Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 15, pp. 2049-2054, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00355-7

EVIDENCE FOR INDUCED DNA BENDING BY THE YEAST ZINC CLUSTER PROTEIN PUT3

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Abstract: We employed helical phasing analysis to study DNA conformation in the absence and presence of PUT3, a member of the Zn_2Cys_6 binuclear cluster family of transcription factors. Our data indicate that the PUT3 target site UAS_{10} does not bend appreciably in the absence of PUT3(1-100), but bends towards the major groove by approximately 46 degrees in the presence of PUT3(1-100). © 1997 Elsevier Science Ltd.

GAL4, PUT3, and PPR1 are members of the Zn_2Cys_6 binuclear cluster family of eukaryotic transcriptional activators.[1] Zn_2Cys_6 binuclear cluster proteins bind as dimers to upstream activation sequences (UAS) that consist of two CGG half sites arranged in an inverted, everted[2] or direct[3,4] repeat and separated by a variable number of base pairs. Like members of other transcription factor families,[5,6] certain Zn_2Cys_6 binuclear cluster proteins display half-site spacing selectivity; that is, they differentiate between target sites on the basis of the number of base pairs separating two half sites. For example, GAL4 prefers 11 base pairs (bp) between two inverted CGG triplets, PPR1 prefers 6 bp, and PUT3 prefers 10 bp (Figure 1). Zn_2Cys_6 binuclear cluster proteins differ not only in their preferred inter-half site spacings but also in their tolerance to insertions or deletions in the spacer.[7] GAL4[8,9] and PPR1[9] tolerate insertions or deletions of a single base pair in the spacer with only minimal losses in binding free energy, whereas PUT3 does not; it binds poorly if at all to target sites containing 11 or 9 bp spacers.[9] Data from X-ray crystallography of the UAS_{11} complex of a minimal GAL4 DNA binding domain (GAL4(1-65)) show the DNA binding domain to consist of a zinc binding module whose residues contact DNA directly, an extended eight amino acid linker, and a coiled coil dimerization domain.[10] Based on the observation that the extended linker in GAL4(1-65) makes no strong contacts to DNA or to other regions of the domain, it was suggested that the linker could contract to permit recognition of target sites with different half-site spacings.[10] The structure of the PPR1•UAS_6 complex supports this proposal; the eight amino acid linker is folded into a β–hairpin that positions the zinc-binding modules in a manner appropriate for recognition of UAS_6.[11]

GAL4 site	PUT3 site	PPR1 site
CGGAGGACTGTCCT**CCG**	**CGG**GAAGCGCTTC**CCG**	**CGG**CAATTG**CCG**

Figure 1. Sequences of consensus PUT3, PPR1 and GAL4 target sites.

bZIP proteins also exhibit half-site spacing specificity. In this case, half-site spacing specificity refers to the ability to discriminate differentially between CRE and AP-1 target sites that differ in the number of base pairs separating ATGA half sites.[12] Like Zn_2Cys_6 binuclear cluster proteins, certain bZIP family members tolerate insertions and deletions in the inter-half site sequence while others do not. GCN4, like PPR1 and GAL4, tolerates insertions or deletions and binds approximately equally to the CRE and AP-1 target sites (Figure 2).[13] CRE-BP1,[14] on the other hand, mimics PUT3 and tolerates deletion of not even a single base pair. The analogies between bZIP proteins and Zn_2Cys_6 binuclear cluster proteins extend to their consensus target sites: natural PUT3 and CRE-BP1 target sites are characterized by a central GC rich sequence, whereas GAL4 and GCN4 target sites are not. It has been suggested that the half-site spacing specificity of CRE-BP1 may be related to the ability to remove the intrinsic major groove bend in the CRE target site, a bend that is absent in the AP-1 target site.[5] In view of the analogies between CRE-BP1 and PUT3 and their respective target sites, we initiated an experiment to investigate intrinsic and induced curvature within the PUT3 consensus target sequence UAS_{10}.

CRE site	AP-1 site
ATGACG**TCAT**	**ATGA**C**TCAT**

Figure 2. Sequences of the consensus CRE and AP-1 target sites for bZIP proteins.

Initially we examined whether the PUT3 target site was curved intrinsically by use of a helical phasing analysis[15,16] analogous to the one used to identify intrinsic curvature in the CRE target site.[5] Phasing analysis is more sensitive to DNA curvature than other gel-based methods, and distinguishes distortions resulting from directed bends from those resulting from isotropic flexibility or other distortions. It also defines the orientation of a directed bend relative to the reference standard incorporated into the fragment. A set of test oligonucleotides was constructed that contained the PUT3 target sequence separated by a variable length linker from a 25-bp sequence containing an A-tract of defined curvature (Figure 3A). The distance between the centers of the PUT3 site and the A-tract varied in steps of 2 or 3 bp over one helical turn, rotating the center of the A-tract once around the DNA axis relative to the center of the PUT3 site. The center-to-center spacing of the CRE and AP-1 test fragments varied between 21 to 30 bp, also rotating the A-tract site over one helical turn.[5] The relative electrophoretic mobilities of the three sets of test fragments are shown in Figure 3B. As expected, the CRE test fragments, but not the AP-1 test fragments, showed considerable phase-dependent variations in electrophoretic mobility (Figure 3B). The PUT3 test fragments showed mobility aberrations intermediate between the two. To determine the orientation and magnitude of bend in the PUT3 site, we plotted the relative mobilities of the five test fragments as a function of the distance in base pairs between the centers of the two sites (Figure 3C). The constructs with the slowest mobility contained a center-to-center spacing of 26 and 35 bp, corresponding to 2.5 or 3.5 helical turns. As the A-tract sequence is known to bend toward the minor groove, this result indicates that the PUT3 target site bends slightly toward the major groove. Analysis of the differences in mobility exhibited by the PUT3 test fragments suggests that the UAS_{10} site bends minimally, by approximately 6^{o}, whereas the bends detected in the CRE and AP-1 sites measure 11^{o} and 3^{o}, respectively.[5]

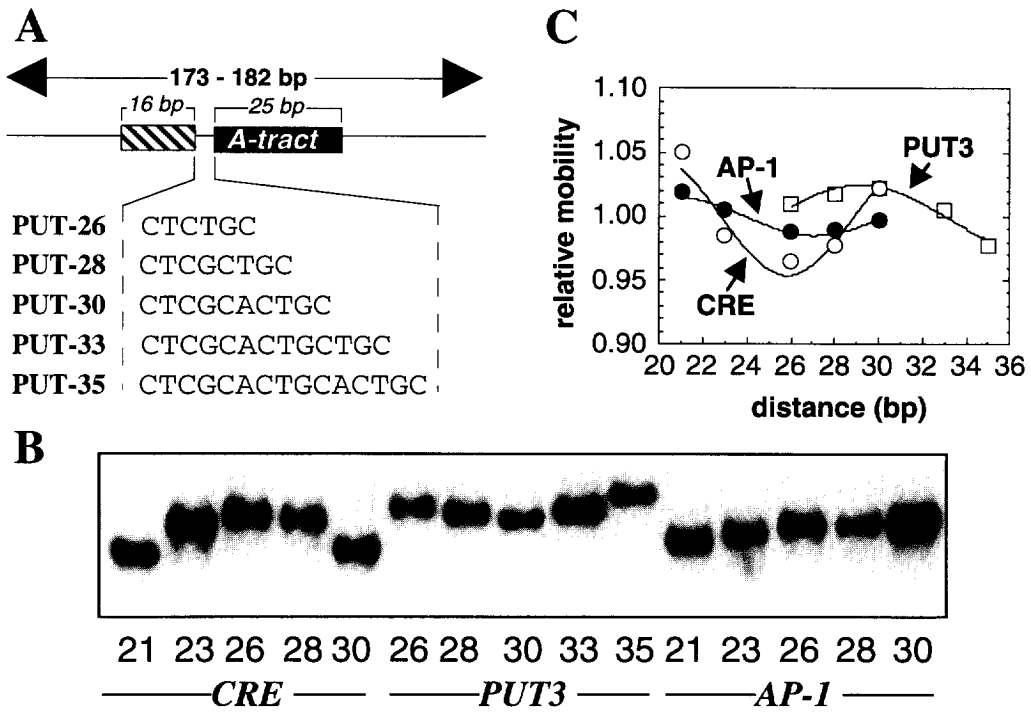

Figure 3. Phasing analysis of DNA bending in the CRE, PUT3 and AP-1 target sequences.[17] (**A**) Set of DNA test fragments used for phasing analysis of the PUT3 target site. Each fragment contains the 16 bp PUT3 site shown in Figure 1 separated by a variable length linker from a 25 bp A-tract sequence. The CRE and AP-1 test fragments have been described previously.[5] The number associated with each probe represents the distance in base pairs between the center of the target site and the 25-bp A-tract. (**B**) Autoradiogram of a nondenaturing 8% polyacrylamide gel illustrating the relative mobilities of CRE, PUT3 and AP-1 test fragments. Gels were prepared in 3/4X TG buffer [18.75 mM Tris, 162 mM glycine pH 8.9] and subject to electrophoresis at 4 °C and 12 $V \cdot cm^{-1}$. Radioactivity was quantified with a Betascope 605 Blot Analyzer and by autoradiography. (**C**) Relative mobilities as a function of the distance in base pairs between the centers of the CRE, PPR-1 and PUT3 target and A-tract sites. The data represent the average of at least four independent experiments. Error bars represent the standard deviation. The points are connected by the calculated best fit of the data to a cosine function.[16]

To examine whether the binding of PUT3 induced additional curvature in UAS_{10}, we analyzed the relative mobilities of the five UAS_{10}•PUT3(1-100) complexes (Figure 4). The five UAS_{10} test fragments showed considerably greater mobility aberrations when bound to PUT3(1-100) than when free, indicating a significant induced DNA bend. Once again, the construct with the slowest mobility contained 26 base pairs, or two and one-half helical turns of DNA, between the center of the PUT3 and A-tract sites, indicating an induced bend towards the major groove. Analysis of the mobilities exhibited by the bound UAS_{10} test fragments suggests there is a bend angle of 51° in the complex, an increase of roughly 46° over that observed in the absence of protein. Recent data from X-ray crystallography confirm that the DNA in the UAS_{10}•PUT3(1-100) complex is bent by approximately 45 degrees toward the major groove.[18,19]

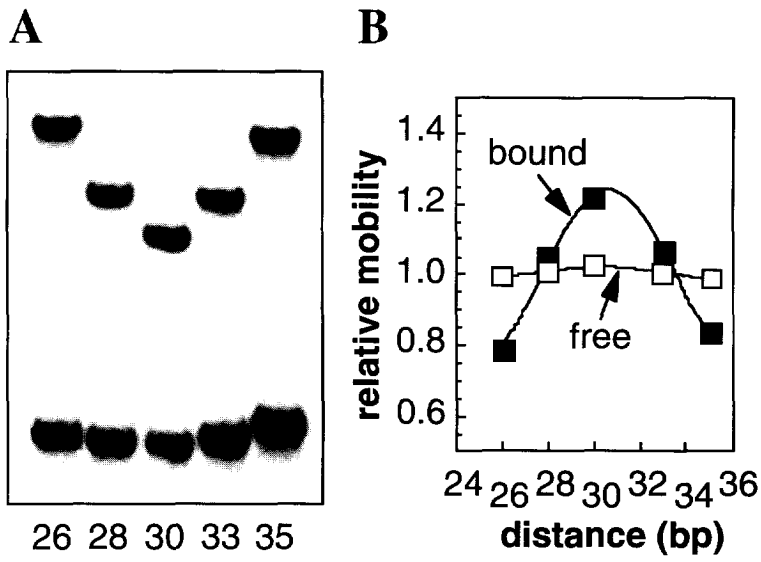

Figure 4. Phasing analysis of UAS_{10}•PUT3(1-100) complexes. (**A**) Autoradiograph of a nondenaturing 8% (32:1) polyacrylamide gel illustrating the relative mobilities of the UAS_{10} phasing fragments bound to PUT3(1-100). The UAS_{10} fragments used for phasing analysis are shown in Figure 3. Binding reactions were performed by incubation of PUT3(1-100) (20 nM) with each of the five UAS_{10} fragments in a final reaction mixture containing 2.7 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.3), 5% glycerol, 1 mM EDTA, 1 mM DTT, 0.05% NP-40 at 4 °C. Free and peptide bound fragments were resolved by electrophoresis at 4 °C and 12 $V \cdot cm^{-1}$. The running buffer contained 19 mM Tris, 162 mM glycine (pH 8.9). (**B**) Relative mobilities of free and peptide bound phasing fragments as a function of the distance in base pairs between the center of the UAS_{10} and A-tract sites. The data represent the average of at least four independent experiments; error bars represent the standard deviation. The points are connected by the calculated best fit of the data to a cosine function.[16]

In summary, we have shown that the Zn_2Cys_6 binuclear cluster protein PUT3(1-100) induces significant curvature in the UAS_{10} site upon binding. This result indicates that the PUT3 dimer is unable to present a recognition surface that is perfectly complementary to the unbound form of UAS_{10}, and the DNA must bend to ensure a proper fit. But if PUT3 requires UAS_{10} to bend to form a high affinity complex, then why does it bind UAS_{11} poorly, when this DNA sequence contains only a single additional base pair in the spacer? One explanation is that UAS_{10} and UAS_{11} differ in intrinsic flexibility; it may cost more energy to bend UAS_{11} than to bend UAS_{10}. However, recent circular permutation experiments on the GAL4(1-149)•UAS_{11} complex indicate that there is a 26° bend induced in UAS_{11} upon the binding of GAL4(1-149); this data suggests that UAS_{11} is not entirely inflexible.[20] A second possibility is that GAL4(1-100) contains a flexible segment whose counterpart in PUT3 is less flexible. A good candidate for this flexible segment is the "linker" sequence which separates the zinc cluster DNA recognition domain from the coiled coil. A third possibility is that PUT3 uses residues within the linker for sequence-specific recognition of the inter-half site sequence found in UAS_{10}.[18] Further structural

and biochemical studies will be necessary to reveal the mechanism of half-site spacing selectivity in Zn_2Cys_6 binuclear cluster proteins.

Acknowledgment. This work was supported by the NIH (GM 52544). We are grateful to Dr. Richard Reece (Harvard University) for a generous gift of PUT3(1-100) and to Dr. Ronen Marmorstein and Dr. Richard Reece for communicating their results prior to publication.

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    5' - CTAGAGAG**CGGGAAGCGCTTCCCG**CTCTGC*AAAAACGGGCAAAAACGGGCAAAAACGC*
    5' - CTAGAGAG**CGGGAAGCGCTTCCCG**CTCGCTGC*AAAAACGGGCAAAAACGGGCAAAAACGC*
    5' - CTAGAGAG**CGGGAAGCGCTTCCCG**CTCGCACTGC*AAAAACGGGCAAAAACGGGCAAAAACGC*
    5' - CTAGAGAG**CGGGAAGCGCTTCCCG**CTCGCACTGCTGC*AAAAACGGGCAAAAACGGGCAAAAACGC*
    5' - CTAGAGAG**CGGGAAGCGCTTCCCG**CTCGCACTGCACTGC*AAAAACGGGCAAAAACGGGCAAAAACGC*
    containing a UAS_{10} site (in bold) and three phased A-tracts (in italics) were cloned into pBEND2[Kim, J.; Zwieb, C.; Wu, C.; Adhya, S., *Gene* **1989**, *85*, 15-23] between the unique Xba I and Sal I restriction sites to generate plasmids pPH-PUT-26, 28, 30, 33 and 35, respectively. Recombinant plasmids were identified after transformation into competent *E. coli* GM2929 cells by screening for loss of the Sal I site. Plasmids were then sequenced by the Sanger dideoxy-mediated chain-termination method. Digestion of the plasmids with Stu I gave the a set of phasing fragments which were purified by agarose gel electrophoresis, amplified by PCR, and labeled on the 5'-end.
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(*Received in USA* 14 *May* 1997; *accepted* 3 *July* 1997)