. 3). A promoter containing two BSAP binding sites (BS2CAT) is maximally active, but promoters containing a single site have only 4% (NMCAT) and 8% (MNCAT) of the activity of BS2CAT, respectively (Fig. 3B). Thus, there is a large synergistic increase in activity observed for two BSAP binding sites versus one site. The synergistic effect is approximately eightfold, since the activity expected for an additive effect is 12 U and the activity observed is 100 U (Fig. 3D). To determine whether the synergistic effect arises as a result of cooperative binding of BSAP-CREB to adjacent BSAP sites, we examined binding of BSAP-CREB to different DNA probes (Fig. 3C). An oligonucleotide containing two BSAP sites (NN) as arranged in BS2CAT was no more efficient than an oligonucleotide containing one site (MN) in competing for BSAP DNA binding (Fig. 3C), demonstrating a lack of cooperativity. Thus, the requirement for two BSAP binding sites for efficient, synergistic activation reflects a requirement for two BSAP-CREB monomers bound to the promoter. The two BSAP binding sites in BS2CAT are on the same face of the DNA helix. Separating the two sites by half a helical turn (BS2+5CAT, 4% of BS2CAT) or a full helical turn (BS2+10CAT, 8% of BS2CAT) strongly reduces activity (Fig. 3B). Moving both sites 10 bases further away from the TATA box (BS2DCAT) has no effect (Fig. 3B). In summary, we conclude that synergistic activation by BSAP-CREB requires two monomers that must be intimately juxtaposed (less than 30 bp apart) at the promoter.

To determine the regions of CREB required for constitutive transcriptional activity, we examined the effect of a number of previously characterized CREB mutations (5, 21, 32, 46). The results are summarized in Fig. 4A, and representative autoradiograms are shown in Fig. 4B. As expected, mutation of the PKA site (S-to-A mutation at position 119 [S119A]) has no effect on BSAP-CREB activity, thus ruling out the possibility that constitutive BSAP-CREB activity arises because of adventitious phosphorylation of the PKA site in the absence of PKA. Several mutations within the DLSSD motif (D140N, D140/144N, and ϕ) that decrease the activity of native CREB up to 20-fold (21) also have no effect on BSAP-CREB activity (Fig. 4B). Thus, two functionally important elements within the KID are dispensable for BSAP-CREB activity. These results demonstrate that the KID (or at least two elements within the KID) is unable to cooperate in *cis* with other activating elements within monomeric CREB.

CREB contains two glutamine-rich activation regions (Q1 and Q2) (5, 21, 28, 32, 46). The function of Q1 and Q2 is further demonstrated by a protein called CREMα, which is highly homologous to CREB but lacks Q1 and Q2 and acts as a repressor (17, 31). CREMα fails to substitute for CREB when fused to the DNA-binding domain of BSAP (Fig. 4B), pointing to a role for Q1 and Q2 in BSAP-CREB activity. Deletion of the entire Q2 region from BSAP-CREB decreases activity 10-fold, while partial deletions of Q2 decrease activity in a manner that roughly correlates with the number of glutamines removed, as recently shown for native CREB (5). Deletion of Q1 (BΔQ1) reduces the activity of BSAP-CREB fivefold, and Q1 weakly activates by itself when fused to BSAP

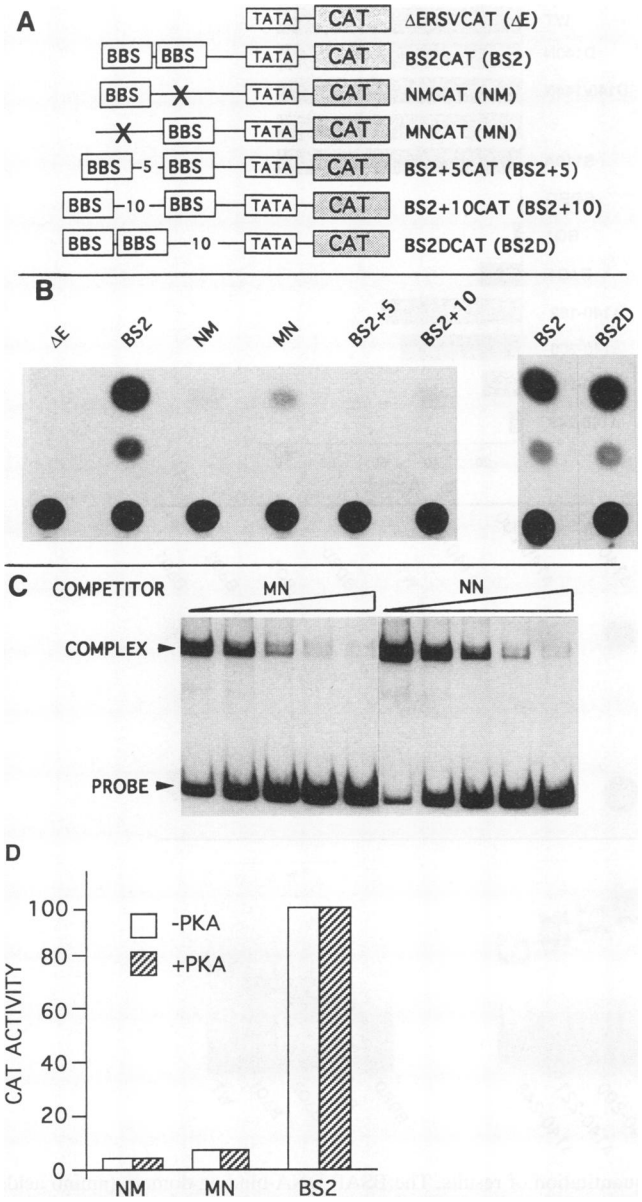

FIG. 3. Determination of promoter requirements for activation by BSAP-CREB. (A) Description of promoter constructs. ΔERSVCAT (53) contains no BSAP binding sites (BBS), and all other constructs are variations of pBS2CAT (BS2) as follows. Single-site reporters have the promoter-proximal (NMCAT) and promoter-distal (MNCAT) BSAP binding sites mutated. The mutated sequence (TCTCCGTAT TACAGTCTTATGTGAAGGTCT) exactly replaces the wild-type sequence in BS2CAT, thereby maintaining the spacing between the single BSAP site and the TATA box. BS2+5CAT and BS2+10CAT have 5 and 10 bp, respectively, inserted between the two BSAP binding sites in BS2CAT. BS2DCAT has 10 bp inserted between the BSAP binding sites and the TATA box. (B) CAT assays. Transfection and CAT assays were performed as described in Materials and Methods, using intact BSAP-CREB protein. Representative autoradiograms are shown. (C) DNA-binding characteristics of BSAP-CREB. Gel mobility shift assays were performed by incubating a ^{32}P-labelled DNA probe containing a single BSAP binding site with nuclear extract containing BSAP-CREB. DNA-protein complexes were resolved on low-ionic-strength neutral gels, and the positions of unbound probe DNA and BSAP-CREB–DNA complexes are indicated on the left. A competition assay was performed in the presence of increasing amounts of unlabelled competitor oligonucleotides of equal length containing one (MN) or two (NN) BSAP binding sites. The amounts of competitor were 10, 20, 40, 80, and 160 ng for MN and 5, 10, 20, 40, and 80 ng for NN. (D) Quantitation of synergistic activation by BSAP-CREB. Reporters (*x* axis) are as described for Fig. 2 and panel A above. The activator is intact BSAP-CREB.

(compare BDBD and BQ1). All of the mutant proteins are expressed and bind to BSAP binding sites at comparable levels (Fig. 4C). Thus, efficient constitutive activation by BSAP-CREB is dependent on two activation domains (Q1 and Q2) that are important for inducible activation by native CREB (5, 21). The involvement of both Q1 and Q2 regions indicates that the requirement for two BSAP-CREB monomers for full constitutive activity does not arise because of partial loss of function created by fusion of CREB to BSAP.

DISCUSSION

Mechanisms of CREB action. The finding that monomeric CREB is a highly efficient constitutive activator demonstrates that CREB has the potential to be utilized as both an inducible and a constitutive activator. This is of physiological significance, in light of the fact that CREB has been shown to be able to activate transcription in vivo independently of signalling pathways that induce phosphorylation (36). In the pancreatic tumor cell line TU6, synergistic activation of the somatostatin promoter by a factor called Isl-1 (which binds adjacent to CREB in the somatostatin promoter) and CREB does not require the PKA phosphoacceptor site of CREB (36). Our findings suggest the possibility that Isl-1 (or an additional factor) could act by direct interaction with CREB so as to perturb CREB structure and allow KID-independent activation. Alternatively, the putative interaction between Isl-1 and CREB may functionally correspond to the direct interaction of CREB with a protein termed CBP (9) that is postulated to function as a PKA-dependent CREB coactivator.

Our findings also have implications for the mechanism by which phosphorylation activates CREB. Efficient, constitutive activation by monomeric CREB indicates that native (dimeric) CREB is in a repressed state such that constitutive activating regions (including Q1 and Q2) are unable to efficiently function. This could result from a steric effect of dimerization or might reflect a specific interaction between CREB monomers. Because activation regions (Q1 and Q2) that are required for PKA-inducible CREB activity are also required for constitutive activity, it seems likely that relief of repression is part of the mechanism that allows CREB to function as an inducible activator. In this event, phosphorylation of CREB would induce a conformational change that unmasks Q1 and Q2 and would facilitate functional interactions with the general transcriptional machinery (16). This suggestion is consistent with the findings that KID phosphorylation does induce a conformational change in CREB (21, 32) and also influences the DNA-binding properties of the bZIP domain (43).

While our experiments suggest an allosteric function for the KID, there is also clear evidence that the KID can play a direct role in transcriptional activation. First, the KID alone has a limited ability to activate when fused to a heterologous DNA-binding domain (5, 32, 46). Second, the KID interacts with the coactivator CBP only when phosphorylated (9). Finally, Brindle et al. recently reported that the KID can act synergistically and in *trans* with the Q2 domain (5). To reconcile the claims that the KID can act both directly and indirectly (our study), it is reasonable to propose that distinct mechanisms for CREB activation are available. This proposal is consistent with the observation that for PKA-inducible promoters containing

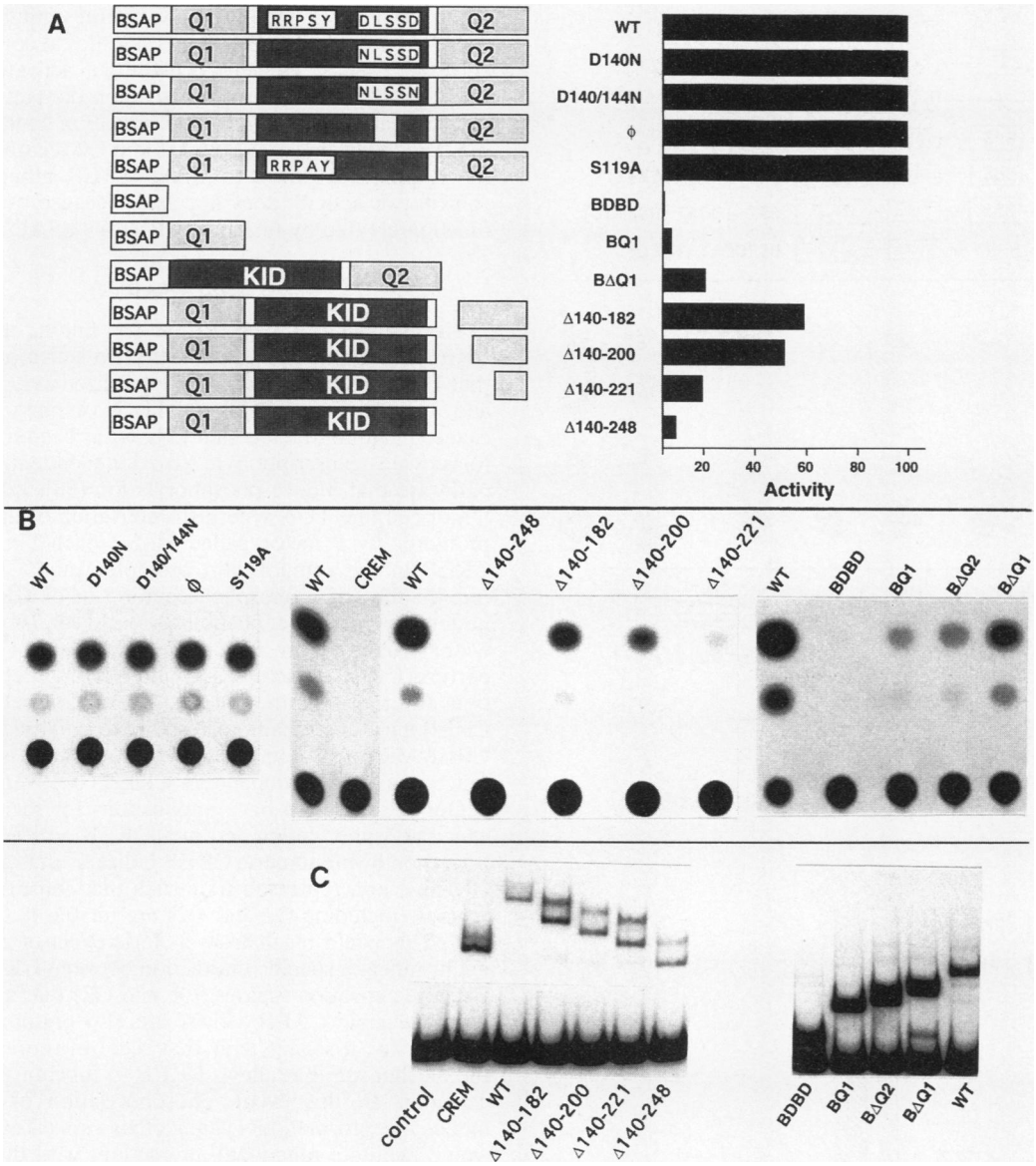

FIG. 4. Mutational analysis of BSAP-CREB. (A) Mutant proteins and quantitation of results. The BSAP DNA-binding domain (amino acids 1 to 163) is at the amino terminus of all fusion proteins. Q1 (amino acids 1 to 87) and Q2 (amino acids 140 to 248) represent the two glutamine-rich activation domains of CREB. The KID (amino acids 101 to 140) includes the PKA phosphoacceptor site (RRPSY) and an element termed β (containing the DLSSD motif). Nomenclature for mutations within the DLSSD motif is as previously described (21). The DNA-binding–dimerization domain (bZIP domain) of CREB resides at the carboxy terminus of CREB (Fig. 1) and is not present in any of the proteins analyzed. For quantitation, activity is shown as percentages of BSAP-CREB wild-type (WT) activity in the absence of PKA. (B) CAT assays. Transfections and CAT assays were performed as described in Materials and Methods, and representative autoradiograms are shown. KID (left panel), Q2 (middle panel), and Q1 (right panel) mutants were analyzed in different experiments, each with a WT control. (C) Expression and DNA binding of mutant BSAP-CREB proteins. Gel mobility shift assays were performed as described in Materials and Methods.

CREB binding sites, there are additional promoter-specific determinants of CREB activity (34, 38, 43). Furthermore, the apparent malleability of CREB provides a basis for understanding how CREB can function in disparate signalling pathways (4, 12, 20, 22, 23, 30, 36, 40, 48, 49) while retaining specificity.

Function of heterodimers. The activity of monomeric CREB suggests constraints on the properties of heterodimeric complexes containing CREB. CREB and two other proteins (CREM and ATF1) form a subfamily of bZIP proteins that can all heterodimerize (24, 26, 39, 42, 47). Several CREM isoforms (CREMα, CREMβ, CREMγ, and S-CREM) act as repressors of cAMP-inducible transcription (13, 17), and these proteins might repress CREB by forming inactive heterodimers. The finding that a single BSAP-CREB monomer has little activity implies that repression through heterodimerization could occur passively because of the lack of activation modules (Q1 and Q2) in the dimerization partner. Because some promoters that are activated by CREB contain only one CREB binding site (34), it is apposite to consider the activation potential of single heterodimers. Considered in this way, a CREB-CREM heterodimer bound to the promoter would be equivalent to a

single BSAP-CREB monomer and would therefore be inactive. Such a mechanism of repression is consistent with the observed lack of specific repressor domains in proteins that act as repressors.

Transcriptional synergy. Our results are of significance to the phenomenon termed synergy, whereby multiple activators synergistically activate transcription via cooperative interactions that do not increase DNA-binding activity (7, 37, 45, 50). The mechanisms of synergy are not well understood, but two features are strongly suggested. First, it is postulated that multiple contacts between activators and their targets in the transcription complex cooperate to facilitate stable complex formation (8, 15, 25). Second, steric constraints and not simply the number of activation domains present appear to be critical for synergistic activation (15, 44). It is intriguing that two BSAP-CREB monomers can synergize only when very closely apposed (<30 bp apart) at the promoter, thereby defining a specific steric requirement for synergy. This observation raises the possibility that dimerization could contribute to synergy by increasing the local concentration of activation domains. Such an effect may be required for efficient coupling of CREB to the general transcription machinery via interaction with the TBP-associated factor TAF_{II} 110 (16). Whatever the mechanistic implications for CREB, the approach that we have employed provides a simple system for the study of other classes of activation domain and synergistic activation in general.

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