Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1210–1213, February 1994
Biochemistry

The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex

(glutamine-rich activator/TATA binding protein-associated factor dTAF110)

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Communicated by Wylie Vale, October 8, 1993

ABSTRACT **cAMP regulates the expression of a number of genes through the protein kinase A-mediated phosphorylation of CREB at Ser-133. The effects of Ser-133 phosphorylation appear to be primarily transmitted through a modulatory kinase-inducible domain in CREB that functions cooperatively with a 120-amino acid glutamine-rich region (Q2) to stimulate transcription. Indeed, the kinase-inducible domain activity alone is not sufficient to sustain a transcriptional response as illustrated by the CREM family of repressors, which contain the kinase-inducible domain but lack the Q2 region. Here we demonstrate that Q2 functions as a potent constitutive activator *in vitro*. The transcription factor TFIID fraction supports transcriptional activation by Q2, although the "TATA" binding protein alone does not, suggesting that other components of the TFIID complex mediate the response to CREB Q2. In fact, Q2 associates with the TATA binding protein-associated factor dTAF$_{II}$110. As the transcriptionally inactive CREM α and β proteins lack sequences in Q2 that are necessary for binding dTAF$_{II}$110, our results suggest that these proteins may repress transcription because they are unable to interact with the basal transcription complex.**

A number of peptide hormones regulate gene expression through stimulatory guanine nucleotide binding protein-coupled receptors that activate the second messenger cAMP. In response to cAMP elevation, the catalytic subunit of cAMP-dependent protein kinase (PKA) dissociates from the regulatory subunit and migrates into the nucleus where it phosphorylates the cAMP-responsive transcription factor CREB at Ser-133 (1). Although the mechanism by which phosphorylation stimulates CREB activity remains elusive, mutagenesis studies have revealed that a 60-amino acid modulatory domain of CREB, termed the kinase-inducible domain (KID), is critical for this effect (2). KID does not act alone to regulate transcription of target genes but rather functions synergistically with an adjacent glutamine-rich region termed Q2 (3, 4) that extends from amino acids 160 to 280 of the CREB protein. This observation is supported by comparison with the recently described CREM repressor proteins (2) that antagonize CREB-dependent transcriptional activation. These factors contain a phosphorylatable KID domain but apparently inhibit transcription by competitive binding to the cAMP response element (CRE).

The assembly of a transcriptionally active complex on RNA polymerase II-dependent templates apparently requires a productive interaction between upstream factors and proteins in the basal transcription complex. In this study, we examine how the Q2 domain in CREB interacts with the general transcription machinery to stimulate transcription. Our results suggest that Q2 associates with at least one component of the TFIID complex and that this association is important for transcriptional activation by CREB.

METHODS

Expression and Purification of Recombinant CREB Polypeptides. Recombinant CREB and CREB-ΔQ2 were expressed in bacteria using pET expression vectors and purified as described (5). DNA fragments encoding Q2 basic-region leucine zipper (bZIP) and bZIP were inserted into pGEX-3X vector (Pharmacia) and expressed in bacteria, and the recombinant proteins were purified on glutathione-Sepharose (Pharmacia) with subsequent elution by factor Xa. For Gal4 fusion proteins (see Fig. 2), DNA fragments encoding amino acids 1–147 of Gal4 and amino acids 160–283 of CREB were inserted into pGEX-KT and expressed in bacteria, and the recombinant proteins were purified on glutathione-Sepharose columns and eluted with thrombin.

***In Vitro* Transcription Assays.** *In vitro* transcription assays and primer-extension analyses were performed as described (5) except that HeLa cell nuclear extracts (120 μg) were used in the place of PC12 nuclear extracts. The majority of the CRE-binding activity was depleted in the extracts by incubation (4°C for 1 h) with anti-CREB antibody 244 and protein A-Sepharose prior to addition to the reaction mixtures. Amounts of recombinant CREB and Gal4–CREB fusion proteins added to the reaction mixtures were based on equivalent levels of binding activity in gel mobility shift assays. The 5×CRE-2.5ΔCAT template, employed in Fig. 1 was constructed by inserting four copies of the somatostatin CRE into the *Bgl* II site at position −71 of 2.5ΔCAT (6). Template DNAs used in Fig. 2*A* were −1.4(Gal4)CAT (6) (250 ng) and pDNAdML (7) (50 ng). −1.4(Gal4)CAT contains 1.4 kb of upstream sequence from the somatostatin promoter in which the central four bases of the CRE were removed and replaced with five Gal4 recognition sites. pDNAdML contains positions −50 to +10 of the adenovirus 2 major late promoter. For assays using fractionated extracts, partially purified TFIIE/F/H/polymerase II and TFIID fractions were prepared as described (8) (through the DE52 step) and optimal concentrations were determined with the minimal adenovirus 2 major late promoter. The TFIIE/F/H/polymerase II fraction contains trace TFIID activity but requires exogenous TFIIB. Recombinant human TFIIB and "TATA" binding protein (TBP) were expressed from bacterial pET vectors and subsequently purified.

TBP-Associated Factor (TAF) 110 Binding Assay. CRE-Sepharose (57 μg of DNA per ml of resin) was synthesized as described (9). *Drosophila* TAF110 mRNA was transcribed from *Xho* I-cut pTβ110 with T7 RNA polymerase and trans-

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Abbreviations: PKA, cAMP-dependent protein kinase; KID, kinase-inducible domain; TBP, TATA binding protein; TAF, TBP-associated factor; CRE, cAMP response element; bZIP, basic-region leucine zipper; DBD, DNA binding domain.

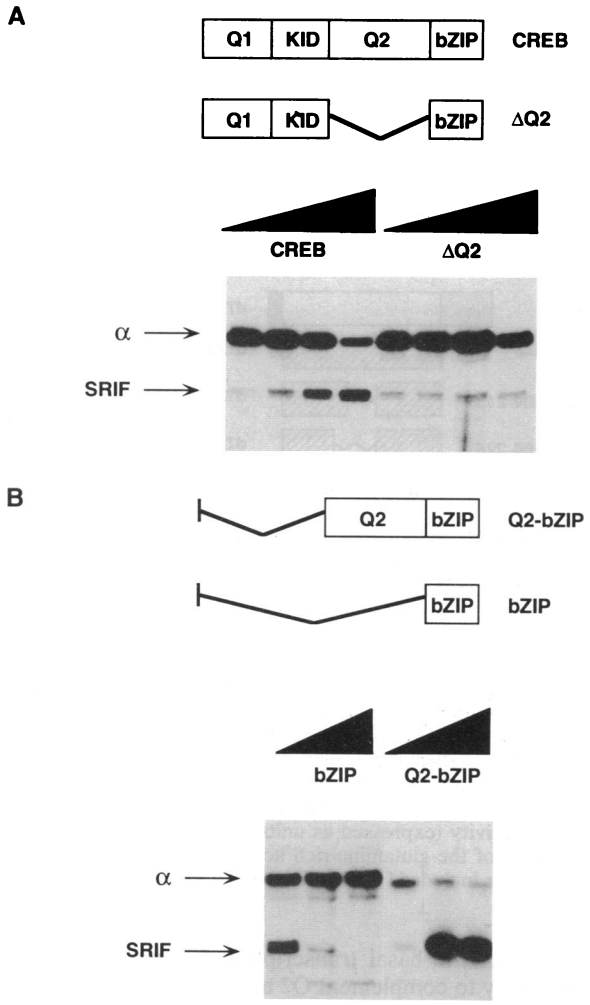

FIG. 1. (*A*) CREB Q2 domain is required for maximal transcriptional activation in *in vitro* assays. The schematic diagram depicts the structural domains of CREB and CREB-ΔQ2 (deletion of amino acids 160–283). Increasing amounts (0, 50, 250, and 1000 ng) of CREB or CREB-ΔQ2 were assayed by *in vitro* transcription assays using the CRE-containing somatostatin promoter–chloramphenicol acetyltransferase fusion gene (SRIF, −71ΔCAT²) and the α-globin gene control template (α) on CREB-immunodepleted extracts. Transcripts were analyzed by primer-extension analysis and autoradiography. (*B*) Activation by Q2 does not require the PKA phosphorylation site. Recombinant CREB deletion mutants bZIP (amino acids 283–341) and Q2–bZIP (amino acids 160–341) were prepared and tested for transcriptional activation (0, 80, and 800 ng) in *in vitro* transcription assays utilizing the somatostatin promoter containing five CREB DNA binding sites (SRIF). Representative assays of each activator are shown.

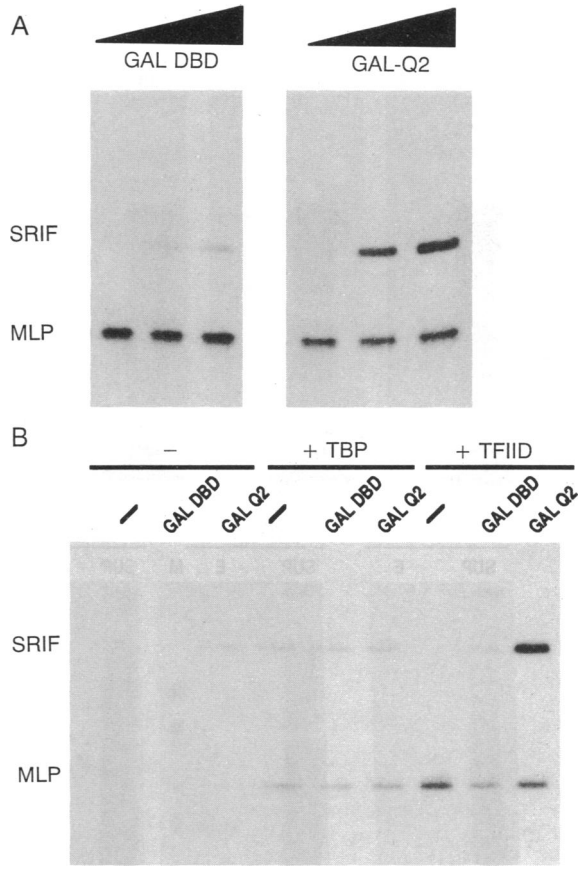

FIG. 2. (*A*) Transcriptional activation by Q2 is independent of other regions of the CREB molecule. Q2 (amino acids 160–283) was expressed as a fusion protein with the DNA binding domain (DBD, amino acids 1–147) of the yeast Gal4 activator. Comparison of the *in vitro* transcriptional activation with increasing amounts (0, 80, and 320 ng) of GAL–DBD alone or GALQ2 was examined utilizing a somatostatin promoter construct containing two Gal4 sites in the place of the CRE. (*B*) TFIID, but not TBP, mediates activation by the Q2 domain. *In vitro* transcription assays were reconstituted with a partially purified TFIIE/F/H/polymerase II fraction and recombinant human TFIIB (−), and with the addition of either recombinant human TBP (+TBP) or a TFIID fraction (+TFIID). GAL–DBD (200 ng) or GAL–Q2 (160 ng) was used. MLP, major late promoter.

lated *in vitro* with rabbit reticulocyte lysate as described (10). Recombinant CREB or CREB-ΔQ2 (50 μg) was incubated at 25°C for 20 min with 50 μl of CRE-Sepharose in 200 μl of PC50+ [20 mM Hepes, pH 7.9/2 mM $MgCl_2$/0.2 mM EDTA/10 mM 2-mercaptoethanol/20% (vol/vol) glycerol/0.05% Nonidet P-40/2 mM dithiothreitol]. After rinsing with 1 ml of PC50+, the resin samples were incubated overnight at 4°C with 200 μl of PC50+ containing ^{35}S-labeled TAF110 in reticulocyte lysate. The CRE-Sepharose samples were washed three times with ice-cold PC50+ and then eluted with PC50+/1 M KCl. The supernatants and eluates were precipitated with methanol/chloroform and electrophoresed on SDS/8% polyacrylamide gels. The gels were subsequently silver stained or soaked in 1 M sodium salicylate, dried, and autoradiographed.

Yeast Two-Hybrid Assay. Expression plasmids were introduced into the yeast strain Y153 and β-galactosidase assays were performed as described (10). The plasmid expressing a fusion of $dTAF_{II}110$ to the DNA binding domain of Gal4 includes the entire coding region of the $dTAF_{II}110$ cDNA; this plasmid and the plasmid expressing the fusion of Sp1B to the acidic activation domain have been described (10). Wild-type CREB Q2 (residues 160–283) and the indicated deletion derivatives (3) were cloned into pGAD1F by PCR. All of the fusion proteins were shown to be expressed in yeast by Western blot analysis using antisera directed against portions of Gal4 (gift of Ivan Sadowski, University of British Columbia, Vancouver).

RESULTS AND DISCUSSION

To characterize factors that functionally interact with CREB, we employed an *in vitro* transcription assay system using purified wild-type or mutant CREB polypeptides (Fig. 1). When added to crude HeLa nuclear extracts, the unphosphorylated CREB protein stimulated transcription from the CRE-containing somatostatin promoter but not a control α-globin template (Fig. 1*A*). In contrast, a mutant CREB

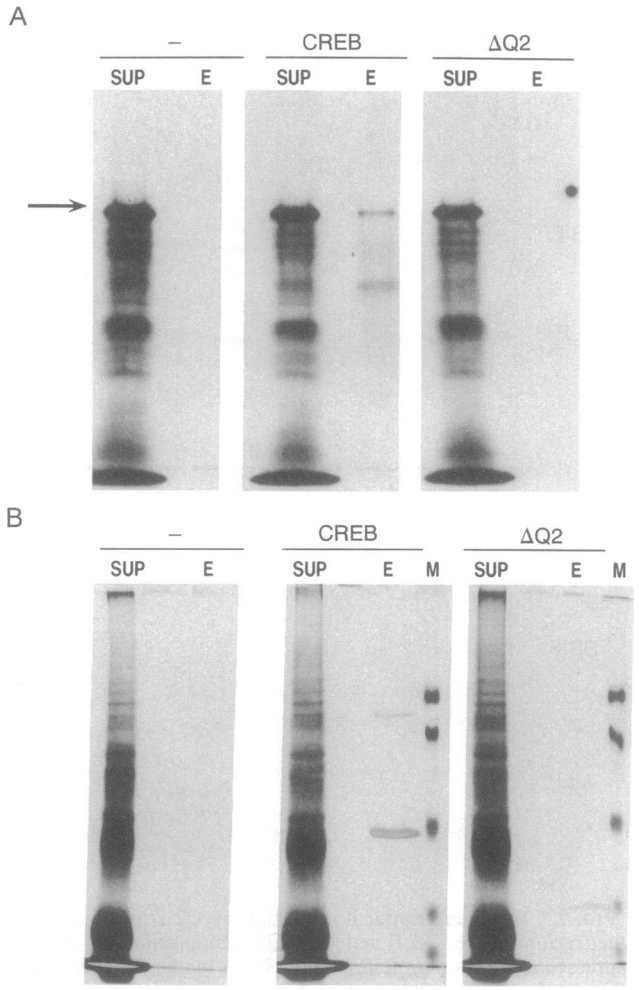

FIG. 3. dTAF$_{II}$110 binds to CREB but not to CREB–ΔQ2. CREB or CREB–ΔQ2 was allowed to bind to CRE-Sepharose prior to incubation with [^{35}S]Met-labeled dTAF110. After washing, the bound complexes were eluted with 1.0 M KCl. The supernatants (lanes SUP) and eluates (lanes E) were then resolved by SDS/PAGE and subjected to autoradiography (*A*) and silver staining (*B*). Arrow points to full-length dTAF$_{II}$110 protein. Molecular mass markers shown in *B* (lanes M) correspond to relative masses of 110, 75, 50, 33, and 27 kDa. Silver-stained bands in eluate (lanes E) lanes correspond to wild-type and ΔQ2 mutant CREB proteins eluted from CRE-affinity column with high salt.

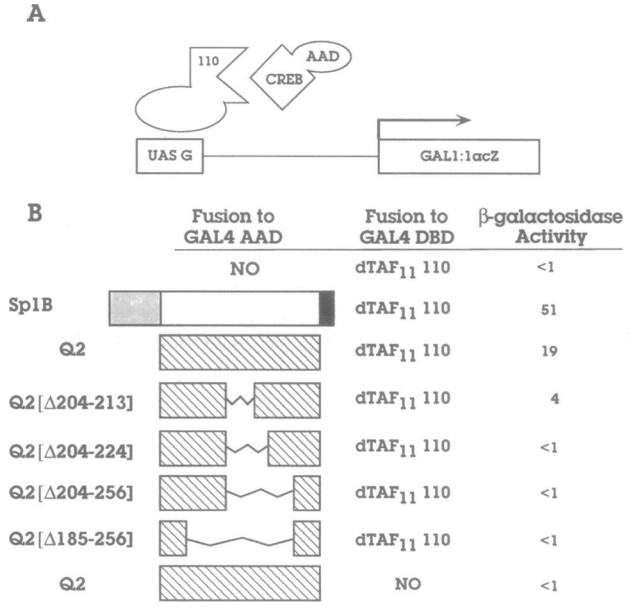

FIG. 4. Interaction between the CREB Q2 domain and dTAF$_{II}$110 requires sequences absent from CREM repressors. Yeast bearing an integrated *GAL1–lacZ* reporter gene were cotransformed with two plasmids: fusions to the acidic activation domain of Gal4 (amino acids 768–881) and fusions to the DBD of Gal4 (amino acids 1–147). (*A*) A schematic drawing of the assay in which interaction between two fusion proteins recruits the acidic activation domain to the promoter bearing Gal4 binding sites. (*B*) The resulting β-galactosidase activity (expressed as units/mg of protein) is shown. The interaction of the glutamine-rich activation domain B of Sp1 with dTAF$_{II}$110 is included for comparison. All assays were performed in triplicate.

polypeptide, resembling the CREM repressor protein ε by its absence of the glutamine-rich Q2 region (ΔQ2), was transcriptionally inactive despite having wild-type DNA binding affinity *in vitro* (data not shown).

To test whether the Q2 domain was sufficient to stimulate somatostatin promoter activity, we purified a recombinant polypeptide containing Q2 and bZIP domains of CREB (Fig. 1*B*). Q2–bZIP strongly stimulated somatostatin transcription whereas bZIP alone was slightly repressive probably due to competition with endogenous CRE binding protein. To avoid this complication, a fusion protein containing the Q2 region attached to the Gal4 DNA binding domain was also tested (Fig. 2*A*). When evaluated on a somatostatin promoter template containing two Gal4 recognition sites, the GAL–Q2 protein stimulated somatostatin promoter activity in a dose-dependent manner, but the Gal4 DNA binding domain alone was minimally active. These results suggest that the Q2 domain functions as a constitutive activator both *in vitro* and *in vivo* (3).

To characterize functional interactions between Q2 and the basal transcription complex, we prepared partially purified fractions of the basal transcription factors and monitored their ability to complement Q2 activity (Fig. 2*B*). Transcription reactions reconstituted with all basal factors except the TFIID fraction showed minimal activity from either the somatostatin or control adenovirus 2 major late promoters. Addition of recombinant TBP restored basal but not GAL–Q2 activator-dependent activity to both templates while the holo-TFIID fraction could fully reconstitute GAL–Q2 transactivation. Within the TFIID fraction, TBP is associated with a number of factors (TAFs) that appear to have coactivator activity (10). These results suggest that some factor in this fraction, in addition to TBP, is required for Q2 function.

Inspection of sequences within Q2 revealed an intriguing resemblance to the hydrophobic and glutamine-rich Sp1 activation domain B. As mutational analysis indicated that this region was important for interaction with dTAF$_{II}$110 (G.G., unpublished data), we tested for similar interactions between Q2 and dTAF$_{II}$110. CREB stimulates transcription of target genes comparably in *Drosophila* and mammalian nuclear extracts (M.M., unpublished observations), thereby permitting us to evaluate interactions between Q2 and the recently cloned *Drosophila* TAFs. Toward this end, we prepared CRE oligonucleotide affinity resins bound by purified CREB or mutant ΔQ2 proteins. We observed that ^{35}S-labeled dTAF$_{II}$110 protein from reticulocyte lysates programmed with dTAF$_{II}$110 RNA could indeed bind to CRE resin containing wild-type CREB, albeit with low efficiency (≈8% by densitometry). But no such binding was observed between dTAF$_{II}$110 protein and ΔQ2 mutant CREB protein, suggesting that the interaction was specific for the Q2 domain (Fig. 3). Indeed, dTAF$_{II}$110 was retained on resins containing GAL–Q2 protein but not the GAL DBD alone (data not shown).

To confirm these *in vitro* assays and begin to map the sequences in Q2 that mediate interaction with $dTAF_{II}110$, we employed the yeast two-hybrid system (11) (Fig. 4). A yeast expression plasmid encoding the Q2 domain fused to the acidic activation domain of Gal4 was cotransformed into a yeast strain with a second vector expressing a $dTAF_{II}110$–Gal4 DBD fusion protein. Interaction of CREBQ2 with $dTAF_{II}110$, monitored by the recruitment of the acidic activation domain onto the promoter of a *GaL1–lacZ* reporter gene, was readily observed. In similar assays, CREB Q2 did not appear to interact with other components of the TFIID complex such as TBP, TAF40, and TAF80.

Based on sequence similarity with Sp1 (amino acids 375–378), we postulated that the putative $dTAF_{II}110$ interaction motif in CREB is located at amino acids 204–208. This motif is absent from the CREM α, β, and ϵ repressors, which lack sequences in Q2 extending from amino acid 183 to 244 of CREB. Similar deletion mutants of CREB (Δ204–214 and Δ204–224) exhibit far lower constitutive and PKA-dependent activity than the wild-type protein in cotransfection assays using a somatostatin–chloramphenicol acetyltransferase reporter vector (3). Consistently, $dTAF_{II}110$ association with these and other inactive CREB deletion mutants was markedly decreased compared to the wild-type Q2 domain (Fig. 4*B*), demonstrating that Q2 activity was indeed correlated with its ability to bind $dTAF_{II}110$.

We have previously observed that PKA-mediated induction of CREB requires two activation domains, KID and Q2, functioning synergistically to stimulate transcription. Our results suggest that Q2 recruits TFIID to cAMP-responsive promoters, probably by interacting with TAF110. Thus the CREM proteins α, β, and ϵ, which lack Q2, may function as repressors because they are unable to form productive interactions with proteins in the basal transcription complex.

The Q2 domain in CREB is fully competent to serve as a strong activator *in vitro*, suggesting that KID may act subsequent to the assembly of a transcription initiation complex. For example, KID may stabilize "open" complex formation by associating with a distinct basal transcription factor and thus promote recycling of RNA polymerase II on activated templates. The use of *in vitro* reconstitution systems should be instrumental in defining the mechanism by which CREB and other phosphorylation-dependent activators interact with the transcriptional machinery to regulate gene transcription.

We thank members of the Montminy lab for helpful discussions, O. Flores for providing reagents, and B. Coyne for technical assistance. This work was supported by National Institutes of Health Grant RO1 GM 37828.

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