DNA determinants in sequence-specific recognition by *Xma*I endonuclease

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Received March 20, 1995; Revised and Accepted June 23, 1995

ABSTRACT

The *Xma*I endonuclease recognizes and cleaves the sequence C↓CCGGG. Magnesium is required for catalysis, however, the enzyme forms stable, specific complexes with DNA in the absence of magnesium. An association constant of $1.2 \times 10^9/\text{M}$ was estimated for the affinity of the enzyme for a specific 195 bp fragment. Competition assays revealed that the site-specific association constant represented an $\sim 10^4$ -fold increase in affinity over that for non-cognate sites. Missing nucleoside analyses suggested an interaction of the enzyme with each of the cytosines and guanines within the recognition site. Recognition of each of the guanines was also indicated by dimethylsulfate interference footprinting assays. The phosphates 5' to the guanines within the recognition site appeared to be the major sites of interaction of *Xma*I with the sugar-phosphate backbone. No significant interaction of the protein was observed with phosphates flanking the recognition sequence. Comparison of the footprinting patterns of *Xma*I with those of the neoschizomer *Sma*I (CCC↓GGG) revealed that the two enzymes utilize the same DNA determinants in their specific interaction with the CCGGG recognition site.

INTRODUCTION

A number of different amino acid sequence motifs and protein secondary structures have now been identified as sequence-specific DNA binding domains (1–3). A critical function of these domains is the ability to discriminate specific nucleotide sequences from a large excess of non-specific binding sites. Recent structural analyses of several protein–DNA complexes has revealed the complex patterns of interactions at the protein–DNA interface that underlie the binding specificity (4–12). Insight into mechanisms of sequence discrimination has also been afforded by analyses of proteins that have the same structural binding domain and that recognize related sequences. For example, the bHLH motif is utilized by a family of transcription factors that recognize the consensus sequence, CANNTG (13). Within the conserved helical DNA binding domain amino acids have been identified that specify recognition of the outer (CA – – TG) base

pairs (14,15). Sequence comparisons between members of this family have further enabled identification of the single amino acid position that confers specificity for either CG or GC at the central bases (16). In a similar manner the specificity of the bZip protein GCN4 can be varied by selective replacement of amino acids within the basic region of the DNA binding domain (17). However, for other DNA binding motifs, such as the helix–turn–helix motif, in which the orientation of the binding domain and the DNA substrate varies between different protein–DNA complexes, identification of amino acids conferring sequence discrimination is more difficult to predict (18). Furthermore, little is known of how different types of protein structures may recognize the same DNA sequence.

The type II restriction endonucleases are frequently used as model systems for analyzing protein–DNA interactions (19–21). However, it has been difficult to define a general relationship between the structure and the recognition and cleavage specificity for this class of DNA binding proteins. Crystal structures of the *Eco*RI–DNA (22), *Eco*RV–DNA (23,24) and *Pvu*II–DNA (25) complexes have revealed distinct protein structures and modes of docking to the DNA for each of these enzymes. Biochemical studies of *Eco*RI and *Eco*RV have further revealed that these enzymes are distinguished by the origin of their specificity: *Eco*RI demonstrates sequence-specific binding in the absence of magnesium (26), whereas *Eco*RV reportedly binds to all DNA sequences with similar affinity (27,28). The cleavage specificity is conferred by the preferential and high affinity binding of magnesium when the protein is bound to the specific recognition site (28,29). Despite the significant differences in the recognition properties, the structures of the catalytic site of several of the enzymes appear to be conserved (24,25,29,30).

Detailed analyses of the mechanism of sequence discrimination by endonucleases has been limited and those that have been studied rigorously, such as *Eco*RI and *Eco*RV, have different recognition specificities. Nonetheless, there exist families of endonucleases comprised of both isoschizomers, having identical recognition and cleavage specificity, and neoschizomers, that recognize the same site but cleave at different positions within the site. These enzymes provide an opportunity to study not only the mechanism(s) by which the endonucleases may interact with the same DNA sequence, but also to determine how the requirements for recognition and catalysis are reconciled.

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The *SmaI* and *XmaI* endonucleases recognize the sequence CCCGGG and cleave at different positions within the sequence to yield blunt and 5' cohesive termini, respectively. Sequence analysis has failed to reveal any primary structural similarity between the enzymes (31,32). We have recently characterized the interaction of *SmaI* with its recognition sequence (33). In the current study the sequence-specific binding of *XmaI* has been examined and the patterns of base and phosphate contacts made by the endonuclease have been identified. Comparison with the *SmaI* endonuclease suggests that despite the different positions of cleavage within the recognition site, these enzymes appear to utilize very similar DNA (base and phosphate) determinants in their specific interactions with the same DNA sequence.

MATERIALS AND METHODS

Enzymes and chemicals

Synthetic oligonucleotides were prepared by solid phase phosphoramidate synthesis at the Macromolecular Structure Facility, Michigan State University. T4 polynucleotide kinase was from New England Biolabs. [γ -³²P]ATP (>3000 Ci/mmol) was obtained from Amersham Corp. NA-45 DEAE membranes, from Schleicher and Schuell, were used according to manufacturer's instructions. Bio-gel P6 was obtained from BioRad. Formic acid, dimethylsulfate and piperidine were obtained from Aldrich. Ethylnitrosourea was purchased from Sigma and hydrazine was supplied by Kodak. Duracryl acrylamide was purchased from Millipore Corp. *XmaI* endonuclease was provided by New England Biolabs. Analysis of the endonuclease preparation by SDS-PAGE and silver staining revealed only one predominant band, which corresponded to the 37 kDa *XmaI* endonuclease. No cognate methylase activity was detected in the endonuclease preparation. The enzyme was diluted in binding buffer immediately before use. The concentration of endonuclease dimer ($[E]_0$) was estimated from the x -axis intercept in Scatchard plots of equilibrium binding data (34).

Preparation of DNA substrates

DNA oligonucleotide substrates used for footprinting analyses were 5'-end-labeled with T4 polynucleotide kinase and purified from truncated fragments by electrophoresis on denaturing 18% polyacrylamide gels. The single-stranded DNA was recovered by electroelution onto NA-45 membranes as previously described (33). The complementary strands were annealed by heating at 90°C for 5 min in a buffer containing 66 mM Tris-HCl pH 7.6, 7 mM MgCl₂, 3 mM DTT and then slowly cooling to room temperature. The sample was desalted by gel filtration on a BioRad P6 column, from which the DNA was eluted with 20% ethanol. The duplex oligonucleotide was dried under vacuum and then resuspended in water immediately before use. The DNA concentration was estimated from the absorbance at 260 nm.

Analysis of protein-DNA complexes

Protein-DNA complexes were formed by incubation of the enzyme with labeled DNA substrate in a binding buffer containing 20 mM HEPES pH 7.0, 50 mM potassium glutamate, 0.5 mM EDTA and 1 mM 2-mercaptoethanol. Reactions were carried out in a 20 μ l volume and the samples were incubated for 1 h at 37°C. At the completion of the reaction 2 μ l 0.1%

bromophenol blue (in binding buffer) were added and the samples were separated by electrophoresis on 7.5% polyacrylamide gels. The gel and electrophoresis running buffer was 20 mM HEPES, pH 7.4, containing 2 mM EDTA. Electrophoresis was carried out at room temperature at a constant voltage of 10 V/cm. The protein-DNA complexes were detected by autoradiography of the dried gel.

Determination of the equilibrium binding constant

The substrate used for the binding assays was a 195 bp fragment, generated by PCR amplification of the sequences surrounding the *XmaI* site in the multiple cloning site of M13mp18. The sequence of the primers (primer set C), the conditions for the amplification reactions and purification of the fragment were carried out as previously described (35). Binding assays were carried out as described above and binding isotherms were generated using 0.75 nM *XmaI* (dimer) and 0.01–7.5 nM DNA. After electrophoresis the distribution of radioactivity between protein-bound and free DNA was quantitated on an AMBIS Radioanalytical Imaging System (AMBIS, San Diego, CA). The association constant was determined from Scatchard analysis of the binding data using Enzyfitter software (Biosoft, Cambridge, UK). The non-specific binding constant was estimated from equilibrium competition assays as described by Terry *et al.* (26). The competitor DNA used in these assays was a 185 bp fragment obtained from PCR amplification of sequences adjacent to the *XmaI* site in M13mp19 and generated as previously described (33).

Interference footprinting assays

The substrate used for footprinting analyses was a 30 bp duplex generated by annealing equimolar concentrations of oligonucleotide I:

5'-GCATGCACATGACTGGCCCGGGATCCAGTA-3',

and oligonucleotide II:

3'-ACGTGTACTGACCGGGCCCTAGGTCATCGT-5'.

Dimethylsulfate modification was carried out according to published procedures (36) using 10 μ g duplex oligonucleotide in which only one of the strands was either 5'- or 3'-end-labeled. The resulting methylated DNA was precipitated with ethanol in the presence of 5 μ g tRNA, washed twice with 80% ethanol and dried under vacuum. Binding of *XmaI* to the methylated DNA was carried out as described above and the protein-DNA complexes and free DNA were separated by electrophoresis on 10% polyacrylamide gels. The DNA species were recovered by electroelution and subsequently cleaved at the sites of modification by incubation of the sample in 100 μ l 10% piperidine at 90°C for 30 min. Samples were lyophilized three times to remove the piperidine, resuspended in loading buffer and electrophoresed on denaturing 20% polyacrylamide gels.

The depurination and depyrimidination (missing nucleoside) reactions were carried out on end-labeled, single-stranded oligonucleotides which were subsequently annealed to the complementary, unmodified strand. The depurination reactions, using formic acid, and the pyrimidine modifications were carried out with 10 μ g substrate DNA essentially as described by Brunelle and Schlieff (37). However, the pyrimidine modification reactions were terminated by the addition of 200 μ l 0.3 M sodium acetate, pH 7.0, containing 1 mM EDTA. *XmaI* binding reactions and subsequent analysis of the protein-bound and free DNA was

Figure 1. Analysis of sequence-specific binding by the *XmaI* endonuclease. (A) Titration of 0.75 nM *XmaI* with increasing concentrations of a specific 195 bp DNA fragment. Lanes 1–7 correspond to 0.05, 0.10, 0.25, 0.5, 1.0, 2.5 and 5 nM DNA, respectively. (B) Binding isotherm (inset) and the derived Scatchard analysis of the binding data shown in (A). (C) Dixon plot representing competition for *XmaI* binding by a 185 bp fragment lacking the CCCGGG recognition site.

carried out as described above. Phosphate alkylation with ethyl nitrosurea was carried out according to published procedures (33).

Band intensities on the sequencing gels used in the interference assays were quantitated after scanning the autoradiograms with a Bio-Image (Kodak) laser scanning densitometer. The data were analyzed with Visage system software (Milligen). Peak areas were used as a measure of image density.

RESULTS

Binding specificity of the *XmaI* endonuclease

The *XmaI* endonuclease forms stable complexes with DNA in the absence of magnesium. Incubation of the enzyme with a 195 bp substrate containing one copy of the CCCGGG recognition site resulted in the appearance of a single protein–DNA complex in gel retardation assays (Fig. 1A). Titration of the enzyme with increasing concentrations of the substrate resulted in a hyperbolic binding isotherm, as shown in Figure 1B. Scatchard analysis of the binding data yielded a value of $1.25 \pm 0.15 \times 10^9/M$ for the specific association constant. No complexes were detected when DNA fragments lacking the recognition site were used as substrates for binding under the same conditions. Further

analyses, by competition assays (Fig. 1C), of the specificity of *XmaI* resulted in a K_i of $2.4 \pm 0.74 \times 10^7/M$ for non-specific binding of *XmaI* to a 185 bp fragment lacking the recognition sequence. However, if each hexanucleotide within this fragment is considered as a potential non-specific binding site the resulting affinity for non-cognate sites ($K_i/\text{number of binding sites}$; 38) is estimated at an average of $1.5 \times 10^5/M$. Consequently, the affinity of *XmaI* for the specific recognition site is $\sim 10^4$ times that of random sequences.

XmaI–DNA complexes were readily observed in gel retardation assays when using either short duplex (22mer and 30mer) oligonucleotide substrates or the longer DNA fragments (such as the 195 bp substrate shown above). However, the ability to detect the complexes was critically dependent upon the conditions employed for electrophoresis. Complexes were not detected when electrophoresis was carried out in buffers of pH >7.5. Furthermore, titration of a fixed concentration of the specific DNA fragment with increasing concentrations of the enzyme often resulted in smearing and a lower electrophoretic mobility of the retarded complex. However, silver staining of retardation assay gels, in which the control lanes contained the same concentrations of *XmaI* but lacked the DNA substrate, revealed that at higher concentrations of the enzyme (>5 nM) smearing

Figure 2. Missing nucleoside analyses of *Xma*I binding to the top (A) and bottom (B) strands of the 30 bp duplex recognition oligonucleotide. Lane (G+A) corresponds to the fragments generated from acid depurination of the substrate; (T+C) represents the products from depyrimidation of the DNA. Fragments in lanes B (bound) and F (free) were generated from the DNA isolated from the protein–DNA complexes and free DNA, respectively. (C) Histogram summary of the missing nucleoside analyses for *Xma*I binding to the recognition oligonucleotide. Relative intensity represents the ratio of intensities of the free to bound sample for each fragment. (D) DMS interference footprinting assays of the *Xma*I–DNA complexes. Lanes G and C correspond to the G- and C-specific (Maxam–Gilbert) sequencing products.

was also observed in these control lanes. The anomalous electrophoretic mobility of the complexes during protein titrations of the DNA, therefore, appears to reflect the electrophoretic behavior of *Xma*I, rather than a property of the protein–DNA complexes. [In contrast, at equivalent protein and DNA concentrations *Sma*I–DNA complexes migrate as a single band in the retardation gels (33). The *Sma*I endonuclease also migrates as a discrete band under the same electrophoretic conditions.] Determination of the affinity constants for *Xma*I was, therefore,

carried out by varying the DNA, rather than the protein, concentration.

Analysis of protein–base interactions

The potential base contacts made by the *Xma*I endonuclease were identified by missing nucleoside interference analyses (37,39). The analyses were carried out on a 30 nt duplex fragment and resulted in footprinting patterns for the top and bottom strands of

the substrate, as exemplified in Figure 2A and B. It should be noted that misincorporation during synthesis of the top strand oligonucleotide generated some fragments containing dG, rather than dC, at the third base of the recognition site. The mixed bases at this position are apparent in the control T+C lane in Figure 2A. However, the presence of the mixed bases does not interfere with the footprinting experiments, since the fragments with the incorrect recognition sequence are not bound by the enzyme. Comparison of the relative intensity of each of the fragments in the bound and free lanes revealed that modification of any of the guanines and cytosines within the recognition site markedly reduced binding by *XmaI*. These observations suggest that *XmaI* interacts with each of the bases within the recognition sequence. In the autoradiogram shown in Figure 2A the reduction in the relative intensity of the fragment corresponding to the second cytosine of the recognition site appears less than that of other bases. However, in other experiments and, as reflected in the histogram summary (Fig. 2C) and the analysis of the complementary strand of the duplex (Fig. 2B), deletion of the second cytosine of the recognition site significantly reduces binding of the enzyme.

The role of the guanines in the *Xma*I–DNA interaction was also investigated by dimethylsulfate interference assays (Fig. 2D). Footprinting analyses revealed that in the protein–DNA complexes there was an absence of fragments corresponding to methylation of any of the guanines within the recognition site. These results are consistent with the missing nucleoside analyses and further implicate interactions between *Xma*I and each of the guanines in the protein–DNA complex. However, steric hindrance, arising from the methyl group, rather than the loss of a hydrogen bond, could also account for the observed footprint.

The histogram summary shown in Figure 2C reveals that in addition to the apparent interaction of *Xma*I with each of the bases within the recognition site, there was also a small increase in the ratio of the relative intensity of the bases immediately flanking the recognition site. However, the effect was considerably less significant than the large changes observed for the recognition site bases. It is possible that removal of the flanking bases results in local structural perturbations at the edges of the recognition site and consequent destabilization of the protein–DNA complex. Furthermore, the observed asymmetry would be consistent with the different base sequences flanking the recognition site.

Analysis of protein–phosphate interactions

Ethylation interference assays were used to identify the phosphate groups important in the *Xma*I–DNA interaction. The footprinting pattern obtained with the ethylnitrosurea-modified substrate is shown in Figure 3. The doublet bands in the sample lanes of the autoradiogram represent the heterogeneous termini (3′-hydroxyl and 3′-ethylphosphate) that are generated by the alkylation and cleavage reaction (39). These species are partially resolved on the 20% sequencing gels used in the footprinting assays. Furthermore, the products migrate ~0.5 positions behind the corresponding base-specific cleavage bands (36). In order to accurately identify the bands in the sample lanes an additional control, i.e. a ladder of fragments terminating in a 3′-hydroxyl generated by phosphodiesterase digestion of the substrate (33), was also separated by electrophoresis on the sequencing gel (not shown).

Three phosphates per strand were identified as necessary for the formation of stable protein-DNA complexes. The endonuclease

Figure 3. Ethylation interference footprints of *Xma*I binding to the top (A) and bottom (B) strands of the recognition oligonucleotide. Lanes (G+A) and (T+C) correspond to the Maxam–Gilbert sequencing products. B and F represent the fragments derived from the protein-bound and free DNA respectively. (C) Histogram summary of the phosphate alkylation interference assays for the top and bottom strands of the duplex substrate.

appeared to interact with the phosphates 5' to each of the guanines within the recognition site. There was no evidence of an interaction of the protein with any of the phosphates flanking the recognition site nor of an interaction with any of the phosphates 5' to the cytosines of the recognition site.

DISCUSSION

Type II restriction endonucleases reportedly fall into two classes: those that exhibit sequence-specific binding in the absence of magnesium and those that recognize all sequences with similar

affinity and demonstrate catalytic specificity in the presence of magnesium (44). *XmaI* belongs to the former class, as evidenced by the sequence-specific DNA recognition in the absence of magnesium. A specific association constant was estimated at $1.25 \pm 0.15 \times 10^9/\text{M}$, which represented a 10^4 -fold greater affinity of *XmaI* for the specific hexanucleotide sequence compared with that for non-specific sites.

XmaI is a member of a family of endonucleases that recognize the sequence CCCGGG. Within this family *XmaI* and *Cfr91* are perfect isoschizomers and cleave the site to produce a 5' 4 bp overhang. The enzymes exhibit ~80% amino acid sequence similarity, which is distributed uniformly throughout the protein (41). The apparent homology would be expected to result in similar binding properties for the two enzymes. However, it has been reported that *Cfr91* fails to discriminate specific from non-specific sequences in the absence of magnesium (42). However, at the enzyme and DNA concentrations used in the assay of *Cfr91*, *XmaI* would also exhibit non-specific binding. It is possible that use of the binding and electrophoretic conditions described above for *XmaI* would facilitate detection of specific binding by *Cfr91*.

The ability to isolate stable, specific *XmaI*-DNA complexes facilitated footprinting assays to identify base and phosphate contacts utilized by the endonuclease. The assays, which only identified the bases within the recognition sequence as potential sites of interaction with the protein, provided further evidence for the specificity of the complexes observed in the gel retardation assays. Interaction of *XmaI* with functional groups in the major groove was suggested from the observed footprint after methylation of the recognition site guanines. Furthermore, missing base analyses revealed that removal of any of the guanines or cytosines within the recognition site significantly destabilized the protein-DNA complex. These observations are consistent with an interaction of *XmaI* with each of the bases within the recognition site. However, the possibility that steric hindrance or subtle changes in the structure of the modified DNA substrates underlies the observed footprints cannot be excluded. Nonetheless, there was no evidence for the absence of interaction of *XmaI* with any of the bases, analogous to the failure to detect an interaction of *TaqI* with the pyrimidines of its TCGA recognition sequence (44). Furthermore, *N*⁴-methylcytosine substitutions within the recognition site also destabilize *XmaI* binding (J.C.Dunbar, unpublished results), suggesting that the protein approaches very closely to, if not making a direct contact with, the cytosines.

Ethylation interference analyses revealed only three phosphates per strand as the major sites of interaction of *XmaI* with the sugar-phosphate backbone. The phosphate contacts appeared symmetrical and included only the phosphates 5' to the guanines within the recognition site.

Details of the topology of endonuclease-DNA complexes is still rather limited. Crystal structures have been solved for three [*EcoRI* (22), *EcoRV* (23,23) and *PvuII* (25)] endonuclease-DNA complexes and the structure of *BamHI* in the absence of DNA has been determined (43). Solution studies of *RsrI* (19), *TaqI* (44-45) and *SmaI* (33) have also been reported. Nonetheless, it has been difficult to discern general rules governing the interaction of endonucleases with DNA. Significant differences are apparent in the origin of specificity and the topology of the protein-DNA complexes for the *SmaI* and *EcoRV* endonucleases, both of which cleave hexanucleotide recognition sequences at the central base pairs. *PvuII* is also a blunt-end cutter, however, comparison of the

crystal structure with that of *EcoRV* has revealed differences in the protein structures and the mode of docking of the endonucleases with their cognate DNA sequences (25). *BamHI* (G↓GATTC) and *EcoRI* (G↓AATTC) have, to date, exhibited the greatest similarity: both endonucleases bind specifically to DNA in the absence of magnesium (26,46) and, despite the lack of sequence conservation, the enzymes contain a common structural core motif (43). The structure of the active site is also similar (43). It appears, then, that for these two enzymes their related recognition and cleavage specificities are reflected in comparable binding and structural properties.

XmaI is analogous to *EcoRI* in that it cleaves after the first base of a hexanucleotide recognition sequence. *XmaI* also resembles *EcoRI* in that it: (i) displays sequence discrimination in the absence of magnesium; (ii) interacts with each of the bases within the recognition sequence; (iii) induces similar DNA conformational changes (35) and contains the 'consensus' catalytic site sequence motif (32). Furthermore, in both of the endonuclease-DNA complexes interactions with three phosphates (per strand) provide the major contacts to the sugar-phosphate backbone. However, the pattern of phosphate interactions is quite distinct. Significantly, the primary clamp for *EcoRI* occurs at the immediate 5'-end of the recognition sequence (pGAATTC). Supplementary clamps occur adjacent to the primary clamp (pNGAATTC) and also at the center of the recognition sequence (GAApTTC) (47). In contrast, the phosphate contacts for *XmaI* are clustered at the 3'-end and are all within the recognition site. It has been suggested that endonucleases generating a 5' 4 bp overhang may contain a common structural core motif (43), such as is observed in the *EcoRI* and *BamHI* endonucleases. A similar pattern of phosphate interactions for *BamHI* and *EcoRI* (L.Jen-Jacobsen, personal communication) would appear to reflect the conservation and similar docking of this motif. However, *XmaI* does not exhibit the pattern of phosphate interactions, despite the similar position of cleavage, within its recognition site. It is possible that an analogous core structure does not exist in *XmaI* or that the *XmaI*-phosphate interactions are constrained by the specific base sequence recognized by the enzyme. Even subtle substitutions of base analogs in the *EcoRI* recognition sequence generates different patterns of *EcoRI*-phosphate interactions (47).

SmaI endonuclease has the same recognition specificity as *XmaI*, but cleaves at the central CpG to generate a blunt-end scission. Although the primary structure of the cognate methylases is well conserved, sequence comparisons have revealed no similarity between the endonucleases (31,32). The lack of sequence similarity, however, does not preclude potential structural similarity, as is well illustrated by the *EcoRI* and *BamHI* endonucleases (43). Comparison of the recognition properties of *XmaI* with *SmaI* reveals a number of similarities in the mechanism by which the endonucleases interact with the same sequence. The enzymes both bind specifically to DNA in the absence of magnesium. Furthermore, when using the same DNA substrates the specific and non-specific association constants determined for *XmaI* are very similar to those reported for *SmaI* (33). Furthermore, the interference footprints obtained from dimethylsulfate and missing nucleoside analyses are analogous for the two enzymes. The missing nucleoside assays, however, would not distinguish interactions of the proteins with different functional groups on a given base. Nonetheless, for each of the cytosines within the recognition site the effect of methylation at

the C5 or N4 position has a comparable effect on the K_m of *SmaI* and *XmaI* (J.C.Dunbar, in preparation). These observations further suggest similarity in the recognition of the DNA functional groups by the two endonucleases. Significantly, the pattern of phosphate interactions, as revealed by ethylation interference analyses, is identical for *SmaI* and *XmaI*.

At the resolution of the techniques employed *SmaI* and *XmaI* appear to dock similarly to the DNA and to utilize a common pattern of phosphate backbone contacts. It remains to be determined whether the observed similarity is a reflection of similar structures of their DNA binding domains. If such is the case it would be anticipated that differences in the relative orientation of the binding and catalytic sites dictates the relative positions of cleavage by *XmaI* and *SmaI* within the same recognition site.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation grant MCB9004611 (to JD). BW was supported in part by a Rumble Graduate Fellowship from Wayne State University. We would like to thank Robert Blumenthal for his helpful review of the manuscript.

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