Electrostatic Mechanism for DNA Bending by bZIP Proteins†

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ABSTRACT: Biology is replete with examples of protein-induced DNA bending, yet the forces responsible for bending have been neither established nor quantified. Mirzabekov and Rich proposed in 1979 that asymmetric neutralization of the anionic phosphodiester backbone by basic histone proteins could provide a thermodynamic driving force for DNA bending in the nucleosome core particle [Mirzabekov, A. D., & Rich, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1118−1121]. Strauss and Maher lent support to this proposal in 1994 by demonstrating that replacement of six proximal phosphate residues with neutral methylphosphonates resulted in DNA bent spontaneously toward the neutralized face [Strauss, J. K., & Maher, L. J., III (1994) *Science 266*, 1829−1834; Strauss, J. K., Prakash, T. P., Roberts, C., Switzer, C., & Maher, L. J., III (1996) *Chem. Biol. 3*, 671−678; Strauss, J. K., Roberts, C.; Nelson, M. G.; Switzer, C., & Maher, J. L., III (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9515−9520]. Here it is shown that bZIP proteins bend DNA via a mechanism involving direct contacts between one or two basic side chains and a symmetry-related pair of unique, nonbridging phosphate oxygens. The locations of these phosphates provide direct experimental support for a protein-induced bending mechanism based on asymmetric charge neutralization. This straightforward mechanism is compatible with many DNA-recognition motifs and may represent a general strategy for the assembly of protein−DNA complexes of defined stereochemistries.

Many catalytic and regulatory DNA-binding proteins bend or otherwise distort DNA when they bind, and in many cases DNA distortion is tied intimately to biological function. For example, certain restriction enzyme isoschizomers bend DNA in opposite directions to access alternative phosphodiester bonds within a common sequence (Withers & Dunbar, 1993). Eukaryotic histone proteins bend DNA to condense the entirety of the genome into the nucleus (Richmond et al., 1984); analogous bacterial proteins condense the smaller prokaryotic genome (Tanaka et al., 1984). Many prokaryotic and eukaryotic transcription factors bend DNA to form specific, high-affinity complexes. DNA bending in these cases may serve an architectural role (Tjian & Maniatis, 1994) to generate scaffolds for the assembly of multiprotein complexes of defined stereochemistries or to increase target site specificity (Schepartz, 1995). Despite the pervasiveness of protein-induced bending in biology, the forces responsible for protein-induced bending have been neither established nor quantified.

Basic region leucine zipper (bZIP) proteins represent one class of eukaryotic transcription factors whose members can bend DNA (Hamm & Schepartz, 1995; Kerppola, 1996; Kerppola & Curran, 1991a,b, 1993; Paolella et al., 1994). Certain bZIP proteins [e.g., CRE-BP1 (Paolella et al., 1994) and CREB (Hamm & Schepartz, 1995)] bend DNA toward the minor groove, while others [GCN4 (Gartenberg et al., 1990; Paolella et al., 1994)] do not bend DNA at all (Figure 1). We noticed that all bZIP proteins that bend DNA toward the minor groove contain a cluster of three basic amino acids at the very NH_2 terminus of the basic segment (Paolella et al., 1994). This cluster is absent in bZIP proteins that bend DNA toward the major groove or do not bend DNA (Paolella et al., 1994) (Figure 2). In the case of CRE-BP1 and the cyclic AMP response element (CRE) target site (ATGACGTCAT), the induced minor groove bend counteracts the intrinsic major groove bend present in this sequence; the result is apparently straight DNA (Paolella et al., 1994). Here we demonstrate that bending of the CRE DNA by bZIP proteins results from direct electrostatic interactions between one or two basic side chains and a symmetry-related pair of unique, nonbridging phosphate oxygens. The simplicity of these interactions suggests that DNA bending through asymmetric phosphate neutralization may represent a common mechanism for the assembly of protein−DNA complexes of defined stereochemistries.

MATERIALS AND METHODS

Peptides. Peptides ggg, ccc, and cgg have been described (Paolella et al., 1994). All other peptides were expressed in *Escherichia coli*. Peptides c5g and c10g were purified by hydroxyapatite chromatography and RP-HPLC. Peptides g1c, g2c, g3c, g5c, c2g2c, and c1g3c were purified by hydroxyapatite and/or cation ion-exchange (SP-Sepharose, Pharmacia) chromatography.

Phasing Analysis. Phasing oligonucleotides CRE-21, -23, -26, -28, and -30 were generated as described (Paolella et al., 1994). Phasing oligonucleotides TmC-21, -23, -26, -28, and -30 were prepared by use of a mutually primed synthesis procedure. Five sets of partially complementary synthetic oligonucleotides containing a single methylphosphonate linkage were prepared using a commercially available methylphosphonamidite monomer (Glen Research, Sterling,

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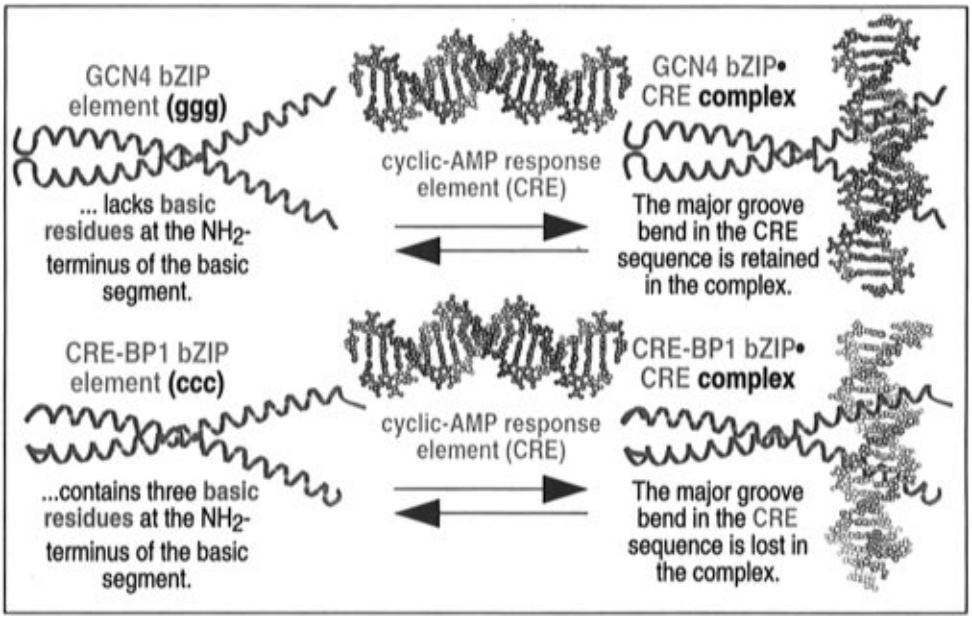

FIGURE 1: Schematic illustration of the effects of GCN4 (top) and CRE-BP1 (bottom) bZIP element peptides on the conformation of the intrinsically bent (Paolella et al., 1994) CRE site. The GCN4 bZIP−CRE complex retains the major groove bend (König & Richmond, 1993; Keller et al., 1995) observed in the free CRE DNA, whereas the CRE-BP1−CRE complex contains apparently straight DNA (Paolella et al., 1994). The five residues in the CRE-BP1 cluster (KRRKF) are colored red.

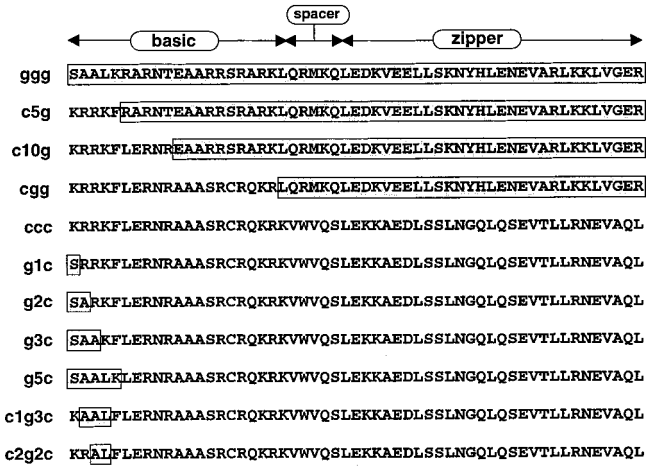

FIGURE 2: bZIP peptides used in this study. Each 55-residue bZIP monomer contains a basic segment containing those residues that contact DNA (Ellenberger et al., 1992; Glover & Harrison, 1995; König & Richmond, 1993; Keller et al., 1995) joined through a six-residue spacer to a zipper segment whose residues mediate protein dimerization (O'Shea et al., 1991).

VA). Oligonucleotides containing methylphosphonate linkages were deprotected and purified according to the procedure described by Hogrefe et al. (1993). Each set of oligonucleotides was complementary over a 26 base pair region which included the methylphosphonate linkages. Extension with Vent (exo$^-$) polymerase generated phasing oligonucleotides TmC-21, -23, -26, -28, and -30, which were identical in length and linker composition to the corresponding CRE phasing oligonucleotides. The methylphosphonate linkage in each TmC oligonucleotide was a racemic mixture of R_P and S_P isomers. Binding reactions were performed as described (Paolella et al., 1994).

Although the issue remains controversial (Hagerman, 1996; Kerppola, 1996), two groups have provided evidence that the phasing analysis may be flawed for the analysis of certain protein-induced DNA bends. Sitlani and Crothers reported that phasing oligonucleotides containing directly adjacent reference and test bends could lead to erroneous protein-induced bend angles because of interactions in the gel between the bound protein and the reference A-tract (Sitlani & Crothers, 1996). To test whether such interactions existed in our system, which contained directly adjacent reference and test bends, we performed phasing analyses with oligonucleotides containing a CRE site whose center was separated from the A-tract center by between five and six helical turns of DNA. These experiments showed clear evidence of intrinsic CRE curvature and straightening of this curvature by CRE-BP1-derived peptides. In addition, minicircle ligation experiments were performed with CRE phasing oligonucleotides (Figure 3A) in the presence and absence of peptides ccc, csg, cgg, and ggg (Figure 2) (Sitlani & Crothers, 1996). Peptides ccc, csg, and cgg decreased the ratio of circular to linear ligation products formed from CRE-26 in a concentration-dependent manner, whereas peptide ggg did not (M. A. Fabian, unpublished results). This decrease is consistent with induced bending of the CRE site toward the minor groove by peptides ccc, csg, and cgg. These two control experiments verify use of the phasing analysis for this system, although we cannot rule out the possibility that our phasing probes underestimate the extent of induced DNA bending (Kerppola, 1996). McCormick et al. reported that the anomalous migration of phasing oligonucleotides bound to bHLH proteins containing leucine zippers of varying length decreased with the length of the zipper (McCormick et al., 1996). The experiments described herein employed a set of peptides containing leucine zippers of identical length.

Ligation Ladder Analysis. Oligodeoxyribonucleotide monomers employed for ligation ladder analysis contained the following sequences: *Bam*HI-AT$_{21}$-A, AAAAAACGCCGGGATCCCGCC; *Bam*HI-AT$_{21}$-B, CGGGATCCCGGCGTTTTTTGG; TmC-AT$_{21}$-A, AAAAAACGCATGACGTmCATCC; TmC-AT$_{21}$-B, ATGACGTmCATGCGTTTTTTGG; CRE-AT$_{21}$-A, AAAAAACGCATGACGTCATCC; CRE-

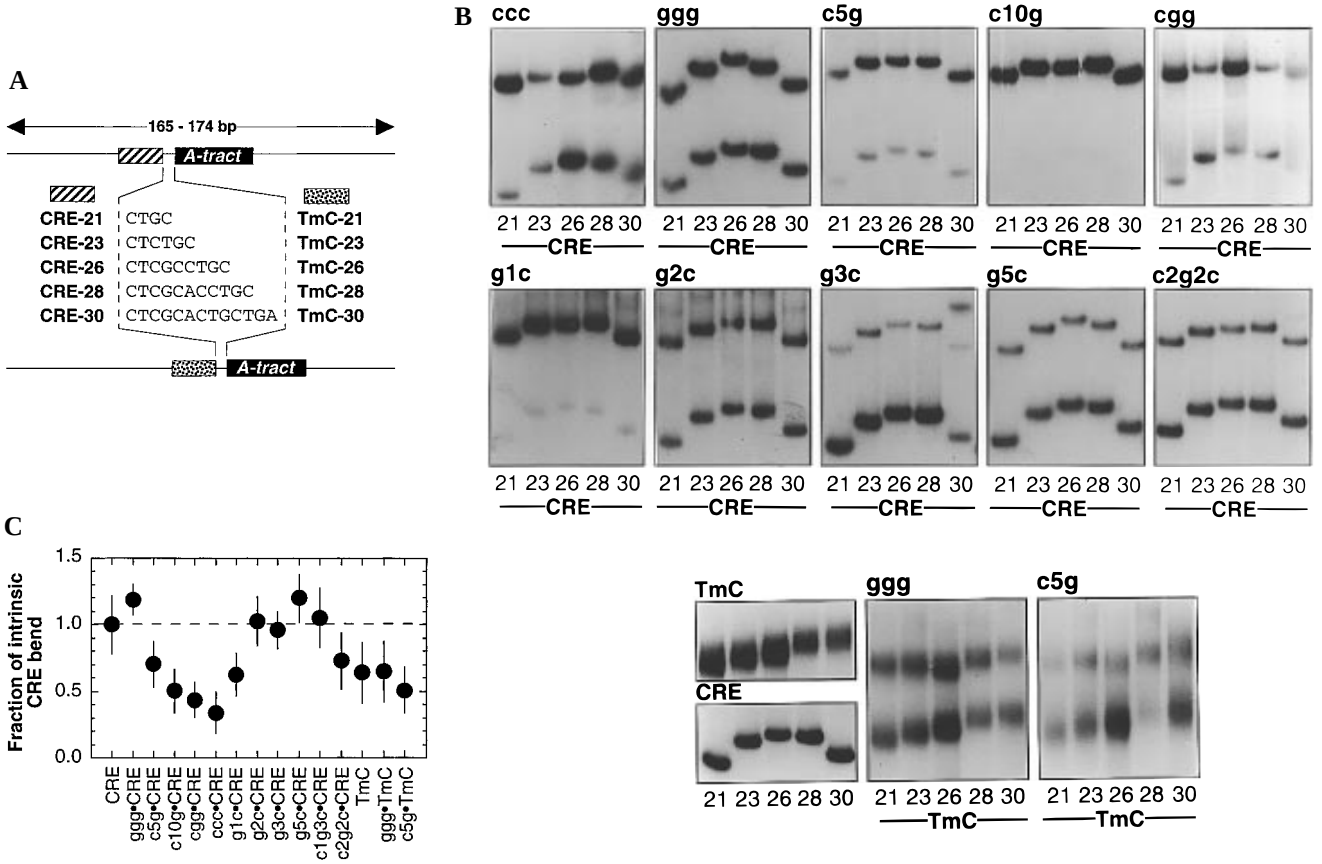

FIGURE 3: Phasing analysis (Kerppola & Curran, 1991a; Paolella et al., 1994; Zinkel & Crothers, 1987) of the CRE (ATGACGTCAT) and TmC (ATGACGTmCAT; m represents methylphosphonate) sequences in the presence and absence of bound bZIP homodimers. (A) Oligonucleotides used for phasing analysis contained a 10-bp CRE (striped box) or TmC (stippled box) sequence separated by a variable length linker from a 25-bp A-tract sequence that bends intrinsically toward the minor groove by approximately 54° (Koo et al., 1990). The number describing each phasing oligonucleotide refers to the distance in base pairs between the centers of the CRE (or TmC) site and the 25-bp A-tract. Outside of the variable linker, all oligonucleotides within each set were the same size and contained the same sequence. (B) Electrophoretic mobility shift analyses of bZIP homodimers bound to phasing analysis oligonucleotides (Paolella et al., 1994). The lower bands in each panel contain free DNA; the upper bands contain bZIP−DNA complexes. (C) Comparison of the extent of bending within the CRE− and TmC−peptide complexes. Relative mobilities and bend angles were calculated from the amplitudes of the corresponding phasing function as described (Kerppola & Curran, 1993; Paolella et al., 1994). The degree of induced bending in each complex is expressed relative to the bend present in the free CRE DNA (Paolella et al., 1994). Peptide-induced minor groove bends are reflected by values <1.0. The data represent the average of at least three independent experiments. Error bars represent the standard deviation.

AT_{21}-B, ATGACGTCATGCGTTTTTTGG; AP1-AT_{21}-A, AAAAAACGCGATGAGTCATCC; AP1-AT_{21}-B, ATGACTCATCGCGTTTTTTGG. Single-stranded oligonucleotides were labeled on the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase and the complementary A and B strands combined and annealed. Ligation reactions were performed by incubating duplex monomers with T4 DNA ligase (New England Biolabs) as described (Koo & Crothers, 1988). Ligation products were resolved with a nondenaturing 8% (32:1) polyacrylamide gel prepared in TBE buffer [80 mM Tris−borate, 2 mM EDTA (pH 8.0)]. Electrophoresis was performed at 4 °C and 7 V·cm^{-1}. Relative mobilities were calculated as described (Paolella et al., 1994).

Molecular Modeling. Our modeling experiment began with the GCN4 bZIP·CRE structure (König & Richmond, 1993; Keller et al., 1995). Replacement of Ala_{228} in each GCN4 monomer with arginine using the program Midas (Ferrin, 1988) initially positioned a guanidinium nitrogen within 6−7 Å of the TpC phosphate in each CRE half-site. Rotation of the Cα−Cβ bond of this residue by any value between −45° and −105° placed a guanidinium nitrogen within 2.5−3.5 Å of a nonbridging phosphate oxygen of the TpC base step without generating unacceptable steric conflicts as judged by the program PROCHECK (Laskowski et al., 1993). Pictures were created using the program SETOR (Evans, 1993).

RESULTS AND DISCUSSION

Residues within the Basic Cluster Are Sufficient To Straighten the CRE Site. To determine if residues within the basic cluster of CRE-BP1 were sufficient to straighten the CRE site, we performed phasing analyses (Kerppola, 1996; Kerppola & Curran, 1991a; Paolella et al., 1994; Zinkel & Crothers, 1987) of the CRE complexes of two chimeric peptides (Figure 2) in which five (c5g) or ten (c10g) residues at the NH_2 terminus of the GCN4 bZIP element (Harrison, 1991) were replaced with the corresponding residues from CRE-BP1. Phasing analyses of the CRE complexes of peptides ccc and ggg, which contained the bZIP elements of CRE-BP1 and GCN4, respectively, were performed for comparison. As seen previously (Paolella et al., 1994), the ccc−CRE complex showed evidence of induced curvature toward the minor groove, whereas the ggg−CRE complex did not (Figure 3). Bending of the CRE DNA toward the minor groove by ccc counteracted the intrinsic major groove bend (11−13°) present in the free DNA (Paolella et al., 1994)

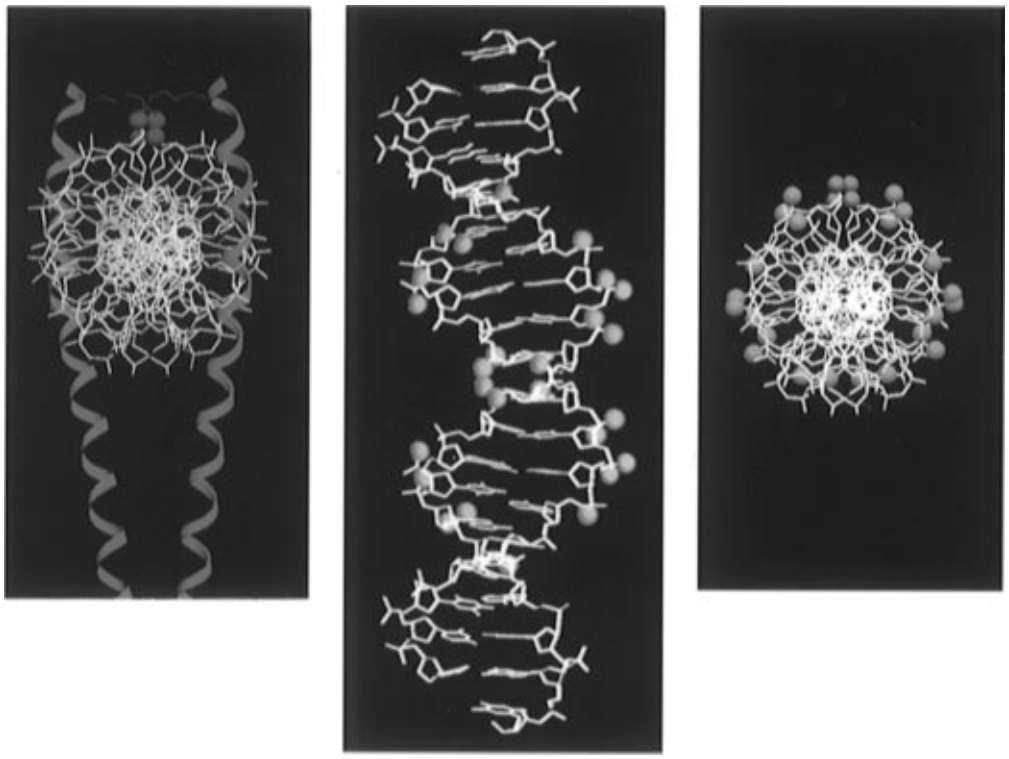

FIGURE 4: (A, left) View of a GCN4−CRE (König & Richmond, 1993) complex with the DNA perpendicular to the plane of the figure, in which Ala_{228} has been replaced with arginine (red). Pink spheres denote symmetry-related TpC phosphates. The ten base pairs of the CRE site are shown in yellow. (B, center, and C, right) View of the DNA within the GCN4 bZIP−CRE complex, with the DNA axis (B) parallel or (C) perpendicular to the plane of the figure. Blue spheres denote phosphates that participate in direct protein contacts.

to produce a complex with no apparent curvature. The absence of bending by ggg yielded a ggg−CRE complex whose curvature (12° toward the major groove) mirrored that seen in the free CRE DNA (Paolella et al., 1994). By contrast, both chimeric peptide−CRE complexes showed evidence of induced curvature toward the minor groove. In the case of c5g, the induced minor groove bend accounted for approximately 50% of the bend induced by ccc; in the case of c10g, the induced bend accounted for 75% of the bend induced by ccc (Figure 3B,C). Peptides c5g and c10g bent the related AP-1 target site (ATGACTCAT) toward the minor groove by comparable amounts (data not shown). Taken together, these results indicate that five residues at the NH_2 terminus of the CRE−BP1 bZIP element (Lys-Arg-Arg-Lys-Phe) are sufficient to induce a significant structural change in the CRE DNA. The relationship between DNA bending and charged residues at the NH_2 terminus of the bZIP element has also been reported by Leonard et al. (1997).

Two Basic Residues Are Required To Straighten the CRE Site. Two experiments were performed to identify the precise number and arrangement of residues within the basic cluster required for bending the CRE site toward the minor groove. First, we performed a phasing analysis of the CRE complexes of a series of chimeric peptides (g1c, g2c, g3c, g5c) in which the five NH_2-terminal residues of ccc (Lys-Arg-Arg-Lys-Phe) were altered systematically into their counterparts from ggg (Ser-Ala-Ala-Leu-Lys) (Figure 2). Replacement of the NH_2-terminal Lys residue of ccc with Ser (g1c) reduced the magnitude of the induced bend by 45%, and replacement of the NH_2-terminal Lys-Arg sequence with Ser-Ala (g2c) reduced the magnitude of the induced bend to zero. The phase-dependent mobility variations of the g3c−CRE and g5c−CRE complexes, like the g2c−CRE complexes, paralleled those of the free CRE DNA (Figure 3B,C). These results indicate that the ability of ccc to bend (i.e., straighten) the CRE site is abolished by loss of two basic residues from the NH_2 terminus of the bZIP basic segment.

To determine if the ability to straighten the CRE site depended on the number of positively charged side chains within the basic segment or to their position along the helix, we compared the abilities of g2c and c2g2c to bend the CRE site. Both peptides contained one lysine residue and one arginine residue within the basic cluster but differed in terms of the placement of these residues along the helix (Figure 2). Bending of the CRE site was induced by c2g2c, which contained the NH_2-terminal sequence Lys-Arg-Ala-Leu-Phe, but not by g2c, which contained the sequence Ser-Ala-Arg-Lys-Phe (Figure 3C). Moreover, a peptide (c1g3c) containing only one basic residue at the first position of the cluster did not bend the CRE site (Figure 3C). Our results indicate that two precisely positioned basic residues located at the NH_2 terminus of the bZIP region provide a gain of function—DNA bending—to both GCN4- and CRE-BP1-derived peptides. These two basic residues are conserved among CREB/ATF bZIP proteins and may play a structural role in the transcriptional regulation of CRE- and certain CRE-like sites.

From the crystal structure of the GCN4 bZIP−CRE complex (König & Richmond, 1993), we predicted which CRE phosphates were most likely to interact with the first two residues within the CRE−BP1 basic cluster. Replacement of Ala_{228} in each GCN4 monomer with arginine positioned a guanidinium nitrogen of this residue within 2.5−3.5 Å of a nonbridging phosphate oxygen of the TpC base step found within each CRE half-site without introducing significant destabilizing interactions (Figure 4A). These two symmetry-related TpC steps are located on the minor groove face of the GCN4−CRE complex, directly opposite the major

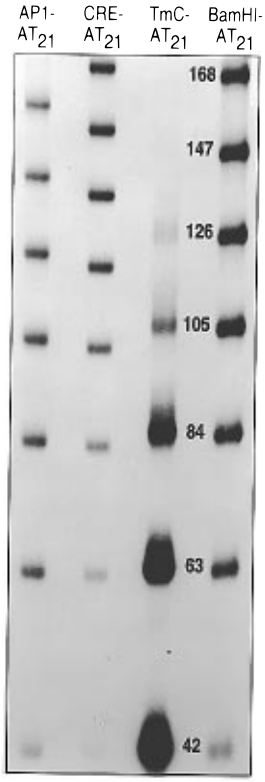

FIGURE 5: Native polyacrylamide gel electrophoretic analysis of DNA conformation. Labeled 21-bp DNA duplexes were joined with DNA ligase to produce multimers whose lengths differed by 21 base pairs. The relative mobilities of multimers of AP1-AT_{21}, CRE-AT_{21}, and TmC-AT_{21} were compared with multimers of *Bam*HI-AT_{21} whose length in bp is indicated.

groove bend, and their phosphates are otherwise devoid of protein contacts (Figure 4B,C) (König & Richmond, 1993). Interaction of these phosphates with one or more basic cluster residues would neutralize negative charge on the convex surface of the bZIP−CRE complex and induce DNA bending toward the minor groove, straightening the intrinsic CRE bend. The location of the two TpC phosphates is therefore consistent with a charge neutralization model for induced DNA bending (Mirzabekov & Rich, 1979; Strauss & Maher, 1994; Strauss et al., 1996a,b) in which electrostatic interactions between one or more bZIP basic cluster residues and the TpC phosphates bend DNA toward the minor groove.

DNA Bending Results from Neutralization of Two TpC Phosphates within Each CRE Half-Site. We employed two assays to determine if neutralization of the TpC phosphate within each CRE half-site was sufficient to remove the intrinsic CRE site bend in the absence of a bound bZIP peptide. First we compared the relative mobilities of multimers of a 21-bp CRE site-containing oligonucleotide (CRE-AT_{21}) to multimers of an analogous 21-bp oligonucleotide in which the two TpC phosphates in the CRE site were replaced by neutral methylphosphonate derivatives (TmC-AT_{21}) (Figure 5) (Koo & Crothers, 1988). To modulate the degree of bending, each 21 base pair monomer sequence also contained an A-tract located in phase with the CRE or TmC site. Monomers containing an AP-1 site (AP1-AT_{21}) or a straight (Koo et al., 1990) *Bam*HI site (*Bam*HI-AT_{21}) in place of the CRE site were prepared as controls. Because the CRE (Paolella et al., 1994) and A-tract sequences (Koo et al., 1990) bend intrinsically in opposite directions and were separated by one helical turn of DNA in CRE-AT_{21}, multimers of this monomer migrated faster than multimers of AP1-AT_{21} or *Bam*HI-AT_{21} monomers whose target sequences were not bent. Each TmC-AT_{21} multimer migrated slower than the corresponding CRE-AT_{21} multimer, and the mobility difference increased with multimer length (Figure 5). The TmC-AT_{21} multimers migrated alongside the corresponding *Bam*HI-AT_{21} multimers, suggesting that the TmC sequence was completely unbent. These data demonstrate that neutralization of the two TpC phosphates within each CRE site is sufficient to significantly alter DNA conformation in the absence of a bound bZIP protein. Further support for this conclusion derives from phasing analysis of DNA fragments containing the TmC site, which revealed the TmC sequence to possess greatly reduced intrinsic curvature (Figure 3B,C). Neutralization of two phosphates located on the convex face of the intrinsic CRE bend reduced the bend magnitude as effectively as the peptide c5g (Figure 3C). These results define two phosphates as a minimal element required for DNA bending by asymmetric charge neutralization. Neutralization bends DNA toward the neutral surface, as predicted (Mirzabekov & Rich, 1979) and observed in a model GC-rich sequence (Strauss & Maher, 1994; Strauss et al., 1996a,b).

The experiments described above demonstrate that neutralization of two symmetry-related phosphates opposite the intrinsic CRE bend straightens the DNA in the absence of a bound bZIP peptide and that specific residues within the c5g NH_2-terminal basic cluster are necessary and sufficient for CRE site bending. Two testable predictions follow from a model invoking direct electrostatic interactions between these basic cluster residues and the TpC phosphate. First, c5g should induce no curvature in the TmC sequence upon binding. Bending should not result because the TmC sequence has been straightened via charge neutralization (Figures 3 and 5) and exists (to a first approximation) in a conformation suitable for c5g. Induced bending is, moreover, impossible because the electrostatic partner within the TmC sequence is absent. Second, ggg should induce no curvature in the TmC sequence upon binding, as ggg lacks basic cluster residues and does not contact the TpC phosphate. Phasing analysis revealed that neither the c5g nor the ggg peptide induced curvature in the TmC DNA (Figure 3B,C). This result supports the proposal that DNA bending by c5g is manifested in full or in part by direct electrostatic interactions with the phosphate backbone of the CRE site at position TpC.

Many transcription factor−DNA complexes contain bent DNA. In certain cases, such as the complexes of TBP (Kim et al., 1993a,b), SRY (Werner et al., 1995), PurR (Schumacher et al., 1994), and Lef-1 (Love et al., 1995), the DNA bends away from a concave protein surface, and the protein−DNA interfaces are distinguished by intercalation of nonpolar side chains into the DNA (Werner et al., 1996). In other cases, such as the DNA complexes of MATa1/α2 (Li et al., 1995), serum response factor (Pellegrini et al., 1995), CAP (Schultz et al., 1991; Warwicker et al., 1987), and the 434 (Aggarwal et al., 1988) and metJ (Somers & Phillips, 1992) repressors, intercalation is not observed. In these cases, the DNA bends toward a convex protein surface, and the protein−DNA interfaces are distinguished by basic side chain−phosphate contacts positioned in a manner consistent with a charge neutralization model for DNA bending. For example, DNA in complex with the yeast MATa1/MATα2

homeodomain heterodimer bends by 60° toward the protein surface (Li et al., 1995), with the bend localized between the a1 and α2 target sites and within the target site for a1. The a1 protein makes numerous contacts with the phosphodiester backbone. Several of these contacts, such as those involving Arg_{13}, Arg_{46}, and Arg_{53}, are positioned to direct DNA bending via charge neutralization and would not take place if the a1 target site were not bent (Li et al., 1995). The observation that the extent of bending is affected only modestly by mutations that alter the flexibility of the a1/α2 dimerization interface emphasizes the importance of interactions between a1 and DNA (Kerppola, 1996). It is possible that the energy gained from these contacts compensates in full or in part for the energy expended by DNA bending (Schepartz, 1995). Similar phosphate interactions are found at the DNA interfaces of the 434 (Aggarwal et al., 1988) and metJ repressors (Somers & Phillips, 1992). A mechanism for protein-induced DNA bending based on one or two direct electrostatic interactions is compatible with a wide range of DNA-recognition motifs. DNA bending via charge neutralization may be employed generally in cases where DNA bends toward a convex transcription factor surface to generate multiprotein complexes of defined stereochemistries.

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