Sequence Determinants of the Intrinsic Bend in the Cyclic AMP Response Element†

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ABSTRACT: The cyclic AMP response element (CRE site, ATGACGTCAT) is the DNA target for transcription factors whose activities are regulated by cyclic AMP (*1*). Recently, we discovered that the CRE site is bent by 10−13° toward the major groove (*2*). Little or no bend is detected in the related AP-1 site (ATGACTCAT), which differs from the CRE site by loss of a single, central, C·G base pair (*2*, *3*). Here we describe experiments designed to identify which base pairs within the CRE site induce the bent structure in an attempt to understand the origins of the dramatically different conformations of the CRE and AP-1 sites. Our data indicate that the intrinsic CRE bend results from distortion within the TGA sequence found in each CRE half site (ATGAC). These two TGA sequences are located in phase with one another in the CRE sequence but are not (completely) in phase in the AP-1 sequence. This difference in phasing leads to the overall difference in bend as detected by gel (*2*) and cyclization methods (S. C. Hockings, J. D. Kahn, and D. M. Crothers, unpublished results; M. A. Fabian and A. Schepartz, unpublished results). Our results confirm earlier predictions of altered structure within TG steps, provide insight into the structural reorganizations induced in DNA by bZIP proteins, and lead to a revision of the relationship between the structures of the free and bZIP-bound forms of the CRE and AP-1 sites.

The possibility that certain DNA molecules might possess B-form structures that differed from that of the Watson−Crick model (*4*) was recognized by the discovery of bent DNA in 1982 (*5*). It was discovered that the kinetoplast minicircles of the *Leishmania tarentolae* parasite contained tracts of four to five dA·dT base pairs (called A-tracts) repeated in phase with the DNA helical repeat (*6*, *7*). Fragments of these minicircles displayed anomalously low mobilities in nondenaturing gels when compared with reference DNA sequences of equivalent length. Subsequent experimentation revealed that the anomalous migration of minicircle DNA resulted from a bend of approximately 17° associated with each A-tract (*8*). When located along the same face of the DNA double helix, the bends produced by each A-tract added constructively to generate a DNA fragment with a significant overall bend. A-tract bending has been studied extensively (for reviews, see refs *9*−*13*) since its discovery because of the significance of DNA structure and structural dynamics for at least three vital biological functions: transcriptional regulation (*14*), control of replication and recombination (*15*, *16*), and the packaging of DNA into nucleosomes (*10*, *17*, *18*). Although a number of intrinsically bent DNA sequences unrelated to A-tracts have been identified (*14*, *19*−*26*), the magnitudes of the bends in these sequences are, with two exceptions (*24*, *26*), small relative to the bend produced by a single A-tract (∼17°) (*8*).

Recently, we discovered that the cyclic AMP response element (CRE site, ATGACGTCAT), the DNA target for transcription factors whose activities are regulated by cyclic AMP (*1*), is bent significantly in solution (*2*). The CRE site consists of two ATGAC half-sites arranged in an inverted repeat and is unrelated to A-tract DNA. The magnitude of the intrinsic CRE bend was estimated at between 10° and 13° toward the major groove (*2*). Thus the CRE bend is similar in magnitude to that of a single A-tract, but the bend is in the opposite direction (*8*). Although the bend in the CRE site was detected initially by use of gel electrophoretic methods (*27*), its existence has been confirmed subsequently by analyzing the rates of minicircle ligation reactions (*28*, *51*) (M. A. Fabian and A. Schepartz, unpublished results) as well as by X-ray crystallography [see reference in Paolella et al. (*2*)]. Here we employ a related gel electrophoretic method to analyze the extent of bending in a series of oligonucleotides related to the CRE site. Our data indicate that the intrinsic CRE bend (*2*) is the additive result of two major groove bends that are each found within the TGA sequence in each CRE half-site (ATGAC). These two TGA sequences are located in phase with one another in the sequence ATGACGTCAT but are not (completely) in phase in the related AP-1 sequence (ATGACTCAT). We propose that this difference in phasing leads to the overall difference in bend as detected by gel-based methods.

MATERIALS AND METHODS

Materials. Enzymes were purchased from New England Biolabs and used with the buffers supplied. Adenosine 5′-[γ-^{32}P]triphosphate was purchased from DuPont. Adenosine 5′-triphosphate monomagnesium salt was purchased from Sigma. Phosphoramidites and other reagents employed in solid-phase oligonucleotide synthesis were purchased from Perceptive Biosystems.

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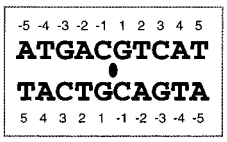
Oligonucleotide Synthesis. Oligonucleotides were synthesized on a 0.2 μmol scale with a Millipore Expedite DNA/RNA Synthesizer Model 8909 and standard phosphoramidite chemistry (*29*) and were purified by preparative denaturing gel electrophoresis. DNA-containing regions of the gel were identified by shadowing (*30*) and excised, and the oligonucleotides were eluted into TE buffer [90 mM Tris (pH 8.0), 1 mM EDTA] at room temperature for at least 10 h. Concentrated eluents were dialyzed against 1 mM Tris (pH 7.4) for 24 h at 25 °C and stored at −20 °C. Oligonucleotides were labeled on the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (*31*), annealed to their complementary strands by heating to 70 °C followed by slow cooling to room temperature, and used directly in ligation ladder experiments.

Ligation Ladder Experiments. ^{32}P end-labeled oligonucleotide duplexes (800 pmol) prepared as described above were treated with T4 DNA ligase (800 units) in 70 μL of reaction buffer [50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 4 mM ATP, 50 μg/mL BSA] for 90 min at 16 °C. The reaction mixtures were washed with phenol/chloroform (*31*) and precipitated with ethanol. The DNA was dried and resuspended in 20 μL of loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 15% glycerol) and applied to an 8% nondenaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide) prepared in 1× TBE buffer [90 mM Tris base, 90 mM boric acid, 2 mM EDTA (pH 8.2)]. Electrophoresis was performed at 300 V for 24 h at 4 °C in 1× TBE buffer. After autoradiography, the relative length (R_L) of each multimer was determined by comparing its migration (in centimeters) to that of a *Bam*HI multimer of identical length (*32*). R_L = (mobility of *Bam*HI multimer)/(mobility of test multimer of identical length).

RESULTS

Ligation Ladder Analysis. The overall bend in each of the sequences shown in Figure 1 was evaluated by comparing the mobilities of multimers of each sequence on nondenaturing gels. This method of assessing the magnitude of an intrinsic DNA bend is referred to as ligation ladder analysis (*33*). Ligation ladder analysis entails the directional ligation of short (10−15 bp) duplex DNA molecules containing the target sequence and resolution of the resulting multimers by nondenaturing gel electrophoresis. The result is a series of multimers that differ in length by a set number of base pairs and appear as a ladder of bands on the gel. An overall bend in the target sequence is detected by comparing the mobilities of target sequence multimers to those of a reference sequence that lacks a bend and displays normal electrophoretic mobility. Ligation ladder analysis, like phasing analysis, is based on the observation that a bent DNA molecule migrates more slowly in a nondenaturing polyacrylamide gel than a straight DNA molecule of equivalent length (*33*, *34*). A bent monomer ligated in phase with the helical repeat—that is, containing an integral number of helical turns of DNA between the centers of the bends—should show increasing retardation in the gel (and a larger relative length) as the length of the multimer increases. This situation arises because consecutive bends arranged in phase with the helical repeat add constructively and the ratio of end-to-end distance to the number of base pairs decreases with increasing length (*33*).



CRE	ATGACGTCAT CTGCAGTATA	$CRE_{3,-3}$	ATCACGTGAT GTGCACTATA
AP-1	ATGACTCATC CTGAGTAGTA	$CRE_{4,-4}$	AAGACGTCTT CTGCAGAATT
CRE^{15}	AGATGACGTCATCTC TACTGCAGTAGAGTC	mCRE	AGTACGTTCT ATGCAAGATC
$AP\text{-}1^{15}$	AGATGACTCATCTCG TACTGAGTAGAGCTC	CRE^{11}	ATGACGCTCAT CTGCGAGTATA
CRE_{GG}	ATGAGGTCAT CTCCAGTATA	CRE^{12}	ATGACGCGTCAT CTGCGCAGTATA
CRE_{CC}	ATGACCTCAT CTGGAGTATA	*Bam*H I	CGGGATCCCG CCTAGGGCGC
$CRE_{1,-1}$	ATGAGCTCAT CTCGAGTATA	*Bam*H I^{11}	CGGGATCCCGC CCTAGGGCGGC
$CRE_{2,-2}$	ATGTCGACAT CACGTGTATA	*Bam*H I^{12}	CGGGATCCCGCG CCTAGGGCGCGC

FIGURE 1: Numbering system and DNA sequences used in this study. Unless otherwise noted by a superscript (for example, CRE^{15}), each duplex DNA molecule subjected to ligation comprised two oligonucleotide decamers. The 10 base pair CRE monomer contains the 10 base pair CRE site. The 10 base pair AP-1 monomer contains the 9 base pair AP-1 site and a terminal C·G base pair. The presence of this base pair produces a TGA step at the AP-1 ligation junction. Previous work using 21 base pair oligonucleotides lacking a C·G base pair at the ligation junction (and the resulting TGA step) demonstrates that the AP-1 sequence exhibits little or no intrinsic curvature as detected by ligation ladder analysis (*35*). Subscripts (for example, $CRE_{1,-1}$) indicate which base pair in the sequence differed from the CRE sequence.

Verification of the Intrinsic Bend in the CRE Site. The relative mobilities of multimers produced by ligation of the 10 bp CRE and AP-1 duplexes were compared with the mobilities of multimers containing a *Bam*HI recognition sequence which is not bent (*33*) (Figure 1). An autoradiogram illustrating the relative mobilities of the multimers is shown in Figure 2A. Graphs illustrating the relative apparent lengths (R_L) of the multimers as a function of length are shown in Figure 2B−G. When compared to the *Bam*HI multimers, the CRE multimers showed large migrational anomalies that increased with multimer length, whereas the AP-1 multimers did not (Figure 2B). These results confirm those obtained from phasing analysis (*2*) and ligation ladder analysis (*35*), as well as minicircle ligation kinetics (*28*) (S. C. Hockings, J. D. Kahn, and D. M. Crothers, unpublished results; M. A. Fabian and A. Schepartz, unpublished results), indicating that the 10 bp CRE site is bent intrinsically and that deletion of a central, single C·G base pair eliminates much of the observed bend.

To verify that the distortion in the CRE site resulted from a unidirectional bend, as opposed to nondirectional flexibility, we examined the relative mobilities of multimers of 15 bp monomers containing the CRE or AP-1 sites (Figure 1). These CRE^{15} and $AP\text{-}1^{15}$ monomers contained the CRE or AP-1 site, respectively, surrounded by flanking sequences that should not, on their own, exhibit an overall bend (*2*). Ligation of these oligonucleotides resulted in multimers containing CRE or AP-1 sites whose centers were separated by approximately one and one-half helical turns of DNA. This spacing placed the target site centers out of phase with the DNA helical repeat. If the CRE site bend resulted from

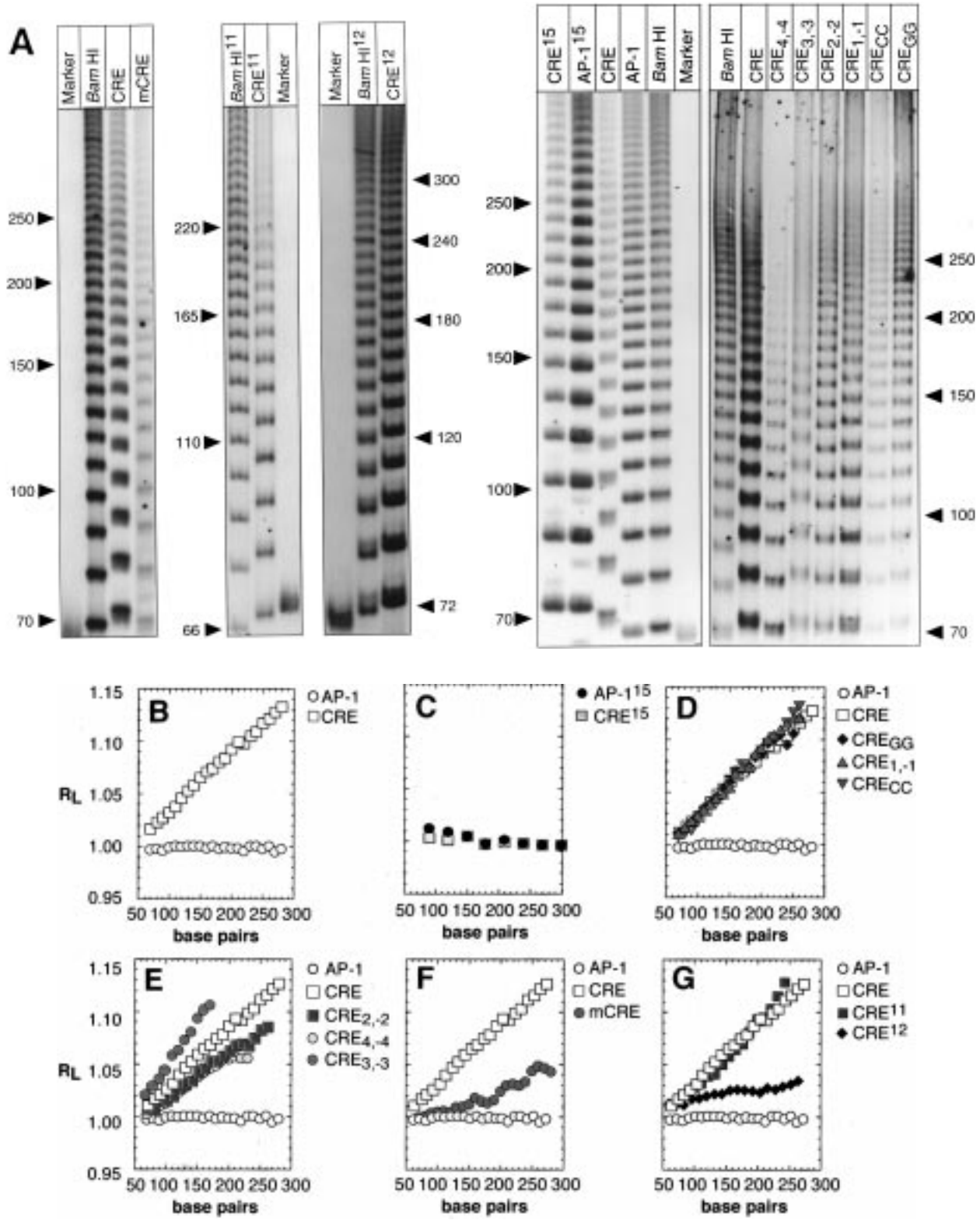


FIGURE 2: (A) Autoradiogram of an 8% nondenaturing gel illustrating the relative electrophoretic mobilities of the multimers of the DNA sequences studied. (B−G) Relative electrophoretic mobilities of (B) CRE and AP-1, (C) CRE^{15} and AP-1^{15}, (D) CRE_{GG}, CRE_{CC}, and $CRE_{1,-1}$, (E) $CRE_{4,-4}$, $CRE_{3,-3}$, and $CRE_{2,-2}$, (F) CRE, AP-1, and mCRE, and (G) CRE, AP-1, CRE^{11}, and CRE^{12} were calculated as described in Materials and Methods.

nondirectional flexibility, then with increasing multimer length, CRE^{15} multimers should show diminishing electrophoretic mobilities relative to AP-1^{15} multimers. If the intrinsic CRE bend was unidirectional, however, then the CRE^{15} and AP-1^{15} multimers should show similar electrophoretic mobilities and neither should show evidence of an overall bend. This situation results because nondirectional flexibility, as opposed to a unidirectional bend, is independent of the phasing between target sites. Our results (Figure 2C) showed comigration of CRE^{15} and AP-1^{15} multimers up to 300 bp in length (the longest multimer that could be distinguished on the gel). Not only did the CRE^{15} and AP-

1^{15} multimers comigrate but their relative mobilities did not increase with multimer length. This result indicates that neither CRE^{15} nor AP-1^{15} multimers exhibit a significant overall bend, as expected. These data provide further evidence for a defined bend within the 10 bp CRE site (*2*).

Which Dinucleotide Steps Contribute to the Intrinsic CRE Bend? A series of duplexes was prepared to identify the sequence determinants of the intrinsic CRE bend (Figure 1). The first set of 10 bp duplexes contained a consensus CRE site or a variant thereof in which one base pair in each ATGAC half-site was inverted. These base pairs are underlined in Figure 1. Each monomer was assigned a name indicating which base pairs were exchanged. For example, $CRE_{2,-2}$ refers to the sequence in which the two symmetry-related A_{-2}•T_2 base pairs were converted into T•A base pairs, respectively. Several other sequences with multiple base pair changes or insertions were also prepared (Figure 1).

Examination of the data in Figure 2 reveals that the sequences fell into three categories depending on whether they exhibited an overall bend that was greater than, less than, or equal to that of the CRE site. Multimers of $CRE_{1,-1}$, CRE_{CC}, and CRE_{GG} displayed mobilities that paralleled those of the CRE multimers (Figure 2D). This result indicated that the central CpG sequence could be changed to GpC, CpC, or GpG without altering the bent conformation of the DNA. We conclude that the intrinsic CRE bend is not determined by the CpG sequence in the center of the site, in contrast to earlier predictions (*36*). Multimers of $CRE_{4,-4}$ and $CRE_{2,-2}$ displayed higher mobilities than the analogous CRE multimers (Figure 2E), illustrating that reversing either the T_4•A_{-4} base pair or the A_{-2}•T_2 base pair decreased the magnitude of the intrinsic bend. Multimers of $CRE_{3,-3}$ displayed lower mobilities than the analogous CRE multimers (Figure 2E), illustrating that exchanging the G_{-3}•C_3 base pair for a C•G base pair increased the magnitude of the intrinsic bend. Taken together, these two results suggest that the intrinsic CRE bend is sensitive to the composition of the $T_{-4}G_{-3}A_{-2}$ sequence found in each ATGAC half-site: $CRE_{4,-4}$ and $CRE_{2,-2}$, which each lacked a TGA sequence, exhibited a reduced overall bend relative to CRE, whereas $CRE_{3,-3}$, which retained a TGA sequence, exhibited an overall bend that was slightly larger than CRE.

Is the Bend in the CRE Site the Product of Two Bends, One in Each TGA Sequence? The sequence mCRE was prepared to test explicitly whether alteration of the TGA sequence in each CRE half-site would eliminate the intrinsic CRE bend (Figure 1). In mCRE the two TGA trinucleotide steps were replaced by GTA steps. An autoradiogram illustrating the relative mobilities of the CRE, AP-1, mCRE, and *Bam*HI multimers is shown in Figure 2A; relative mobilities are plotted in Figure 2F. The data show that mCRE multimers migrated more rapidly than analogous CRE multimers, indicating that the mCRE sequence possessed a considerably smaller overall bend than the CRE sequence. The mCRE sequence displayed less bending than either $CRE_{4,-4}$ or $CRE_{2,-2}$ (Figure 2E,F), suggesting that each of these three base pairs contributed to the observed bend. The small bend remaining in mCRE may result from the pair of phased AG steps in this sequence (*20*, *37*).

If both the CRE Site and the AP-1 Site Contain TGA Sequences, Then Why Do the Two Sites Differ in Overall Bend? Although removal of the two phased TGA steps in CRE eliminated much of the intrinsic bend, the presence of this sequence cannot be the sole determinant of the CRE bend, since it is also found in the AP-1 site which exhibits little or no overall bend (*2*). In the CRE site, the centers of the two TGA sequences are separated by 5 base pairs and located on opposite DNA strands. For a DNA duplex with a helical repeat of 10.5 base pairs, this spacing places the two bends in position to add constructively, producing a molecule with roughly twice the bend of each constituent half. In the AP-1 site, the two TGA sequences are separated by 4 base pairs, which is not the correct orientation for full addition of the constituent bends. To determine whether the phasing between the two TGA sequences is responsible for the different electrophoretic mobilities of the CRE and AP-1 sites, we analyzed the relative mobilities of multimers of two new duplexes containing one (CRE^{11}) or two (CRE^{12}) additional base pairs between the two ATGA half-sites (Figure 1). If the phasing between the two TGA sequences is responsible for the observed electrophoretic mobility differences between CRE and AP-1, then multimers of CRE^{11} should exhibit mobilities that are comparable to those of CRE because both sets of multimers contain TGA sequences located closely in phase with the helical repeat. Multimers of CRE^{12} should exhibit different mobilities because they contain TGA sequences that are not closely in phase with the helical repeat. An autoradiogram illustrating the relative mobilities of CRE, AP-1, CRE^{11}, and CRE^{12} multimers is shown in Figure 2A. Plots of relative mobility versus multimer length are shown in Figure 2G. As predicted, multimers of CRE^{11} migrate through the gel much like multimers of CRE, whereas multimers of CRE^{12} migrate more rapidly. This result indicates that changing the phasing between two TGA sequences changes the overall bend of the DNA. The lack of an observed bend in CRE^{12} supports the hypothesis that the intrinsic CRE bend results from two smaller bends, centered in the TGA sequence, that add in the context of the CRE site because they are oriented in phase with the helical repeat.

DISCUSSION

Here we describe experiments aimed at understanding the elements of sequence that produce the dramatically different conformations of the CRE and AP-1 sites, two major DNA targets for bZIP transcription factors. Our data suggest that the intrinsic CRE bend (*2*) is the additive result of two major groove bends found within the TGA sequence in each CRE half-site (ATGAC). These two sequences are located in phase with one another in the CRE sequence ATGACGTCAT but are not (completely) in phase in the related AP-1 sequence ATGACTCAT. Our results indicate that this difference in phasing produces the overall difference in bend detected by gel methods and cyclization kinetics.

Our conclusion that the TGA sequence in each CRE half-site bends intrinsically toward the major groove is consistent with a large body of previous research that has detected noncanonical B-form structure within TG steps. The TG step exhibits rapid exchange of the thymine imino proton (*38*) and is highly reactive toward oxidation by hydroxyl radical and $KMnO_4$ (*39*) and cleavage by DNase I (*37*). These experiments are consistent with a distortion involving static or dynamic base unstacking at TG steps. Evidence for increased flexibility, or kinking, of the DNA at TG steps

derives from gel electrophoresis and minicircle cyclization experiments (*25*, *40*, *41*). Considerable evidence indicates that the distortion at TG steps represents compression of the major groove, as suggested initially by Monte Carlo calculations (*42*). For example, the presence of a TG step one-half helical turn away from the center of an A-tract increases the overall bend of the DNA (*39*). Moreover, DNA that has been selected for maximal bending contains TG steps significantly over-represented in the sequences between successive A-tracts (*43*). Finally, the structure of the catabolite activator protein (CAP) bound to DNA contains a 40° kink toward the major groove at each of the two TG steps (*44*), and ligation kinetics indicate that a fraction of the CAP-induced bend exists in the free DNA (*45*, *46*).

The conclusion that the intrinsic CRE bend results from distortion in the TGA sequence is also supported by experiments aimed at understanding the mechanism by which this bend is removed (and the CRE site straightened) by bZIP proteins in the CREB/ATF subfamily. Paolella and coworkers made use of a series of peptides related to the CREB/ATF family member CRE-BP1 to test the prediction (*2*) that CRE straightening results from direct electrostatic interactions between the phosphodiester backbone and a cluster of basic residues conserved among these proteins. This experiment pointed to a direct interaction between one or two basic side chains and a single phosphate within the base-paired $T_{-4}G_{-3}A_{-2}$ /$T_2C_3A_4$ sequence (*35*). The single phosphate was localized to between T_2 and C_3. These interactions neutralize charge on the convex surface of the bend, leading to an induced bend toward the minor groove and ostensibly straight DNA. Moreover, replacement of the anionic T_2-pC_3 phosphate with a neutral methylphosphonate analogue straightened the CRE site in the absence of a bound bZIP protein. This result confirms that the CRE bend depends critically on distortion within the TGA sequence.

In the GCN4 bZIP·CRE structure, the two TGA sequences exhibit standard B-form structural parameters, whereas the central CG sequence does not (*47*, *48*). The DNA contains an overall bend of approximately 20° into the major groove—close to the 10−13° bend in the free DNA (*2*)—that results from several distortions within the central CpG sequence: the deoxyribose of C_{-1} exists in a C3′ *endo* conformation; the distance between the phosphates of the central $C_{-1}G_1$ dinucleotide step is 5.9 Å; and the central $C_{-1}G_1$ base pairs display high positive roll and large inclinations (*48*). By contrast, the base pairs of the $T_{-4}G_{-3}A_{-2}$ sequence display B-form structural parameters such as those in the GCN4·AP-1 (*49*) and fos-jun·AP-1 structures (*50*). Our data indicate that the central C·G base pairs are not distorted in the free CRE DNA: exchanging the central CpG sequence for a GpG, CpC, or GpC sequence has no effect on the overall DNA bend. Thus, our results suggest the GCN4 bZIP induces two structural changes within the CRE site: the major groove bend in the TGA sequence is straightened and the central CpG sequence is bent toward the major groove. These two structural changes compensate for one another, resulting in no change in the overall bend as detected by gel or minicircle cyclization methods.

Which interactions between the GCN4 bZIP and the CRE site might cause these structural changes? In the GCN4 bZIP·CRE structure (*47*, *48*), the side chain guanidinium groups of Arg_{240} and Arg_{243}, buttressed by Glu_{237}, bridge the phosphates of the central $C_{-1}G_1$ base step. These interactions enforce the short (5.9 Å) inter-phosphate spacing that is characteristic of A-form DNA. The AP-1 sequence lacks a central CG base step, and thus the equivalent interactions cannot form. We suggest that interactions made by GCN4 with the center of the CRE site position the basic segment helices so that they enter the adjacent major grooves, with a concomitant widening (and straightening) of the TGA sequence. Coincidentally, this structural reorganization alters the location of the bend (from the TGA sequence to the CG sequence) but has only a small effect on its overall magnitude. Hence, straightening of the TGA sequence without bending of the central CG sequence results in the observed lack of a bend within the GCN4 bZIP·AP-1 (*49*) and fos-jun·AP-1 structures (*50*).

SUMMARY

Here we describe ligation ladder experiments designed to identify which base pairs within the CRE site are responsible for the observed intrinsic bend in an attempt to understand the origins of the dramatically different conformations of the two sites. Our data indicate that the intrinsic CRE bend results from distortion within the sequence TGA found within each CRE half-site. These two sequences are located in phase with one another in the sequence ATGACGTCAT but are not in phase in the related AP-1 sequence. This difference in phasing leads to the overall difference in bend as detected by gel-based methods. Our results confirm earlier predictions based on theory and experiment of altered structure within TG steps, provide insight into the structural reorganizations caused by bZIP proteins when they bind and bend DNA, and lead to a revision of the relationship between the structures of the free and bZIP-bound forms of the CRE and AP-1 sites.

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