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*Ban*I Restriction Endonuclease Binds in the Major Groove of DNA: An Inhibition Kinetic Study Using Substrates with Mismatch Basepairs

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Structural information on *Ban*I–DNA interaction was obtained from simple inhibition kinetic assays using altered substrates. Self-complementary 13-mer oligodeoxynucleotides with or without mismatch basepairs in the *Ban*I recognition sequence (GGPy-PuCC) were synthesized. UV melting curves and CD spectra indicated double-stranded B-DNA structure for all the oligomers. Among the seven oligomers with recognition sequences, GGTACC, GGTGCC, GGTATC, GGCACC, GGAGCC, GGTAAC, and GGATCC, only the first two were cleaved with *Ban*I. Kinetics of *Ban*I cleavage of the native substrate was inhibited competitively by all of the other oligomers except the one with sequence GGCACC. From inhibition kinetic analysis in presence of a fixed concentration of the inhibitor, apparent K_m and K_I were determined. The data were analyzed in the context of alterations made in the hydrogen bonding potential in the major and minor groove of DNA within the recognition sequence due to basepair mismatches. Such analyses led to the conclusion that *Ban*I, like *Bam*HI, binds in the major groove and the central thymines make important contact with the protein. © 2000 Academic Press

Molecular basis for the sequence specificity of recognition and catalysis by Type II restriction endonucleases has emerged only in a few cases where protein-DNA X-ray cocrystal structures have been resolved [see reviews 1, 2]. Mutational studies [3–5] and biochemical studies with substrates altered by incorporation of base analogues [6, 7] or mismatch basepairs [8, 9] have greatly supplemented the information from crystallography in understanding the structural and mechanistic aspects of recognition and catalysis. Mismatch basepair approach has the advantage of avoiding difficult chemical synthesis and has enhanced our understanding of the two most well studied enzymes viz. *Eco*RI [10] and *Bam*HI [11]. We report here our attempt to map the DNA contact sites for *Ban*I restriction enzyme using oligodeoxynucleotides with or without mismatch basepairs either at the center or next to the scissile phosphodiester bonds in the *Ban*I recognition sequence. *Ban*I enzyme has been cloned, sequenced [12] and even overexpressed but no structural information is yet available. *Ban*I and *Bam*HI recognize similar sequences, GGTACC and GGATCC respectively and cleave the DNA identically between the two G's. It is important to understand why a simple positional reversal of the two central A · T basepairs in the recognition sequence would completely change the specificity of recognition from *Bam*HI to *Ban*I. We show here that from cleavage analysis and inhibition kinetic studies using altered substrates, we can conclude that *Ban*I like *Bam*HI, binds in the major groove of DNA and the central thymines make important contact with the protein. We also infer that position reversal of A · T pairs in *Ban*I vis-a-vis *Bam*HI sequences requires that recognition and catalytic domains would also be reversed in the *Ban*I protein compared to the *Bam*HI enzyme.

MATERIALS AND METHODS

The various oligodeoxynucleotides (for sequence and two-letter codes, see Table 1) used in this study were obtained as reverse-phase purified material from Macromolecular Resources, U.S.A. They were further purified by polyacrylamide gel electrophoresis. The chain length and purity of each oligomer was verified by gel electrophoresis of 5′-end radiolabeled material. Oligonucleotide concentration was taken as 1 OD = 33 μg. R.*Ban*I of high specific activity was used for the cleavage and inhibition kinetic studies and was from New England Biolabs, U.S.A.

UV melting curves were recorded with a heating rate of 1°C/min in a Shimadzu UV 160A (Japan) spectrophotometer fitted with a temperature programmer (CE-247 Cecil, UK). About 0.2 OD of the lyophilized oligonucleotide was dissolved in 400 μl *Ban*I digestion buffer (10 mM Tris–acetate (pH 7.9) and 50 mM potassium acetate) for UV and CD measurement. CD spectra were recorded at 30°C using a Jasco J-700 spectropolarometer (Japan).

Cleavage analysis. Each substrate (5 pmol) was 5′-end-radiolabeled by usual procedures using [γ-^{32}P]ATP and T4 polynu-

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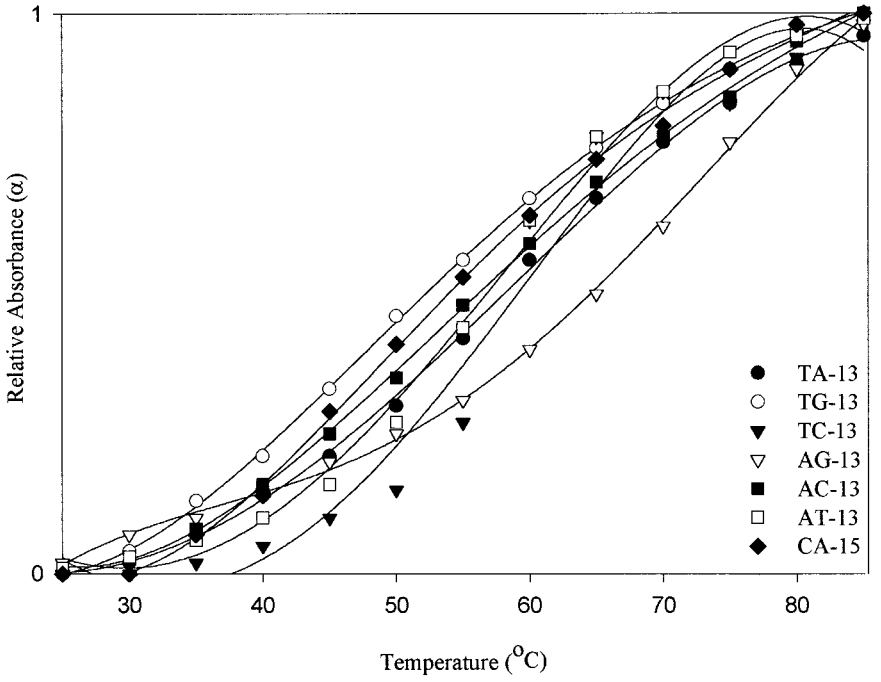

FIG. 1. UV melting curves for oligodeoxynucleotide substrates.

cleotide kinase. The labeled oligonucleotides were separated from unincorporated ATP by electrophoresis on a 20% polyacrylamide gel using standard procedures [13]. The labeled substrate was digested with 50 units of *Ban*I in a 30 μl reaction mixture containing 10 mM Tris–acetate (pH 7.9), 10 mM $MgCl_2$, 50 mM potassium acetate, 1 mM DTT at 37°C for 6 hours or overnight in some cases. The reaction was stopped by immersing the reaction tubes in boiling water for 2 min. The cleavage products were analyzed on a 20% urea-polyacrylamide gel (1 mm) on Resolmax electrophoresis apparatus (Atto Corp., Japan) at 40 mA constant current.

Inhibition kinetic studies (competitive inhibition assays). A fixed amount of the inhibitor (7.5 μM) oligomer and variable amounts of the cognate substrate TA-13 were incubated with 40 units of *Ban*I endonuclease in 40 μl of standard *Ban* I digestion buffer at 37°C. Substrate concentrations were varied by using a fixed small amount of labeled substrate (1 pmole) and varying concentrations of cold unlabeled substrate (1.25 μM to 5 μM) and analytically correcting for carrier dilution (14). For the TG-13 mismatch inhibitor only two concentrations, 3.75 μM and 7.5 μM respectively, of the inhibitor were used. For each kinetic assay, aliquots of 6.4 μ were taken at intervals of 0, 2, 5, 10, 30, and 60 min and transferred to a tube containing 6 μl stop-mix (80% formamide, 5 mM EDTA, 20% glycerol). They were then heated to 90°C for 2 min, quick-chilled on ice and subjected to 20% denaturing polyacrylamide gel electrophoresis followed by autoradiography. Aliquots of a control reaction without the inhibitor were also taken similarly. By monitoring the hydrolysis of varying concentrations (1.25, 1.75, 2.5, 5 μM) of the cognate 13-mer in the presence of 7.5 μM of each of the other oligodeoxyribonucleotides as inhibitor (TG-13, AT-13, AG-13, AC-13, TC-13 and CA-15), the relative K_I values were determined using the relation $K_m(\text{apparent}) = K_m(\text{actual})(1 + [I]/K_I)$ (15).

RESULTS AND DISCUSSION

The thermal transition data (Fig. 1) and the CD spectra (Fig. 2) of the mismatch substrates indicate that all the oligodeoxynucleotides are in the double-stranded DNA conformation under our experimental conditions thus satisfying the primary requirement for

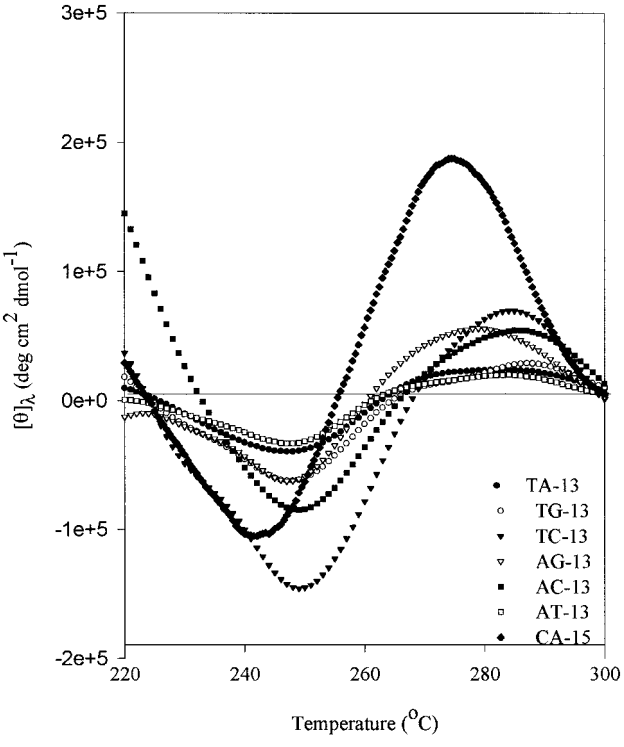

FIG. 2. CD spectra for the various oligodeoxynucleotide substrates.

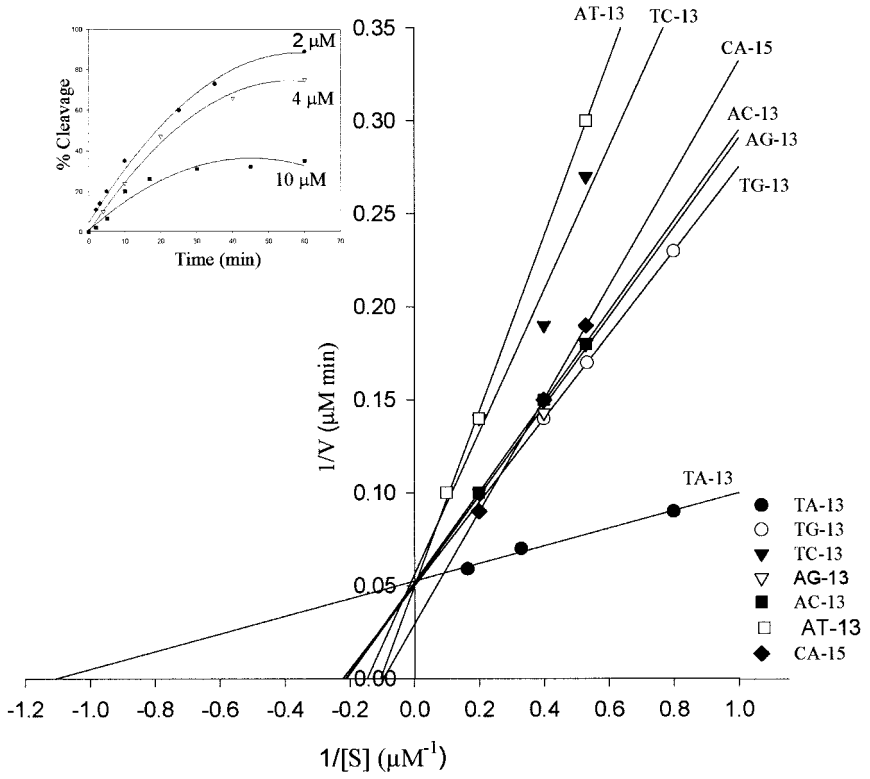

FIG. 3. Lineweaver–Burk plot for inhibition kinetics of the various oligodeoxynucleotide substrates. Inset shows the velocity curves for cognate substrate, TA-13 in absence of inhibitor.

recognition and cleavage by a type II restriction enzyme like *Ban*I. The cleavage experiments however showed that among the seven oligomers, only the cognate TA and the TG mismatch substrates were cleaved normally but at very different efficiencies, without any change in cleavage specificity (data not shown). The enzyme obviously binds to the sequences that are cleaved, but failure of cleavage does not necessarily suggest failure to bind (7, 15). Kinetic parameters were obtained for the native substrate, the self-complimentary oligomer d(AGTGCGGTACCGC), in the concentration range 0.1–4 μM with a K_m of 1.14 μM and a k_{cat} of 18.9 min^{-1}. The rate of phosphodiester bond hydrolysis was linear from 2–6 min, and initial velocities could be obtained from this portion of the assays (Fig. 3, inset).

To determine whether the endonuclease did bind to our altered substrate oligodeoxynucleotides which themselves were not cleaved but would act as a competitive inhibitor, inhibition studies were done. The Lineweaver–Burk plot for the inhibition kinetics indicated that competitive inhibition occurred with all the mismatch substrates except the CA-15 sequence which shows noncompetitive inhibition (Fig. 3). These data suggests that the different inhibitors are able to bind with varying affinities proportional to their K_i (Table 1). As our objective was to obtain only a relative idea about the strength and specificity of

TABLE 1

Sequence notation	Sequence	T_m (°C)	Apparent K_m[a] (μM)	K_I (μM)
TA-13	5′-ATGGCGGTACCGC-3′	58	1.140	—
TG-13	5′-ATGGCGGTGCCGC-3′	50	3.745	2.403
AG-13	5′-ATGGCGGAGCCGC-3′	54	4.545	1.875
AC-13	5′-ATGGCGGTAACGC-3′	53.5	4.831	1.738
TC-13	5′-ATGGCGGTATCGC-3′	53.5	6.802	1.156
AT-13	5′-ATGGCGGATCCGC-3′	58.5	9.345	0.808
CA-15 (pH 5.5)	5′-TCTAGAGGCACCTCT-3′	55	—	—
CA-15 (pH 7.9)	5′-TCTAGAGGCACCTCT-3′	52	11.490	0.644

[a] For TA-13, the value is for actual K_m done in absence of inhibitor.

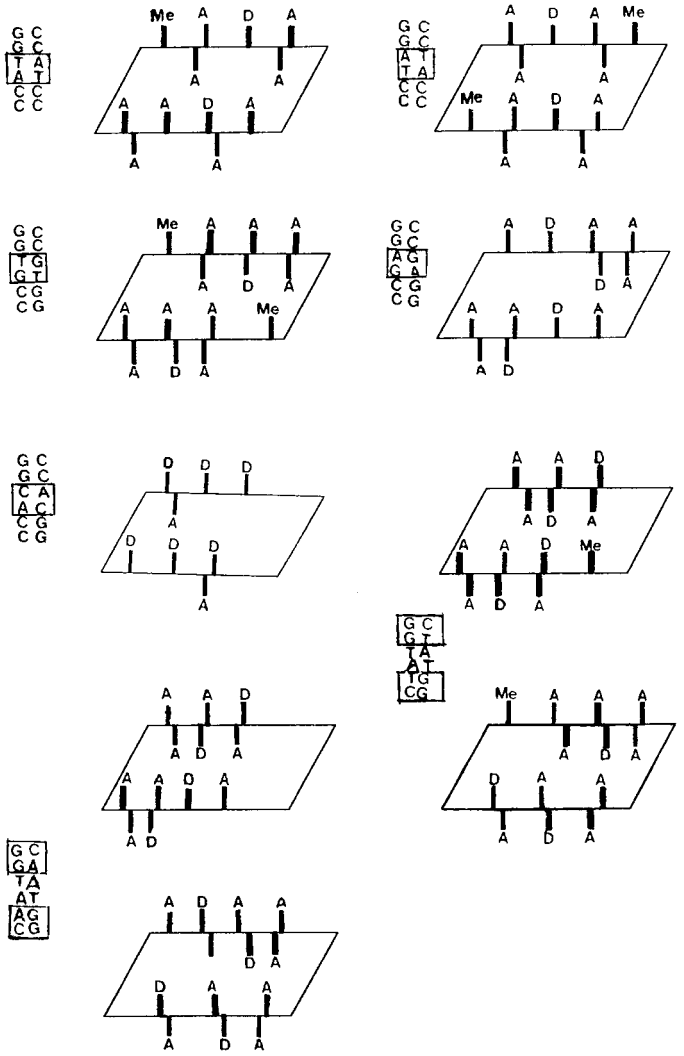

FIG. 4. Schematics of various basepairs (boxed) in our designed as well as the cognate recognition hexamers.

binding of various mismatch substrates, inhibition kinetics were done at only at one fixed inhibitor concentration. Within experimental error identical V_{max} values were obtained with and without the inhibitor. From the plot, the varying affinities of the oligodeoxynucleotides for *Ban*I are in the order TA-13 > TG-13 > AG-13 > AC-13 > TC-13 > AT-13; AT-13 is the most and TG-13 is the least potent inhibitor of *Ban* I cleavage (Fig. 3). A schematic diagram showing potential contact sites in the major and minor groove of the mismatch as well as the cognate recognition sequence, which might be involved in interaction with *Ban*I is shown in Fig. 4. The cleavage and kinetic data for the mismatch sequences and the canonical −TA and −AT sequences are analyzed in the context of Fig. 4 and is discussed below to understand the direct readout in the recognition process of *Ban*I.

No Mismatch, TA/AT Sequences

The hydrogen bonding contact sites in the central TA basepairs in *Ban*I and central AT basepairs in *Bam*HI recognition sequences are identical but positionally reversed in the major groove (Fig. 4). Considering the twofold symmetry of the network of interactions with the dimeric protein, it was shown earlier (11) that *Bam*HI binds to both the −AT and −TA sequences in the major groove, although the latter was not cleaved. Here, we observe that *Ban*I also binds and cleaves the −TA sequence but not −AT sequence. The −AT sequence is the strongest potent inhibitor of *Ban*I cleavage action on the −TA substrate. This suggest that both −AT and −TA sequences bind to the same active site in similar fashion as *Bam*HI. In other words, *Ban*I like *Bam*HI binds in the major groove of DNA.

TG Sequence—Mismatch but Cleaved

Comparison of functional group pattern in the major and minor grooves of −TG and −TA sequences, which may be involved in direct readout, shows little difference. A hydrogen bond donor group (exocyclic NH_2 of adenine in −TA sequence) in the major groove is shifted to the minor groove (exocyclic NH_2 of guanine in the −TG sequence) and an acceptor group (guanine O4) occupies that position. Poor inhibitor property and very reduced cleavage of −TG sequence can possibly be explained by the fact that the −TG sequence contains two consecutive G · T mismatches at the center. This can cause significant distortion or even kinks/bends in the DNA substrate at the center. Such kink was although helpful to form a productive binding in case of *Eco*RI (16), in case of *Bam*HI (17), the DNA remained straight whereas protein had to make conformational adjustment to form a productive complex. If *Ban*I binding is similar to that of *Bam*HI, such bent substrate will not only weaken binding but also severely compromise the formation of transition state complex leading to drastic reduction in catalytic activity as we see here. So, *Ban*I possibly behaves similarly as *Bam*HI.

CA Sequence

For the −CA mismatch the adenine is thought to be protonated and forms two hydrogen bonds with cytosine (18). The crystal structure of an RNA octamer 5′-CGCCAGCG-3′ has shown that tandem C · A mispairs comprise an internal loop in the middle of the duplex which is easily accommodated with little distortion of the A-form double helix (19). Our UV melting curve and CD data for −CA sequence indicate duplex structure, which is more like B-form DNA, yet this sequence despite being cognate type is neither cleaved nor acts as competitive inhibitor in the *Ban*I cleavage reaction. Rather it behaves as a noncompetitive inhibitor changing both K_m and V_{max} (mixed type inhibition)

[15] which suggest that −CA sequence binds to the protein but differently from the substrate binding. We presume this has to do with the bent structure of the −CA sequence. Also, the disposition of the hydrogen bond donor-acceptor groups and van der Waal's contacts in the major and minor groove is totally different from that in the cognate −TA sequence (Fig. 4), which predicts no binding at the active site and hence no cleavage occurs.

AC and TC Sequences

In these two sequences alterations have been made in the basepairs next to the scissile phosphodiester bonds; the −AC oligomer has two G · A mispairs and −TC oligomer has two G · T mispairs. Relative positions of the donor (cytosine or adenine amino N6) and acceptor (guanine N7 and O6) sites in the major groove are similar in G · C and G · A (syn) basepairs, with an extra acceptor site (adenine N1) in the latter (Fig. 4). Thus potential contact sites in the major groove is not altered when 'CC' is replaced by 'AC' in the oligomer sequence. The minor groove on the other hand, has lost an acceptor site (cytosine O2). The competitive inhibition by this sequence is then suggestive of binding in the major groove. However, introduction of a G · A (syn) mispair next to the scissile phosphodiester bond may introduce sufficient local distortion in the DNA backbone compromising proper alignment of the scissile bond with respect to the catalytic moieties in the active site thus preventing hydrolysis. In contrast, the −TC oligomer is a more potent competitive inhibitor of *Ban*I cleavage than the AC-13 oligomer, implying that binding of the enzyme to this sequence is more efficient than with −AC oligomer. Although the G · T wobble basepair in this sequence replaces the donor (cytosine 6-amino) with an acceptor (thymine O4) group and an extra methyl group of thymine is also introduced in the major groove when 'CC' is replaced with 'TC', the minor groove functional group pattern is not altered. Surprisingly, when same alteration was made in the *Bam*HI recognition sequence, the enzyme *Bam*HI was found not to bind to this sequence at all and it was concluded that 6-amino-group of cytosine must be making an important contact with protein [11], which was indeed found in the X-ray crystal structure of *Bam*HI-DNA complex [17]. The loss of this donor site and the steric hindrance by thymine methyl group was thought to have prevented binding of *Bam*HI to the DNA there. In case of *Ban*I, binding to −TC oligomer is efficient which implies that the donor group of inner cytosine is not important here and the newly introduced thymine methyl group probably makes a van der Waals' contact leading to enhanced affinity. The binding, however, may be non-productive due to the local distortion of the G · T mispairs next to the scissile bonds, which prevents catalysis.

AG Sequence

As shown in Fig. 4, GGAGCC has the same relative positions of the donor and acceptor groups in the major groove as in GGATCC. Only the thymine methyl group is replaced by an acceptor group (guanine N7). The minor groove, on the other hand, has lost an acceptor site and a donor group is introduced close to the other acceptor site. Thus functional group pattern is drastically altered in the minor groove but not in the major groove. Binding of *Ban*I to −AG− sequence is therefore expected if the endonuclease interacts through the major groove as it binds to both −TA and −AT sequences. Inhibition kinetics shows −AG sequence is as good inhibitor as the −TG sequence, which is a cognate sequence and is cleaved. This suggest that −AG sequence binds to the same site as the cognate −TA and −TG sequence and hence must be interacting through the major groove.

This similarity between *Ban*I and *Bam*HI is strengthened by our analysis of the secondary structure of *Ban*I. We have identified a putative active site (the amino-acid region 173–211) in *Ban*I through Chou-Fasman secondary structure analysis, which looks similar to that seen in *Bam*HI secondary structure, only change observed is the order in which the catalytic domain (central β-sheet) and recognition domain (adjacent α-helix) are arranged, the order in *Ban*I is reverse to that observed in *Bam*HI crystal structure. Considering the symmetry of the dimeric protein bound to DNA, this reversal is perhaps necessary to compensate for the reversal of the two central A–T basepairs in the recognition sequence of *Ban*I vis a vis the *Bam*HI site. In *Bam*HI-DNA complex only the thymine bases of the central A · T pairs make direct contact with the protein. Each subunit makes a hydrogen bond and a van der Waals contact with the thymine carbonyl and the methyl group respectively but in a crossover manner (17); i.e., L-subunit makes most of its base contacts to the R-half site. We predict that in *Ban*I-DNA complex this crossover manner of contacts will be maintained as the positions of both thymines as well as the positions of the catalytic and the binding domains seem to be reversed in *Ban*I when compared with the corresponding situations in *Bam*HI. In view of the above discussion, we conclude that *Ban*I like *Eco*RI and *Bam*HI interacts through the major groove forming a similar symmetric complex of the DNA and the dimeric protein and the central thymines make important contacts with *Ban*I protein.

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REFERENCES

1. Pingoud, A., and Jeltsch, A. (1997) *Eur. J. Biochem.* **246,** 1–34.
2. Heitman, J. (1992) *BioEssays* **14,** 445–454.
3. Selent, U. Ruter, T., Kohler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschlager, T., Wolfes, H., Peters, F., and Pingoud, A. (1992) *Biochemistry* **31,** 4808–4815.
4. Flores, H., Osuna, J., Heitman, J., and Soberon, X. (1995) *Gene* **157,** 295–301.
5. Xu, Y., and Scildkraut, I. (1991) *J. Biol. Chem.* **266,** 4423–4429.
6. Aiken, C. R., and Gumport, R. I. (1991) *Methods Enzymol.* **208,** 433–457.
7. McLaughlin, L., Benseler, F., Graeser, E., Piel, N., and Scholtissek, S. (1987) *Biochemistry* **26,** 7238–7245.
8. Germann, M. W., Kalisch, B. W., Varnum, J. M., Vogel, H. J., and van de Sande, J. H. (1998) *Biochem. Cell Biol.* **76,** 391–402.
9. Alves, J., Selent, U., and Wolfes, H. (1995) *Biochemistry* **34,** 11191–11197.
10. Thielking, V., Alves, J., Fliess, A., Maass, G., and Pingoud, A. (1990) *Biochemistry* **29,** 4682–4691.
11. Roy, K. B., and Dave, V. (1995) *Biochem. Mol. Biol. Int.* **36**(4),759–770.
12. Maekawa, Y, Yasukawa, H., and Kawakami, B. (1990) *J. Biochem.* **107,** 645–649.
13. Maniatis,T., Sambrook, J., and Fritsch, E. F. (1989) *in* Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Roy, K. B., Dave, V., and Jayaram, B. (1994) *Anal. Biochem.* **220,** 160–164.
15. Fersht, A. R. (1985) *in* Enzyme Structure and Mechanism, 2nd ed., Chap. 12. Freeman, New York.
16. Rosenberg, J. M. (1991) *Curr. Opin. Struct. Biol.* **1,** 104–113.
17. Newman, M., Strzelecka, T., Dorner, L. F. Schildkraut, I., and Aggarwal, A. K. (1995) *Science* **269,** 656–663.
18. Pan, B., Mitra, S. N., and Sundaralingam, M. (1998) *J. Mol. Biol.* **283,** 977–984.
19. Jang, L. (1998) *Biochemistry* **37,** 11726–11731.