Cyclic AMP Response Element-Binding Protein (CREB) and CAAT/Enhancer-Binding Protein β (C/EBPβ) Bind Chimeric DNA Sites with High Affinity†

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ABSTRACT: Basic region leucine zipper (bZIP) proteins are transcription factors that interact selectively with duplex DNA to regulate gene expression. Specifically, the cAMP response element-binding protein (CREB) interacts with the cAMP response element (CRE) DNA site with high affinity, while it binds the CAAT/enhancer-binding protein (CEBP) DNA site with low affinity. Despite the selectivity of CREB for the CRE site, CREB-dependent transcription is observed via chimeric DNA sites with similarities to both CRE and CEBP sites. Because CRE/CEBP and CEBP/CRE chimeric DNA are relevant for transcription regulation but have not been rigorously characterized, quantitative electrophoretic mobility shift assays were used to characterize the binding affinity and specificity of CREB to the sites. In addition to CREB, C/EBPβ was tested because chimeric DNA was shown to stabilize CREB−C/EBPβ heterodimerization. Despite previous work, no CREB−C/EBPβ heterodimer was observed in the presence of chimeric DNA; only CREB and C/EBPβ homodimers were seen. The CREB homodimer bound to the chimeric sites with high affinity, demonstrating that the presence of one CRE half-site is sufficient for high-affinity interaction. A comparison of CREB and C/EBPβ homodimers indicated that they bind the chimeric sites with similar, high affinity. Whereas the CRE and CEBP sites preferentially interact with CREB and C/EBPβ, respectively, the chimeric sites bind CREB and C/EBPβ competitively. Because DNA binding correlates with transcription regulation, the results suggest that gene expression from chimeric sites can be altered by small changes in relative bZIP concentrations or bZIP accessory factors.

Basic region leucine zipper (bZIP)[1] proteins are transcription factors that specifically bind to DNA to regulate transcription (*1*). Because of their fundamental role in gene expression, bZIP proteins are associated with a variety of biological events. Notably, the bZIP protein cyclic AMP response element-binding protein (CREB) is involved in cellular proliferation (*2*), insulin response in the liver (*3*), and brain functions, including circadian rhythms, drug addiction, and memory potentiation (*4*, *5*). In addition, the bZIP protein CAAT/enhancer-binding protein β (C/EBPβ) is associated with memory consolidation (*6*), liver regeneration (*7*), and adipocyte differentiation (*8*). bZIP proteins mediate their biological role by interacting with genomic DNA to recruit coactivators, such as the CREB-binding protein (CBP), to augment transcription (*9*).

The bZIP DNA-binding domain is an unusually simple motif consisting of a continuous α helix that binds to double-stranded DNA via a basic region and dimerizes in the form of a coiled-coil leucine zipper (*10*, *11*). Despite its structural simplicity, the bZIP DNA-binding element distinguishes between a variety of 9−10 base-pair sequences (*12*). Specifically, CREB binds with high affinity to the cyclic AMP response element (CRE) site comprised of two ATGA half-sites in an inverted pair, separated by two G/C base pairs (**ATGA**CG**TCAT**) (*13*). CREB prefers the CRE site to other bZIP DNA sites (*14*), including the CEBP site, which contains two inverted ATTG half-sites (**ATTG**CG**CAAT**) (*14*−*16*). In contrast to CREB, C/EBPβ preferentially interacts with the CEBP site compared with the CRE site but with significantly less specificity; variations in the CEBP sequence are tolerated with C/EBPβ (*17*). Because bZIP DNA-binding specificity correlates with transcriptional activation, the specificity of CREB and C/EBPβ to recognize their consensus sequences is important for their transcription activity in vivo (*18*, *19*).

Given that CREB is highly specific for the CRE site compared to the CEBP site, we were interested to discover that multiple DNA sequences bound by CREB in vivo are chimeras of a CRE and CEBP site, comprised of half-sites from each. For example, the expression of the serotonin

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[1] Abbreviations: bZIP, basic region leucine zipper; CREB, cyclic AMP response element-binding protein; CBP, CREB-binding protein; CRE, cyclic AMP response element; C/EBP, CAAT/enhancer-binding protein; NAT, serotonin *N*-acetyltransferase; HBV, hepatitis B virus; HBV pX, HBV X protein; PPT-I, proprotachykinin I; EMSA, electrophoretic mobility shift assays; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; HTLV-1, human T-cell leukemia virus type 1.

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N-acetyltransferase (NAT) gene, which encodes an enzyme necessary for the conversion of serotonin to melatonin, a hormone linked to regulating sleep cycles (*20*), is controlled by a CREB-dependent CEBP/CRE chimera DNA site (**AT-TG**GG**TCA**G) (*21*, *22*). The hepatitis B virus X protein (HBV pX) gene encodes for the pX protein involved in the development of liver cancer in infected patients and is regulated by a CREB-dependent chimeric CRE/CEBP site (C**TGA**CG**CAA**C) (*23*, *24*). Finally, expression of the proprotachykinin I (PPT-I) gene, which encodes several peptides that have been associated with depression, pain, and other non-neuronal functions (*25*), is controlled by a CREB-dependent chimeric CEBP/CRE site (**ATTG**CG**TCAT**) (*26*). The facts that CRE and CEBP site chimeras bind CREB and regulate gene expression suggest a role for chimeric DNA sites in biological events. In fact, a chimeric CEBP/CRE site (C**TT**ACT**TCA**C) was selected in an in vitro assay as a potent promoter of gene expression, suggesting that chimeric DNA sites are relevant for transcriptional activation (*27*).

Despite the significance of chimeric DNA sites in CREB-regulated gene expression, the binding affinity and specificity of CREB to chimeric DNA sites has been studied only modestly in vitro. CREB was shown to interact with the CRE/CEBP (G**TGA**CG**CAAT**) and CEBP/CRE (G**TTA**CG**T-CA**G) chimeric sites (*28*, *29*), although equilibrium binding affinities were not assessed. Interestingly, a chimeric CEBP/CRE site (G**TTA**CG**TCA**G) bound to CREB and C/EBPβ simultaneously, likely as a heterodimer (*29*). Even though chimeric DNA sites regulate gene expression, the DNA-binding affinity and specificity of bZIP proteins to chimeric DNA sites has not been rigorously characterized.

To assess the binding affinity and specificity of CREB to chimeric CRE/CEBP and CEBP/CRE sites, electrophoretic mobility shift assays (EMSA) were performed and binding isotherms were generated with full-length CREB protein (amino acids 1−341). We also monitored the DNA binding of the bZIP element peptide of C/EBPβ (amino acids 224−285) to probe the possibility of heterodimerization. We found that CREB and C/EBPβ homodimers bound to the chimeric DNA sites with high affinity, indicating that the presence of only one consensus half-site is sufficient to promote binding. Despite previous work indicating that CREB and C/EBPβ heterodimerize on chimeric DNA (*29*), only CREB and C/EBPβ homodimers were observed here. Interestingly, CREB and C/EBPβ homodimers bound to the chimeric DNA sites with comparable affinities. The results suggest that CREB and C/EBPβ bind competitively to chimeric sites in vitro, in contrast with specific binding of CREB and C/EBPβ to the consensus sites.

MATERIALS AND METHODS

Protein Expression and Purification. The full-length protein of CREB (amino acids 1−341) was prepared from pET-15b-CREB/Ser as previously reported (*30*). Briefly, the plasmid pET-15b-CREB/Ser was used to transform BL21-(DE3) *Escherichia coli* cells. Cells were grown in Luria broth supplemented with ampicillin and 4 g/L dextrose at 37 °C with shaking until the optical density was 0.6 absorbance at 595 nm. A final concentration of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression, and the cells were incubated for 3.5 h at 37 °C with shaking. The cells were harvested by centrifugation, washed with 10 mM Tris-HCl buffer at pH 8.0 containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF), and lysed by heating to 75 °C for 10 min. CREB was further purified from the lysate supernatant using preswollen DE53 (diethylamino)ethyl (DEAE) anion-exchange resin (Whatman) and BioRad's BioLogic LP protein purification system at 4 °C. CREB was eluted using a 0−500 mM NaCl gradient in 10 mM Tris-HCl buffer at pH 8.0, 1 mM EDTA, and 1 mM DTT. Fractions containing CREB were concentrated using Amicon YM-30 Centriplus membranes and dialyzed into 25 mM Tris at pH 7.15, 50 mM NaCl, and 1 mM DDT at 4 °C. The identity and purity of CREB was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (see Supporting Information), as previously reported (*30*). The final protein concentration was determined by Bradford (Biorad) and Advanced Protein (Cytoskeleton) Assays. The protein was stored at −80 °C as a 10% glycerol solution to stabilize the full-length protein, as previously described (*30*).

The bZIP element DNA-binding domain of C/EBPβ (amino acids 224−285) was prepared using the plasmid pET-11a-C/EBPβ, as previously described (*31*). Briefly, BL21-(DE3) *E. coli* cells were transformed with pET-11a-C/EBPβ. Cells were grown in Luria broth supplemented with ampicillin at 37 °C with shaking until the optical density was 0.6 absorbance at 595 nm. A final concentration of 0.8 mM of IPTG was added to induce protein expression, and the cells were incubated for 3.5 h at 37 °C with shaking. The cells were harvested by centrifugation and washed with 20 mM Tris at pH 7.5, 1 mM EDTA, and 20 mM β-mercaptoethanol (buffer A), containing 1 mM protease inhibitor cocktail (Calbiochem) and 1 mM PMSF. The cells were lysed by heating to 75 °C for 10 min, and the insoluble fraction was obtained by centrifugation for 20 min at 12 000 rpm. After resuspension of the insoluble lysate pellet in buffer A containing 6 M urea, the urea-soluble supernatant containing C/EBPβ was applied to SP Sepharose ion-exchange resin (Amersham). The column was washed with 10 column volumes of buffer A containing 6 M urea and then with 10 column volumes of buffer A alone to renature the bound C/EBPβ. The renatured C/EBPβ eluted at 0.7 M NaCl using a gradient of 0−1 M NaCl in buffer A. The fractions containing C/EBPβ were concentrated using Amicon YM-3 Centriplus membranes and dialyzed against water. The identity and purity of C/EBPβ was confirmed by SDS−PAGE (see the Supporting Information), as previously reported (*30*). The final protein concentration was determined using the Advanced Protein Assay. The purified peptide was stored at −80 °C as a dry solid.

DNA Purification and ^{32}P-End Labeling. Synthesized DNA (IDT) was purified on a denaturing 12% (19:1 acrylamide/bisacrylamide) 0.8 mm polyacrylamide gel with 8 M urea in 1× TBE buffer (45 mM Tris-base, 45 mM boric acid, and 1 mM EDTA at pH 8.0). The running buffer was also 1× TBE. The gel was prerun for 1 h prior to the addition of the DNA. Each of the oligonucleotide fragments was combined with an equal volume of formamide loading buffer (98% deionized formamide, 10 mM EDTA at pH 8.0, 0.025% xylene cylanol FF, and 0.025% bromophenol blue) and loaded onto the gel without an electric current. The gel

was then run for 130 W h. The largest band (as visualized with UV light) was eluted with 1 mL of water for 12 h at room temperature. Each DNA strand was dialyzed, individually, with water. The DNA concentration was determined by absorbance at 260 nm and stored at −20 °C.

To radioactively label the 5′ end of the DNA, 10 pmol of gel-purified oligonucleotide, kinase buffer (70 mM Tris-HCl at pH 7.6, 10 mM $MgCl_2$, and 5 mM DTT), 10 μCi of [γ-^{32}P]-ATP (New England Nuclear), and 10 units of T4 polynucleotide kinase (Promega) were incubated for 2 h at 37 °C before purification (QIAquick Nucleotide Removal Kit, QIAGEN). To produce double-stranded DNA for the DNA-binding experiments, the complementary strands was added in slight excess to the ^{32}P-end-labeled DNA and annealed by heating to 95 °C for 2 min followed by slow cooling over 2 h.

Quantitative EMSA. The assay was performed as previously described (*32*, *33*). Briefly, binding reactions contained <50 pM duplex ^{32}P-labeled DNA (CRE, CEBP, CRE/CEBP, or CEBP/CRE) and between 3.2 μM and 0.78 nM total protein (CREB or C/EBPβ) in phosphate-buffered saline (PBS)-binding buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 at pH 7.4), containing 1 mM EDTA, 1.25 mM DTT, 0.1% Igepal, 0.5 mg/mL BSA (New England Biolabs), 0.4 μg/mL poly(dI−dC)−poly(dI−dC) (Sigma), and 5% glycerol. Binding reactions were incubated for 30 min at room temperature, and the free and protein-bound DNA were separated using 5% nondenaturing polyacrylamide gel (79:1 acrylamide/bisacrylamide for CREB and 29:1 acrylamide/bisacrylamide for C/EBPβ), prepared in 0.25× TBE. If magnesium was included, a final concentration of 10 mM $MgCl_2$ was added to the binding and gel buffers, as previously described (*16*). The electrophoresis running buffer was 0.25× TBE. Gels were pre-equilibrated for 30 min at 350 V and were maintained at 25 °C by immersion in a circulating, temperature-controlled water bath. The gels were run for 300 V h and dried by vacuum at 80 °C. Dried gels were exposed to a storage phosphor screen and scanned using a Storm 860 PhosphorImager (Molecular Dynamics). The amount of complexed and free DNA was quantified using ImageQuant 5.0 software (Molecular Dynamics). The fraction of protein-bound DNA was plotted versus the total concentration of bZIP protein (CREB or C/EBPβ) and fit to an equation describing a 1:2 stoichiometry of DNA/protein [fraction DNA bound $= 1/(1 + K_{app}/(\text{protein})_{total}^2)$] to determine the equilibrium dissociation constant, K_{app} (*32*). At least five independent binding experiments were averaged, and the standard error is shown with error bars (*34*). ΔG_{obs} values were calculated from the equation: $\Delta G_{obs} = -RT \ln(1/K_{app})$, where $R = 0.001\,987$ kcal mol^{-1} K^{-1} and $T = 298$ K.

RESULTS

To assess the DNA-binding affinity and specificity of bZIP protein binding to chimeric DNA sites, the full-length protein of CREB (amino acids 1−341) and bZIP element peptide of C/EBPβ (amino acids 224−285) were overexpressed and purified (see the Supporting Information). The differing sizes of CREB and CEBPβ were important to facilitate analysis of heterodimerization (*29*). The bZIP element domain of C/EBPβ comprises the C-terminal amino acids of mouse C/EBPβ (*31*), and contains all of the amino acids necessary for DNA binding (*12*). The CREB protein is a rat/human hybrid, where the human bZIP element DNA-binding domain with three cysteine to serine mutations at residues Cys^{300}, Cys^{310}, and Cys^{337} was subcloned into the full-length rat CREB341 gene (*30*). The CREB mutant demonstrated indistinguishable structural and DNA-binding properties compared to wild-type CREB but displayed improved solubility and yield during bacterial purification (*30*, *33*, *35*, *36*).

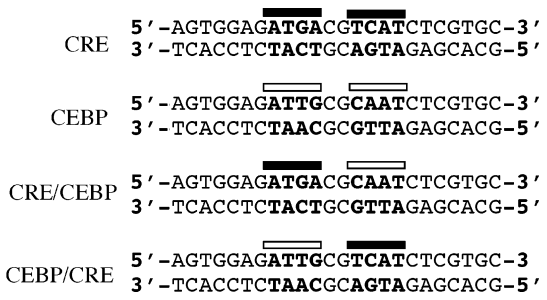

FIGURE 1: Sequences of consensus (CRE and CEBP) and chimeric (CEBP/CRE and CRE/CEBP) DNA sites used in this study are shown. The black boxes above each sequence indicate the CRE half-site, while the white boxes above each sequence indicate the CEBP half-sites.

We also synthesized four double-stranded DNA sites for the binding assays (Figure 1). The two consensus DNA sites (CRE and CEBP) maintain similar half-site spacing but contain different inverted half-sites, as previously reported (*32*, *33*). Chimeric DNA sites (CRE/CEBP and CEBP/CRE) are comprised of half-sites from each consensus DNA site in inverted pairs.

Quantitative EMSA. To assess the DNA-binding affinity and specificity of CREB and C/EBPβ for the four DNA sites, we employed quantitative EMSA, as previously described (*32*, *33*). EMSA were selected to facilitate discrimination of DNA-bound CREB and C/EBPβ homo- and heterodimers (*29*). Initially, EMSA were employed to characterize the DNA-binding specificity and affinity of CREB and C/EBPβ homodimers. Binding isotherms are displayed in Figure 2, and binding curves fit to an equation describing a 2:1 protein/DNA complex are shown in Figure 3. Calculated values for dissociation constants and Gibbs free energy are shown in Table 1.

The CREB−CRE complex exhibited an equilibrium dissociation constant (K_{app}) of $(6.7 \pm 0.4) \times 10^{-18}$ M^2, corresponding to a Gibbs free energy (ΔG_{obs}) of −23.4 kcal mol^{-1}. The EMSA data are consistent with previous solution fluorescent anisotropy experiments showing a half-maximal binding of 2 ± 1 nM for CREB and the CRE site (*30*). CREB binding to the CEBP site was not observed even at 2 μM protein concentration, indicating that CREB prefers the CRE site to the CEBP site by at least 7.9 kcal mol^{-1}. The affinity of CREB for the chimeric DNA sites was reduced compared with the CRE site with a K_{app} of $(7.3 \pm 1.3) \times 10^{-17}$ M^2 for CEBP/CRE ($\Delta G_{obs} = -22.0$ kcal mol^{-1}) and $(8.2 \pm 2.8) \times 10^{-16}$ M^2 for CRE/CEBP ($\Delta G_{obs} = -20.6$ kcal mol^{-1}). CREB prefers the CRE target site to the CRE/CEBP or CEBP/CRE sites with a differential Gibbs free energy ($\Delta\Delta G_{obs}$) of 1.4 and 2.8 kcal mol^{-1}, respectively. However, CREB binds significantly better to the chimeric sites compared to the CEBP consensus site. Despite the clear

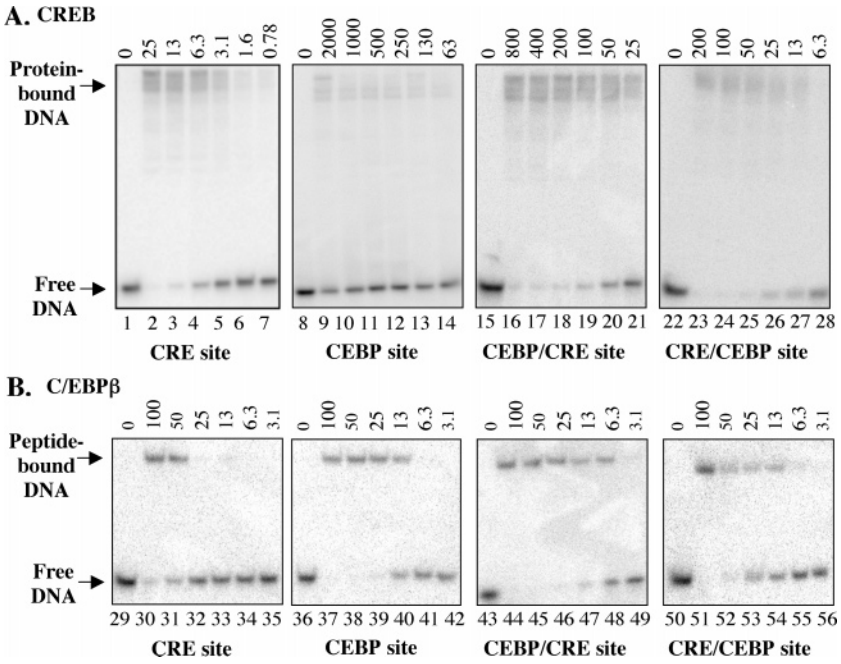

FIGURE 2: Equilibrium binding of the four DNA sites shown in Figure 1 with decreasing concentrations of CREB (A; CRE, lanes 1−7; CEBP, lanes 8−14; CEBP/CRE, lanes 15−21; and CRE/CEBP, lanes 22−28) or C/EBPβ (B; CRE, lanes 29−35; CEBP, lanes 36−42; CEBP/CRE, lanes 43−49; and CRE/CEBP, lanes 50−56). The concentration (in nanomolars) of CREB or C/EBPβ used in each binding reaction is indicated above each lane.

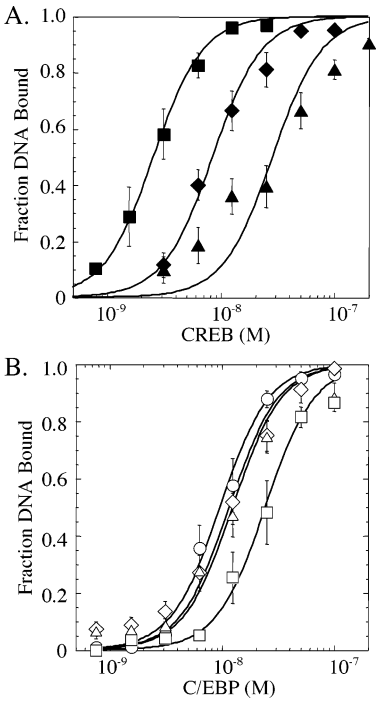

FIGURE 3: Equilibrium binding isotherm for CREB (A) or C/EBPβ (B) bound to the CRE site (■ and □), CEBP site (● and ○), CEBP/CRE site (◆ and ◇), and CRE/CEBP site (▲ and △). Protein concentrations are indicated in Figure 2. CREB did not show appreciable binding to the CEBP site at 2 μM concentration (see Figure 2A). The binding curves were calculated using the equation (fraction bound = $1/(1 + K_{app}/(\text{protein})^2)$, where K_{app} is the adjustable parameter. At least five independent binding experiments were averaged, and the standard error is shown as error bars.

specificity of CREB for the CRE half-site, CREB maintains high affinity for the chimeric sites, which contain one CEBP half-site. Therefore, the presence of only one CRE half-site is sufficient for high-affinity binding of CREB to DNA. The data are consistent with previous work, demonstrating that the GCN4 bZIP protein interacts with high affinity with a DNA site containing only a single half-site (*37*).

The complex of C/EBPβ and the CEBP site demonstrated an apparent dissociation constant (K_{app}) of $(9.5 \pm 1.0) \times 10^{-17}$ M^2, corresponding to a ΔG_{obs} of −21.8 kcal mol^{-1}. The binding affinity of C/EBPβ for the CRE site was reduced, with a K_{app} of $(6.1 \pm 0.7) \times 10^{-16}$ M^2 (ΔG_{obs} = −20.7 kcal mol^{-1}). As expected, C/EBPβ prefers the CEBP site to the CRE site by a $\Delta\Delta G_{obs}$ of 1.1 kcal mol^{-1} (*38*). C/EBPβ bound to the two chimeric DNA sites with similar affinity or a K_{app} of $(1.4 \pm 0.2) \times 10^{-16}$ M^2 for CEBP/CRE (ΔG_{obs} = −21.6 kcal mol^{-1}) and $(1.6 \pm 0.3) \times 10^{-16}$ M^2 for CRE/CEBP (ΔG_{obs} = −21.5 kcal mol^{-1}). These data indicate that C/EBPβ bound the two chimeric sites with only slightly reduced affinity compared to the consensus CEBP site or a $\Delta\Delta G_{obs}$ of 0.2 or 0.3 kcal mol^{-1}. Therefore, the presence of one CRE half-site only modestly influences the affinity of C/EBPβ to DNA. The data are consistent with previous work, indicating that DNA sequence variations are allowed by C/EBPβ (*17*).

Competitive EMSA with CREB and C/EBPβ. Previous work in nuclear extracts indicated that CREB and C/EBPβ interact simultaneously with a chimeric CREB/CRE site (*29*). In that case, CREB−C/EBPβ heterodimerization was used to rationalize the simultaneous interaction with the DNA.

To explore the possibility that CREB and C/EBPβ heterodimerize on chimeric DNA, we performed EMSA with the chimeric DNA sites in the presence of equal concentrations of CREB and C/EBPβ (Figure 4). The full-length CREB protein and the bZIP element peptide of C/EBPβ were useful in theses studies because the presence of a CREB−C/EBPβ heterodimer bound to the DNA would produce a protein−DNA complex of intermediate migration on the gel compared with the CREB or C/EBPβ homodimers. As observed in Figure 4, the CREB and C/EBPβ homodimers

Table 1: DNA-Binding Constants for the CREB and C/EBPβ Homodimers

	CREB			C/EBPβ			CREB C/EBPβ
DNA site	K_{app} (M^2)[a]	ΔG_{obs}[b]	$\Delta\Delta G_{obs}$[c]	K_{app} (M^2)[a]	ΔG_{obs}[b]	$\Delta\Delta G_{obs}$[c]	$\Delta\Delta G$[d]
CRE	$6.7 \pm 0.4 \times 10^{-18}$	−23.4		$6.1 \pm 0.7 \times 10^{-16}$	−20.7	1.1	−2.7
CEBP	$>4.0 \times 10^{-12}$ [e]	< −15.5	>7.9	$9.5 \pm 1.0 \times 10^{-17}$	−21.8		6.3
CEBP/CRE	$7.3 \pm 1.3 \times 10^{-17}$	−22.0	1.4	$1.4 \pm 0.2 \times 10^{-16}$	−21.6	0.2	−0.4
CRE/CEBP	$8.2 \pm 2.8 \times 10^{-16}$	−20.6	2.8	$1.6 \pm 0.3 \times 10^{-16}$	−21.5	0.3	1.0

[a] All values reported represent the mean of at least five determinations, with standard error indicated. [b] ΔG_{obs} is equal to $-RT \ln(1/K_{app})$, where T is 298 K and R is 0.001 987 kcal mol^{-1} K^{-1}. [c] $\Delta\Delta G_{obs}$ is equal to $-RT \ln(1/(K_{app(CRE)}/K_{app(DNA)}))$ for CREB and $-RT \ln(1/(K_{app(CEBP)}/K_{app(DNA)}))$ for C/EBPβ. [d] $\Delta\Delta G$ is equal to $-RT \ln(1/(K_{app(C/EBP\beta)}/K_{app(CREB)}))$ for each DNA site, where all complexes are compared to the CREB−DNA complex. [e] Estimated on the basis of the failure to observe a CREB−CEBP complex at a concentration of 2 μM.

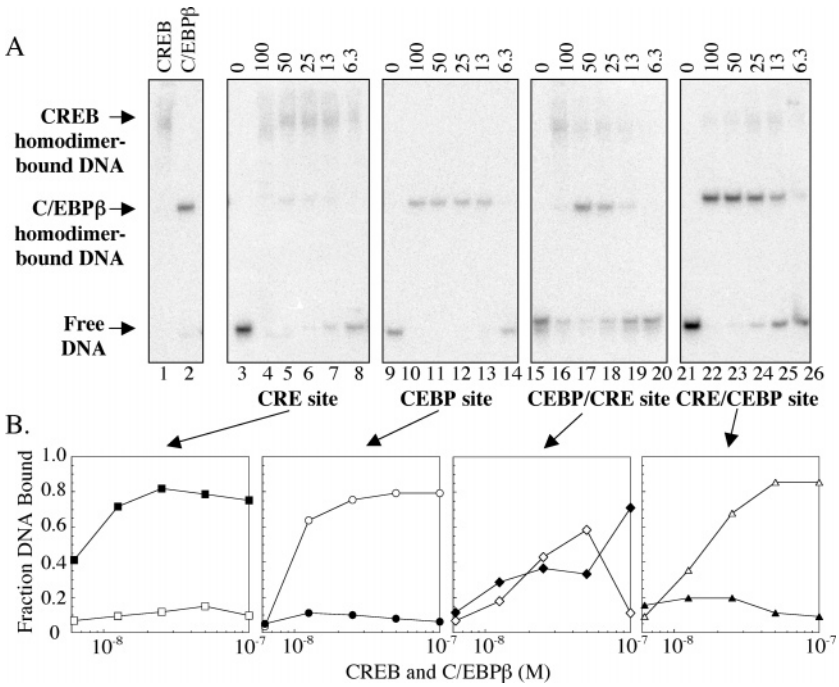

FIGURE 4: (A) Equilibrium binding of 100 nM CREB protein with the CRE site (lane 1), 100 nM C/EBPβ peptide with the CRE site (lane 2), or a decreasing concentration of equal parts CREB and C/EBPβ (lanes 3−26) bound to the four DNA sites shown in Figure 1 (CRE, lanes 3−8; CEBP, lanes 9−14; CEBP/CRE, lanes 15−20; and CRE/CEBP, lanes 21−26). The individual concentrations of CREB and C/EBPβ (in nanomolars) included in the competitive binding reactions are indicated above each lane. For example, 100 indicates that 100 nM CREB and 100 nM C/EBPβ were included in the binding reaction, for a combined total of 200 nM protein. (B) Equilibrium binding isotherm of gels in A for CREB (black) or C/EBPβ (white) bound to the CRE site (■ and □), CEBP site (● and ○), CEBP/CRE site (◆ and ◇), and CRE/CEBP site (▲ and △).

produced observable protein−DNA complexes on the gel. However, no protein−DNA complex of intermediate migration was observed. The data indicate that homodimers of CREB and C/EBPβ preferentially formed stable complexes with the DNA; no heterodimeric complexes were seen.

Although heterodimerization was not observed, the DNA-binding data offer a view of the competitive interaction of CREB and C/EBPβ with DNA. As expected, the CREB homodimer preferentially interacts with the CRE site in the presence of the C/EBPβ peptide (lanes 4−8 in Figure 4A), while the C/EBPβ homodimer preferentially binds to the CEBP site in the presence of the CREB protein (lanes 10−14 in Figure 4A). In contrast, the interactions of CREB and C/EBPβ with the chimeric sites were less selective (lanes 15−26 in Figure 4A); CREB and C/EBPβ homodimers are seen with both sites, indicating that the DNA partially interacts with each homodimer simultaneously. In the case of the CEBP/CRE site, protein−DNA complexes comprised of CREB and C/EBPβ homodimers were observed simultaneously at lower concentrations (lanes 17−18 in Figure 4A), although CREB homodimers were preferentially seen at the highest concentration (lane 16 in Figure 4A). Quantification of the gels (Figure 4B) confirmed the qualitative analysis; only at the highest concentration of protein does the CREB homodimer·DNA complex predominate. With the CRE/CEBP site, while both homodimers are observed simultaneously at all concentrations, a significantly greater fraction of DNA is bound by C/EBPβ (lanes 22−26 in Figure 4A), particularly at higher concentrations. The competitive binding assays confirm the observation with quantitative EMSA that the chimeric sites differ from the consensus sites in their ability to discriminate CREB and C/EBPβ.

Quantitative EMSA in the Presence of $MgCl_2$. Crystallographic data of CREB bound to DNA indicate that a hexahydrated Mg^{2+} ion binds between the CREB homodimer and DNA, making a water-mediated contact with DNA (*11*). In addition, previous papers showed that inclusion of Mg^{2+} salts in the reaction and EMSA gel significantly influenced the binding affinity and specificity of CREB (*11*, *16*, *35*, *39*). To assess the influence of Mg^{2+} on the DNA-binding

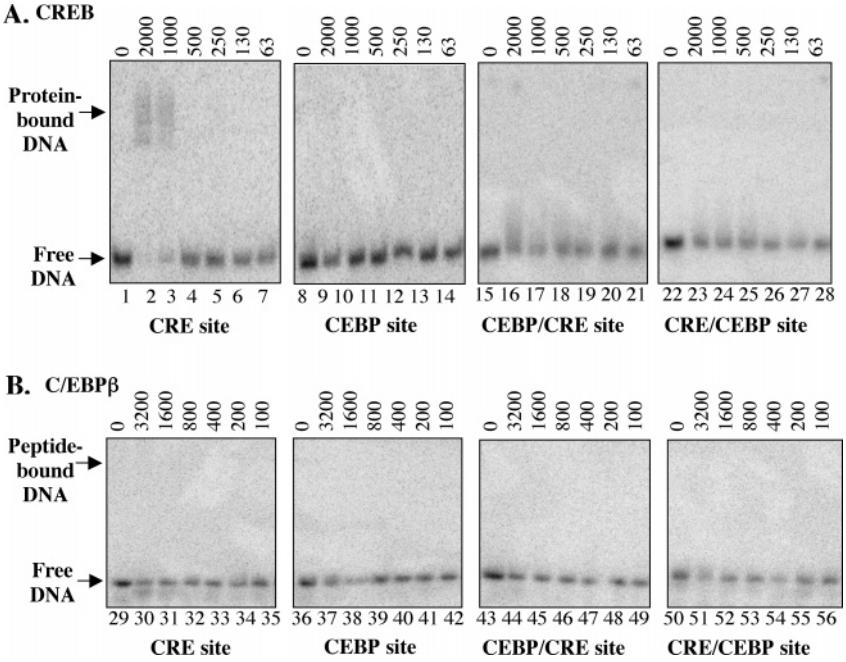

FIGURE 5: Equilibrium binding of a decreasing concentration of CREB bZIP element homodimers (A) to the four DNA sites shown in Figure 1 (CRE, lanes 1−7; CEBP, lanes 8−14; CEBP/CRE, lanes 15−21; and CRE/CEBP, lanes 22−28) or C/EBPβ homodimers (B) to the DNA sites shown in Figure 1 (CRE, lanes 29−35; CEBP, lanes 36−42; CEBP−CRE, lanes 43−49; and CRE/CEBP, lanes 50−56) in the presence of 10 mM $MgCl_2$. $MgCl_2$ was included in the reaction binding buffer and gel buffer but not in the electrophoresis running buffer, as previously reported (*16*). The peptide concentration (in nanomolars) used in each binding reaction is indicated above each lane.

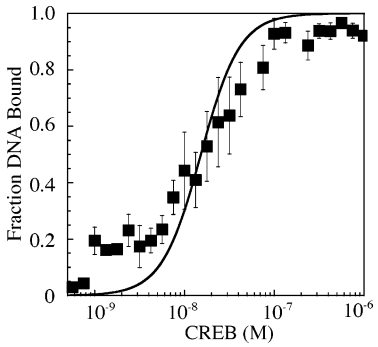

FIGURE 6: Equilibrium binding isotherm for CREB bound to the CRE site in the presence of 10 mM $MgCl_2$. The protein concentrations range from 1000 to 0.56 nM with 0.75 serial dilutions (1000, 750, 560, 420, 320, 240, 130, 100, 75, 42, 32, 24, 18, 13, 10, 7.5, 5.6, 4.2, 3.2, 2.4, 1.8, 1.3, 1.0, 0.75, and 0.56 nM). CREB did not show appreciable binding to the other DNA sites even at 2 μM concentration (see Figure 5A). Please see the caption of Figure 3 for additional details.

affinity and specificity of CREB for the chimeric sites, we performed electrophoretic mobility assays in the presence of $MgCl_2$, as previously described (*16*). Binding isotherms are displayed in Figure 5, and the binding curve is shown in Figure 6.

In the presence of $MgCl_2$, the affinities of C/EBPβ and CREB for DNA were significantly reduced. In fact, the only complex observed was the CREB−CRE complex, with a K_{app} of $(2.3 \pm 0.5) \times 10^{-16}$ M^2 ($\Delta G_{obs} = -21.3$ kcal mol^{-1}). No other complexes with DNA were observed even with 2 μM CREB or 3.2 μM C/EBPβ. The data indicated that the affinity of CREB for the CRE site was reduced by $\Delta\Delta G = 2.1$ kcal mol^{-1}, while the affinity of C/EBPβ for the CEBP site was reduced by at least $\Delta\Delta G = 6.9$ kcal mol^{-1}. The results suggest that $MgCl_2$ has a significant influence on the affinities of CREB and C/EBPβ for DNA.

DISCUSSION

Chimeric DNA comprised of abutted half-sites from CRE and CEBP are found upstream of multiple genes regulated by CREB (*21*−*24*, *26*). Despite the likely significance in gene expression, the binding affinity and specificity of CREB to chimeric DNA sites has not been rigorously explored (*28*, *29*).

We began the assessment of DNA binding by comparing the affinities of CREB and C/EBPβ for the consensus CRE and CEBP sites. The data indicated that CREB discriminates the CRE and CEBP sites to a larger extent than C/EBPβ. CREB prefers the CRE site to the CEBP site by a $\Delta\Delta G = 7.9$ kcal mol^{-1}, while C/EBPβ binds to the CEBP site in preference to the CRE site by $\Delta\Delta G = 1.1$ kcal mol^{-1}. In fact, the CREB protein binds the CRE site with the highest affinity of all combinations tested, $\Delta G_{obs} = -23.4$ kcal mol^{-1}. The data are consistent with previous work, demonstrating the relatively high affinity and specificity of CREB for its CRE consensus site (*30*, *33*, *40*). In addition, the data showed that the CRE and CEBP consensus sites predominantly bind their bZIP subfamily protein. Specifically, the CRE site prefers binding with CREB to a greater extent than C/EBPβ by $\Delta\Delta G = -2.7$ kcal mol^{-1}, while the CEBP site binds with higher affinity to C/EBPβ than CREB by $\Delta\Delta G = 6.3$ kcal mol^{-1}. Consistent with the consensus site selectivity, competitive binding experiments (Figure 4) indicated that CREB preferentially interacts with the CRE site, even in the presence of equal concentrations of C/EBPβ. Likewise, C/EBPβ preferentially binds to the CEBP site, even in the presence of equal concentrations of CREB. Therefore, the data suggest that CREB and C/EBPβ interact preferentially with their subfamily consensus sequences to regulate transcription.

In contrast to data with the consensus CRE and CEBP sites, CREB and C/EBPβ bind to the chimeric sites with

relatively similar affinities. CREB binds the CEBP/CRE site with a slight preference compared with C/EBPβ or $\Delta\Delta G = -0.4$ kcal mol^{-1}. In the case of the CRE/CEBP site, C/EBPβ binds with modestly higher affinity compared to CREB or $\Delta\Delta G = 1.0$ kcal mol^{-1}. Whereas the CRE and CEBP consensus sites predominantly bind their respective subfamily protein ($\Delta\Delta G = 2.7$ and -6.3 kcal mol^{-1}), the chimeric DNA sites bind competitively to CREB and C/EBPβ. Interestingly, dependent upon the half-site positioned at the 3′ end of the binding site, either CREB or C/EBPβ binds with modestly higher affinity. When the CRE half-site is positioned at the 3′ end (CEBP/CRE), CREB binds with slightly higher affinity; when the CEBP site is present at the 3′ end (CRE/CEBP), C/EBPβ binds with modestly higher affinity.

Crystallographic analysis of CREB bound to the CRE site indicated that a Mg^{2+} ion is positioned in the protein−DNA interface of the complex (*11*). In addition, in vitro binding experiments demonstrated that inclusion of 10 mM $MgCl_2$ in the binding reactions resulted in a significant alteration in protein−DNA complex stability (*11*, *16*, *35*, *39*). Specifically, several studies noted that 10 mM $MgCl_2$ increases CREB binding to the CRE site by 2−25-fold (*11*, *35*, *39*), while the most recent work reported that the presence of 10 mM $MgCl_2$ decreases the binding of CREB to the CRE site by 3-fold (*16*). Because unbound Mg^{2+} concentrations are known to fluctuate in vivo between 0.5 and 2 mM, previous data suggest that Mg^{2+} may influence DNA binding and transcription regulation (*16*).

Because of the likely role of Mg^{2+} in DNA affinity and selectivity, we performed quantitative EMSA in the presence of $MgCl_2$. As expected, 10 mM $MgCl_2$ significantly influences the DNA-binding affinity of CREB and C/EBPβ. In this case, the binding affinity of CREB for the CRE site is reduced by 15-fold or $\Delta\Delta G = 2.1$ kcal mol^{-1}. Because no DNA complexes were observed even at 2 μM protein concentration, the binding selectivity of the CREB−CRE complex compared to all other protein−DNA complexes tested is greater than $\Delta\Delta G = 5.8$ kcal mol^{-1}. The data here are consistent with recent work demonstrating that 10 mM $MgCl_2$ decreases the binding of CREB to the CRE site (*16*) but contrary to previous work showing enhanced CREB−CRE stability in the presence of 10 mM $MgCl_2$ (*11*, *35*, *39*). A possible explanation for the observed differences is the varying concentrations of salt included in the buffers in these papers; however, no correlation in results exists between the studies using 50 mM NaCl (*11*, *39*) and those using 150 mM NaCl (*16*, *35*), suggesting that salt cannot explain the disparities. Similar to the salt concentration, the discrepancy also does not correlate with the method of analysis, EMSA (*16*, *35*) or fluorescence anisotropy (*11*, *39*). Another difference is the use of double-stranded hairpin loops in two of the papers demonstrating increased CREB−CRE affinity in the presence of $MgCl_2$ (*11*, *39*), instead of linear sequences (*16*, *35*). Perhaps the flanking regions of DNA significantly influence the DNA-binding affinities of CREB and CEBPβ in the presence of $MgCl_2$. Despite the differences, all papers consistently demonstrate that $MgCl_2$ significantly influences the CREB−CRE complex stability.

Previous work documented that a CREB−C/EBPβ complex binds to chimeric DNA (*29*, *41*). In contrast, multiple studies in the absence of DNA indicated that CREB and C/EBPβ do not form stable heterodimers via their bZIP element (*42*, *43*). The combined data suggest that DNA has the potential to stabilize bZIP element-mediated heterodimerization. To test the possibility that DNA and particularly chimeric DNA has the ability to stabilize CREB−C/EBPβ heterodimerizers, we incubated full-length CREB protein and the bZIP element peptide of C/EBPβ with the consensus CRE and CEBP sites and the chimeric CRE/CEBP and CEBP/CRE sites. In contrast to previous studies indicating that CREB and C/EBPβ heterodimerize in the presence of a CEBP/CRE chimeric site (*29*), no evidence of CREB−C/EBPβ heterodimers was observed with any DNA site tested; homodimers formed preferentially in all cases. One possible reason for the discrepancy is the use of different DNA sites; previous work involved a minimal CEBP/CRE site (G**T-TA**CG**TCA**G) compared to studies performed here (**AT-TG**CG**TCAT**). In addition, the DNA sequences flanking the CEBP/CRE sites were variable. The discrepancy suggests that the role of base pairs at the 5′ or 3′ termini or the flanking regions of the DNA may influence the stability of protein−DNA complexes. Another possible explanation is the identity of the CREB and C/EBPβ proteins used. The proteins were prepared using reticulocyte lysates in previous work (*29*) and in bacteria in the present work. It is possible that protein post-translational modification or associated proteins only available using eukaryotic expression systems influence heterodimerization. Consistent with this possibility, CREB and C/EBPβ are known to interact in a phosphorylation-dependent manner (*44*). In fact, the phosphorylation-dependent CREB−C/EBPβ complex is attributed to interactions with the CREB activation domain, Q1, as well as the leucine zipper (*44*). Therefore, a third explanation for the variability in CREB−C/EBPβ heterodimerization is cooperative interactions between areas of the proteins outside of the bZIP element. In fact, CREB−C/EBPβ heterodimerization was observed previously using the full-length CREB and C/EBPβ proteins (*29*). In this scenario, it is possible that the bZIP element peptide alone is not sufficient to mediate heterodimerization. Because heterodimerization propensities likely influence bZIP-mediated transcriptional regulation, dissecting DNA-dependent from DNA-independent bZIP heterodimerization will enlighten the role of bZIP proteins in gene expression.

While binding assays in the presence of CREB and C/EBPβ were not useful for validating bZIP heterodimerization, they were helpful by providing insights into the competitive interactions of CREB and C/EBPβ homodimers with the chimeric DNA sites. Consistent with quantitative EMSA, the competitive binding assays revealed that chimeric DNA sites bind CREB and C/EBPβ competitively; in both cases, the homodimeric CREB−DNA and C/EBPβ−DNA complexes are observed simultaneously (Figure 4). Interestingly, a greater fraction of the CEBP/CRE chimeric site binds CREB at high concentrations, consistent with the slight selectivity of CREB for the CEBP/CRE site observed in the quantitative EMSA analysis ($\Delta\Delta G = -0.4$ kcal mol^{-1}). In addition, a greater proportion of the CRE/CEBP chimeric site binds C/EBPβ, consistent with quantitative EMSA data, demonstrating that C/EBPβ binds the CRE/CEBP site with modest selectivity ($\Delta\Delta G = 1.0$ kcal mol^{-1}). In total, the competition data provide corroborating evidence that CREB and C/EBPβ homodimers bind the chimeric sites with

comparable affinity in vitro, suggesting that competitive binding of CREB and C/EBPβ to chimeric sites occurs in vivo to regulate gene expression.

The fact that CREB and C/EBPβ interact with chimeric DNA sites with comparable affinities has implications for transcription. The data are consistent with a model where C/EBPβ contributes to transcription regulation from chimeric DNA sites by competing for CREB binding. According to this model, the relative in vivo concentrations of CREB and C/EBPβ ultimately dictate which protein is binding and regulating transcription. The proposed model is distinct from the scenario with CRE or CEBP consensus sites, where each site preferentially interacts with specific subfamily members to regulate transcription. Consistent with the proposed model, chimeric DNA comprised of half-sites from the CRE and PAR sites were shown to interact with members of the PAR bZIP subfamily with high affinity, suggesting that competitive interactions between CREB and PAR bZIP proteins regulate transcription from CRE and PAR chimeric sites (*45*).

The proposed model also suggests that minor alterations in the DNA-binding ability of CREB or C/EBPβ can profoundly influence the fraction of chimeric DNA-bound CREB or C/EBPβ. Previous work indicated that bZIP accessory factors influence the DNA-binding affinities and specificities of bZIP proteins. For example, the HBV pX perturbs the binding affinity and specificity of CREB for the CRE site (*18*, *23*, *24*). In addition, the Tax protein from the human T-cell leukemia virus type 1 (HTLV-1) influences the DNA-binding ability of CREB (*46*, *47*). The data presented here suggest that viral accessory factors alter gene expression by perturbing the competitive binding of bZIP proteins, such as CREB and C/EBPβ, to chimeric DNA enhancer sites. Interestingly, the DNA enhancer sequence controlling the expression of HBV pX is a chimeric CRE/CEBP site (*23*, *24*). Therefore, it is attractive to speculate that HBV pX perturbs the competition between CREB and C/EBPβ for binding to the chimeric DNA site, ultimately resulting in changes in its own gene expression. Because HBV pX is associated with heptocellular carcinoma (*48*), deciphering the role of HBV pX in dictating DNA-binding specificity and gene expression from chimeric sites will aid in the characterization of HBV-induced liver cancer.

In addition to the HBV pX enhancer sequence, several other chimeric CRE and CEBP sites are associated with CREB-dependent transcription regulation. For example, CRE and CEBP site chimeras direct the transcription of various neuronal genes, including the NAT gene, which encodes an enzyme necessary for the conversion of serotonin to melatonin (*20*), and the PPT-I gene, which encodes several peptides associated with depression, pain, and other non-neuronal functions (*25*). With a role in the transcription of such diverse genes, characterizing the transcriptional regulatory functions of chimeric bZIP DNA sites will enhance the understanding of bZIP protein-mediated gene expression.

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SUPPORTING INFORMATION AVAILABLE

Figure S1, denaturing SDS−PAGE displaying the purification of CREB (A) and C/EBPβ (B) using Coomassie staining. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Hurst, H. C. (1994) Transcription factors 1: bZIP proteins, *Protein Profile 1*, 123−168.
2. Della Fazia, M. A., Servillo, G., and Sassone-Corsi, P. (1997) Cyclic AMP signalling and cellular proliferation: Regulation of CREB and CREM, *FEBS Lett. 410*, 22−24.
3. Klemm, D. J., Roesler, W. J., Boras, T., Colton, L. A., Felder, K., and Reusch, J. E. B. (1998) Insulin stimulates cAMP-response element binding protein activity in HepG2 and 3T3-L1 cell lines, *J. Biol. Chem. 273*, 917−923.
4. Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S., and Greenberg, M. E. (1993) Regulation of CREB phosphorylation on the suprachiasmatic nucleus by light and a circadian clock, *Science 260*, 238−241.
5. Maldonado, R., Blendy, J. A., Tzavara, E., Gass, P., Roques, B. P., Hanoune, J., and Schütz, G. (1996) Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB, *Science 273*, 657−659.
6. Taubenfeld, S. M., Milekic, M. H., Monti, B., and Alberini, C. M. (2001) The consolidation of new but not reactivated memory requires hippocampal C/EBPβ, *Nat. Neurosci. 4*, 813−818.
7. Luedde, T., Duderstadt, M., Streetz, K. L., Tacke, F., Kubicka, S., Manns, M. P., and Trautwein, C. (2004) C/EBP β isoforms LIP and LAP modulate progression of the cell cycle in the regenerating mouse liver, *Hepatology 40*, 356−365.
8. Tang, Q.-Q., Gronborg, M., Huang, H., Kim, J.-W., Otto, T. C., Pandey, A., and Lane, M. D. (2005) Sequential phosphorylation of CCAAT enhancer-binding protein β by MAPK and glycogen synthase kinase 3β is required for adipogenesis, *Proc. Natl. Acad. Sci. U.S.A. 102*, 9766−9771.
9. Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D., and Montminy, M. (1997) Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors, *Gene Dev. 11*, 738−747.
10. McKnight, S. L. (1991) Molecular zippers in gene regulation, *Sci. Am. 264*, 54−64.
11. Schumacher, M. A., Goodman, R. H., and Brennan, R. G. (2000) The structure of a CREB bZIP−somatostatin CRE complex reveals the basis for selective dimerization and divalent cation-enhanced DNA binding, *J. Biol. Chem. 275*, 35242−35247.
12. Hope, I., and Struhl, K. (1986) Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast, *Cell 46*, 885−894.
13. Hai, T., Liu, F., Allegretto, E. A., Karin, M., and Green, M. R. (1988) A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1, *Genes Dev. 2*, 1216−1226.
14. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) Isolation of a recombinant copy of the gene encoding C/EBP, *Genes Dev. 2*, 786−800.
15. Agre, P., Johnson, P., and McKnight, S. (1989) Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP, *Science 246*, 922−926.
16. Moll, J. R., Acharya, A., Gal, J., Mir, A. A., and Vinson, C. (2002) Magnesium is required for specific DNA binding of the CREB B-ZIP domain, *Nucleic Acids Res. 30*, 1240−1246.
17. Falvey, E., Marcacci, L., and Schibler, U. (1996) DNA-binding specificity of PAR and C/EBP leucine zipper proteins: A single amino acid substitution in the C/EBP DNA-binding domain confers PAR-like specificity to C/EBP, *Biol. Chem. 377*, 797−809.
18. Pflum, M. K. H., Schneider, T., Hall, D., and Schepartz, A. (2001) Hepatitis B virus X protein activates transcription by bypassing CREB phosphorylation, not by stabilizing bZIP−DNA complexes, *Biochemistry 40*, 693−703.
19. Thiel, G., Al Sarraj, J., Vinson, C., Stefano, L., and Bach, K. (2005) Role of basic region leucine zipper transcription factors cyclic AMP response element binding protein (CREB), CREB2, activating transcription factor 2 and CAAT/enhancer binding protein in

cyclic AMP response element-mediated transcription, *J. Neurochem. 92*, 321−336.
20. Borjigin, J., Li, X., and Snyder, S. H. (1999) The pineal gland and melatonin: Molecular and pharmacologic regulation, *Annu. Rev. Pharmacol. Toxicol. 39*, 53−65.
21. Baler, R., Covington, S., and Klein, D. C. (1997) The rat arylalkylamine *N*-acetyltransferase gene promoter, *J. Biol. Chem. 272*, 6979−6985.
22. Burke, Z., Wells, T., Carter, D., Klein, D., and Baler, R. (1999) Genetic targeting: The serotonin *N*-acetyltransferase promoter imparts circadian expression selectively in the pineal gland and retina of transgenic rats, *J. Neurochem. 73*, 1343−1349.
23. Maguire, H. F., Hoeffler, J. P., and Siddiqui, A. (1991) HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein−protein interactions, *Science 252*, 842−844.
24. Williams, J. S., and Andrisani, O. M. (1995) The hepatitis B virus X protein targets the basic region-leucine zipper domain of CREB, *Proc. Natl. Acad. Sci. U.S.A. 92*, 3819−3923.
25. Kramer, M. S., Cutler, N., Feighner, J., Shrivastava, R., Carman, J., Sramek, J. J., Reines, S. A., Liu, G., Snavely, D., Wyatt-Knowles, E., Hale, J. J., Mills, S. G., MacCoss, M., Swain, C. J., Harrison, T., Hill, R. G., Hefti, F., Scolnick, E. M., Cascieri, M. A., Chicchi, G. G., Sadowski, S., Williams, A. R., Hewson, L., Smith, D., Carlson, E. J., Hargreaves, R. J., and Rupniak, N. M. J. (1998) Distinct mechanism for antidepressant activity by blockade of central substance P receptors, *Science 281*, 1640−1645.
26. Qian, J., Yehia, G., Molina, C. A., Fernandes, A., Donnelly, R. J., Anjaria, D. J., Gascon, P., and Rameshwar, P. (2001) Cloning of human preprotachykinin-I promoter and the role of cyclic adenosine 5′-monophosphate response elements in its expression by IL-1 and stem cell factors, *J. Immunol. 166*, 2553−2561.
27. Edelman, G. M., Meech, R., Owens, G. C., and Jones, F. S. (2000) Synthetic promoter elements obtained by nucleotide sequence variation and selection for activity, *Proc. Natl. Acad. Sci. U.S.A. 97*, 3038−3043.
28. Shuman, J. D., Cheong, J., and Coligan, J. E. (1997) ATF-2 and C/EBPα can form a heterodimeric DNA binding complex in vitro: Functional implications for transcriptional regulation, *J. Biol. Chem 272*, 12793−12800.
29. Tsukada, J., Saito, K., Waterman, W. R., Webb, A. C., and Auron, P. E. (1994) Transcription factors NF-IL6 and CREB recognize a common essential site in the fuman prointerleukin 1β gene, *Mol. Cell. Biol. 14*, 7285−7297.
30. Richards, J. P., Bachinger, H. P., Goodman, R. H., and Brennan, R. G. (1996) Analysis of the structural properties of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB, *J. Biol. Chem. 271*, 13716−13723.
31. Podust, L. M., Krezel, A. M., and Kim, Y. (2001) Crystal structure of the CCAAT box/enhancer-binding protein β activating transcription factor-4 basic region leucine zipper heterodimer in the absence of DNA, *J. Biol. Chem. 276*, 505−513.
32. Metallo, S. J., and Schepartz, A. (1994) Distribution of labor among CRE−BP1 bZIP segments in the control of DNA affinity and specificity, *Chem. Biol. 1*, 143−151.
33. Hamm, M. K., and Schepartz, A. (1995) Studies on the formation of DNA−protein interfaces: DNA specificity and straightening by CREB, *Biol. Med. Chem. Lett. 5*, 1621−1626.
34. Devore, J. L. (1987) *Probability and Statistics for Engineering and the Sciences*, Brooks/Cole, Monterey, CA.
35. Montclare, J. K., Sloan, L. S., and Schepartz, A. (2001) Electrostatic control of CRE/AP-1 specificity by bZIP proteins, *Nucleic Acids Res. 29*, 3311−3319.
36. Santiago-Rivera, Z. I., Williams, J. S., Gorenstein, D. G., and Andrisani, O. M. (1993) Bacterial expression and characterization of the CREB bZIP module: Circular dichroism and 2D-^{1}H NMR studies, *Protein Sci. 2*, 1461−1471.
37. Hollenbeck, J. J., and Oakley, M. G. (2000) GCN4 binds with high affinity to DNA sequences containing a single consensus half-site, *Biochemistry 39*, 6380−6389.
38. Bakker, O., and Parker, M. G. (1991) CAAT enhancer binding protein is able to bind to ATF CRE elements, *Nucleic Acids Res. 19*, 1213−1217.
39. Craig, J. C., Schumacher, M. A., Mansoor, S. E., Farrens, D. L., Brennan, R. G., and Goodman, R. H. (2001) Consensus and variant cAMP-regulated enhancers have distinct CREB-binding properties, *J. Biol. Chem. 276*, 11719−11728.
40. Williams, J. S., Dixon, J. E., and Andrisani, O. M. (1993) Binding constant determination studies utilizing recombinant δCREB protein, *DNA Cell Biol. 12*, 183−190.
41. Dumais, N., Bounou, S., Olivier, M., and Tremblay, M. J. (2002) Prostaglandin E2-mediated activation of HIV-1 long terminal repeat transcription in human T cells necessitates CCAAT/enhancer binding protein (C/EBP) binding sites in addition to cooperative interactions between C/EBPβ and cyclic adenosine 5′-monophosphate response element binding protein, *J. Immunol. 168*, 274−282.
42. Newman, J. R. S., and Keating, A. E. (2003) Comprehensive identification of human bZIP interactions with coiled-coil arrays, *Science 300*, 2097−2101.
43. Vinson, C., Myakishev, M., Acharya, A., Mir, A. A., Moll, J. R., and Bonovich, M. (2002) Classification of human B-ZIP proteins based on dimerization properties, *Mol. Cell. Biol. 22*, 6321−6335.
44. Chen, Y., Zhuang, S., Cassenaer, S., Casteel, D. E., Gudi, T., Boss, G. R., and Pilz, R. B. (2003) Synergism between calcium and cyclic GMP in cyclic AMP response element-dependent transcriptional regulation requires cooperation between CREB and C/EBP-β, *Mol. Cell. Biol. 23*, 4066−4082.
45. Haas, N. B., Cantwell, C. A., Johnson, P. F., and Burch, J. B. E. (1995) DNA-binding specificity of the PAR basic leucine zipper protein VBP partially overlaps those of the C/EBP and CREB/ATF families and is influenced by domains that flank the core basic region, *Mol. Cell. Biol. 15*, 1923−1932.
46. Perini, G., Wagner, S., and Green, M. R. (1995) Recognition of bZIP proteins by the human T-cell leukaemia virus transactivator Tax, *Nature 376*, 602−605.
47. Baranger, A. M., Palmer, C. R., Hamm, M. K., Giebler, H. A., Brauweiler, A., Nyborg, J. K., and Schepartz, A. (1995) Mechanism of DNA binding enhancement by the HTLV-I Tax protein, *Nature 376*, 606−608.
48. Kim, C.-M., Koike, K., Saito, I., Miyamura, T., and Jay, G. (1991) HBx gene of hepatitis B virus induced liver cancer in transgenic mice, *Nature 351*, 317−320.

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