Consensus and Variant cAMP-regulated Enhancers Have Distinct CRE-binding Properties*

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Recent determination of the cAMP response element-binding protein (CREB) basic leucine zipper (bZIP) consensus CRE crystal structure revealed key dimerization and DNA binding features that are conserved among members of the CREB/CREM/ATF-1 family of transcription factors. Dimerization appeared to be mediated by a Tyr307–Glu312 interhelical hydrogen bond and a Glu319–Arg314 electrostatic interaction. An unexpected hexahydrated Mg2+ ion was centered above the CRE in the dimer cavity. In the present study, we related these features to CREB dimerization and DNA binding. A Y307F substitution reduced dimer stability and DNA binding affinity, whereas a Y307R mutation produced a stabilizing effect. Mutation of Glu319 to Ala or Lys attenuated dimerization and DNA binding. Mg2+ ions enhanced the binding affinity of wild-type CREB to the palindromic CRE by ~20-fold but did not do so for divergent CREs. Similarly, mutation of Lyg304, which mediates the CREB interaction with the hydrated Mg2+, blocked CREB binding to the palindromic but not the variant CRE sequences. The distinct binding characteristics of the K304A mutants to the consensus and variant CRE sequences indicate that CREB binding to these elements is differentially regulated by Mg2+ ions. We suggest that CREB binds the consensus and variant CRE sequences through fundamentally distinct mechanisms.

The cAMP response element-binding protein (CREB)† is a 43-kDa basic leucine zipper (bZIP) transcription factor that couples gene activation to a wide variety of cellular signals (for review, see Ref. 1). Initially identified as a mediator of the cAMP pathway, CREB is now recognized to respond to calcium/calmodulin and growth factor pathways as well (2–4). The prototypical target sequence for CREB is the palindromic CRE by only a single nucleotide. CREB family members are notable in that they recognize the CRE exclusively, or nearly so. One caveat in this characterization, however, is that the defining features of the CRE have not been established unequivocally.

CREB binds as a dimer to the CRE with an affinity of ~1–2 nM (15). Dimerization and DNA binding are mediated by the adjacent basic and leucine zipper domains (7, 8). At the extreme C-terminal end of the protein is the leucine zipper, characterized by a conserved heptad repeat of seven residues, denoted a–g. The residues at positions a and d typically form a hydrophobic interface, with conserved leucines at position d. As with other bZIP proteins, this configuration in CREB allows for the formation of a two-stranded parallel coiled-coil and, along with charged residues at positions e and g, presents a plane for dimerization specificity (12, 16–19). In addition, dimerization of the CREB monomers apposes the basic regions in a parallel orientation to the DNA, allowing the dimer to bind at a right angle to the DNA helical axis. DNA binding elicits an a-helical conformation of the basic region that facilitates DNA recognition. The basic region of CREB abuts the amino terminus of the leucine zipper. This segment mediates DNA half-site sequence recognition and binding by passing through the major groove and forming several direct and indirect protein-DNA contacts.

Insights into the mechanisms underlying CREB dimerization and DNA binding were obtained from the crystal structure of the CREB bZIP-CRE complex (20). Two features of this structure were unexpected. First was a hexahydrated Mg2+ ion centered on the CRE between the two bZIP monomers at the dimer fork. This hexahydrated Mg2+ ion is not found in related instances, functional studies have indicated that these sequences are required for second messenger-directed transcriptional responses (for review, see Ref. 6). Whether variations of the CRE from the consensus sequence have functional consequences has not been determined.

Two general categories of factors can recognize the CRE (1, 7, 8). The first is composed of factors that can dimerize with CREB. This group includes the CRE modulator CREMα (and the multitude of splice variants that contain the CREMα DNA binding domain) and the activating transcription factor-1 (ATF-1). CREMα and ATF-1 recognize the CRE as homodimers and as heterodimers with CREB (9–11). The second category of CRE binding proteins includes factors that cannot heterodimerize with CREB, such as c-Jun, some of the other ATFs, and members of the CAAT/enhancer-binding protein family (12, 13). In addition to their inability to heterodimerize with CREB, these factors appear to be capable of recognizing DNA sequences that are distinct from the CRE (14). This promiscuity in DNA binding is not entirely unexpected, because some of these distinct elements, such as the AP-1 site, differ from the CRE by only a single nucleotide. CREB family members are notable in that they recognize the CRE exclusively, or nearly so. One caveat in this characterization, however, is that the defining features of the CRE have not been established unequivocally.

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‡ The abbreviations used are: CREB, cAMP response element-binding protein; bZIP, basic leucine zipper; CRE, cAMP response element; CREM, CRE modulator; ATF-1, activating transcription factor-1; TAT, tyrosine aminotransferase; PEPCK, phosphoenolpyruvate carboxykinase.

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bZIP-DNA complexes and participates in a water-mediated contact between Lys<sup>304</sup> in the CREB basic domain and the CRE. Structurally, the hexahydrated Mg<sup>2+</sup> ion was proposed to function by positioning and stabilizing the CREB basic region in the CRE major groove. It may also impart additional rigidity to the CREB basic domain, which may prevent binding to shorter or longer divergent CRE sequences. Fluorescence anisotropy assays revealed that CREB bZIP binding to the CRE was dramatically regulated by Mg<sup>2+</sup> and other divalent cations. In the absence of divalent cation, binding decreased by at least 20-fold. The divalent cation concentration required for half-maximal binding was 340 μM, approximately the concentration of free Mg<sup>2+</sup> ions in many types of cells. Thus, it was proposed that the concentration of free Mg<sup>2+</sup> might regulate the level of CREB binding to the CRE.

The second unexpected feature of the complex was the mechanism underlying the specificity of CREB dimerization. In addition to predicted electrostatic interactions between Glu<sup>319</sup> on one monomer with Arg<sup>314</sup> on the other, a critical interhelical hydrogen bond was found between Tyr<sup>307</sup> and Glu<sup>312</sup> (Fig. 1).

**Fig. 1.** Views of the CREB bZIP-DNA complex depicting key protein-protein and protein-divalent cation interactions. a, view of the CREB bZIP and the side chains of the residues selected for study (Glu<sup>319</sup>–Arg<sup>314</sup>, Tyr<sup>307</sup>–Glu<sup>312</sup>, and Lys<sup>304</sup>), along with their contact distances (Å). The hydrated magnesium ion (gray) complexed with six water molecules occupies the dimer cavity. b, magnification of the dimer cavity with a view of the hexahydrated magnesium ion contacted by the Lys<sup>304</sup> side chains, centered above the CRE illustrated at the base of the dimer fork as a Cory-Pauling-Kolton space-filling model.

The combination of Glu<sup>319</sup>–Arg<sup>314</sup> and Tyr<sup>307</sup>–Glu<sup>312</sup> is unique to members of the CREB family and was proposed to be the primary determinant of CREB, CREMs, and ATF-1 heterodimerization. Of note, although dimerization is generally attributed to leucine zipper sequences, the Tyr<sup>307</sup> is located at the C-terminal end of the basic region.

Studies in this paper were designed to test the major predictions of the CREB bZIP-CRE crystal structure. First, we wanted to test the specificity of the divergent cation effect by determining whether the interaction of Lys<sup>304</sup> with the hexahydrated Mg<sup>2+</sup> ion was essential for CREB binding to DNA. Next, we asked whether Mg<sup>2+</sup> ions regulated the binding of CREB to consensus and nonconsensus CREs. Finally, we tested the functional importance of the residues predicted to direct CREB dimerization specificity. Our studies confirm the predictions of the CREB-consensus CRE crystal structure and suggest that consensus and nonconsensus CREs interact with CREB through fundamentally distinct mechanisms.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Protein Purification**—Construction of the wild-type full-length and CREB bZIP expression vectors was described by Richards et al. (15). The three cysteine residues (Cys<sup>300</sup>, Cys<sup>310</sup>, and Cys<sup>337</sup>) in the bZIP region of rat CREB (residues 285–341) were mutated to serine. These mutations improved protein solubility but did not alter DNA binding, as demonstrated by the similar relative affinities of CREB/Ser and wild-type CREB for the CRE (15). The following mutations were introduced into CREB bZIP/Ser by site-directed mutagenesis using oligonucleotide primers from Life Technologies, Inc.: Y307F, Y307R, E319A, E319K, and K304A. Purification of CREB bZIP/Ser proteins was described by Schumacher et al. (20). Purification of full-length CREB protein was described by Richards et al. (15). Protein concentrations were determined by Bradford protein assay (Bio-Rad).

**Thermal Stability Measurements**—The stability of each bZIP protein was assessed in thermal melting assays by monitoring fluorescence as previously described (21). In brief, the fluorescence of the two tyrosine residues (positions 307 and 336) in wild-type CREB bZIP was monitored as a function of temperature. Measurements were taken on 1.3 ml of a sample containing 5 μM CREB bZIP in HEPES buffer (pH 7.6; with 50 mM or 150 mM NaCl in the presence or absence of 10 mM Mg<sup>2+</sup>) in a 6-mm cuvette. Temperature was monitored using a thermistor element/controller device (Omega Engineering Inc.; model no. DP25-TH-A), with the thermistor element positioned in the solution inside the cuvette. Excitation was set at 280 nm, and emission was monitored at 300 nm using 3-nm band pass settings. The emission data were collected by ramping the temperature from 4 to 85 °C at 2 °C/min. The temperature was ramped back down to 4 °C, and the process was repeated for each sample to confirm reversibility of protein folding. The fluorescence intensity data were fit using a nonlinear least squares regression analysis (Sigma Plot; Jandel Scientific). The thermodynamic relationship between the folded and unfolded states of the protein, represented by the melting temperature (T<sub>m</sub>), was used to compare protein stability and is represented as follows,

\[
T_m = \Delta H / \Delta S_m
\]

(Eq. 1)

where ΔH<sub>m</sub> is the enthalpy and ΔS<sub>m</sub> is the entropy of unfolding (22, 23). Tyrosine emission (300 nm; y axis) was plotted as a function of temper-
**Mg^{2+} Regulation of CREB Binding**

![Graph](image)

**FIG. 2. Involvement of Mg^{2+} and Lys^{304} for binding to the consensus CRE.** a, Representative plot of the thermal denaturation of wild-type CREB (closed circles, with Mg^{2+}, $T_m = 45.0$ °C; open triangles, without Mg^{2+}, $T_m = 43.0$ °C) and the K304A mutant (open squares; $T_m = 46.0$ °C). $F$ represents the emission intensity at 300 nm (y axis) plotted against temperature (Celsius; x axis). $F_{	ext{min}}$ and $F_{	ext{max}}$ represent the observed minimum and maximum tyrosine fluorescence values. Each sample was remelted after the protein was cooled and refolded, and each condition was repeated at least twice. b, Representative DNA binding isotherms of the wild-type (open circles, with Mg^{2+}, $K_d = 1.8$ nM; open triangles, without Mg^{2+}, $K_d > 20$ nM) and K304A proteins (open squares, $K_d$ not determined) bound to the consensus CRE. Measurements, reported as millipolarization units (nF; y axis), were normalized and plotted against CREB bZIP concentration (nM; x axis). The binding curves were repeated at least three times for each condition.

In the thermal melting experiments, the sharp upward slopes represent the transitions between protein folding and unfolding. Pretransition state fluorescence defines the fluorescence of the folded state of the protein (represented by the left tails of the melting curves). Post-transition state fluorescence defines the fluorescence characteristic of the unfolded state (represented by the right tails of the melting curves). By measuring free tyrosine in solution, we confirmed that the downward slopes observed for the pre- and post-transition states were due to the temperature sensitivity of tyrosine fluorescence (data not shown). Assays were performed on the wild-type CREB bZIP, and the mutants K304A, E319A, E319K, Y307F, and Y307R. Note that in the case of the Y307F and Y307R mutants, the emission of only one tyrosine was measured. Although the signal was reduced, the single tyrosine was adequate to measure the transition state of the mutants. Selected proteins were also denatured with urea, yielding profiles that were similar to those produced by thermal melting (data not shown), indicating that heat is a satisfactory denaturing reagent for measuring tyrosine emission.

**Fluorescence Polarization Measurements**—The binding affinities of CREB bZIP proteins for DNA were determined in solution using a PanVera Beacon 2000 fluorescence polarization system. 5'-Fluorescein-labeled oligonucleotides corresponding to the somatostatin (5'-CTCGACGGCCCTCAGTCAGGG-3'), rat c-Fos (5'-AGTACGTAG-GCCCCCTACGTACT-3'), phosphonopyruvate carboxykinase (PEPCk; 5'-CTCTACGTAGCCCTGACGTAAAGG-3'), and the tyrosine aminotransferase (TAT; 5'-CTCGTGTCACGCCCTGACGCG-A3') CREs were purchased from Life Technologies, Inc. The CRE binding sites are indicated in boldface type. Each oligonucleotide was designed to form a hairpin with a 5-nucleotide loop and was self-annealed by heating to 95 °C and snap-cooled on ice. Binding experiments with the somatostatin CRE were performed with wild-type and mutant CREB bZIP (residues 283–341) and wild-type full-length CREB (residues 1–341) proteins. Binding reactions with the PEPCk CRE were performed with wild-type and K304A bZIP and wild-type full-length CREB, and those with the c-Fos and TAT CREs were performed with wild-type and K304A CREB bZIP. Protein was titrated into 0.99 ml solutions that contained 1 nM fluorescein-labeled oligonucleotide in 25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, 6 mM bovine serum albumin, 10 mM MgCl_2, and 0.01% Tween 20. Protein concentration was measured at 350 nm. The binding curves were fit with a nonlinear least squares regression analysis using Sigma Plot (Jandel Scientific). In addition to the temperature sensitivity of tyrosine fluorescence, the curves showed a linear increase in polarization at higher protein concentrations. For a curve that begins at $y_0$ (minimum polarization) and rises to $y_1$ (maximum polarization), the following equation was used,

$$f = y_0 + (y_1 - y_0)x/(b + x) + cx$$

(Eq. 2)

where $b$ represents $K_d$ as a function of $x = [CREB]$, and $c$ represents the nonspecific constant as a linear additive function ($cx$).

**TABLE I**

Melting temperatures in the presence or absence of Mg^{2+} (10 mM)

| CREB | Without Mg^{2+} | With Mg^{2+} |
|------|----------------|-------------|
| °C   | 43.2 ± 0.4     | 45.0 ± 0.2  |
| Wild type |                |             |
| K304A |                |             |

**TABLE II**

The effects of Mg^{2+} (10 mM) on the affinity of CREB bZIP for various CREs

|       | + Mg^{2+} | - Mg^{2+} |
|-------|-----------|-----------|
| Consensus | c-Fos | TAT | c-Fos | TAT | c-Fos | TAT |
| +      | -        |          | +      | -        |          | +      | -        |          |
| Wild type | 1.2 ± 0.4 | 1.4 ± 0.2 | 5.0 ± 0.3 | 4.5 ± 0.2 | 10.0 ± 1.1 | 9.8 ± 0.2 |
| K304A    | 2.0 ± 0.4 | 2.0 ± 0.2 | 5.5 ± 0.4 | 5.7 ± 0.1 | 6.3 ± 0.4 | 6.1 ± 0.1 |

Values represent $K_d$ ± S.E. For not determined (ND) values, no specific binding was detected up to the concentrations indicated in the figures.
RESULTS

Fig. 1 depicts two magnified views of a portion of the CREB bZIP-consensus CRE complex as reported by Schumacher et al. (20). Key residues in the basic and leucine zipper domains are highlighted. The continuous α-helical chains are symmetrically apposed at the C-terminal leucine zipper and are fastened by an electrostatic interaction between Arg314 and Glu319 as well as hydrogen bonds between Tyr307 and Glu312. The helices pass across the DNA through the major groove, where several residues from the basic region make protein-DNA contacts. The most extraordinary feature of the complex is the hydrated Mg2+ ion, which is juxtaposed between the two α-helices and appears to stabilize the dimer fork on the CRE sequence.

A lysine residue (Lys304) in the basic region of each subunit provides a hydrogen bond to one of the coordination waters of the Mg2+ ion (Fig. 1b). Thus, we hypothesized that Lys304 was essential for Mg2+ ion binding. To test this idea, we mutated Lys304 to Ala and performed thermal stability and fluorescence polarization assays on the mutated and wild-type CREB proteins in the presence or absence of Mg2+ ions. Alanine was chosen for this substitution because it is predicted to maintain the helical structure of CREB bZIP. Moreover, the related factor CAAT/enhancer-binding protein α contains an Ala in the corresponding position. The thermal stability of each CREB protein was determined by measuring the intrinsic tyrosine fluorescence (contributed by Tyr307 and Tyr336) as a function of temperature. These studies indicated that the conformational stability of the wild-type protein was unaffected by the presence or absence of Mg2+ ions, as indicated by the unchanged $T_m$ values (Fig. 2a and Table I; $T_m = 45.0 \pm 0.2 \, ^\circ\text{C}$ with Mg2+, $T_m = 43.2 \pm 0.4 \, ^\circ\text{C}$ without Mg2+). Similarly, the K304A mutation did not have an adverse effect on the conformational stability of the protein (Fig. 2a and Table I; $T_m = 47.0 \pm 0.3 \, ^\circ\text{C}$ with Mg2+, $T_m = 46.7 \pm 0.3 \, ^\circ\text{C}$ without Mg2+), indicating that divalent ions are not necessary for maintaining structure in the absence of DNA. In contrast, fluorescence polarization measurements indicated that the absence of Mg2+ ions markedly decreased the binding affinity of wild-type CREB for the CRE (Fig. 2b and Table II; $K_d = 1.8 \pm 0.2 \, \text{nM}$ with Mg2+, $K_d > 20 \, \text{nM}$ without Mg2+), as reported previously (20). Moreover, no binding of CREB bZIP K304A to the CRE was detected even in the presence of Mg2+ ions (Fig. 2b). Thus, the enhancement of CREB binding to the consensus CRE mediated by Mg2+ depends upon the integrity of Lys304.

With the exception of the tyrosine aminotransferase (TAT) CRE, the binding properties of CREB for nonconsensus CRE sequences have been poorly explored. We recently reported that CREB binds the nonconsensus PEPCK CRE with high affinity (24). This finding is not altogether surprising, because this sequence differs from the somatostatin CRE by only a single nucleotide (TACGTCA; the single change in boldface type). However, these studies were performed under standard conditions, in the presence of Mg2+ ions. To test whether CREB binding to nonconsensus CREs similarly required Mg2+, we reanalyzed binding to the PEPCK element in the presence and absence of divalent cation. High affinity binding was detected in both cases (Fig. 3, a and b, and Table II; $K_d = 1.2 \pm 0.4 \, \text{nM}$ with Mg2+, $K_d = 2.0 \, \text{nM}$ without Mg2+). Similar findings were obtained in binding assays performed using full-length CREB.

Fig. 3. CREB binding to the high affinity, nonconsensus PEPCK CRE sequence does not require Mg2+. Representative fluorescence polarization plots are shown of the wild-type CREB bZIP with Mg2+ ($K_d = 1.2 \, \text{nM}$) (a) and without Mg2+ ($K_d = 1.4 \, \text{nM}$) (b) and the K304A mutant (with or without Mg2+, $K_d = 1.4 \pm 0.2 \, \text{nM}$ without Mg2+).
CREB (data not shown). Consistent with these observations, the K304A mutant bound equally to the PEPCK CRE in the presence or absence of Mg$^{2+}$ ions (Fig. 3c and Table II; $K_d = 2.0 \pm 0.4$ nM with Mg$^{2+}$, $K_d = 2.0 \pm 0.2$ nM without Mg$^{2+}$). These studies indicated that the mechanisms of CREB binding to the PEPCK and consensus CREs must be fundamentally different.

Additional binding studies were performed using the c-Fos and TAT CREs. The rat c-Fos promoter contains a CRE element that differs from the somatostatin CRE by two nucleotides, in the seventh and eighth positions (TGACGT$^\text{AG}$). The binding affinity of CREB for the c-Fos CRE had not been determined using equilibrium measurements in solution. The TAT promoter contains a CRE that differs from the consensus sequence by three nucleotides (CTGCGTCA) and has been characterized as a prototypical low affinity binding site (25). The binding affinity of CREB for the TAT CRE was determined to be 11 nM by Richards et al. (15). We analyzed the TAT and c-Fos CREs in the presence and absence of Mg$^{2+}$ ions and found that in both instances CREB bZIP binding did not require Mg$^{2+}$ (Figs. 4 and 5; $K_d = 5$ nM for the c-Fos CRE and 10 nM for the TAT CRE in the presence or absence of Mg$^{2+}$ ions). This Mg$^{2+}$ independence of binding to the c-Fos and TAT elements was supported by the ability of CREB bZIP K304A to bind to both sequences irrespective of Mg$^{2+}$ (Figs. 4c and 5c).

Two key determinants of dimerization unique to CREB family members are interactions between Glu$^{319}$–Arg$^{314}$ and Tyr$^{307}$–Glu$^{312}$. To investigate the contributions of these interactions to protein stability and DNA binding, we utilized CREB derivatives generated by mutating Tyr$^{307}$ to Phe or Arg and Glu$^{319}$ to Ala or Lys. The Y307F substitution was designed to test the importance of the Tyr$^{307}$–Glu$^{312}$ hydrogen bond (Fig. 1a). The Y307R substitution was designed to replace the hydrogen bond with an electrostatic interaction, which was predicted to increase the stability of a potential dimer structure. As compared with wild-type CREB (Fig. 2a), the Y307F mutant shows a dramatic reduction in thermal stability in 50 mM NaCl (Fig. 6a and Table III; $T_m = 24.3 \pm 0.6 ^\circ C$), while the Y307R mutation had a stabilizing effect under this ionic condition (Fig. 6b and Table III; $T_m = 55.3 \pm 0.3 ^\circ C$). However, the Y307R mutant was irreversibly denatured at physiological ionic strength (150 mM NaCl), indicating that this concentration of NaCl can interfere with the formation of the Arg$^{307}$–Glu$^{312}$ salt bridge (data not shown). Fig. 7b shows that the Y307F mutant also exhibited a temperature-dependent decrease in binding to the CRE, as determined by fluorescence polarization assays. Compared with wild-type CREB (Fig. 7a and Table IV; $K_d = 2.0 \pm 0.1$ nM at 25 $^\circ C$, $K_d = 15 \pm 1.1$ nM at 37 $^\circ C$), DNA binding of the Y307F protein was greatly diminished at 25 $^\circ C$ and was abolished at 37 $^\circ C$ (Fig. 7b and Table IV). Thus, the interaction between Tyr$^{307}$ and Glu$^{312}$ is crucial for dimerization and subsequent DNA binding.

The importance of the Glu$^{319}$–Arg$^{314}$ interaction to dimer stability and specificity was tested by measuring the thermal stability of proteins containing mutations of Glu$^{319}$ to Ala or Lys. As shown in Fig. 8, both mutations reduced CREB protein stability compared with the wild type CREB bZIP control (Fig. 2a). The E319A substitution reduced dimer stability in an ionic strength-independent manner (Fig. 8a and Table III; $T_m = 23.6 \pm 0.4 ^\circ C$), DNA binding (Fig. 5a and Table IV). Therefore, the interaction between Tyr$^{307}$ and Glu$^{312}$ is crucial for dimerization and subsequent DNA binding.

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Fig. 4. CREB binding to the moderate affinity, nonconsensus rat c-Fos CRE does not require Mg$^{2+}$. Representative fluorescence polarization plots are shown of the wild-type CREB bZIP with Mg$^{2+}$ ($K_d = 5.0$ nM) (a) or without Mg$^{2+}$ ($K_d = 4.5$ nM) (b) and the K304A mutant (with or without Mg$^{2+}$, $K_d = 5.5$ nM) (c) bound to the c-Fos CRE. Measurements were plotted as in Fig. 2. The binding curves were repeated at least three times for each condition.
Despite the lowered thermal stability, both derivatives were able to bind to the consensus CRE, in the presence of Mg\(^{2+}\) ions, although with greatly reduced affinity at 25 °C (Fig. 9a and Table IV, E319A K\(_d\) 565.0 nM; Fig. 9b, E319K K\(_d\) 250 nM). Consistent with their T\(_m\) values, binding of both Glu319 substitutions to the CRE was completely abolished at 37 °C (Fig. 9, a and b, and Table IV).

Fig. 6. Contribution of the Tyr\(^{307}\)-Glu\(^{312}\) hydrogen bonds to CREB stability. Representative thermal melting plots of Y307F (T\(_m\) = 25.0 °C) (a) and Y307R (T\(_m\) = 55.0 °C) (b) are shown. Tyrosine fluorescence was measured and plotted as in Fig. 2a. Each sample was remelted after the protein was cooled and refolded, and each condition was repeated at least twice.

29.5 ± 0.8 °C; and data not shown), and the E319K mutant appeared completely unfolded at 4 °C, regardless of the ionic strength of the buffer (Fig. 8b and data not shown).

Despite the lowered thermal stability, both derivatives were able to bind to the consensus CRE, in the presence of Mg\(^{2+}\) ions, although with greatly reduced affinity at 25 °C (Fig. 9a and Table IV, E319A K\(_d\) = 65.0 nM ± 1.1; Fig. 9b, E319K K\(_d\) > 250 nM). Consistent with their T\(_m\) values, binding of both Glu\(^{319}\) substitutions to the CRE was completely abolished at 37 °C (Fig. 9, a and b, and Table IV).

Fig. 5. CREB binding to the low affinity, nonconsensus TAT CRE does not require Mg\(^{2+}\). Representative fluorescence polarization plots are shown of the wild-type CREB bZIP with Mg\(^{2+}\) (K\(_d\) = 10.0 nM (a) or without Mg\(^{2+}\) (K\(_d\) = 10.0 nM) (b) and the K304A mutant (with or without Mg\(^{2+}\), K\(_d\) = 6.3 nM) (c) bound to the TAT CRE. Measurements were plotted as in Fig. 2. The binding curves were repeated at least three times for each condition.
DISCUSSION

In this study, we examined the contributions of key residues of the CREB bZIP to structural stability and DNA binding. For these determinations, we utilized thermal melting and fluorescence polarization assays. The melting assays measured the transitions of the mutant and wild-type CREB bZIP proteins from the folded to the unfolded states during thermal denaturation by tracking the emission spectra from tyrosine residues.

In the context of the folded protein, the tyrosine fluorescence is quenched, presumably by neighboring residues (26). Upon thermal unfolding of the protein, this quenching is removed, and the tyrosine fluorescence increases. Indirect measurements of bZIP dimer association (using circular dichroism in the absence of DNA) indicate that dimer formation occurs at micromolar concentrations of peptide (27, 28). However, there has been some disagreement as to whether CREB exists as a dimer or monomer in solution in the absence of DNA. Previously, our laboratory used fluorescence resonance energy transfer to determine that the K_d for CREB dimerization in the absence of DNA was 0.6 nM (29), but equilibrium sedimentation studies suggested that CREB exists primarily in a monomeric form (30). The melting curves in the current study were carried out using relatively high concentrations of CREB protein (5 μM), and we have interpreted these results assuming that, under our experimental conditions, CREB exists in the dimeric form. Consistent with this interpretation, mutations that were expected to disrupt dimerization caused observable changes in T_m and reductions in DNA binding, while the substitution in the basic region (K304A) did not alter the T_m value from that obtained for wild-type CREB bZIP. Thus, the thermal melting analyses described herein can be interpreted reasonably as an indirect measure of dimer stability. The finding that the thermally unstable E319K mutant binds to the CRE with low affinity (K_d ~ 250 nM) suggests that if this form is monomeric, then a single bZIP helix interacts rather poorly with DNA. Alternately, it may be that the CRE can recruit and stabilize the very low concentration of the dimeric form of this mutant, or that it overcomes the interhelical repulsion between Lys319 and Lys314 at high protein concentrations.

Role of Mg^{2+} Ions on CREB bZIP Stability and DNA Binding—We utilized fluorescence polarization assays to obtain equilibrium measurements of protein-DNA binding in solution. Precise quantitation of these interactions might not be achieved by using the more standard gel mobility shift assays, because the proteins can dissociate from the DNA within the gel matrix, and the ionic environment is difficult to control during electrophoresis.

We first tested whether Lys^{319}, which interacts with the hexahydrated Mg^{2+} ion, was necessary for CREB dimerization and DNA binding. The importance of Lys^{319} in CREB function was tested previously by Dwarki et al. (31). Their study exam-
ined the properties of a CREB K304E mutant in an effort to
determine whether the predicted electrostatic repulsion be-
tween the Glu and the negatively charged phosphate backbone
of the CRE would be deleterious for CREB function. As pre-
predicted, this mutant failed to bind the CRE in vitro or to activate
transcription in vivo. The present study took advantage of the
structure of the CREB bZIP-
ion-mediated DNA se-
quence recognition. We mutated the Lys 304 to Ala, the residue
that occupies this position in the related transcription factor
CAAT/enhancer-binding protein α. The thermal stability of the
CREB bZIP K304A mutant was the same as that of the wild-
type CREB protein, irrespective of Mg2+
(summarized in Table I). Therefore, we suspect that the stability of the mutant dimer
is not impaired. However, binding of wild-type CREB bZIP to
the consensus CRE was highly dependent upon Mg2+ (summa-
rized in Table II). In the absence of Mg2+, CREB bound poorly
to the consensus CRE, and no binding was detected with
K304A, regardless of whether Mg2+ was included in the buffer.
It is possible for Lys304 to interact with the phosphate backbone

**FIG. 8.** Contribution of the Glu319–Arg314 electrostatic interaction to CREB stability. Representative thermal melting plots are shown of E319A (Tm = 29.5 °C) (a) and E319K (Tm not determined (n.d.)) (b). Tyrosine fluorescence was measured and plotted as in Fig. 2. Each sample was remelted after the protein was cooled and refolded, and each condition was repeated at least twice.

**FIG. 9.** Glu319 mutants decrease DNA binding. Representative fluorescence polarization plots are shown of E319A (open circles, 25 °C, Kd = 65 nM; open squares, 37 °C, Kd not determined (n.d.)) (a) and E319K (open circles, 25 °C, Kd > 250 nM; open squares = 37 °C, Kd not determined (n.d.)) (b) bound to the consensus CRE. Measurements were plotted as in Fig. 2. The binding curves were repeated at least three times for each condition.
of the DNA in the absence of the hexahydrated Mg$^{2+}$ ion (determined by manipulation of the complex in RASMOL), which would be consistent with the ability of the wild-type CREB protein to bind to the CRE at low affinity in the absence of Mg$^{2+}$. Elimination of the positive charges contributed by the Lys$^{304}$ side chain would diminish its interaction with DNA, regardless of the presence of Mg$^{2+}$. Alternately, the presence of a Mg$^{2+}$ ion might physically obstruct the binding of the K304A mutant to the consensus CRE, if the Ala substitution effectively removed a Mg$^{2+}$-dependent spacing requirement from the dimer fork. Nonetheless, our data indicate that both a Mg$^{2+}$ ion and Lys$^{304}$ are required for CREB to bind with high affinity to the consensus CRE but do not contribute to CREB stability.

Role of Mg$^{2+}$ Ions for CREB bZIP Binding Specificity—The next set of experiments tested whether the Mg$^{2+}$ ion requirement for CREB binding was unique to the consensus CRE (Table II). The PEPCK, TAT, and c-Fos genes are all regulated by CREB via nonconsensus CREs. The PEPCK CRE differs from the consensus CRE at the −3 base position (−3, −2, −1/1, 2, 3, 4 corresponds to TGAC/GTCA), in which there is a pyrimidine for purine substitution (TTACGTCA), in which there is a pyrimidine for purine substitution in the −3 base position (CTGCCGTTCA), and the rat c-Fos CRE contains substitutions in the 3 and 4 base positions (TGACGTAG), with a purine for pyrimidine substitution at the 3 base position. The binding affinities of CREB for the PEPCK and TAT elements were determined previously to be 2.0 and 11.0 nM, respectively (see Refs. 24 and 15, respectively). The binding affinity of CREB for the c-Fos element was determined in the present study to be 4.5–5.0 nM, approximately 3-fold lower than that for the consensus CRE. The importance of the interhelical hydrogen bond formed between Tyr$^{307}$ and Glu$^{312}$ was tested by mutating Tyr$^{307}$ to Phe and Arg. The Phe substitution decreased protein stability, and the Arg substitution increased protein stability (although only at the lower salt concentration), as predicted (Table III). Furthermore, the unstable CREB Y307F mutant had a reduced ability to bind to the CRE (Table IV). These data support a dual role for the Tyr$^{307}$-Glu$^{312}$ interaction, one that serves to promote dimerization specificity and a second that stabilizes the dimer on the DNA.

Conclusions—Our studies support the principal predictions of the CREB bZIP-CRE crystal structure and confirm the functional roles of the key residues that define the unique features of transcription factors in the CREB gene family. The importance of Mg$^{2+}$ ions in the association of CREB with the consensus CRE suggests that this divalent cation may have a regulatory role for discrimination at the promoter level. How the difference in Mg$^{2+}$ sensitivity of consensus and nonconsensus CREs might affect the regulation of specific genes remains to be determined, however. In neurons, depolarization induces calcium fluxes that cause a 3–10-fold increase in the concentration of free Mg$^{2+}$ (34, 35). It is possible that these Mg$^{2+}$ changes could regulate the binding of CREB to some cellular genes, but not others, depending on the presence of particular CRE sequences in the relevant transcriptional control regions. This complexity and the potential increase in regulatory control have not been appreciated previously.

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