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DNA bending by the a1 and α2 homeodomain proteins from yeast

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ABSTRACT

Structural and biochemical studies of monomer homeodomain–DNA complexes have not so far revealed any cases of pronounced DNA distortion. In this paper we show that multimeric complexes of the yeast homeodomain proteins a1 and α2 induce significant bends in their operators upon binding. Based on a series of circular permutation experiments, we found that a dimer of α2 bound to operator DNA produced a mild bend in the DNA, whereas the α2–MCM1–DNA and the a1–α2–DNA complexes exhibited much sharper bends. As these latter two complexes represent the *in vivo* form of DNA-bound a1 and α2, we conclude that, in the cell, these homeodomain proteins are associated with pronounced bends in DNA. We discuss possible roles for these bends in transcriptional repression.

INTRODUCTION

Since the introduction of the circular permutation assay (1), many sequence-specific DNA binding proteins have been proposed to bend DNA (for reviews, see 2–6). In some cases, protein-induced bending facilitates the formation of nucleoprotein complexes by juxtaposing, with fixed angles and phasing, multiple sites along DNA. These nucleoprotein complexes range from those responsible for site-specific recombination in bacteria to those involved in transcriptional activation in mammalian cells (7–12). It has also been proposed that the energy stored in a bend could be reused later, for example, to facilitate escape of *E.coli* RNA polymerase from the promoter (13). In other cases, DNA bending functions as a negative transcriptional regulator, inhibiting the formation of an activating structure (14,15). Finally, bending in some cases may simply be a consequence of an extended protein–DNA interface and may serve no additional role in the cell.

Although a number of helix–turn–helix proteins are known to bend DNA (see reviews cited above), the evidence, so far, suggests that homeodomain proteins, a large family of helix–turn–helix proteins, do not. X-ray studies (16,17) have revealed some irregularities in the DNA complexed with homeodomain proteins, but have not revealed any pronounced bends. In addition, circular permutation experiments have shown that the homeodomains of several POU class proteins also do not produce a significant bend upon DNA binding (18). It should be noted, however, that all of these studies have utilized homeodomain monomers. Ceska *et al.* (19) have suggested, based on model-building studies, that interaction of an LFB1 homeodomain dimer with DNA may require the DNA to be bent.

We wished to determine whether two homeodomain proteins from yeast—a1 and α2—produced a bend upon specific DNA binding. Since these two homeodomain proteins have obligate protein partners (for review, see 20), we monitored DNA bending in the presence of these partner proteins. We utilized two naturally occurring operators for these studies. The first, the a-specific gene (asg) operator from the STE6 gene, is bound cooperatively by a dimer of α2 and a dimer of MCM1, and the binding of both proteins is required to turn off transcription of STE6. MCM1, a yeast protein related to the human SRF proteins, binds DNA using a so-called MADS domain (21), about which structural information is currently lacking. We monitored the asg operator for possible bending by an α2 dimer, by an MCM1 dimer, and by the combination of the two. The second operator, the haploid-specific gene (hsg) consensus operator (22), is bound cooperatively by the two homeodomain proteins a1 and α2. The binding of these proteins to the hsg operator, which occurs as a heterodimer, turns off transcription of adjacent genes. We tested for bending of the hsg operator produced both by a dimer of α2 and by the a1–α2 heterodimer.

MATERIALS AND METHODS

Hydroxyl radical protection

Hydroxyl radical protection reactions were carried out in a buffer containing 10 mM Tris–HCl (pH 7), 0.1 mM EDTA, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 2.5 mg/ml calf-thymus DNA and 50 mg/ml BSA, using the procedures of Tullius and Dombroski (23) [see Sauer *et al.* (29) for autoradiographs of this experiment]. The autoradiographs were scanned on an Ephortec, Joyce-Lobel densitometer with the assistance of Joe Day in the laboratory of Herbert Boyer.

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Synthetic operators and plasmid constructions

The WT operator was created by synthesizing a duplex corresponding to the sequence of the STE 6 asg operator: TCGACATGTAATTACCTAATAGGGAAATTTACACGC. The nucleotide sequence of the right half-site is TCGACATAGGGAAATTTACACGC and that of the left half-site is TCGACATGTAATTACCTAATAGC. The sequences of Δ1–Δ13 are given in Figure 3A and Smith and Johnson (24). Both strands of all operators were designed to leave 5′ TCGA overhangs on each end so that when hybridized, each duplex has one *Sal*I end and one *Xho*I end. Duplexed operators were cloned into the *Xho*I site of pGD579X. pGD579X is a derivative of pGD579. pGD579 was provided by Bob Sauer and is pBR322 with a tandem repeat of base pairs 1–375 and a 20 bp polylinker, GAGCTCCCGGGTCTAGATC, between the repeats; pGD579X has a *Xho*I linker was inserted into the *Sma*I site of the polylinker. Recombinant plasmids were transformed into *E.coli* and identified by colony hybridization, using one strand of the duplex as a probe. A single copy insert was verified by double-strand sequencing with Sequenase, using a primer that hybridizes to the linker region between the 375 bp repeats.

Operators were labeled with ^{32}P for use as probes by cutting plasmids with appropriate enzymes and filling in 5′ overhangs (4 bp for all but the fragments cut with *Eco*RV) with Klenow fragment.

Proteins

α2 protein, a gift of Arkady Mak, was purified according to (29). Fragments 1–96 of MCM1, a gift of Drew Vershon, was purified according to (41) and a1, a gift of Caroline Goutte, was purified according to (22).

Mobility shift assays

For Figures 2 and 3, position permutation experiments were performed in the absence of protein. Reactions were electrophoresed on 6%, 1 × TBE, native polyacrylamide gels run at 200 V for 7 h at 4°C. Gels were then stained with ethidium bromide and photographed.

For Figure 4A and B, gel mobility shift assays were performed with added protein in a buffer containing: 20 mM Tris, pH 8; 0.1 mM EDTA; 10 mg/ml BSA (fraction V); 5% glycerol and 10 ng/ml *E.coli* DNA cut with *Hae*III. The appropriate end-labeled operator DNA was added (to ~0.5 nM) along with purified α2, and/or MCM1, at the concentrations given in the figure legends. The reactions were mixed and incubated at room temperature for 60 min. These experiments were electrophoresed through 6%, 1 × TBE, native polyacrylamide gels at 200 V for 2 h at room temperature. Gels were then dried and autoradiographed.

RESULTS

The asg operator contains a sequence directed bend

Experiments in which naked operator DNA was cleaved by hydroxyl radicals suggested that the asg operator was slightly bent in the absence of bound proteins. Hydroxyl radicals, which cleave at sugar residues along the DNA backbone, normally show little or no base sequence preference; however, cleavage of bent DNA produces a characteristic non-uniform cleavage pattern (23). With the α2 operator, a small area of decreased cutting in both the top and the bottom (see bottom panel of Fig. 1) strands was observed. One explanation for the abnormality is the existence of a bend in the DNA.

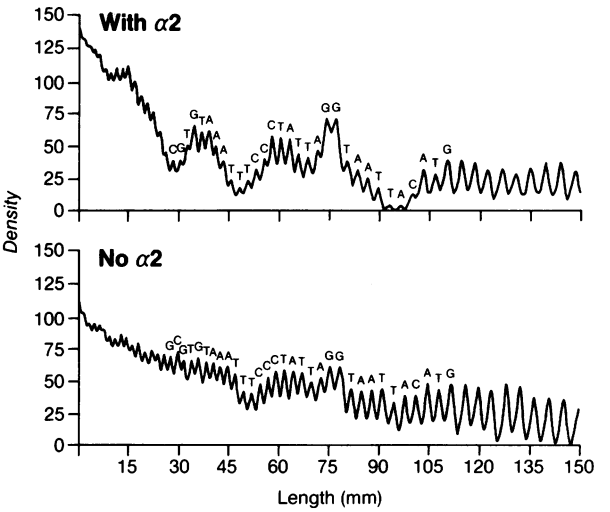

Figure 1. The STE6 operator shows a discontinuity in hydroxyl radical cleavage. Densitometer scans of the hydroxyl cleavage pattern of the top strand of the STE6 operator in the presence (**A**) or the absence (**B**) of α2 protein. The letters above each peak represent the base whose attached deoxyribose was cleaved. The area of lowest density in the 'no α2' panel corresponds to a region in the right half-site of the operator.

In order to test this idea, we performed a position permutation experiment (1). The asg operator was placed at different positions within DNA fragments of identical size and composition, and the relative mobility of these fragments through an agarose gel was measured. A fragment where the operator was positioned in the middle migrated more slowly through gels than those with the operator positioned at either end (see Fig. 2A and B). Analysis of the entire set (Fig. 2C) suggests that the DNA is bent at a position within the operator, near the right half-site. This explanation is consistent with our interpretation of the hydroxyl radical experiments which also suggested a bend near the right half-site.

In order to identify more directly which bases contribute to DNA bending, similar experiments were performed with a series of mutant operators in which successive 2 base pair (bp) deletions were made beginning with the center of the operator: Δ1, Δ3, Δ5, Δ7, Δ9, Δ11 and Δ13, where Δ1 refers to a 1 bp deletion, Δ3 a 3 bp deletion, etc. (see Fig. 3A for operator sequences). Mutant operators containing only the left or the right α2 half-sites were also tested. These experiments are summarized in Figure 3B and C. Starting with Δ9, significant loss the position-dependent migration is seen and with the deletion of an additional 2 or 4 bp (Δ11, Δ13), it is completely lost. The isolated left half-site exhibits no apparent bend and the right half-site appears to be bent to the same degree as the whole operator. In summary, these experiments indicate that there is a modest bend in the right half of the asg operator and the effect of this bend is fully retained with Δ7 but is disrupted with Δ9 and completely lost with Δ11. This would place the bend near or within the AT-rich sequence of the right half-site.

DNA bending of the asg operator by α2 and MCM1

The α2 and MCM1 proteins bind cooperatively to the asg operator (see Introduction), and we next determined how the

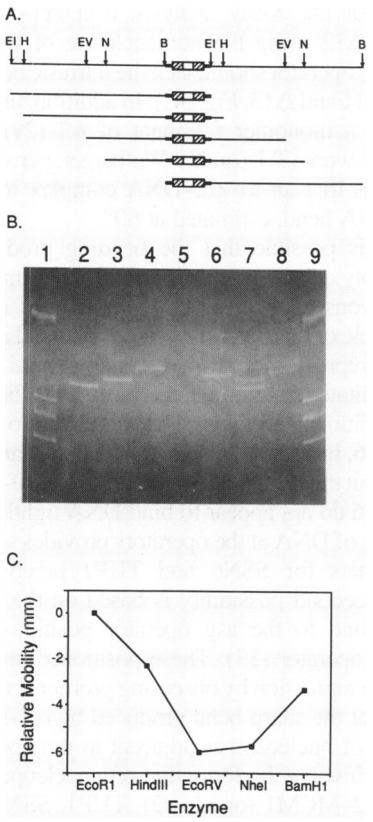

Figure 2. The STE6 operator exhibits a slight bend. The STE6 operator was placed at different positions within fragments of identical size and composition. Each fragment is ~440 bp long including the first 375 bp of pBR322, 24 bp of linker DNA and a 36 bp wild type operator. (**A**) indicates where the operator is positioned in fragments cut with five different restriction enzymes. Letters indicate the following restriction sites: EI, *Eco*RI; H, *Hin*dIII; EV, *Eco*RV; N, *Nhe*I and B, *Bam*HI. Lanes 2–9, in (**B**) show the mobility of DNA cut with the following enzymes: lane 2, *Eco*RI; lane 3, *Hin*dIII; lane 4, *Eco*RV; lane 5, *Nhe*I and lane 6, *Bam*HI. Lane 7 contains a mixture of DNA fragments cut with *Eco*RI, *Hin*dIII and *Eco*RV. Lane 8 contains a mixture of fragments cut with *Nhe*I and *Bam*HI. Lanes 1 and 9 show φX174 DNA cut with *Hae*III as molecular weight markers. (**C**) is a graphic depiction of the data shown in (B). The probable center of bending lies somewhere in the right half-site.

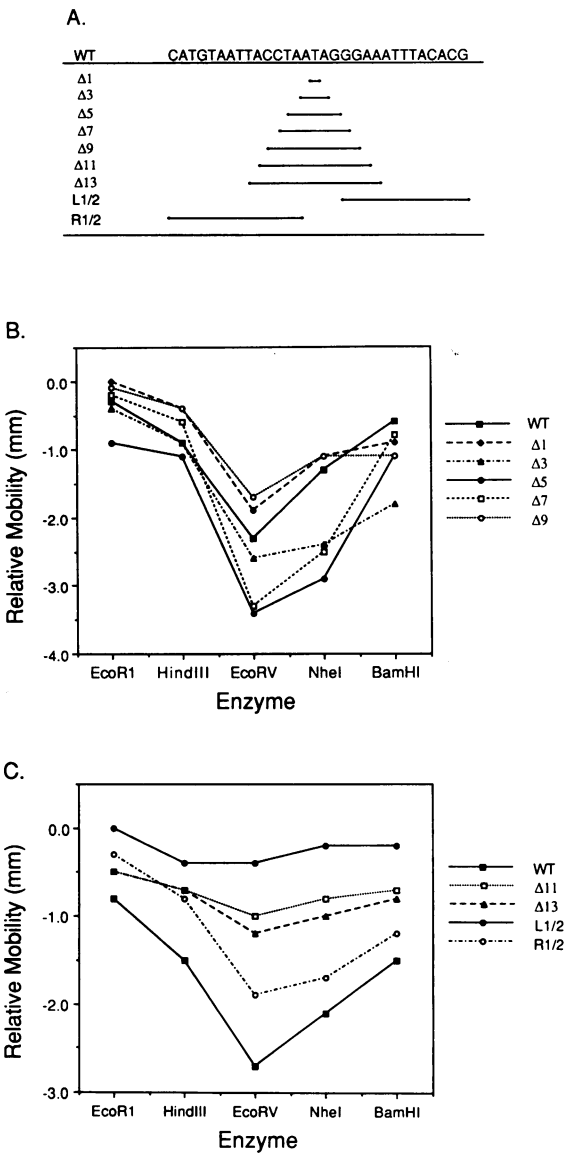

Figure 3. The bend in the STE6 operator maps to the right half site. The same experiment as described and analyzed in Figure 2 was performed, this time using fragments bearing various mutant operators. (**A**) shows the sequence of the wild type STE6 operator. Below this sequence, bars are drawn to indicate the bases deleted in the mutant operators. (**B** and **C**) graph the mobility seen for operator-bearing fragments cut with each of the five enzymes indicated. The wild type (WT) operator is grouped with the Δ1, Δ3, Δ5, Δ7 and Δ9 operators in (B). In a separate experiment, shown in (**C**), the WT operator is grouped with the Δ11, Δ13, the isolated left half-site and the isolated right half-site operators.

binding of α2, MCM1 or a combination of both proteins changed the bend angle of the operator. As indicated by the data in Figure 4A, the binding of an α2 dimer (24) induces further bending at approximately the same position that is bent in naked DNA, and a dimer of MCM1 alone introduces a much larger bend, also apparently at the same position. Both proteins together induce a bend somewhat more extreme than that seen with MCM1 alone. We roughly estimated the protein-induced DNA bending angle from the circular permutation experiment pictured in Figure 4A using the empirical relationship of Thompson and Landy (25). Accordingly, we estimate that the unoccupied asg operator is bent by 20°, the α2 dimer–operator complex by 45° and the MCM1 dimer–operator complex by 120°. When MCM1 and α2 dimers are bound together, the calculated bend angle is 145°. It is important to note that these bend angles should be regarded as crude estimates, particularly since the latter is outside the suggested limits of the Thompson and Landy relationship (25). The DNA bending by MCM1 alone is consistent with the results of Gustafsen, *et al.* (26), who showed that the binding of the related human protein SRF to DNA induces a pronounced bend.

DNA bending of the hsg operator by a1 and α2

Unlike the asg operator, the hsg operator uncomplexed with protein does not appear to be naturally bent. As shown in Figure 4 (lanes 1–4), a dimer of α2 slightly bends the hsg operator, and the a1–α2 heterodimer (lanes 5–8) induces a more pronounced bend. The nominal estimates, based on the Thompson and Landy relationship (25), are 45° and 100° respectively. We next

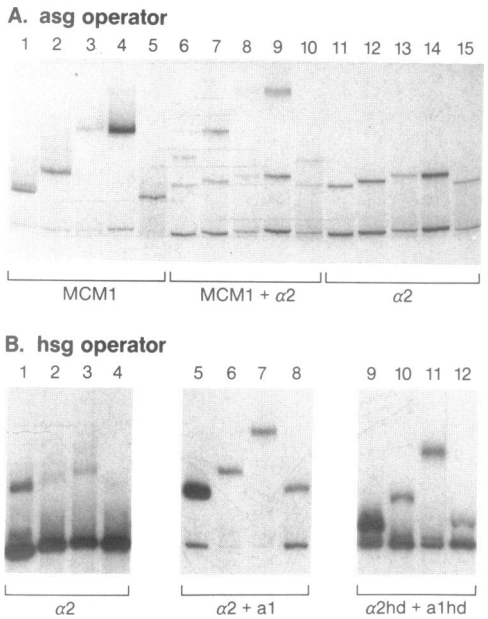

Figure 4. (**A**) DNA bending by α2, MCM1 and the α2–MCM1 combination. Fragments bearing the STE6 operator at various positions were incubated with α2 at 6.8×10^{-9} M (lanes 11–15), MCM1 (a fragment of MCM1 containing amino acids 1–96) at 1.9×10^{-6} M (lanes 1–5) or a combination of α2 at 6.8×10^{-9} M and MCM1 at 9.8×10^{-8} M (lanes 6–10). Fragments were made by cutting with *Eco*RI (lanes 1, 6 and 11), *Hin*dIII (lanes 2, 7 and 12), *Eco*RV (lanes 3,8 and 13), *Nhe*I (lanes 4, 9 and 14) or *Bam*HI (lanes 5, 10 and 15). (**B**) DNA bending by a1 and α2. Fragments bearing the consensus hsg operator (22) at various positions were incubated with α2 and a1 at equimolar concentrations ($\sim 3.0 \times 10^{-8}$ M). Fragments were made by cutting with *Eco*R1 (lanes 1, 5 and 9); *Hin*dIII (lanes 2, 6 and 10); *Nhe*I (lanes 3, 7 and 11) or *Bam*HI (lanes 4, 8 and 12). Operators were labeled with ^{32}P and gel mobility assays were performed as described above with α2, a combination of α2 and a1, or a combination of α2 and a1 homeodomain fragments.

determined whether this bend was produced directly by the homeodomains of a1 and α2 or whether it was dependent on other regions of the proteins which could, in principle, also contact DNA. The minimal fragments required for tight cooperative binding of a1 and α2 are the homeodomain of a1 and a fragment of α2 that contains the homeodomain plus a 20-amino acid 'tail' that contacts a1 (22,27,28). We found that interaction of these fragments with the hsg operator induced a bend of approximately the same magnitude as did the full-length proteins (Fig. 4, lanes 9–12). As we are unable to reliably detect specific binding of a1 alone to the hsg operator (22) we were unable to monitor the effects of a1 bound in isolation to DNA.

DISCUSSION

We have shown that a dimer of the yeast homeodomain protein α2 modestly bends both the asg and hsg operators, and a dimer of α2 in combination with a dimer of MCM1 induces a much more pronounced bend into the asg operator. An a1–α2 heterodimer sharply bends the hsg operator, and we showed that this bending is attributable to the homeodomain portions of a1 and α2.

On the surface, these results seem at odds with the crystallographic model of α2 bound to DNA (17) where some DNA distortion was noted but no indication of a bend was seen. However, the two sets of observations were made using significantly different DNA and protein components and are easily reconcilable. For the X-ray analysis, a center-deleted operator (equivalent to Δ12 using the nomenclature of Figure 3A) was utilized, and this operator should lack the intrinsic bend of the intact operator (see Δ11 and Δ13, Fig. 3C). In addition, the crystals were obtained using a monomer fragment of α2 (29). More recent crystallographic work (T. Li and C. Wolberger, personal communication) indicates that an a1–α2–DNA complex indeed shows a pronounced DNA bend, estimated at 60°.

Although it is possible that the bending produced by these proteins is simply a feature of the protein–DNA interface and has no additional consequence, several possibilities do exist for a physiological role of this bending. α2–MCM1 and a1–α2 are both transcriptional repressors and there exists several ways that the bend could facilitate repression. First, these DNA binding proteins recruit two additional proteins, TUP1 and SSN6, to the DNA. TUP1 and SSN6, in addition to carrying determinants that interact with α2, carry out the actual repression reaction (30–32). Although TUP1 and SSN6 do not appear to bind DNA tightly, it is possible that the bending of DNA at the operators provides some additional favorable contacts for SSN6 and TUP1 hereby aiding their recruitment. A second possibility is based on the discovery that α2–MCM1 bound to the asg operator positions nucleosomes adjacent to the operator (33). These positioned nucleosomes are thought to aid in repression by obscuring promoter sequences (34); it is possible that the sharp bend produced by α2–MCM1 aids in the positioning of nucleosomes adjacent to the operator. Thirdly, repression may involve the formation of a nucleoprotein complex composed of α2–MCM1 (or a1–α2), TUP1, SSN6 and perhaps some of the general transcription factors that assemble around the start point of transcription (35,36). DNA bending could promote the assembly of such a repressing complex by facilitating protein nucleic acid interactions, as previously demonstrated for recombination and transcriptional complexes (8,9,11,12,37,38). Fourthly, several cases have been described in bacteria (14,15) where a correctly phased bend inhibits transcription by preventing the formation of a competing, activating complex. However, since repression brought about by the asg operator is independent of its position and orientation (30,39,40), this model seems unlikely to apply for α2. Despite these diverse possibilities, we do know that the α2–MCM1-directed bend in the operator DNA is not sufficient for repression *in vivo*. Yeast strains deleted for SSN6 are completely deficient in α2–MCM1-mediated repression, yet α2 and MCM1 are still bound to the operator *in vivo* as determined by DMS protection experiments (30).

In summary, we have shown that the DNA binding of two homeodomain proteins from yeast, a1 and α2, is associated with pronounced bends in the DNA. For the case of the hsg operator, we have shown that the bending angle can be induced by minimal homeodomain fragments of a1 and α2. Although the DNA bends are not sufficient for transcriptional repression, induction of a structural distortion in the bound DNA may facilitate the mechanism by which these proteins repress transcription.

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