STRUCTURAL STUDIES OF THE FLP RECOMBINASE OF *SACCHAROMYCES CEREVISIAE* COMPLEXED WITH DNA: IMPLICATIONS FOR THE MECHANISM OF SITE-SPECIFIC RECOMBINATION

by

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ABSTRACT

The Flp site-specific recombinase of *Saccharomyces cerevisiae* induces DNA bending upon specific binding to DNA and catalyzes recombination which involves a complex manipulation of DNA strands. The minimal Flp target site consists of two sequence-specific binding sites which flank a core region. I have defined the position and direction of Flp-induced DNA bends. The bend induced by the noncovalent binding of two Flp molecules to the target site was positioned in the middle of the core region and directed toward the major groove. The position and direction of bends in complexes where one strand was cleaved by Flp differed depending on whether the top or bottom strand of the target site was cleaved. A model is proposed which associates the variable position of the type II bends with a defined order of strand exchanges in the recombination reaction (Chapter 2).

Mutation of the central AT base-pair in the core region altered the position of the bend induced by binding of two molecules of Flp to the FRT site as compared to an unmodified target site complexed

with Flp. This mutation also affected Flp-mediated cleavage and recombination activities. The position of the DNA bend appears to be important to the functionality of the site (Chapter 3).

Examination of complexes in which one of the two Flp molecules was covalently bound to the top or bottom strands revealed cleavage-dependent conformational changes in the DNA of these complexes. An adenine residue opposite the site of cleavage was specifically protected from methylation within the cleaved, covalently bound complexes. Cleavage was also associated with changes in the interaction between the binding site and the noncovalently-bound Flp monomer in the cleavage complex. Flp-mediated cleavages of the top and bottom strands of the target site were differentially interfered with by methylation of bases opposite each cleavage site. This may provide a means to order the cleavage and subsequent strand exchange events (Chapter 4).

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LIST OF ABBREVIATIONS

A	adenosine
ATP	adenosine triphosphate
bp	base pair(s)
C	cytosine
dCTP	deoxycytidine triphosphate
DMS	dimethyl sulfate
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetraacetic acid
FRT	Flp recognition target
G	guanosine
λ Int	lambda integrase
nt	nucleotide(s)
P-tyr	phosphotyrosine
SDS	sodium dodecyl sulphate
T	thymidine
TBE	tris-borate EDTA buffer
tyr	tyrosine
Tet	tetracycline

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CHAPTER 1.

GENERAL INTRODUCTION

1. INTRODUCTION

Recombination is an enzyme-mediated process in which segments of DNA are rearranged. The reshuffling of genetic information carried by chromosomes alters the linkage relationship between genetic loci. Recombination is thus a fundamental cellular mechanism regulating genetic change and is essential for such varied processes as the development of biological diversity, resolution of transposition intermediates, viral integration and excision, monomerization of multimeric bacterial plasmids and chromosomes, and regulation of plasmid copy number.

Recombination events are divided into two major categories: Homologous (general) recombination and site-specific recombination. Homologous recombination occurs between regions of DNA that share extensive sequence homology. This type of recombination was first identified by Morgan (1911) in *Drosophila melanogaster*. Homologous recombination has allowed genetic mapping through linkage analysis of genetic loci on a chromosome.

Site-specific recombination can be further divided into at least two sub-classes: transposition and conservative site-specific recombination. (i) Transposition is the process by which a specific segment of DNA moves to another site and was first discovered by McClintock (1951). Transpositional recombination may occur by a replicative mechanism involving the formation of an intermediary cointegrate structure in which the donor DNA backbone, the target DNA, the parental transposon copy and a replicated transposon copy are linked. The integration and replication of phage Mu in the

Escherichia coli chromosome is an example of this type of transposition. Alternatively, transpositional recombination may occur by a non-replicative, cut-and-paste mechanism involving excision of the transposon from the donor DNA *via* double-strand breaks, followed by integration into a DNA target site (Hagemann and Craig, 1993). (ii) Conservative site-specific recombination was first discovered by Campbell (1962). It is mediated by sequence-specific DNA binding proteins that break and rejoin DNA strands of two copies of a distinct target site to yield novel combinations.

This thesis describes biochemical studies of the Flp site-specific recombinase complexed with DNA. The Flp protein is encoded by the Flp gene on the 2 μm plasmid of *Saccharomyces cerevisiae.*

2. CONSERVATIVE SITE-SPECIFIC RECOMBINATION

Conservative site-specific recombination has several characteristic features. Recombination occurs at specific sites with short regions of homology (less than 10 bp), proceeds *via* breakage and rejoining of DNA at precise locations within the target sites, and is a reciprocal event. This recombination is called conservative because it does not involve synthesis or loss of any DNA (Campbell, 1981). No high-energy cofactor is required since the covalent recombinase:DNA intermediate conserves the energy required for the subsequent ligation of the cleaved DNA strands in a protein:phosphate linkage (Sadowski, 1986). Conservative site-specific recombinases fall into

two families: the resolvase-invertase family and the integrase family.

(i) The resolvase-invertase family

The resolvase-invertase family contains about 85 members which have been identified by sequence homology. Resolvases are responsible for resolution of cointegrate intermediates that are formed during transpositional recombination. γδ resolvase of *Escherichia coli* is an example of a recombinase which facilitates this process (Reed, 1981). Invertases mediate the inversion of segments of DNA which regulate gene expression. For example, the Hin recombinase controls the alternate expression of two flagellin genes by catalyzing the inversion of a promoter-containing segment in the chromosome of *Salmonella typhimurium* (Glasgow et al., 1989; Johnson, 1991). The resulting variation in the flagellin structure likely aids in the evasion of host immune responses (Craig, 1988).

Members of the resolvase-invertase family bring about recombination using a mechanism which has several distinguishing characteristics. The substrate DNA must be supercoiled. Target sites are oriented in a prescribed manner such that resolvases only act on directly oriented sites to excise the DNA between the sites and invertases only act on inverted sites to invert the DNA between the sites. A nucleophilic serine catalyzes cleavage of the DNA and becomes covalently attached to the 5'-phosphoryl end.

(ii) The integrase family

The integrase family contains more than 81 members which overall share weak sequence similarity but which all contain four

absolutely conserved amino acids (Argos et al., 1986; Abremski and Hoess, 1992; Kwon et al., 1997; Esposito and Scocca, 1997). These residues (arginine 191, histidine 305, arginine 308, tyrosine 343, where numbers indicate amino acid numbers of the Flp protein of the 2 μm plasmid from *Saccharomyces cerevisiae*) are thought to facilitate a common catalytic mechanism of site-specific recombination. The integrases bring about strand cleavage by using the conserved tyrosine to link the protein covalently to the 3'-phosphoryl DNA terminus and generate a free 5'-hydroxyl group at the nick (Sadowski, 1986; Craig, 1988; Stark et al., 1992; Sadowski, 1993; Jayaram, 1994). The other three conserved residues are implicated in the cleavage, strand exchange and ligation steps of the recombination reaction (Parsons et al., 1988; Friesen and Sadowski, 1992; Pan et al., 1993). Sequence alignment and evolutionary analysis of 81 integrase family members indicated the presence of subfamilies with significant sequence similarity aside from the four absolutely conserved residues; these subfamilies are consistent with different biological roles (Esposito and Scocca, 1997). The six eukaryotic yeast-derived recombinases including Flp could not be aligned with the rest of the integrase family suggesting that they form a distinct group distant in evolution from the prokaryotic group (Esposito and Scocca, 1997).

The most studied members of the integrase family are integrase from phage lambda (λ Int), *E. coli* XerC and XerD, Cre from phage P1 and Flp. Site-specific binding by each of these proteins to its respective target site create bends in the DNA (Thompson and Landy,

1988; Blakely and Sherratt, 1996a; Guo et al., 1997; Schwartz and Sadowski, 1989).

λ Int is the enzyme responsible for the integration and excision of the λ chromosome into and out of the *E. coli* chromosome. Three other accessory proteins are involved in λ site-specific recombination, all of which are DNA-bending proteins. These are excisionase (Xis), a virally-encoded protein, the host-encoded factor for inversion stimulation (Fis), and integration host factor (IHF; Craig, 1988).

The crystal structure of the catalytic domain of λ Int shows that the four invariant amino acids of the integrase family are located on the surface of λ Int that is proposed to interact with DNA (Kwon et al., 1997). Other conserved, functionally important residues are buried and clustered around the four catalytic residues whereas few of the exposed residues away from the presumed active site are conserved (Kwon et al., 1997).

Xer site-specific recombination at the DNA target site *dif* in *E. coli* is implicated in ensuring faithful segregation of the chromosome to daughter cells at cell division by converting chromosome dimers to monomers (Blakely et al., 1991). XerC and XerD also convert plasmid multimers to monomers by binding to the target sites *cer* and *psi* in the plasmids ColE1 and pSC101, respectively, thereby ensuring stable inheritance of these plasmids (Summers and Sherratt, 1984; Cornet et al., 1994). The involvement of two related recombinases, XerC and XerD (37% identity), distinguishes Xer site-specific recombination from other well characterized integrase family members (Blakely et al., 1993).

Comparison of the crystal structures of λ Int, HP1 Int and XerD reveals that the fold of the catalytic domain is similar in all three structures and unique to the integrase family (Kwon et al., 1997; Hickman et al., 1997; Subramanya et al., 1997). Within the catalytic domain, however, the region (22-30 residues) containing the active site tyrosine is structurally different in each of the three structures. The active site of the XerD structure is buried by the NH_2-terminal domain; access of the active site to DNA requires a conformational change in the protein (Subramanya et al., 1997). In comparison, the active sites of the λ and HP1 integrases are exposed. It is unclear whether the differences in the active site region of the three proteins reflects differences in the catalytic mechanism or different conformational states in the enzyme of each crystal structure (Subramanya et al., 1997).

The Cre recombinase of phage P1 reduces P1 dimers to plasmid monomers to aid in the faithful segregation and stable maintenance of the plasmid in *E. coli* (Austin et al., 1981; Abremski et al., 1983). It has been suggested that Cre-mediated recombination also helps to stabilize P1 copy number by mediating P1 plasmid amplification (Adams et al., 1992). Each P1 plasmid carries a single binding site for Cre called *loxP*. Cre resolves a P1 dimer by binding to the two *loxP* sites and catalyzing a site-specific recombination event between the two sites. The co-crystal structure of a synaptic complex in which four Cre monomers are bound to four half- *lox* sites has been reported; two of the monomers are covalently bound to the DNA (Guo et al., 1997). This structure shows that Cre induces an ~100° bend in the DNA which is positioned centrally in the core

region of the *lox* site (Guo et al., 1997). Mechanistic insights into the recombination reaction that are revealed by the structure are described below.

Flp site-specific recombination facilitates amplification of the 2 μm plasmid (Figure 1-1) of *S. cerevisiae* to high copy number (Volkert and Broach, 1986; Reynolds et al., 1987). Accessory proteins are not required for Flp site-specific recombination *in vitro*. The role of Flp in amplification of the 2 μm plasmid was first proposed as a model by Futcher (1986; Fig. 1-2). Initial bidirectional replication beginning at the origin of the 2 μm plasmid generates a theta intermediate containing three Flp recognition target (FRT) sites. Flp-mediated recombination between two of the FRT sites inverts a large segment of the plasmid thereby changing the relative orientation of the two replication forks which are now in direct orientation with respect to each other. Continued replication gives rise to multimers of the 2 μm plasmid. Additional recombination events restore the bidirectional orientation of the replication forks and reduce plasmid multimers to monomers. The high copy number of the 2 μm plasmid is maintained although the origin of replication is activated only once during the cell cycle.

Similarities between the site-specific recombination pathways mediated by Cre and Flp are evident. Both Cre and Flp are DNA bending proteins which bind to simple target sites consisting of inverted binding elements which flank a core region. Recombination events mediated by Cre and Flp proceed without the need for accessory proteins.

3. FLP SITE-SPECIFIC RECOMBINATION

Since the establishment of the first *in vitro* assay for the Flp recombinase by Vetter et al. (1983), further biochemical studies have added to the understanding of the mechanism of the Flp-mediated recombination reaction. Several *in vitro* assays have permitted the study of partial activities of Flp including DNA binding, DNA bending, synapsis, DNA strand exchange, DNA strand ligation and resolution of Holliday intermediates. High-resolution structural models of XerD and the catalytic domains of λ Int, HP1 integrase and the synaptic Cre:*lox* complex provide additional new insights into the structure and function of the integrases (Subramanya et al., 1997; Kwon et al., 1997; Hickman et al., 1997; Guo et al., 1997). Flp has not been conducive to crystallographic studies however, owing to the insolubility of the Flp protein at high concentrations (D. Kuntz, unpublished observations).

(i) The Flp recognition target site

The sequence of the 2 μm plasmid of *S. cerevisiae* includes two 599 bp inverted repeats each of which contains one FRT site. The FRT site is comprised of three 13 bp symmetry elements (*a*, *b* and *c*), which flank an 8 bp core region; elements *b* and *c* are directly oriented repeats which lie on one side of the core region while element *a* lies on the other side of the core region in an inverted orientation with respect to elements *b* and *c* (Figure 1-3). Symmetry element *c* is not essential for recombination as it can be deleted with no effect on recombination activity *in vivo* or *in vitro*

(Jayaram, 1985; Gronostajski and Sadowski, 1985a; Proteau et al., 1986). The Flp cleavage sites are in the top strand at the junction of the core and symmetry element *b* and in the bottom strand at the junction of the core and symmetry element *a*. Two polypyrimidine tracts present in the top and bottom strand of the FRT site are thought to be important for recombination (Umlauf and Cox, 1988). These tracts meet in the core region and extend outward into the *a* and *b* symmetry elements.

The minimal FRT site (*a* and *b* symmetry elements flanking the core region) contains two asymmetrical features. These are a single base-pair mismatch between the otherwise identical *a* and *b* symmetry elements (positions -6 and +6, Fig. 1-3) and the core region which is asymmetrical. Asymmetry in the core region imparts directionality to the recombination reaction. FRT sites only yield recombinant products from the alignment of two sites in parallel ensuring that recombination between two sites in inverted orientation results in the inversion of the DNA segment between the two sites and that recombination between two directly oriented sites results in the excision of the DNA segment between the two sites. FRT sites with symmetrical core regions, however, yield recombinant products from both a parallel and an antiparallel alignment; the DNA segment between two FRT sites may be inverted or excised (Senecoff and Cox, 1986).

(ii) The Flp protein

The Flp gene encodes a 45 kDa protein of 423 amino acids. Partial proteolysis of the Flp protein resulted in a COOH-terminal domain of

32 kDa (P32), an internal 21 kDa (P21) domain and a NH_2-terminal domain of 13 kDa (P13; Pan et al., 1991; Pan and Sadowski, 1993). The NH_2-terminal P13 domain interacts with the core-proximal 4 bp of the symmetry element while the COOH-terminal P32 domain interacts with the core-distal 9 bp of the symmetry element (Panigrahi and Sadowski, 1994).

(iii) Steps of the Flp-mediated recombination reaction

The steps of the Flp-mediated recombination reaction on a minimal FRT site are shown in Fig. 1-4. The reaction pathway is initiated by binding of Flp to the FRT site (Andrews et al., 1985). Flp makes extensive contacts with residues of both the major and minor grooves of each symmetry element (Panigrahi et al., 1992). No contacts with bases in the core region, however, are associated with DNA binding (Bruckner and Cox, 1986; Beatty and Sadowski, 1988; Schwartz and Sadowski, 1989; Panigrahi et al., 1992). Phosphate contacts which are associated with recombination are clustered opposite the cleavage sites (Bruckner and Cox, 1986; Beatty and Sadowski, 1988).

Binding is accompanied by Flp-induced DNA bending (Schwartz and Sadowski, 1989). Binding of a single molecule of Flp to one symmetry element induces a bend of 60° (type I bend). Association of two molecules of Flp with the FRT site creates a DNA bend of greater than 140° (type II bend; Schwartz and Sadowski, 1990). Hypermethylation of a guanine residue by dimethylsulfate in the bottom strand of the core region is associated with Flp-induced

bending (Schwartz and Sadowski, 1989). Several Flp mutants are defective in both DNA-bending and recombination implicating a role for Flp-induced DNA bending in the recombination reaction (Schwartz and Sadowski, 1989; Chen et al., 1991, 1992a; Kulpa et al., 1993).

Flp mediates DNA strand cleavage by using the active site tyrosine 343 to covalently link the protein to the 3'-DNA terminus and generate a free 5'-hydroxyl group at the nick (Gronostajski and Sadowski, 1985b; Prasad et al., 1987; Evans et al., 1990). The Flp active site is thought to be assembled from partial active sites contributed by two Flp monomers leading to cleavage of the DNA in *trans* (Chen et al., 1992b). The Flp monomer which contributes the nucleophilic tyrosine is not bound to the symmetry element immediately adjacent to the site of cleavage. Cleavage may proceed by a *trans*-horizontal mechanism (Lee et al., 1994), i.e. the Flp monomer contributing the nucleophilic tyrosine is positioned on the same substrate molecule as the phosphodiester bond that is to be cleaved. Only one of two potential active sites is assembled at any one time limiting strand cleavage to a single event (Qian et al., 1990; Friesen and Sadowski, 1992).

λ Int and Xer recombinases, however, cleave their target sites in *cis* when assayed on Holliday intermediates (Nunes-Düby et al., 1994; Arciszewska and Sherratt, 1995). The recent synaptic Cre/*lox* crystal structure (Guo et al., 1997) shows that Cre also facilitates *cis* cleavage of the site although another enzymatic study implied *trans* cleavage (Shaikh and Sadowski, 1997). Furthermore, the conserved amino acids which form the active site are all donated

from the same Cre molecule indicating that a shared active site is not formed by Cre (Guo et al., 1997). It is unresolved whether Flp is divergent from λ Int, XerC/XerD and Cre in the ability to carry out cleavage in *trans* (Blakely and Sherratt, 1996b; Jayaram, 1997). The evolutionary distance indicated by the sequence dissimilarity of the eukaryotic Flp-like recombinases from the remaining integrases of prokaryotic origin may support the divergence in the cleavage mechanism (Esposito and Scocca, 1997).

Synapsis between two substrate molecules occupied by Flp is not thought to be required for strand cleavage (Voziyanov et al., 1996). Bound FRT sites assemble in a synaptic structure through intermolecular protein:protein interactions (Amin et al., 1990). In the Cre/*lox* structure, synapsis is facilitated by the interaction of a short COOH-terminal helix of one Cre molecule in the hydrophobic pocket of the adjacent Cre molecule, as well as, interactions between two helices of the NH_2-terminal domains which form a parallel helix-helix interface (Guo et al., 1997).

Following synapsis, the free 5'-hydroxyl group of the nick in each of the two FRT sites acts as a nucleophile to attack the Flp:DNA covalent linkage in the partner FRT site resulting in intermolecular strand ligation. The Cre/*lox* structure shows that the ~100° DNA bend in the core region is associated with three unpaired nucleotides adjacent to the cleaved site. In the structure these nucleotides have a high degree of mobility and are moved toward the center of the strand exchange cavity suggesting that the bend in the DNA facilitates strand exchange following DNA cleavage (Guo et al., 1997). The initial strand exchange forms a four-armed DNA

intermediate called a Holliday intermediate or χ-structure (Holliday, 1964). The Holliday intermediate is resolved by a second pair of cleavages at the remaining sites on the other side of the core region followed by the exchange and ligation of DNA strands to yield two recombinant products.

Resolution of the Holliday intermediate was long thought to require extensive isomerization of the four-armed structure and branch migration to bring the remaining pair of cleavage sites into proximity for the second strand exchange event (Sobell, 1974; Meselson and Radding, 1975). In contrast to such models, Dixon et al. (1995) proposed that resolution required only a limited migration of the branch point to 3 bp from the initial cleavage sites. The Cre/*lox* structure shows that following the first set of cleavages the four active sites in the synaptic structure are symmetrically positioned around the cleavage sites with similar inter-active site distances providing support for the model that resolution occurs in the absence of extensive isomerization and branch migration.

4. PROTEIN-INDUCED DNA BENDING

DNA bending is a common although not a universal consequence of proteins binding to DNA. DNA bending proteins are often regulators of basic biological processes including site-specific recombination, transcription, DNA replication and DNA repair. Biochemical approaches to studying protein-induced DNA bending include ligase-mediated DNA cyclization (Kotlarz et al., 1986; Crothers et al., 1992) and the electrophoretic methods of circular permutation and

phasing analysis (Zinkel and Crothers, 1987). Other less commonly used methods include electron microscopy (Gronenborn et al., 1984; Shi et al., 1992) and scanning force microscopy (Erie et al., 1994). Crystallographic studies of protein:DNA complexes are adding to the understanding of the mechanisms by which proteins induce DNA bending.

(i) Biological function of protein-induced DNA bending

Several examples of DNA bending proteins whose biological function in transcription or site-specific recombination is understood are reviewed below. These include catabolite activator protein (CAP), MerR and IHF.

A well studied DNA bending protein is CAP of *E. coli.* CAP is a transcriptional activator which increases transcription at the *lac*, *gal* and many other promoters. The magnitude of the overall DNA bend in the CAP:DNA complex is ~90° and is comprised almost entirely of two 40° kinks as revealed by two crystallographic studies (Schultz et al., 1991; Parkinson et al., 1996). This agrees with the bend angle of ~100° estimated from the electrophoretic mobilities of CAP:DNA complexes (Zinkel and Crothers, 1990). The CAP binding site can be replaced with intrinsically bent DNA and transcriptional activation from the *gal* promoter *in vivo* and from the *lac* promoter *in vitro* is retained at levels comparable to the unmodified promoters (Bracco et al., 1989; Gartenberg and Crothers, 1991). CAP-induced bending activates transcription by increasing the affinity of RNA polymerase for the regulatory region (Zinkel and Crothers, 1991).

The MerR protein of *E. coli* is a transcription factor that is required for expression of the mercury-detoxification proteins encoded by the *mer* operon. In the repressed state both MerR and RNA polymerase bind to the promoter region and the complex remains in an inactive closed form (Frantz and O'Halloran, 1990; Heltzel et al., 1990). In this complex MerR induces a DNA bend of 25°±10° as estimated from circular permutation analysis (Ansari et al., 1992). Binding of mercuric ion to the MerR protein, however, converts the transcription complex to an active open form (O'Halloran et al., 1989; Frantz and O'Halloran, 1990; Heltzel et al., 1990). The transition from the closed to the open transcriptionally active complex is accompanied by Hg-MerR-induced unbending and unwinding of the operator DNA (Ansari et al., 1992; Ansari et al., 1995). Unbending of the operator DNA is experimentally supported by the observed loss of the phase-dependent variation in electrophoretic mobility of Hg-MerR complexes relative to MerR complexes, as well as, the decreased rate of DNase I cleavage in the Hg-MerR complex (Ansari et al., 1995).

IHF of *E. coli* is a site-specific DNA bending protein that is essential to site-specific recombination of phage λ DNA with the bacterial chromosome. The crystal structure of IHF complexed with DNA shows that the DNA is U-shaped with an overall bend angle of at least 160° (Rice et al., 1996). λ site-specific recombination proceeds by the assembly of a multiprotein:DNA complex called the intasome which contains several copies of lambda integrase (λ Int) and IHF bound to *attP* (the attachment site on phage λ DNA). The ability to replace one of the three IHF-induced bends in *attP* with

intrinsically bent DNA or a CAP-induced bend and retain significant recombination activity indicated that IHF has an architectural role in the assembly of the intasome (Goodman and Nash, 1989). IHF-induced DNA bending enables a single λ Int monomer which contains two autonomous DNA binding domains to interact simultaneously with two distinct and otherwise distant binding sites in *attP* (Moitoso de Vargas et al., 1989).

(ii) Electrophoretic methods for studying protein-induced DNA bending

Experimental techniques based on the electrophoretic mobility of protein:DNA complexes on nondenaturing polyacrylamide gels have become a widely used approach to studying protein-induced DNA bending. The electrophoretic mobility of a DNA molecule through a gel is dependent on its mean square end-to-end distance (Lerman and Frisch, 1982; Lumpkin and Zimm, 1982). DNA molecules containing a bend thus show a retarded mobility as compared to unbent DNA molecules. This forms the basis for both circular permutation and phasing analysis.

Wu and Crothers (1984) developed a method for locating DNA bends by comparing the electrophoretic mobilities of DNA molecules that differ from each other only by the order of the sequence along the fragment (permuted). The DNA molecule with the most retarded mobility has the DNA bend closest to its middle; the DNA molecule with the least retarded mobility has the DNA bend closest to its end. Several vectors have been designed in which direct repeats that contain a number of restriction sites flank a unique cloning site

(Kim et al., 1989; Zwieb et al., 1989). Cloning of the binding site of the protein of interest into the unique site followed by digestion of the plasmid obtained with several restriction enzymes that cut within the direct repeats generates circularly permuted DNA fragments (Fig. 1-5A). These fragments differ only in the position of the protein binding site and otherwise have the same length and sequence. If the protein of interest induces a DNA bend, the electrophoretic mobility of the bound fragments will show a dependence on the position of the binding site on the fragment (Fig. 1-5B). Plotting of the relative mobilities of the bound circularly permuted fragments as a function of the distance between the protein binding site and the end of the fragment followed by extrapolation of the linear portions of the curve enables the estimation of the position of the protein-induced DNA bend. The circular permutation assay may also be used to estimate the magnitude of a protein-induced DNA bend by comparing the relative mobilities of bound circularly permuted fragments to a set of DNA standards which have known degrees of intrinsic curvature (Thompson and Landy, 1988).

Use of the circular permutation assay, however, is subject to some assumptions. In addition to DNA bending, the assay is sensitive to other protein-induced effects such as changes to the flexibility or conformation of the DNA (Van der Vliet and Verrijzer, 1993). Conformational flexibility in the protein, as shown for the transcriptional activator GCN4, can also influence the mobility of complexes in the absence of DNA bending (Gartenberg et al., 1990).

For these reasons protein-induced DNA bending is usually confirmed by an independent experimental approach.

A second electrophoretic method, phasing analysis, was developed by Zinkel and Crothers (1987) to determine the relative direction of a DNA bend. In this approach, an intrinsic sequence-directed bend of known direction and magnitude (usually a set of A tracts) and the protein binding site are separated by a set of linkers that vary in length between 10 to 20 base-pairs (Fig. 1-6A). In the case where the protein of interest induces a DNA bend, the electrophoretic mobility of the bound fragments will show a dependence on the phasing between the two bends. The length of the linker establishes the relative orientation of the intrinsic bend and the protein-induced DNA bend. In the complex with the most retarded mobility the two bends are in phase and cooperate to increase the overall bend angle. The two bends are out-of-phase and counteract each other to reduce the overall bend angle in the complex with the maximal mobility (Fig. 1-6B). The direction of the protein-induced DNA bend is determined by calculating the number of helical turns between the centres of the intrinsic bend which has a known bend direction and the protein-induced bend.

An advantage of phasing analysis is that the position of the protein binding site relative to the ends of the DNA molecule is close to constant. This assay may thus be used to confirm the presence of a protein-induced DNA bend that is suggested by the results of a circular permutation assay. Failure of this assay to substantiate the circular permutation assay may mean that the

protein causes a position-dependent change in the electrophoretic mobility of a fragment that is not due to DNA bending.

(iii) Mechanisms of protein-induced DNA bending

At least two distinct mechanisms of protein-induced DNA bending have emerged. These are the intercalation of a hydrophobic side-chain(s) of the protein into the minor groove of the DNA helix and the asymmetric neutralization of phosphate charges of the DNA backbone at the protein:DNA interface. These two mechanisms are utilized to varying extents by different DNA-bending proteins as is evident from crystallographic studies of proteins complexed with DNA.

The hydrophobic side-chain(s) of a protein inserted into the minor groove acts as a wedge to pry open a single base step and distort the DNA. This results in widening of the minor groove, introduction of a significant positive roll angle between the two stacked base-pairs and a reduction in the helical twist at the site of intercalation into the DNA. Overall intercalation results in the introduction of a sharp, localized bend (kink) into the DNA. Return to B-DNA geometry usually occurs within two to five base steps on either side of the intercalation site (Werner et al., 1996).

Some proteins utilize a mechanism of asymmetric neutralization of phosphate charges on the DNA backbone to induce a bend in the DNA. Electrostatic interactions between the protein and a localized group of DNA phosphates results in an imbalance of the negative charges along the DNA backbone. The electrostatic repulsion between the remaining phosphate charges leads to a spontaneous collapse of the DNA toward the neutralized DNA surface at the protein:DNA

interface. The mechanism of DNA bending by asymmetric phosphate neutralization was initially proposed by Mirzabekov and Rich (1979) and has been demonstrated experimentally in the absence of protein (Strauss and Maher, 1994; Strauss et al., 1996).

The crystal structure of the IHF:DNA complex shows that both of these mechanisms maintain the ~160° DNA bend (Rice et al., 1996). The DNA wraps around a compact core formed by two subunits of IHF. Two β arms extend out from the central core of IHF to the outside of the DNA bend. A proline residue located on the tip of each β arm intercalates into the helix and opens the minor grooves. The central core of the IHF molecule forms electrostatic interactions with phosphates of the narrowed grooves on the inside curve of the bent DNA (Rice et al., 1996; Travers, 1997). The structure of the CAP:DNA complex indicates that CAP relys solely on charge neutralization on the inner DNA surface to induce DNA bending while TATA-binding protein (TBP) induces the DNA bend entirely through minor groove intercalation on the outer surface of the bend (Schultz et al., 1991; Kim, Y. et al., 1993; Kim, J.L. et al., 1993).

5. THESIS OUTLINE

This thesis describes biochemical experiments which investigate the structure of the DNA in Flp:DNA complexes. When these studies were initiated it was known that binding of Flp to the FRT site induced DNA bending (Schwartz and Sadowski, 1989). Binding of a Flp monomer to a single symmetry element generates a DNA bend of ~60° (type I bend) while binding of two Flp monomers to the FRT site

generates a DNA bend of >140° (type II bend; Schwartz and Sadowski, 1990). Mutations in Flp at several positions caused defects in both the type II bend and recombination which suggested that DNA bending has an essential role in the recombination pathway (Schwartz and Sadowski, 1989; Chen et al., 1991, 1992a; Kulpa et al., 1993). Flp-mediated bending was investigated further to better understand the functional role of the DNA structure in the recombination pathway.

In chapter 2, I describe experiments which define the position and direction of Flp-induced DNA bends using the electrophoretic methods of circular permutation and phasing analysis. The type II bend was positioned in the middle of the core region and directed toward the major groove in noncovalently bound Flp:DNA complexes. The position and direction of the type II bends in complexes where either the top or bottom strand was cleaved varied depending on which strand was cleaved. A model is proposed in which the variable position of the type II bends is associated with a defined order of strand exchanges in the recombination reaction. The position of the type I bend differed depending upon whether the substrate contained one or two Flp binding elements. A model in which a single Flp monomer interacts with both symmetry elements of a single FRT site is proposed.

The influence of the core sequence on the positioning of the type II bend is investigated in chapter 3. Substitution of the central AT base-pair in the core region altered the position of the type II bend and affected Flp-mediated cleavage and recombination activities. Thus, the central AT base-pair in the core is important to the position of the type II bend and the function of the site.

Experiments described in chapter 4 examine the interaction of Flp with the FRT site in covalently bound complexes which were cleaved in the top or bottom strands. The FRT sites contained a 5'-bridging phosphorothioate linkage at the site of Flp cleavage to enable the accumulation of covalent complexes. Flp-mediated cleavage is associated with methylation protection of two adenine residues that are opposite the sites of cleavage and covalent attachment by Flp. Cleavage and covalent attachment are accompanied by differences in the contacts of Flp with each of the two cleavage sites and with the surrounding symmetry elements. Flp-mediated cleavage and covalent attachment are apparently associated with conformational changes in the Flp:FRT complexes.

In chapter 5 the preceding chapters are summarized and experiments which extend from this body of work are described.

The model presented in chapter 2 in which a single Flp monomer interacts with both symmetry elements of a single FRT site is addressed and disproved by experiments presented in appendix I. It is suggested that the movement of a Flp monomer between the *a* and *b* elements of one FRT site during electrophoresis influences the position of the type I bend on a two-element FRT site. The type I bend position also appears to be influenced by the DNA sequences flanking the site.

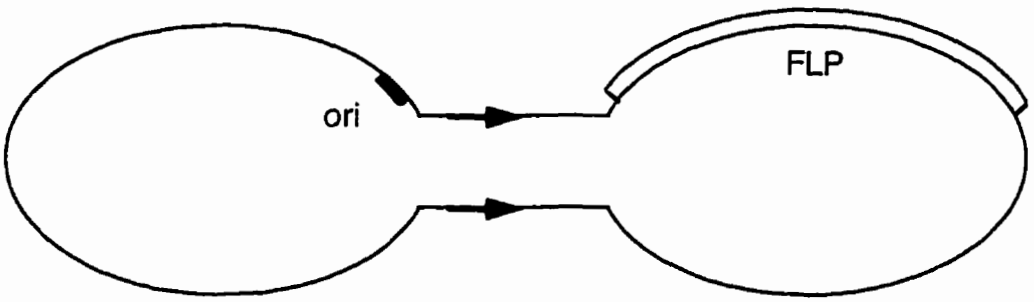


Figure 1-1. The 2 μm plasmid of *Saccharomyces cerevisiae.* The dumb-bell representation of the 2 μm plasmid indicates two unique regions separated by two inverted repeats of 599 bp (parallel horizontal lines). One FRT site (arrow) is located within each of the larger inverted repeats. The origin of replication (ori) and the Flp gene encoded by the plasmid are shown.

Figure 1-2. Futcher model of Flp-mediated amplification of the 2 μm plasmid of *Saccharomyces cerevisiae* (Futcher, 1986). **A.** The 2 μm plasmid contains two FRT site (thick arrows) in inverted orientation. **B.** Bidirectional DNA replication (thin arrows) begins at the origin of the 2 μm plasmid and initially proceeds through one of the FRT sites to generate an intermediate with three FRT sites. **C.** Flp mediates recombination between two of the FRT sites and inverts a large segment of the plasmid. The replication forks are now oriented in the same direction. **D. E.** Further DNA replication generates multimers of the plasmid. Additional Flp-mediated recombination events between two directly oriented FRT sites resolve the multimers to monomers.

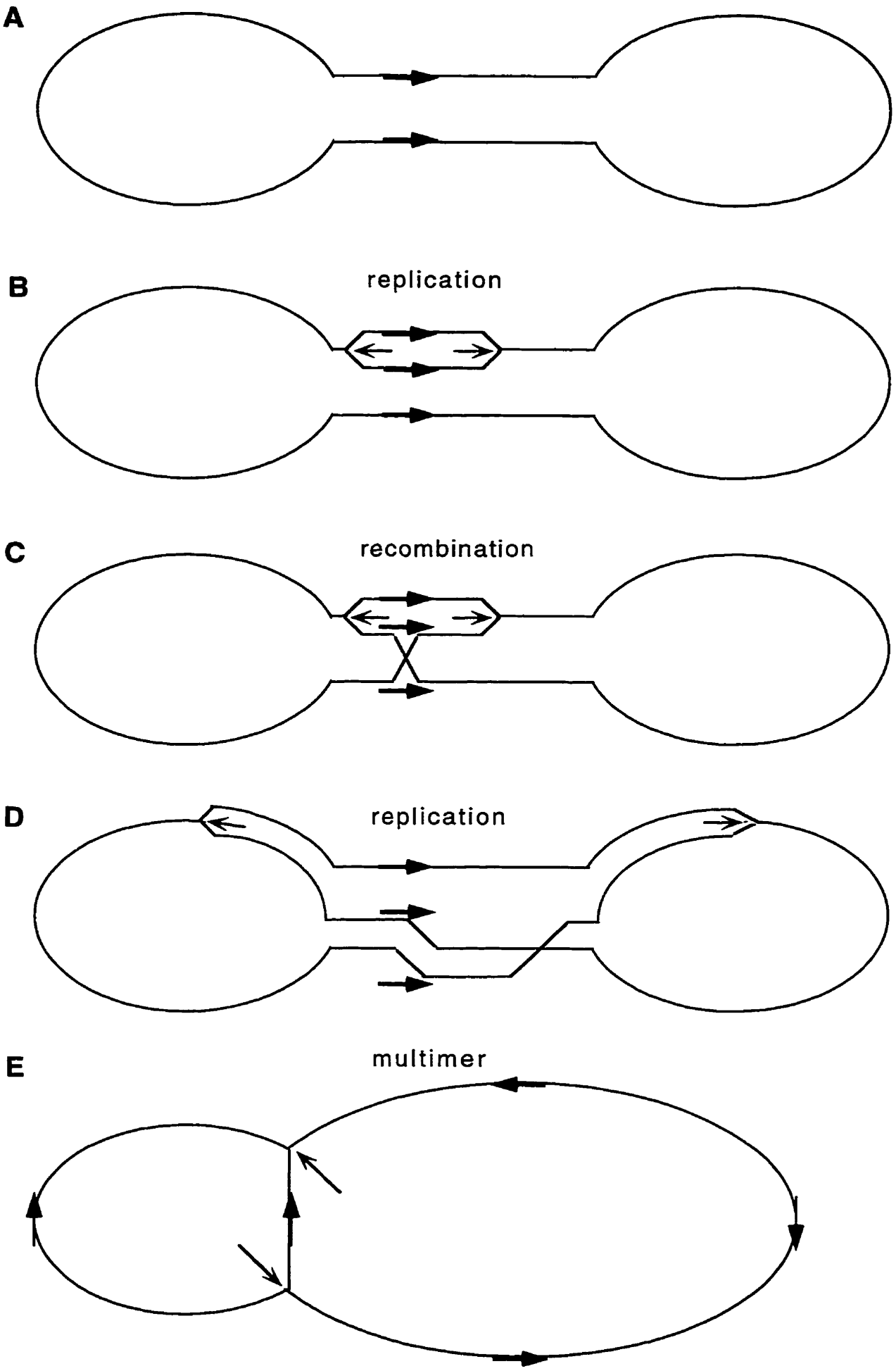

A
B
replication
C
recombination
D
replication
E
multimer

Figure 1-3. The Flp recognition target (FRT) site. The 13 base-pair symmetry elements (*a*, *b* and *c*) are indicated by horizontal arrows and the 8 base-pair core, by an open rectangle. The vertical arrows indicate the sites of Flp cleavage in the top and bottom strands. The stippled boxes indicate the polypyrimidine tracts present in the FRT site.

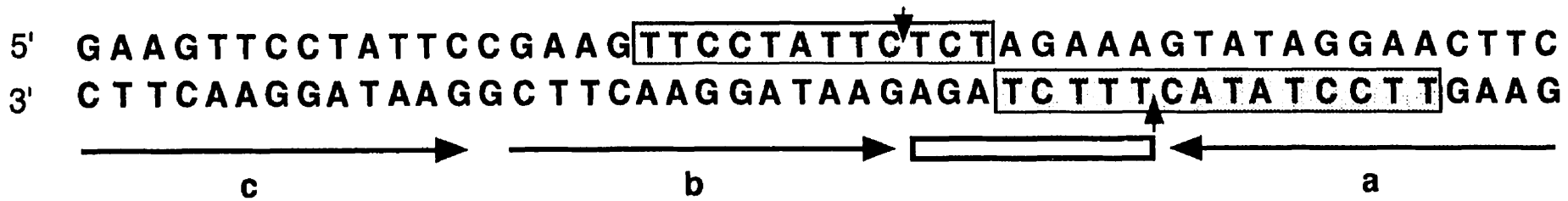
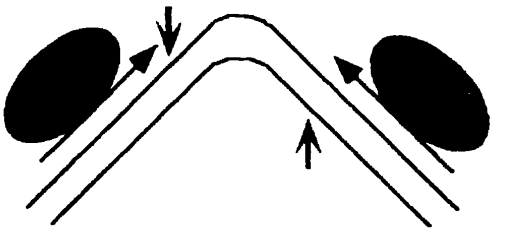

5' GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC
3' CTTCAAGGATAAGGCTTCAAGGATAAGAGATCTTTCATATCCTTGAAG
c
b
a

Figure 1-4. Steps of the Flp-mediated recombination reaction. **A.** A minimal FRT site (parallel lines) containing two inverted binding elements (long arrows) which flank a core region is shown. The vertical arrows indicate the sites of Flp cleavage. One molecule of Flp (purple oval) binds to each of the binding elements. Flp induces DNA bending. **B.** Flp cleaves one strand of the DNA and becomes covalently attached to the 3' terminus (purple line). A 5'-hydroxyl group is present at the nick. **C.** FRT sites bound by Flp assemble in a synaptic complex through protein:protein interactions. **D.** A Holliday intermediate (left) is generated by a reciprocal exchange and ligation of DNA strands between the two FRT sites at the site of the nick. The 5'-hydroxyl group at the nick of each FRT site is ligated to the 3' terminus of the partner FRT site reversing the covalent linkage of Flp to the DNA. The DNA bends and the bound Flp monomers are not shown for the sake of simplicity. Flp-mediated cleavage of the remaining two DNA strands followed by reciprocal strand exchange and ligation resolves the Holliday intermediate into two recombinant DNA molecules (right).

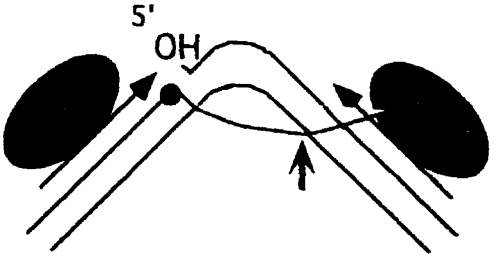
A

DNA binding and DNA bending



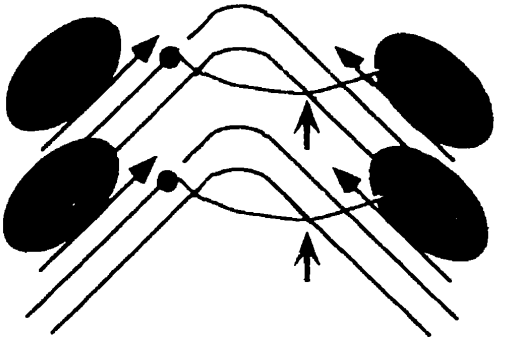
B

DNA cleavage and
covalent attachment



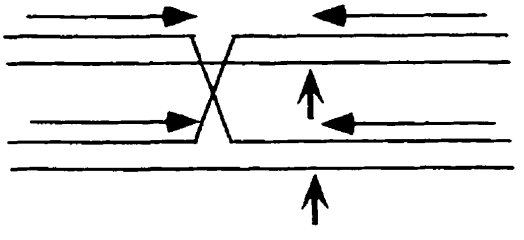
C

synapsis



D

strand exchange and ligation



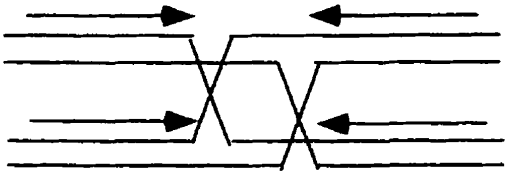
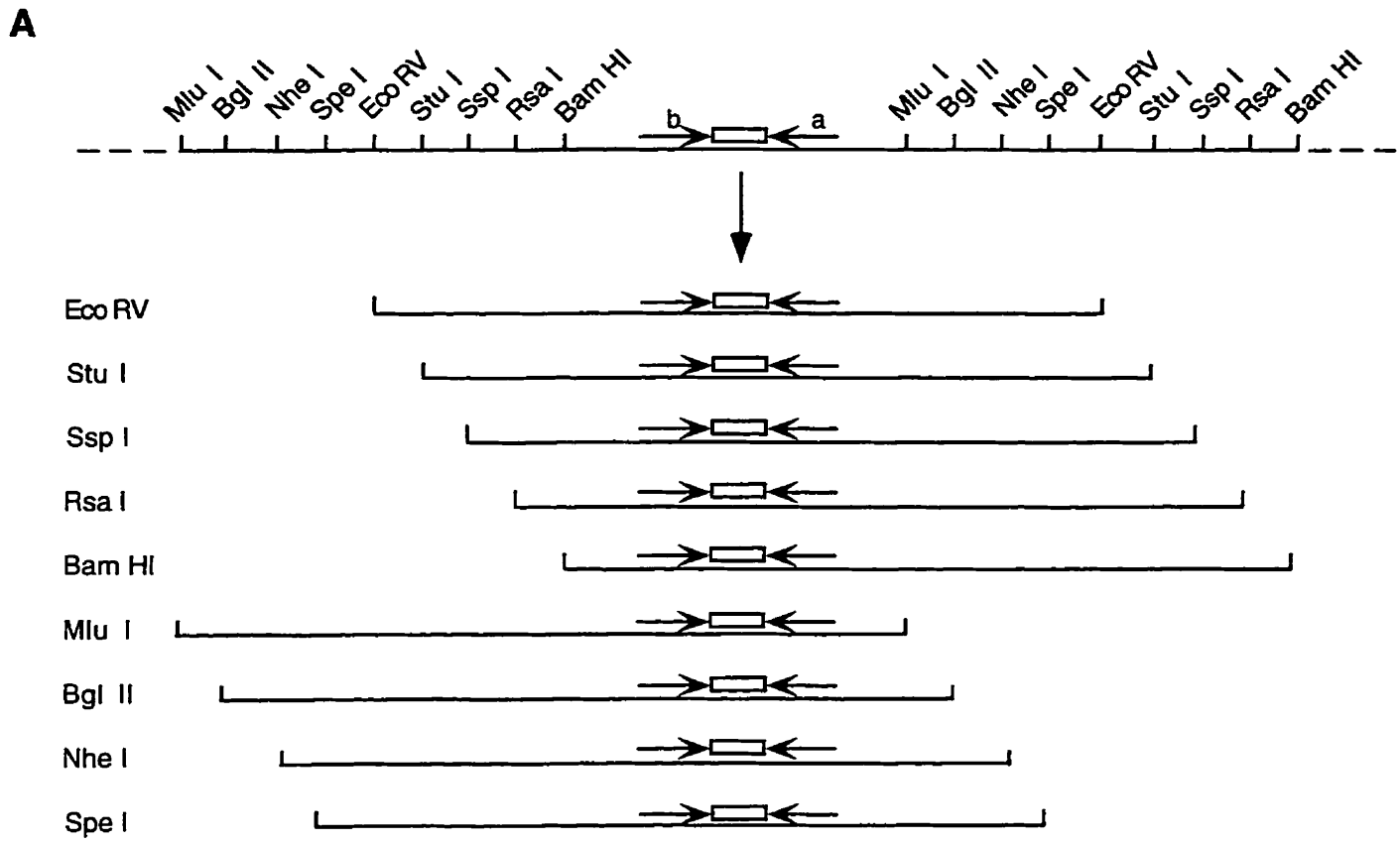


Figure 1-5. Circular permutation analysis. **A.** The protein binding site (two horizontal arrows flanking an open rectangle) is contained in the pBEND2 plasmid (Zwieb et al., 1989) in which it is flanked by two tandemly repeated sets of restriction enzyme sites (top). Restriction enzyme digestion of the plasmid generates a series of fragments which differ in the position of the protein binding site but are otherwise of identical length and nucleotide composition. **B.** The protein:DNA complex with the slowest mobility consists of a DNA bending protein bound to a DNA fragment with the protein binding site closest to the middle. The protein:DNA complex with the fastest mobility consists of a bound DNA fragment containing the binding site closest to the end.


A
Mlu I
Bgl II
Nhe I
Spe I
Eco RV
Stu I
Ssp I
Rsa I
Bam HI
b
a
Eco RV
Stu I
Ssp I
Rsa I
Bam HI
Mlu I
Bgl II
Nhe I
Spe I

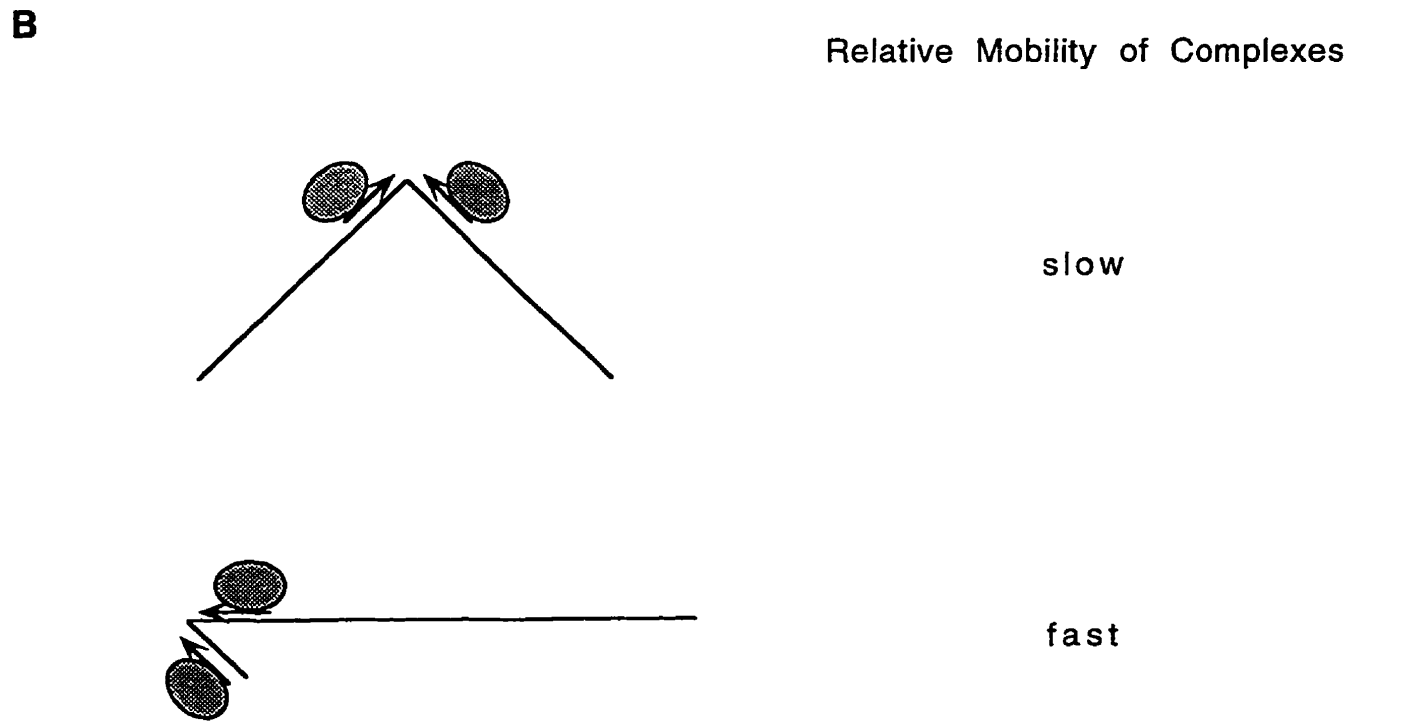
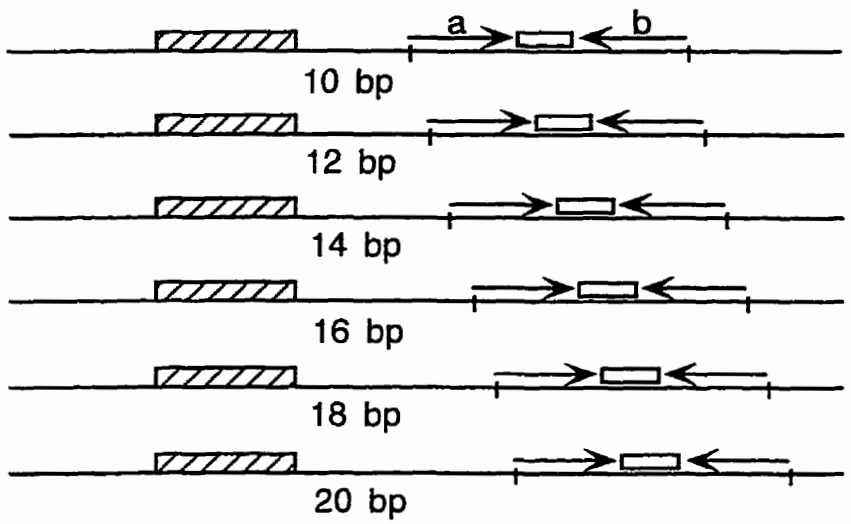

B
Relative Mobility of Complexes
slow
fast

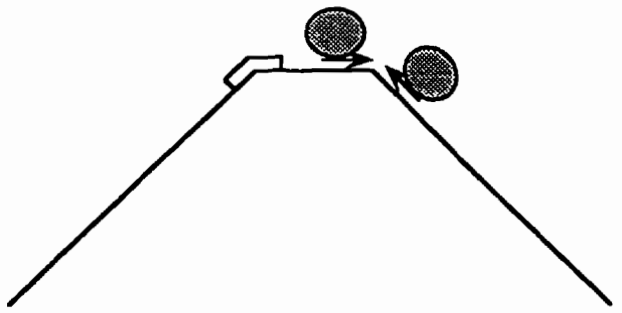
Figure 1-6. Phasing analysis. **A.** The substrates for phasing analysis. DNA fragments contain a sequence-directed bend (striped box) of known direction and magnitude and a protein binding site (two arrows flanking an open rectangle) which are separated by a linker that varies in length (10-20 bp) over one helical turn of DNA. **B.** The protein:DNA complex with the slowest mobility contains a protein-induced DNA bend which is in phase with the intrinsic sequence-directed bend. The protein:DNA complex with the fastest mobility contains a protein-induced bend which is out of phase with the intrinsic bend.

A

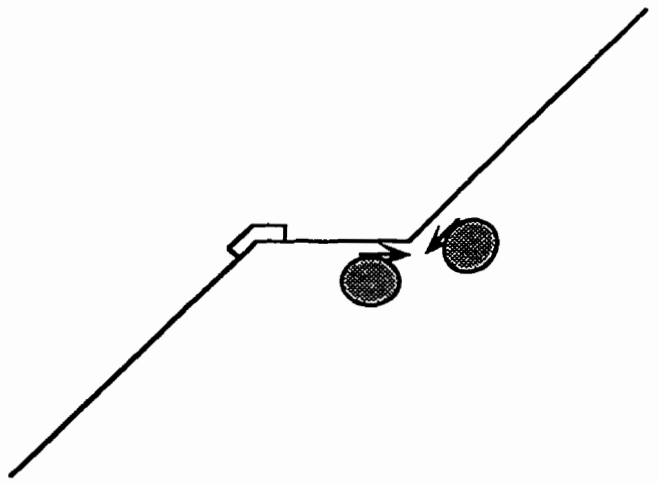


B

Relative Mobility of Complexes



slow (bends in phase)



fast (bends out of phase)

CHAPTER 2.

THE POSITION AND DIRECTION OF FLP-INDUCED DNA BENDS

Material in this chapter has been published in Luetke, K.H. and Sadowski, P.D. (1995). *J. Mol. Biol.* **251**, 493-506.

1. INTRODUCTION

The minimal Flp recognition target site contains two Flp binding sequences flanking an 8-bp core region; binding of Flp results in the formation of two Flp:DNA complexes (complex I and complex II). Binding of a Flp monomer to a single symmetry element generates a DNA bend of about 60° (a type I bend), whereas binding of two Flp monomers to the FRT site generates a DNA bend of >140° (a type II bend; Schwartz and Sadowski, 1990).

I have used circular permutation and phasing analysis to define the position and direction of the type I and type II DNA bends induced by Flp, mutant Flp proteins and the Flp peptides P27 (27 kDa; amino acids 124 to 346) and P32 (32 kDa; amino acids 124 to 423).

The type II bend induced by the noncovalent binding of two Flp molecules to the target site was located in the middle of the core region and directed toward the major groove. Ligation-defective Flp proteins were used to determine the position and direction of the type II bends in complexes where either the top or bottom strand was cleaved. The position and direction of bends in such complexes depended upon which strand was cleaved. In a covalently-bound complex in which the bottom strand was cleaved, the type II bend was similarly positioned in the middle of the core region and directed toward the major groove; the type II bend in a covalently-bound complex in which the top strand was cleaved was positioned in the core-proximal end of the *b* element and directed toward the minor groove. A model is proposed which associates the position of

Flp-induced type II bends with a defined order of strand exchanges in the recombination reaction.

The position of the type I bend depended upon whether the substrate contained one or two Flp binding elements. When the substrate contained one symmetry element, the type I bend was positioned at the core-distal end of the *b* element. It was located at the core-proximal end of the *b* element, however, when the substrate contained two Flp-binding elements. The P27 and P32 Flp peptides, which lack the NH_2-terminal 13 kDa region of Flp do not show this behavior. It was deduced that the 13 kDa region of Flp is critical for positioning of the type I bend centre on a minimal FRT site. A model is proposed in which a single molecule of Flp interacts with two symmetry elements to account for these results. This model is addressed by experiments reported in appendix I. The type I bend was directed toward the major groove when the substrate contained a single symmetry element, however, it was directed toward the minor groove on a two-element FRT site.

2. MATERIALS AND METHODS

(i) Plasmid construction and DNA fragments

The pB2Flp(b) plasmid was constructed by ligating the annealed, complementary oligonucleotides 5' TCGAGAATAGGAACTT 3'/3' CTTATCCTTGAAGATC 5', containing the *b* symmetry element of the FRT site into *XbaI and SalI* digested pBEND2 (Zwieb et al., 1989). In this plasmid the *b* symmetry element is inserted between two tandemly repeated sets of restriction enzyme sites. Circularly

permuted DNA substrates used to determine the centre of Flp, Flp mutant or Flp peptide-induced type I DNA bends were obtained by digestion of pB2Flp(b) with each of *Eco*RV, *Stu*I, *Ssp*I, *Rsa*I, *Bam*HI, *Mlu*I, *Bgl*II, *Nhe*I and *Spe*I. The pB2Flp(ba) plasmid was constructed by ligating the annealed, complementary oligonucleotides 5' TCGAGAAGTTCCTATACTTTCTAGAGAATAGGAACTT 3'/3' CTTCAAGGATATGAAAGATCTCTTATCCTTGAAGATC 5' containing the *b* and *a* symmetry elements flanking the core region of the FRT site into *Xba*I and *Sal*I digested pBEND2 (Zwieb et al., 1989). Circularly permuted DNA substrates were obtained by digestion of pB2Flp(ba), as described above, to determine the bend centres of type I or type II DNA bends on a two-element FRT site.

The plasmids pK10Flp(b), pK12Flp(b), pK14Flp(b), pK16Flp(b), pK18Flp(b) and pK20Flp(b) were used to generate DNA substrates used for phasing analysis of Flp-induced type I DNA bends. These plasmids were constructed by cloning the 267 bp *Bam*HI-*Pst*I DNA fragment from plasmid pCS40 (Schwartz and Sadowski, 1990) containing the *c* element and 4 bp of the adjacent *b* symmetry element into each of *Bam*HI and *Pst*I digested pK10, pK12, pK14, pK16, pK18 and pK20 plasmids (Zinkel and Crothers, 1987). Phasing substrates were obtained by isolating the *Rsa*I-*Pvu*II fragments (325-335 bp) from pK10Flp(b), pK12Flp(b), pK14Flp(b), pK16Flp(b), pK18Flp(b) and pK20Flp(b). The plasmids pK10Flp(ba), pK12Flp(ba), pK14Flp(ba), pK16Flp(ba), pK18Flp(ba) and pK20Flp(ba) were used to generate DNA substrates used for phasing analysis of Flp-induced type II DNA bends. These plasmids were constructed by excising the 156 bp *Bam*HI-*Bam*HI DNA fragment from the plasmids pK10, pK12,

pK14, pK16, pK18 and pK20 and ligating in the annealed, complementary oligonucleotides 5' GATCCGAAGTTCCTATTCTCTAGA AAGTATAGGAACTTC 3'/3' GCTTCAAGGATAAGAGATCTTTCATATCCTT GAAGCTAG 5' containing the *b* and *a* symmetry elements flanking the core region of the FRT site. Phasing substrates were obtained by isolating the *Rsa*I-*Pvu*II fragments (297-307 bp) from pK10Flp(ba), pK12Flp(ba), pK14Flp(ba), pK16Flp(ba), pK18Flp(ba) and pK20Flp(ba). Each of the pKFlp(ba) plasmids contains the two-element FRT site oriented in the same direction, such that the *a* symmetry element of the site is closest to the sequence-directed bend. All oligonucleotides were synthesized by the Carbohydrate Research Centre, University of Toronto.

(ii) Flp preparations

Flp and mutant Flp proteins were purified as described by Pan et al. (1991) or Kulpa et al. (1993). Wild-type protein was >90% pure. The mutant Flp proteins were approximately 10-50% pure. The concentration of Flp was estimated by comparison with highly purified Flp standards on Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. The Bradford (1976) assay was used to determine the concentration of the homogeneous Flp standards. Flp genes with the mutations H305L and Y343F were obtained from M. Jayaram. Mutant Flp proteins are named as the amino acid (expressed as a single letter) present in the wild-type protein, followed by the amino acid number, followed by the amino acid present in the mutant Flp protein. Flp TA232 is a four amino acid insertion mutant at amino acid position 115 in Flp. Flps R191K

and TA232 were isolated in our laboratory (Friesen and Sadowski, 1992; Amin and Sadowski, 1989).

Flp peptide P32 was generated by partial proteolysis of intact Flp, purified and sequenced as described previously (Pan and Sadowski, 1993). The P27 Flp peptide (27 kDa; amino acids 124-346 of Flp) was obtained in the same way except a shorter period of digestion with proteinase K (1.5 min at 0°C) was employed (Kuntz and Sadowski, unpublished).

(iii) Gel mobility shift assays

0.02 pmol of ^{32}P end-labeled DNA substrates were incubated with 0.06-2.0 μM Flp protein or Flp peptide (as indicated in the figure legends) in a 20 μl volume containing 50 mM Tris-HCl (pH 7.4), 33 mM NaCl, 1 mM EDTA and 100 μg calf thymus DNA/ml. Reaction mixtures were incubated at 30°C for 30 min. 2.2 μl of dye mixture were added and reaction mixtures were subjected to electrophoresis on a native 5% (or 8%) polyacrylamide gel (acrylamide:bis-acrylamide ratio of 30:1) which had been pre-run for 30 min at 4°C. The running buffer was 1xTBE (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA, pH 8.3). Electrophoresis was performed at 200v (4°C) until the xylene cyanol dye front had migrated 14 cm (circular permutation analysis) or 17.5 cm (phasing analysis) from the gel wells (7-9 hrs). Dried gels were subjected to autoradiography.

(iv) Circular permutation analysis

Circular permutation substrates containing the *b* symmetry element of the FRT site (131 bp), obtained by digestion of the pB2Flp(b)

plasmid (as described above), were used to determine the centre of type I DNA bends. Circular permutation substrates containing the *b* and *a* symmetry elements flanking the core region of the FRT site (152 bp), obtained by digestion of the pB2Flp(ba) plasmid (as described above), were used to determine the centre of type I and type II DNA bends. ^{32}P end-labeled substrates were used in gel mobility shift assays as described above. The relative electrophoretic mobility of a protein:DNA complex was calculated as the mobility of the complex divided by the mobility of the unbound substrate to correct for small variations in the mobility of unbound substrates resulting from intrinsic sequence-directed bends present in the fragment. Relative mobilities of the complexes (y-axis) were plotted against the distance (bp) of the left-hand end of each substrate to the left-hand *Eco*RV site on the DNA fragment. The curve was plotted using polynomial curve fitting software (Cricketgraph). The bend centre was estimated by extrapolating the linear portions of the curve to find the position at which the curve reached a minimum.

(v) Phasing analysis

Phasing substrates containing the *b* symmetry element of the FRT site (325-335 bp) were used to determine the bend direction of Flp-induced type I bends. Phasing substrates containing the *b* and *a* symmetry elements flanking the core region of the FRT site (297-307 bp) were used to determine the bend direction of Flp-induced type II bends. The relative mobilities of the complexes, obtained as described above, were normalized by dividing by the average

mobility of all complexes. Normalized relative complex mobilities (y-axis) were plotted as a function of the linker length (bp) of each substrate (x-axis). The linker length is defined as the distance (bp) from the middle of the sequence-directed DNA bend to the middle of the Flp-induced DNA bend. The curve was plotted as the best fit using Cricketgraph software.

3. RESULTS

(i) Flp and the cleavage-defective Flp Y343F show a type II bend centre located in the core region

The location of the type II bend centre has been localized approximately to the core region of the FRT site (Schwartz and Sadowski, 1989). We wished to define the location of the type II bend more precisely, and to determine whether the location of the type II bend is dependent on cleavage of the DNA. The two-element circular permutation substrates (Fig. 2-2A) were used for these determinations. The relative mobilities of the complex IIs, formed when Flp binds to both elements *a* and *b* were measured and analyzed as described above (Fig. 2-2B and 2-2C). In agreement with previous results, the type II bend centre was located in the core region at position 0 (Fig. 2-1). Interestingly, the position of this bend centre corresponds to the junction of the two polypyrimidine tracts present in the FRT site and which are diagrammed in Figure 2-1. This result suggests these tracts may have a role in positioning of the type II bend centre prior to DNA cleavage.

Flp Y343F shows the same location of the type II bend centre as wild-type Flp (position 0 in the core region, Fig. 2-1). The mutant protein lacks the nucleophilic tyrosine residue and is unable to cleave and covalently attach to the DNA (Evans et al., 1990). Thus I conclude that the position of the type II bend centre is independent of DNA cleavage.

To confirm that the complex IIs used to determine the location of the type II bend centre did not contain Flp covalently attached to the DNA, the sensitivity of the major Flp-generated complex II band to heparin was determined. The complex II was sensitive to heparin, confirming that the complex II from which the location of the type II bend centre was determined represented a complex in which Flp was not covalently bound to the DNA (Fig. 2-3, lanes 2 and 4).

(ii) Covalently attached mutant Flp proteins induce two type II bends with different bend centre locations

We sought to determine the location of type II bend centres in complexes containing covalently bound Flp proteins. Such covalent complexes are detectable in the Flp recombination reaction (Andrews et al., 1985) but are present in insufficient quantity to be characterized. The Flp R191K mutant protein is capable of cleaving DNA but is defective in ligation activity; therefore covalent intermediates accumulate in which the protein is covalently attached at one of the two cleavage sites (Friesen and Sadowski, 1992; Parsons et al., 1988; Pan et al., 1993). Cleavage by Flp R191K yields a complex II (cIIa) which is nicked in the bottom strand (at the *a* symmetry element/core junction) and in which one of the

bound monomers is covalently attached at the nick; cleavage in the top strand (at the *b* symmetry element/core junction) yields a similar complex (cIIb; Friesen and Sadowski, 1992). Complex IIa and complex IIb appear as two distinct protein:DNA complexes on a native polyacrylamide gel (Friesen and Sadowski, 1992; Fig. 2-3) and can be distinguished from each other by their relative intensities on a native polyacrylamide gel. Flp R191K cleaves the bottom strand at the *a* symmetry element/core junction about twice as efficiently as the top strand at the *b* symmetry element/core junction giving about twice as much complex IIa as complex IIb (Friesen and Sadowski, 1992; Jayaram et al., 1988; Fig. 2-3, Fig. 2-4A).

The results of circular permutation experiments using Flp R191K are shown in Figures 2-4A and 2-4B. The same results were obtained with Flp H305L (data not shown). The bend centre of complex IIa was at position +2 (Fig. 2-1). The bend centre of complex IIb, however, was at position -8 (Fig. 2-1) i.e. 10 bp away from the bend centre of complex IIa.

To verify that complex IIa and complex IIb generated by the ligation-defective Flp R191K are not complexes unique to these proteins, a binding reaction in which wild-type Flp was bound to a two-element FRT site was treated with heparin (Fig. 2-3). This experiment shows that wild-type Flp generates a small amount of two distinct, heparin-resistant complexes (Fig. 2-3, lane 4) which migrate slightly above and slightly below the major complex II generated by Flp (Fig. 2-3, lane 2) and which migrate close to complex IIa and complex IIb (Fig. 2-3, lanes 3 and 5). These

complexes apparently represent the equivalent of complex IIa and complex IIb.

(iii) The Flp-induced type I bend centre shows a substrate-dependent location within the b symmetry element

The migration of a DNA fragment during gel electrophoresis is dependent on its mean square end-to-end distance (Lerman and Frisch, 1982). This forms the basis of circular permutation analysis (Wu and Crothers, 1984), which were used to locate the centre of Flp-induced DNA bends. The Flp-induced distortions are treated as if they are simple directed bends, without specifying the microscopic nature of the actual structure of the distortion caused by the binding of Flp to DNA.

Although the centre of the type II bend has been localized in the core region of the FRT site (Schwartz and Sadowski, 1990), the centre of the type I bend was not determined directly. In order to measure the centres of Flp-induced type I bends, a series of circularly permuted substrates was obtained by digestion of the pB2Flp(b) plasmid with various restriction enzymes. These fragments contain a single *b* symmetry element at different positions along the length of the fragment (Fig. 2-5A). Figure 2-5B and 2-5C shows that the Flp:DNA complexes formed with the *Bgl*II and *Eco*RV fragments have the greatest and the least relative mobilities, respectively, indicating that the Flp-induced bend centre is closest to the *Bgl*II site. The bend centre can be precisely located from a plot of relative mobility of the complexes *versus* the molecular end of the substrate (Wu and Crothers, 1984; Fig. 2-5C).

Extrapolation of the linear portions of this curve indicated that the Flp-induced DNA bend centre on a substrate containing a single *b* element was at the core-distal end of the *b* element at position -16, with an estimated error of ±2 bp (Fig. 1, Table 2-1). The type I bend centre was positioned at nucleotide -16 (with a range of -15 to -17) from the average results of four individual experiments (Fig. 2-1, Table 2-1) derived from 5% (w/v) gels run for varying lengths of time.

To determine whether the centre of the type I bend changed when a recombinationally competent FRT site was used, a second circular permutation analysis was performed using a series of substrates obtained by digestion of the plasmid, pB2Flp(ba). These substrates contain a FRT site consisting of the *a* and *b* elements in inverted orientation flanking the 8 bp core region (Fig. 2-2A). Complex I is formed by a single Flp monomer bound to a single symmetry element, and complex II is formed when Flp binds to both elements (Andrews et al., 1987; Fig. 2-2B). Measurement of the relative mobilities of the complex Is allowed the determination of the location of the centre of the type I bend induced by Flp in a two-element FRT site (Fig. 2-2C). From three replicate experiments, this bend centre was located at the core-proximal end of the *b* element at position -5 (Fig. 2-1; Table 2-1). Thus the centres of Flp-induced type I bends were located 11 bp apart, depending on whether the substrate contained a single symmetry element or two inverted symmetry elements. The implication of this substrate-dependent location of the type I bend centre is discussed below.

(iv) Type I bend centres induced by the P32 and P27 Flp peptides are independent of the FRT substrate

Four peptides have been obtained by partial proteolysis of Flp with proteinase K: an amino-terminal 13 kDa peptide (P13; amino acids 2-123 of Flp), an internal 21 kDa peptide (P21; amino acids 148-346 of Flp), and internal 27 kDa peptide (P27; amino acids 124-346 of Flp), and a carboxyl-terminal 32 kDa peptide (P32; amino acids 124-423 of Flp) (Pan et al., 1991; Kuntz and Sadowski, unpublished; Pan and Sadowski, 1993). Cross-linking and footprinting studies have shown that the P13 peptide binds to the core-proximal 4 bp of each symmetry element whereas the P32 peptide binds in a site-specific manner to the core-distal 9 bp of each symmetry element (Panigrahi and Sadowski, 1994). P32 generates a type I bend of 55°, comparable to that generated by intact Flp (63°), but is unable to generate a type II bend (Pan and Sadowski, 1993).

We were interested to know whether the P32 and P27 Flp peptides which lack the amino-terminal P13 region of Flp show the same type I bend centres as the intact protein. We note that P27 lacks ~75 amino acids of the COOH-terminus but has the same NH_2-terminus as P32 (Kuntz and Sadowski, unpubiished).

The centre of the type I bends induced by both P32 and P27 were approximately the same, at positions -18 to -22, whether the substrate contained one or two symmetry elements (Fig. 2-1; Table 2-1). Since the P32 and P27 peptides show the same type I bend centre, we conclude that the COOH-terminus of Flp, which is absent in P27 but present in P32, does not affect the positioning of the type I bend centre. Both P32 and P27 lack the NH_2-terminal ~13 kDa

region of Flp; we deduce that the apparent difference in the type I bend centre observed with intact Flp on the one-element *versus* the two-element FRT site is dependent upon the NH_2-terminal 13 kDa of Flp.

(v) Direction of Flp-induced type I and type II bends

Phasing analysis was used to determine the direction of Flp-induced DNA bends (Zinkel and Crothers, 1987). Phasing analysis, unlike circular permutation analysis, is used to distinguish a directed bend from non-directed protein-induced flexure of B-DNA and from shape changes of proteins that may cause altered gel mobility by permutation analysis (Zinkel and Crothers, 1990; Van der Vliet and Verrijzer, 1993). This analysis is based on the fact that the electrophoretic mobility of a DNA fragment containing a sequence-directed bend and a protein-induced bend is dependent on the helical phasing between the bends. In the most slowly migrating complex, the protein-induced and sequence-directed bends are cooperatively in phase; i.e. they have the same bend directions and thus the DNA molecule has the shortest end-to end distance. In the complex with the greatest mobility, the two bends are out of phase; i.e. they have opposite bend directions and thus the DNA molecule has the greatest end-to-end distance. Six fragments in which the length of the spacer sequence between the sequence-directed bend and the FRT site varies in 2 bp increments were used. Therefore, the relative position of the two bends is varied over one turn of the DNA helix.

The phasing experiment carried out with Flp and the one-element phasing substrates described above is shown in Figure 2-6A. The

normalized relative mobilities of the complexes were plotted (Fig. 2-6B) to yield a curve with the predicted sine wave function and showing 10 bp periodicity that is observed to be characteristic of protein-induced DNA bends (Zinkel and Crothers, 1987). Thus this result also confirms that the type I bend is a directional bend.

In Figure 2-6B one can see that the maximum relative mobility, corresponding to the complex in which the two bends are out of phase, is shown by the complex formed with the K12(b) substrate; i.e. the one containing a linker length of 99 bp. For a distance of 99 bp possible helical turns of 9, 9.5 and 10 between the two bend centres were considered. These would correspond to helical repeats of 11, 10.4 and 10 bp per turn respectively. Since 10.4 bp per turn is closest to the 10.5-10.6 bp helical repeat for B-DNA in solution it was deduced that the sequence-directed and Flp-induced type I bend are separated by a half-integral number of turns (Rhodes and Klug, 1981; Peck and Wang, 1981). Since the sequence-directed bend has a known overall bend direction towards the major groove and the two out-of-phase bends are separated by a half-integral number of turns, it was concluded that the direction of the Flp-induced type I bend is toward the major groove (Zinkel and Crothers, 1987). This result is confirmed by analysis of the in-phase complex (linker length of 105 bp, diamond, Fig. 2-6B).

The direction of Flp-induced type I and type II bends on substrates containing a two-element FRT site were also determined. The type I bend formed on a two-element FRT site was directed toward the minor groove based on a helical repeat of 10.7 bp per turn (data not shown). Thus it is in the direction opposite to that found

with a one-element site. The type II bend is directed toward the major groove based on a helical repeat of 10.8 bp per turn (data not shown). The curve obtained from plotting the normalized relative mobilities of complex IIs showed a sine wave function with 10 bp periodicity confirming that the type II bend is also a directional bend.

I also determined whether the direction of the type II bend changed after the substrate had been cleaved by Flp R191K. The phasing experiment in which two-element phasing substrates were used is shown in Figure 2-7A. The normalized relative mobilities of complex IIs (cIIa, cIIb, Fig. 2-7A) were plotted in Figure 2-7B. The plot in Figure 2-7B showed that the type II bend in cIIa is directed toward the major groove whereas the type II bend in cIIb is directed toward the minor groove based on helical repeats of 10.7 and 10.9 bp per turn respectively. It was concluded that the type II bend remained directed toward the major groove after cleavage in the bottom strand. Cleavage of the top strand, however, was accompanied by a change in direction of the bend toward the minor groove. Deviations of the experimentally determined helical repeats from the helical repeat for B-DNA may be due to the presence of polypyrimidine tracts in the linker sequences (see Discussion).

4. DISCUSSION

(i) The location of Flp-induced type II bend centres differs in nicked, covalently bound complex IIs and in noncovalently bound complex IIs

Circular permutation experiments showed that the location of type II bend centres was in the middle of the core region. The major complex II generated by Flp was destroyed by heparin, indicating that Flp is not covalently bound to the DNA in this complex. The position of the bend centre in a complex IIa is at a similar position to the bend centre generated by both wild-type Flp and Flp Y343F i.e. position 0 (Fig. 2-1; Table 2-1). However, the position of a type II bend centre in a complex IIb is in the middle of the *b* element (Fig. 2-1). Heparin treatment of a wild-type Flp binding reaction showed a small amount of two complexes which comigrated with complexes IIa and IIb. These results form the basis of a model (Fig. 2-8) in which it is proposed that the position of the type II bend centre determines the fate of each of the two nicked, covalently bound complex IIs in the recombination reaction.

An uncleaved, noncovalently bound complex II has a type II bend centre in the middle of the core region (Fig. 2-8, top). This complex may be cleaved in the bottom strand (cIIa) or it may be cleaved in the top strand (cIIb). A complex IIa retains the bend centre with the associated distortion of the DNA in the core (Schwartz and Sadowski, 1989; Kimball, 1990). I propose that the bend in the core may partially unwind the duplex core or distort the core region sufficiently to displace the 5' hydroxyl group of the nick and prevent

religation. Therefore, in a complex IIa the 5' hydroxyl terminus of the nick can only participate in the initial strand exchange event of the recombination reaction. The bend centre in a complex IIb, however, is in the *b* element and thus retains a double-stranded, undistorted core. Such a complex would be more likely to undergo religation than engage in strand exchange with a partner duplex. According to this model, cleavage in the bottom strand results in the initial strand exchange while cleavage in the top strand occurs subsequently and results in the second strand exchange in the recombination reaction (Jayaram et al., 1988; Parsons et al., 1988; Meyer-Leon et al., 1988, 1990; Dixon and Sadowski, 1993, 1994). It might be argued that strand displacement as shown in the model might introduce a flexure into the FRT site due to single strandedness of the core. However the strong cross-core protein:protein interactions may stabilize the complex II and account for our ability to measure a protein-directed bend by both the circular permutation and phasing experiments.

Gel electrophoresis techniques measure the overall curvature of a DNA molecule but tell us little about the microscopic detail at the actual site of the bends. Although, for simplicity, we have drawn the distortions in Figure 2-8 as simple planar bends, it is possible that the actual trajectory of the DNA around the protein molecule is more complicated than represented.

The recent crystal structure of the synaptic Cre/*lox*A complex provides support for part of this model. The structure shows that the ~100° DNA bend positioned in the middle of the core is associated with unpairing of three base-pairs between the bend and

the site of cleavage such that the free 5'-hydroxyl group of the nick is displaced from the helix and is moved into the strand exchange cavity in the synaptic complex (Guo et al., 1997). The structure is a model of the initial strand exchange leading to formation of the Holliday intermediate. It is feasible that Flp-mediated cleavage of bent DNA facilitates the initial strand exchange in the same way. It is interesting to speculate whether such a bend-coupled strand exchange mechanism is common to the integrase family. It is known that λ Int, XerC and XerD also induce bends in their target sites (Thompson and Landy, 1988; Blakely and Sherratt, 1996a; Subramanya et al., 1997).

The Cre/*lox*A structure suggests that the position of the bend in the core region and its proximity to the nick may be important for unpairing of the base pairs between the nick and the DNA bend. It is plausible that positioning of the bend in the symmetry element on the 3' side of the nick or at the margin of the core region furthest from the site of the nick would not mediate 'fraying' of the 5' end of the nick and would thus inhibit strand exchange and favour religation.

Previous work has shown that λ site-specific recombination proceeds by an ordered sequence of strand exchanges (Nunes-Düby et al., 1987; Kitts and Nash, 1987, 1988a, 1988b). In contrast to the model proposed here, however, the strand-exchange bias in the λ integrase system is dictated by elements in the arms of the phage attachment site and not by the local environment around the strand exchange point in the core of *attP* (Kitts and Nash, 1988a). Characterization of the Holliday intermediate formed by the Cre

recombinase of bacteriophage P1, also showed that one set of strands was always exchanged preferentially before the other although the basis of this preference is unknown (Hoess et al., 1987).

So far there is no evidence for a bias in the initiation of strand exchange by Flp analogous to that observed with the λ integrase or Cre proteins (Nunes-Düby et al., 1987; Kitts and Nash, 1987, 1988a, 1988b; Hoess et al., 1987). Recombination of a linear FRT site with a half-FRT site reveals no bias (Chen et al., 1991, 1992a). This may be due to the fact that the recombination of a half-FRT site with a full FRT-site is more permissive than recombination between two full FRT-sites (Chen et al., 1991, 1992a). Alternatively it is not certain that end products observed in half-site by full-site crosses actually reflects the initiation event in these reactions.

(ii) Substrate-dependent position of the Flp-induced type I bend

Flp showed a substrate-dependent location of the type I bend centre. It was located at the core-distal end of the *b* element (Fig. 2-1, bp position -16) when the substrate contained a single symmetry element but was located at the core-proximal end of the *b* element (Fig. 2-1, bp position -5) when the substrate contained a two-element FRT site. Interestingly, the bend centre induced by the P32 and P27 Flp peptides was the same irrespective or whether the one-element or two-element FRT site was used; it was located close to the core-distal end of the *b* element near the Flp-induced type I bend on a one-element substrate.

Several possible explanations for this substrate-dependent shift in the centre of the type I bend were considered. Complex I (one molecule of Flp bound to the FRT site) could represent a stable mixed population comprised of one species in which the *b* element is occupied by Flp and another species in which the *a* element is occupied by Flp. These two species, however, would be expected to migrate differently from each other in the circular permutation analysis used here.

The differences in length and sequence content between the two-element and one-element circular permutation substrates employed may have been responsible. Since the P32 peptide showed no substrate-dependent difference in the location of the type I bend centre, we think that this explanation is unlikely.

It is possible that the complex Is generated using the two-element substrate are derived from complex IIs in which one Flp monomer had fallen off. The apparent shift in the bend centre could therefore be the result of the protein:protein interactions that occurred before one Flp molecule was lost. To test this possibility, a circular permutation experiment using the two-element substrate was done at very low Flp concentrations, such that the predominant complex formed was complex I (data not shown). This experiment also showed a type I bend centre at the core-proximal end of the *b* element. Thus it seems unlikely that the change in the position of the type I bend centre arose from prior formation of a complex II.

It is also possible that the type I bend centre with a two-element substrate represents an average of bend centres resulting form a rapid equilibrium wherein Flp molecules are exchanging between the

a and *b* elements during electrophoresis. Such a mechanism has been suggested previously to explain results of exonuclease footprinting experiments (Beatty and Sadowski, 1988). Although circular permutation analysis with a single *a* element has not been done, it is reasonable to assume that the bend centre in an *a* element would also be at the core-distal end of the element since the *a* and *b* symmetry elements differ by only a single base pair. If the *a* and *b* elements were occupied by Flp an equal amount of the time, then one would expect the apparent type I bend centre to be in the middle of the core instead of at the core-proximal end of the *b* element. Alternatively the type I bend centre at the core-proximal end of the *b* element could be explained if, in a rapid equilibrium, the *b* element were occupied a greater amount of the time than the *a* element. However, if Flp were capable of such a rapid equilibrium, we might expect P32 to exhibit such a rapid exchange. Since P32 showed no substrate-dependent difference in the position of the type I bend, this explanation seems less plausible.

Kleinschmidt et al. (1991) have observed that the presence of a second protein binding site, in *cis*, on the DNA fragment, can actually appear to cause the disappearance of a complex formed by a single protein binding to one of the binding sites (analogous to complex I). This phenomenon is called "disproportionation" and results from dissociation of a protein from a single binding site and reassociation to a DNA molecule that already contains one bound protein molecule. This phenomenon depends on the dissociation rate constant and time of electrophoresis of the protein:DNA complex. However in the Appendix I describe experiments that renders less

likely the explanation that the apparent shift in the bend centre is due to "disproportionation" between Flp molecules bound to the *a* or *b* symmetry elements.

To explain the substrate-dependent change in the position of the type I bends, we propose that the mode of binding of a Flp monomer to a single element substrate is different from the mode of binding of a Flp monomer to a two-element FRT site. Flp is known to possess a bipartite DNA binding structure (Pan and Sadowski, 1993; Panigrahi and Sadowski, 1994). P32 recognizes the outer (core-distal) 9 bp of the 13 bp symmetry element and P13 is needed for protection of the inner (core-proximal) 4 bp of each symmetry element. A model for the binding of one Flp monomer to a two-element FRT site is shown in Figure 2-9. In the model, the 32 kDa COOH-terminal (P32) and the 13 kDa NH_2-terminal (P13) regions of Flp represent two independent DNA binding domains linked by a flexible hinge. It is proposed that when a single Flp monomer is bound to a two-element FRT site, the P32 domain binds to one element and the P13 domain binds, across the core, to the inner 4 bp of the other symmetry element resulting in the shifted bend centre observed with Flp on a two-element substrate. Appendix I describes experiments that further address this model.

Panigrahi et al. (1992) showed by missing contact interference probing that each base of a single symmetry element is necessary for Flp binding. P13 seems to bind DNA non-specifically and yet it covers four specific base pairs of a symmetry element in concert with P32 (Pan and Sadowski, 1993; Panigrahi and Sadowski, 1994). It is possible that P13 contacts a single symmetry element non-

specifically but binds specifically across the core on a two-element substrate.

Since P32 alone is unable to induce a type II bend, P13 has been implicated in the formation of protein:protein interactions between monomers bound to the *a* and *b* elements (Pan and Sadowski, 1993). We speculate that the bipartite binding of Flp to the FRT site with associated protein:protein interactions between Flp monomers may be instrumental in initiating the severe deformation of DNA observed in a type II bend (bend angle >140°, Schwartz and Sadowski, 1990).

It is interesting to note that mutations of tyrosine at position 60 in Flp (within the P13 region) cause a recombination-defective phenotype that is accompanied by severe defects in the type II bend (Chen et al., 1991, 1992a). Flp TA232 contains a four amino acid insertion at position 115 in Flp. This protein is defective in positioning of the type I bend centre on a two-element substrate (data not shown).

Two other members of the integrase family, the lambda and Tn916 integrases, also possess two DNA binding domains, allowing them to bind distinct and widely separated 'arm-type' and 'core-type' DNA sequences (Moitoso de Vargas et al., 1988; Lu and Churchward, 1994). The proposed model for Flp binding differs somewhat in that it is suggested that a single monomer may span two binding sites spaced 12 bp apart.

(iii) Flp-induced bends are directed toward the major and minor grooves

Our results show that Flp can bend the DNA of the FRT site toward the major or minor groove, depending upon the substrate (complex I) or which strand was cleaved (complex II). Several sources of error are associated with analysis of phasing experiments. First of all, determination of bend direction is subject to the error associated with determining the centre of the protein-induced bend. Secondly, interpretation of phasing experiments assumes that the linker sequence is comprised of regular B-DNA. While it is unlikely that the linker sequence will deviate from B-DNA over the entire length of the linker, local deviations due to atypical sequences such as polypurine:polypyrimidine tracts or protein-induced deformations are possible. Polypurine:polypyrimidine double helices adopt non B-DNA structures associated with A-DNA like conformations (Wells et al., 1988; Rajagopal and Feigon, 1989; Hunter, 1993; Ivanov et al., 1995). The helical repeats that were determined experimentally (10.7-10.9 bp per turn) may reflect the presence of 12-22 bp of one or two polypurine:polypyrimidine tracts in their linker sequences. The linker in the out of phase complex IIb shows the largest deviation from B-DNA (10.9 bp per turn) and contains the longest polypyrimidine stretches consisting of one tract of 14 bp and a second tract of 8 bp.

The type I bend is directed toward the major groove on a single element substrate but is directed toward the minor groove on a two-element substrate. A comparable flexibility in orientation of a protein-induced DNA bend is shown by Fos:Jun heterodimers and Jun

homodimers which, while binding to the same site, bend the DNA in opposite directions (Kerppola and Curran, 1991). This supports our hypothesis (based on the substrate dependent position of the type I bend centre) that the mode of Flp binding to a single element substrate differs from the mode of Flp binding to a two-element substrate (see Fig. 2-9).

The type II bend was directed toward the major groove. Type II bends in nicked, covalently bound complexes IIa and IIb were directed toward the major groove and minor groove respectively. Thus the direction of the type II bend did not change with Flp cleavage of the bottom strand but did change, along with the position of the type II bend, with cleavage of the top strand. The relative bend direction may also contribute to the differing fate of each of the two nicked, covalently bound complex IIs as proposed in the model described above (Fig. 2-8).

Table 2-1. Location of type I and type II bend centres induced by FLP and FLP peptides

FLP protein or peptide	Type I bend centre [a] [1 element substrate]	Type I bend centre [b] [2 element substrate]	Type II bend centre [c] [2 element substrate]	Type II [d] bend <
FLP	-16 (-15, -17, -16, -16)	-5 (-6, -4, -4)	0 (-1, +1, -1)	>140 °
P27	-19	-22 (-21, -22)	-21 (-19, -22)	ND[e]
P32	-21	-18 (-15, -21)	-17 (-14, -19)	56°

[a,b,c] The composition of the circular permutation substrate used to determine the bend centre is given in square brackets. Numbers indicate the bp position of the bend centre obtained from 1-4 experiments, according to the scale of the FRT site shown in Figure 2-1. Numbers in round brackets indicate the results of individual experiments. Errors of ±2 bp and ±3 bp are associated with determination of bend centres induced by Flp proteins and Flp peptides respectively.

[d] Magnitude of type II bends (degrees; Schwartz and Sadowski, 1990).

[e] ND, not done.

Figure 2-1. The position of Flp-induced DNA bends in the FRT site. The location of type I bend centres within the FRT site are indicated above the sequence. For type I bend centres, the composition of the circular permutation substrate used for the determination is given in brackets. The positions of the type II bend centres are indicated below. For type II bend centres, the specific complex which was characterized is given in brackets. The location of bend centres is based on the results of 1-4 experiments (Table 2-1). The location of Flp R191K and Flp H305L bend centres each represent the average of two individual experiments with an associated error of ± 2 bp.

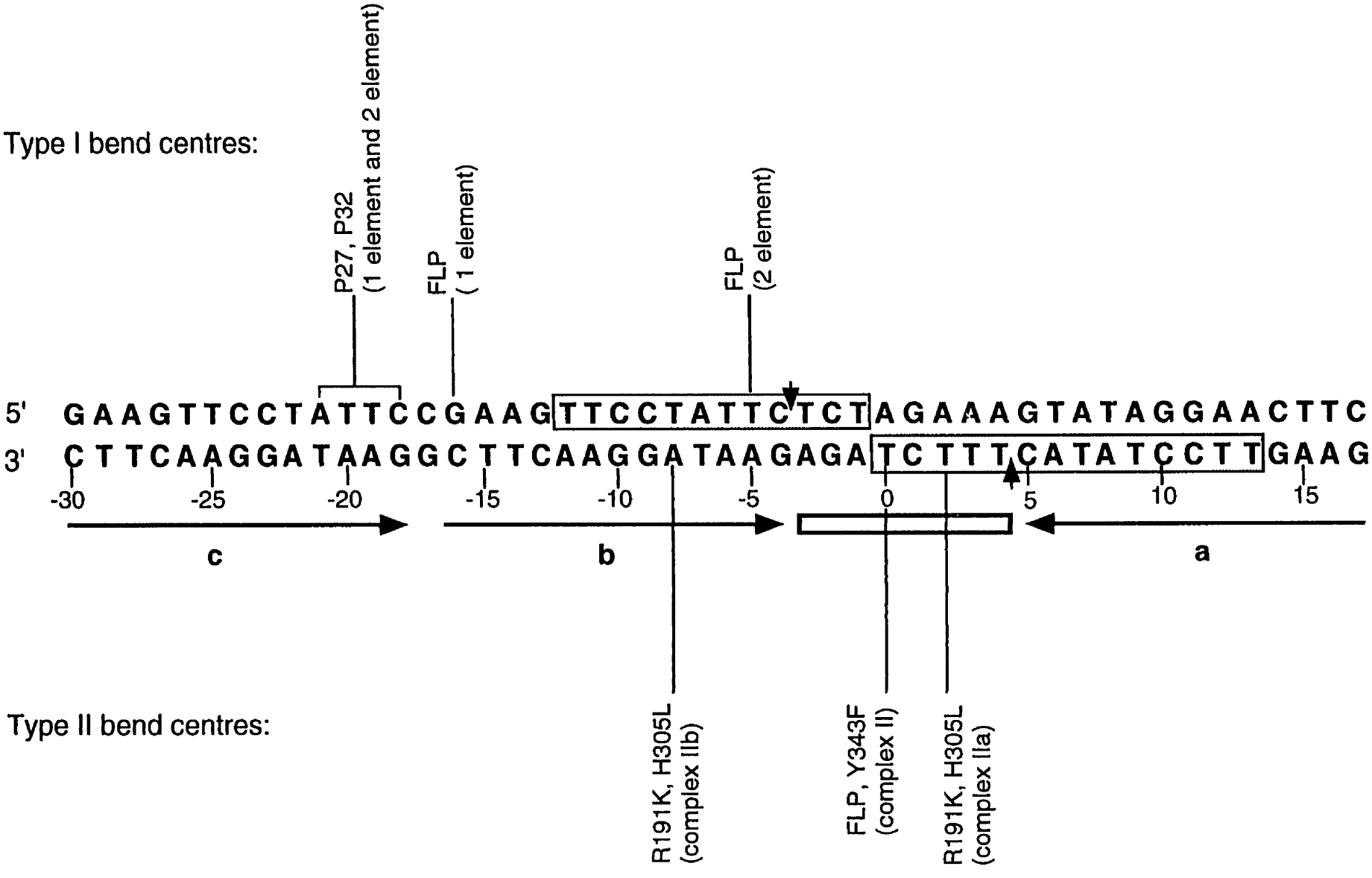
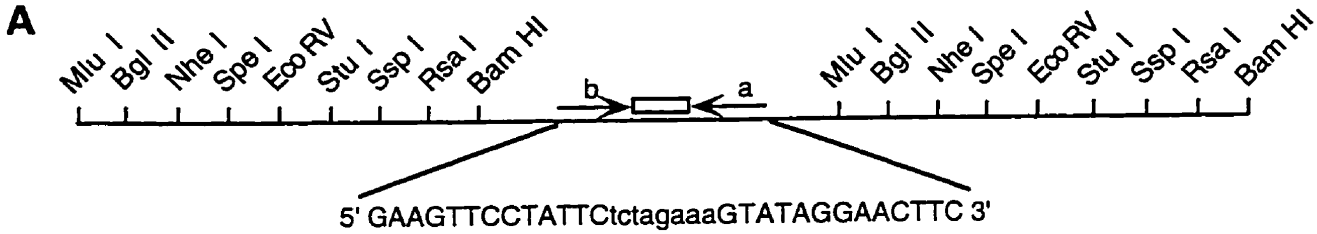
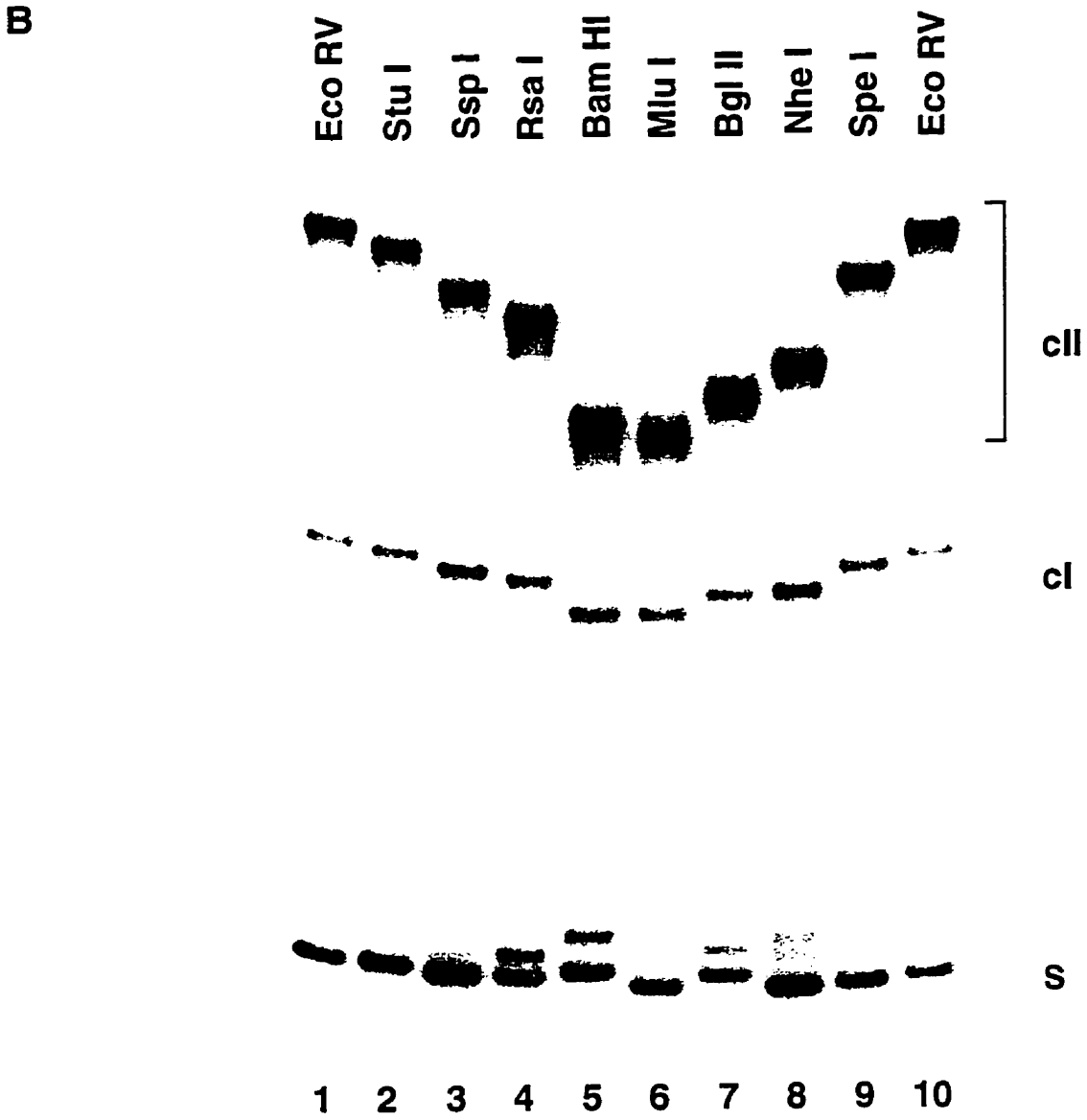

Type I bend centres:
P27, P32
(1 element and 2 element)
FLP
(1 element)
FLP
(2 element)
5' GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC
3' CTTCAAGGATAAGGCTTCAAGGATAAGAGATCTTTCATATCCTTGAAG
-30
-25
-20
-15
-10
-5
0
5
10
15
c
b
a
Type II bend centres:
R191K, H305L
(complex IIb)
FLP, Y343F
(complex II)
R191K, H305L
(complex IIa)

Figure 2-2. Determination of locations of type I and type II bend centres using substrates containing two-element FRT sites. **A.** DNA used to obtain circular permutation substrates. The substrates (152 bp) were generated by cleavage of the DNA fragment, containing a two-element FRT site, at the different restriction sites. Each substrate is named according to the restriction enzyme used to obtain it. The *a* and *b* symmetry elements are indicated by the horizontal arrows flanking the core region of the FRT site, denoted by the open box. The sequence of the two-element FRT site is given below with large and small letters indicating the sequence of the symmetry elements and core region, respectively. **B.** Gel mobility shift assay of the two-element circular permutation substrates bound by Flp. Experimental conditions are as described in Figure 2-5B. S, unbound substrate; cI, complex I; cII, complex II. **C.** Mapping of the type I and type II bend centres to the two-element FRT site. The bend centres were obtained as described in Figure 2-5C. The type I bend centre on a two-element substrate at the core-proximal end of the *b* element is indicated by the open square below the graph. The type II bend centre in the core region is indicated by the solid square.


A
Mlu I
Bgl II
Nhe I
Spe I
Eco RV
Stu I
Ssp I
Rsa I
Bam HI
b
a
Mlu I
Bgl II
Nhe I
Spe I
Eco RV
Stu I
Ssp I
Rsa I
Bam HI
5' GAAGTTCCTATTCtctagaaaGTATAGGAACTTC 3'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Eco RV
Stu I
Ssp I
Rsa I
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Mlu I
Bgl II
Nhe I
Spe I
Eco RV
cII
cI
S
1 2 3 4 5 6 7 8 9 10

C

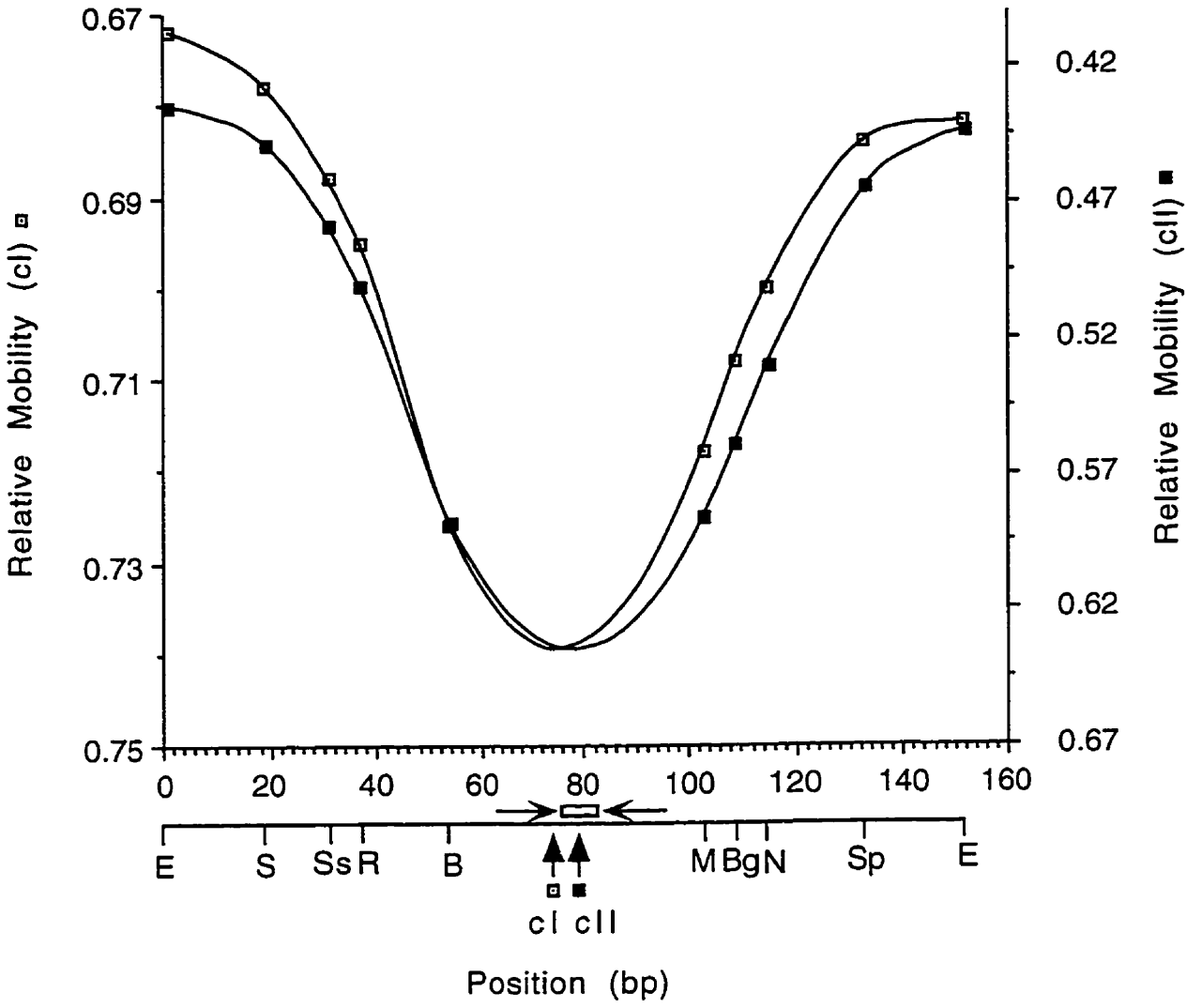

0.67
0.69
0.71
0.73
0.75
Relative Mobility (cI) □
0.42
0.47
0.52
0.57
0.62
0.67
Relative Mobility (cII) ■
0
20
40
60
80
100
120
140
160
E
S
Ss
R
B
M
Bg
N
Sp
E
cI
cII
Position (bp)

Figure 2-3. Sensitivity of Flp:DNA complexes to heparin. Flp (0.06 μM) or Flp R191K (0.49 μM) were incubated with 0.02 pmol of an end-labeled DNA fragment (300 bp) isolated from the plasmid pCS38 (Schwartz and Sadowski, 1990) and containing a two symmetry element FRT site in the middle of the fragment. Reactions were analyzed on a 5% native polyacrylamide gel. Proteins added: lane 1, none; lanes 2 and 4, Flp; lanes 3 and 5, Flp R191K. After 30 min at 22°C, reactions in lanes 4 and 5 were treated with 250 μg/ml of heparin (hep) for an additional 30 min at 22°C. S, unbound substrate; cI, complex I; NC cII (solid circle), noncovalent complex II; cIIa (solid square), complex IIa; cIIb (solid triangle), complex IIb.

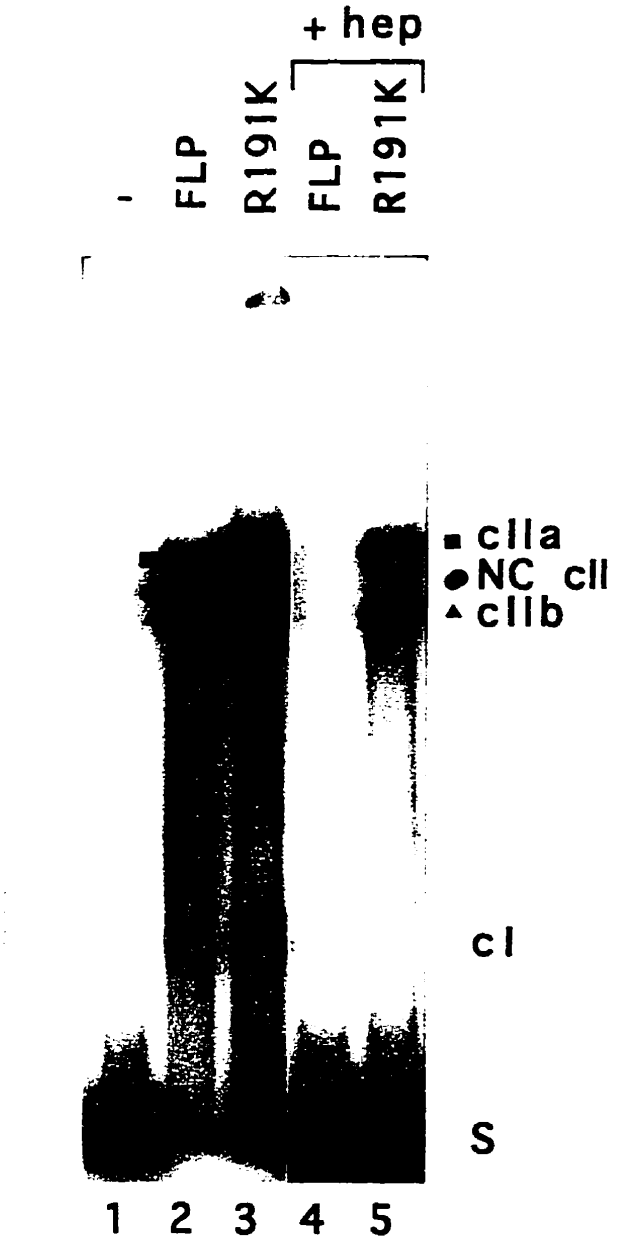
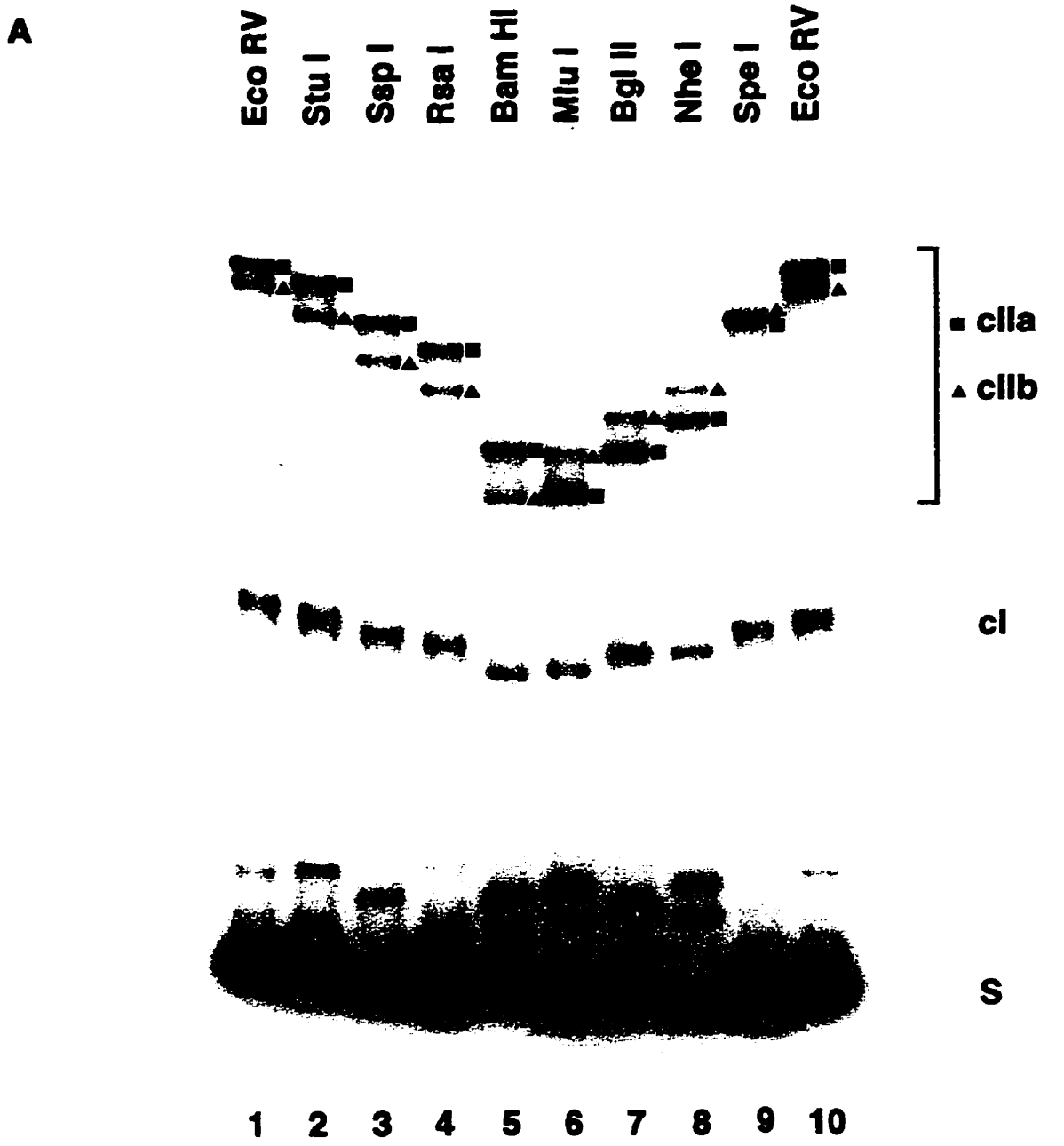

+ hep
-
FLP
R191K
FLP
R191K
cIIa
NC cII
cIIb
cI
S
1 2 3 4 5

Figure 2-4. Positions of type II bend centres induced by Flp R191K. **A.** Gel mobility shift assay of the two-element circular permutation substrates bound by Flp R191K. Experimental conditions are as described in Materials and Methods. Circular permutation substrates employed are detailed in Figure 2-2A. 0.02 pmol of end-labeled DNA substrates were incubated with Flp R191K (2.0 μM), the complexes were separated on a 5% native polyacrylamide gel and the results were visualized by autoradiography. Complex IIa and complex IIb are identified as the darker and lighter upper bands, respectively in each lane resulting from an approximately two-fold bias in cleavage of the bottom strand over the top strand of the FRT site (Friesen and Sadowski, 1992). S, unbound substrate; cI, complex I; cIIa (solid square), complex IIa; cIIb (solid triangle), complex IIb. The bands running above the substrate (S) in some lanes are present in the substrate and are not Flp-dependent. **B.** Mapping of the type II bend centres in complex IIa and complex IIb to the two-element FRT site. The bend centres were obtained as described in Figure 2-5C. The type II bend centre in complex IIa is indicated by the solid square below the graph. The type II bend centre in complex IIb is indicated by the solid triangle.


A
Eco RV
Stu I
Sap I
Rsa I
Bam HI
Mlu I
Bgl II
Nhe I
Spe I
Eco RV
cIIa
cIIb
cI
S
1 2 3 4 5 6 7 8 9 10

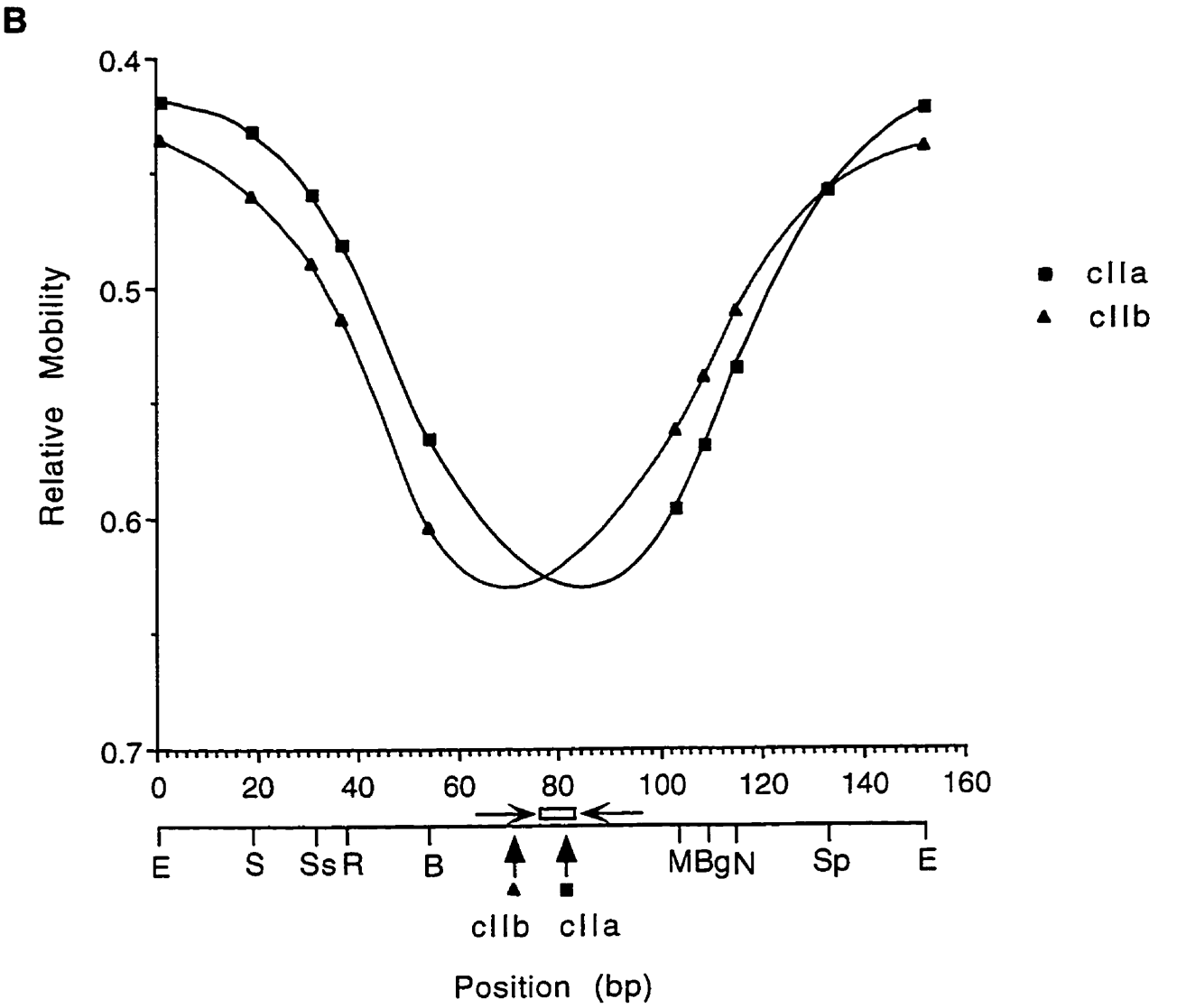
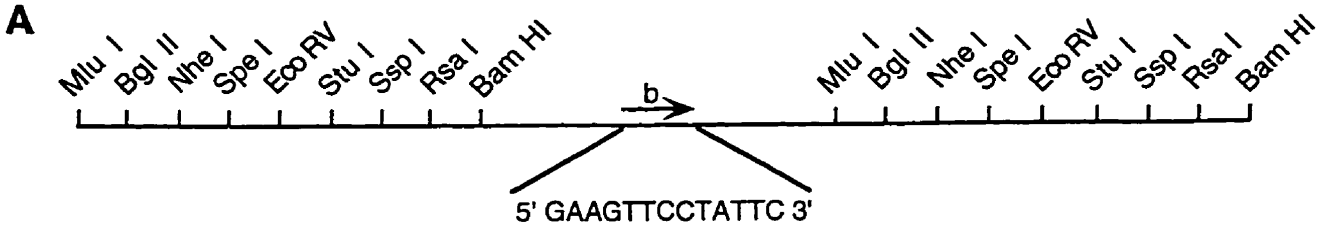
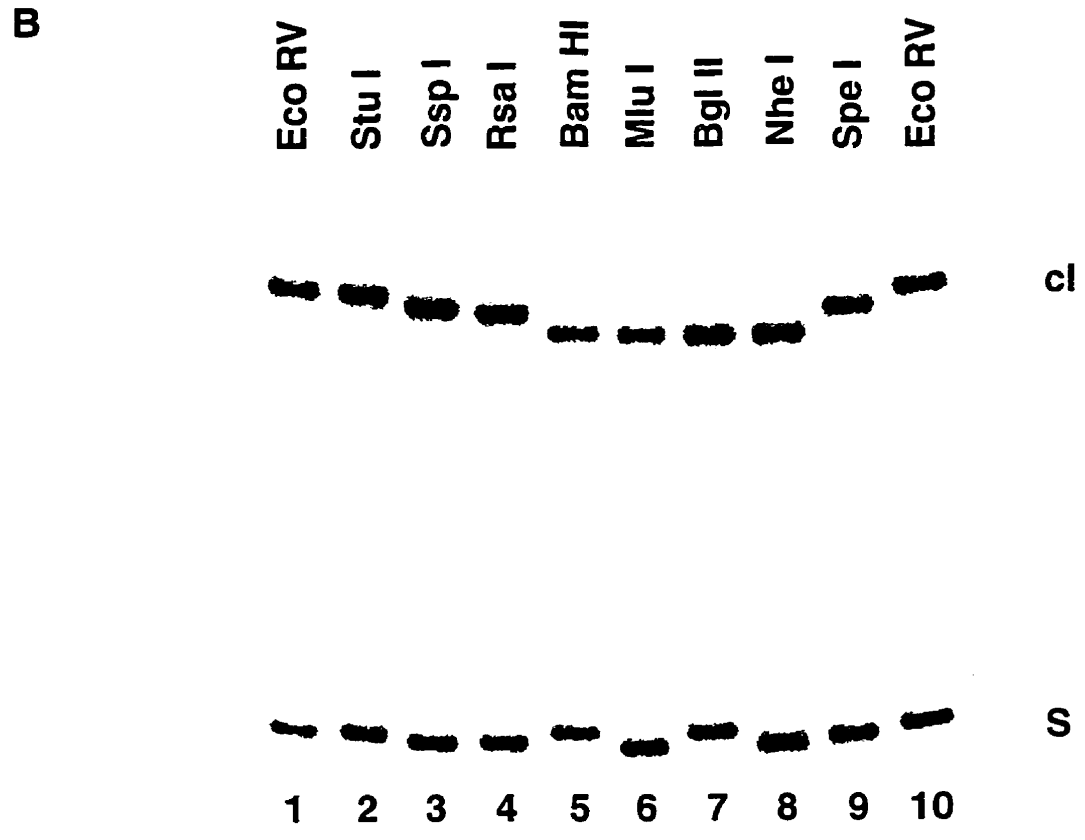

B
0.4
0.5
0.6
0.7
Relative Mobility
0
20
40
60
80
100
120
140
160
E
S
Ss
R
B
MBgN
Sp
E
cIIb
cIIa
Position (bp)
cIIa
cIIb

Figure 2-5. Location of the type I bend centre using single symmetry element substrates. **A.** DNA used to obtain circular permutation substrates. The substrates were generated by cleavage of the DNA with each different restriction enzyme. The DNA contains the *b* symmetry element flanked by two tandemly repeated sets of restriction enzyme sites. Each substrate is named according to the restriction enzyme used to obtain it. The substrates obtained each have the same length (131 bp) and sequence composition. The single element binding site is indicated by the horizontal arrow and its sequence is given below. **B.** Gel mobility shift assay of circular permutation substrates bound by Flp. Experimental conditions are as described in Materials and Methods. 0.02 pmol of end-labeled DNA substrates were incubated with Flp protein (0.07 μM), the complexes were separated on a 5% native polyacrylamide gel and the results were visualized by autoradiography. S, unbound substrate; cI, complex I. **C.** Mapping the type I bend centre to the *b* element binding site. The relative mobilities of the Flp:DNA complexes were plotted as a function of the position of the *b* element binding site relative to the end of the substrate. The x-axis scale indicates the distance (bp) of the left-hand end of each substrate to the left-hand *Eco*RV site on the DNA fragment. The linear portions of the curve were extrapolated to a point on the DNA fragment to allow estimation of the bend centre. The bend centre was determined to be at the core-distal end of the *b* element and is indicated by the arrow below the graph. Abbreviations for restriction enzyme sites are: E, *Eco*RV; S, *Stu*I; Ss, *Ssp*I; R, *Rsa*I; B, *Bam*HI; M, *Mlu*I; Bg, *Bgl*II; N, *Nhe*I; Sp, *Spe*I.


A
Mlu I
Bgl II
Nhe I
Spe I
Eco RV
Stu I
Ssp I
Rsa I
Bam HI
b
5' GAAGTTCCTATTC 3'


B
Eco RV
Stu I
Ssp I
Rsa I
Bam HI
Mlu I
Bgl II
Nhe I
Spe I
Eco RV
cI
S
1 2 3 4 5 6 7 8 9 10

C

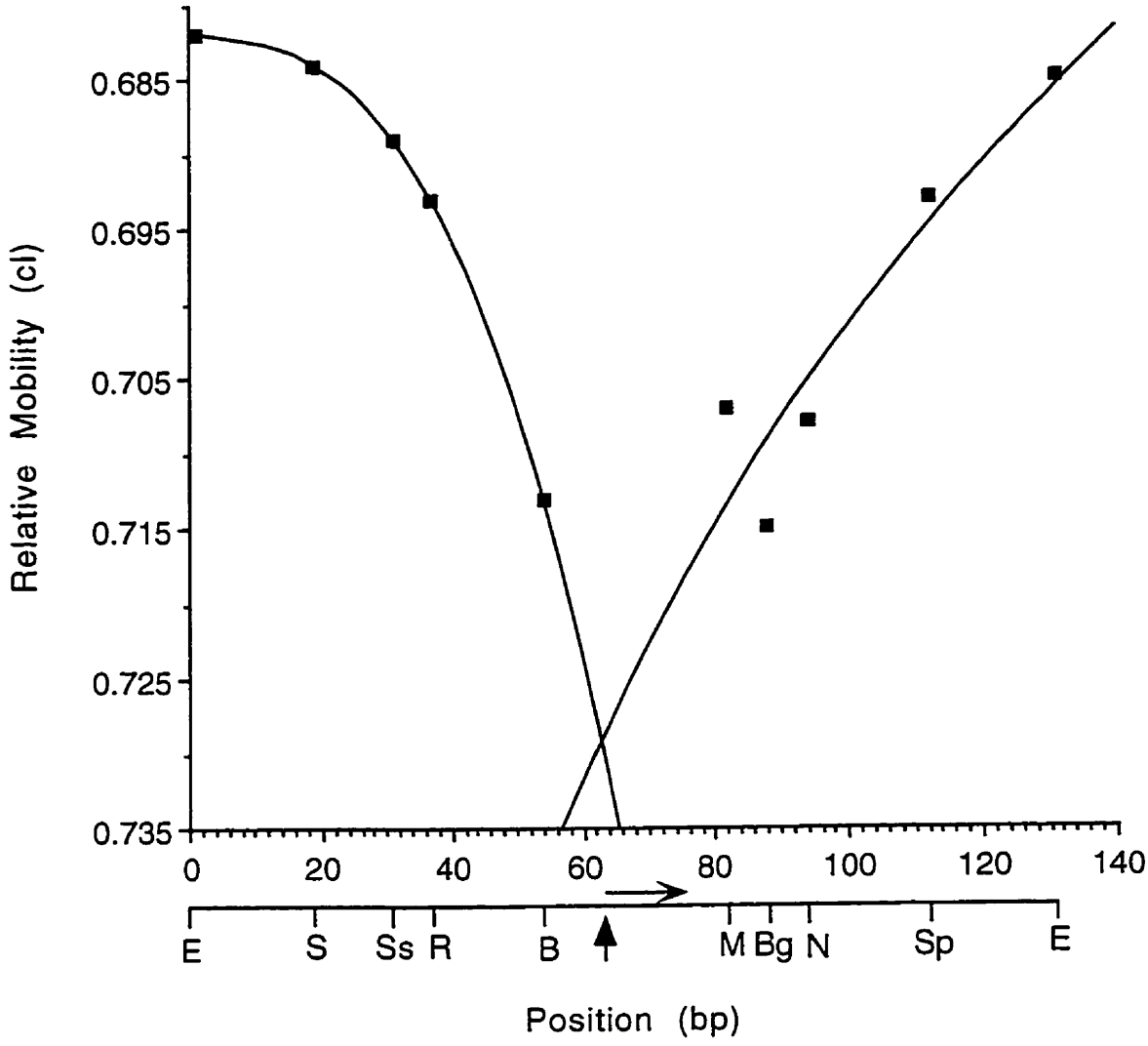
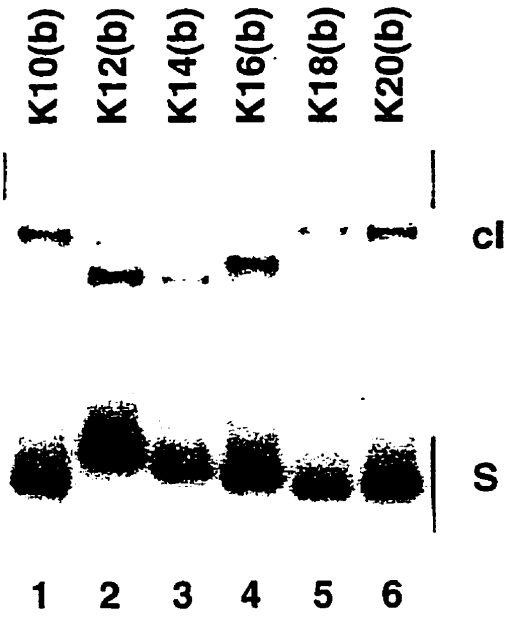

Relative Mobility (cI)
0.685
0.695
0.705
0.715
0.725
0.735
0
20
40
60
80
100
120
140
E
S
Ss
R
B
M
Bg
N
Sp
E
Position (bp)

Figure 2-6. Phasing analysis of the Flp-induced type I bend using single symmetry element substrates. **A.** Gel mobility shift assay of single element phasing substrates bound by Flp. Experimental conditions are as described in Figure 2-5B. S, unbound substrate; cI, complex I. **B.** Determination of the bend direction. The relative mobilities of the Flp:DNA complexes were plotted as a function of the linker length (bp) of each substrate. The linker length is defined as the distance (bp) from the middle of the sequence-directed DNA bend to the middle of the Flp induced DNA bend (determined by circular permutation analysis). The Flp:DNA complex in which the protein-induced and sequence-directed bends are cooperatively in phase (they have the same bend direction) is indicated by the asterisk. The Flp:DNA complex in which the two bends are out of phase (they have opposite bend directions) is indicated by the open diamond.

A


K10(b)
K12(b)
K14(b)
K16(b)
K18(b)
K20(b)
cl
S
1 2 3 4 5 6

B

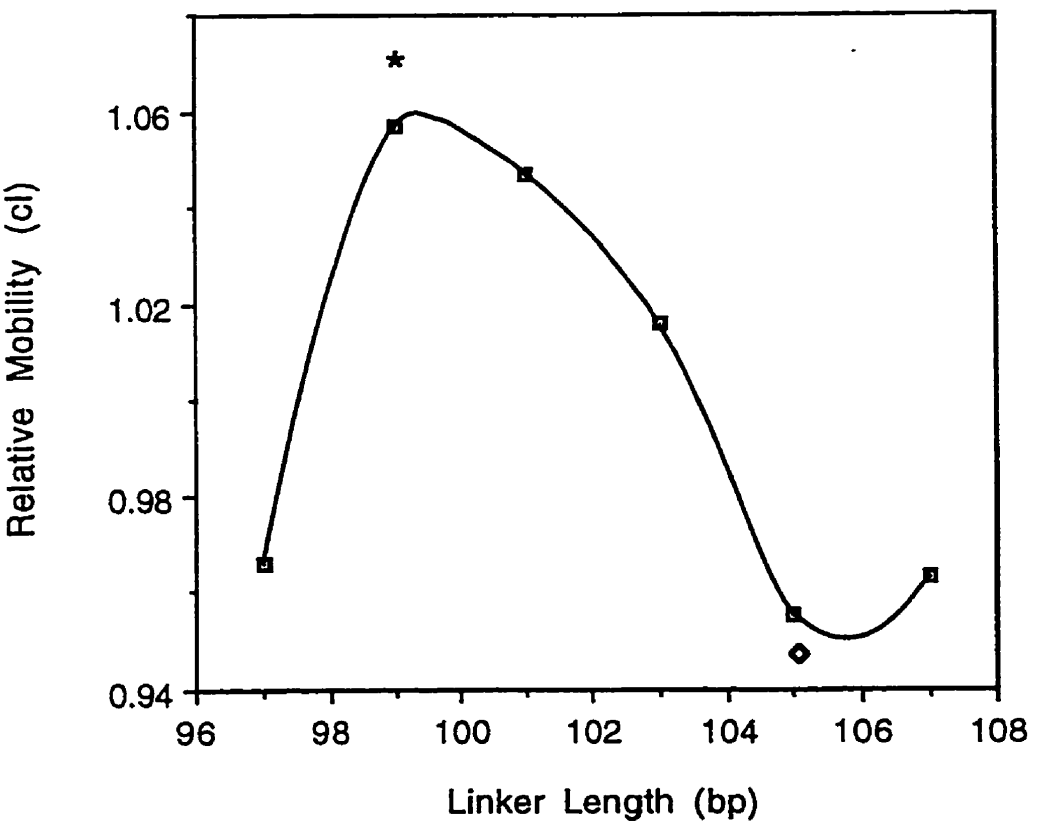

Relative Mobility (cl)
1.06
1.02
0.98
0.94
96
98
100
102
104
106
108
Linker Length (bp)

Figure 2-7. Phasing analysis of type II bends induced by Flp R191K using substrates containing two-element FRT sites. **A.** Gel mobility shift assay of two-element phasing substrates bound by Flp R191K. Experimental conditions are as described in Figure 2-5B. S, unbound substrate; cI, complex I; cIIa (solid square), complex IIa; cIIb (solid triangle), complex IIb. **B.** Determination of bend directions of cIIa and cIIb. Complexes were analyzed as described in Figure 2-6B. The Flp:DNA complexes in which the protein-induced and sequence-directed bends are cooperatively in phase in indicated by the asterisk. The Flp:DNA complexes in which the two bends are out of phase is indicated by the open diamond.


A
K10(ba)
K12(ba)
K14(ba)
K16(ba)
K18(ba)
K20(ba)
cIIa ■
cIIb ▲
cI
S
1 2 3 4 5 6

B

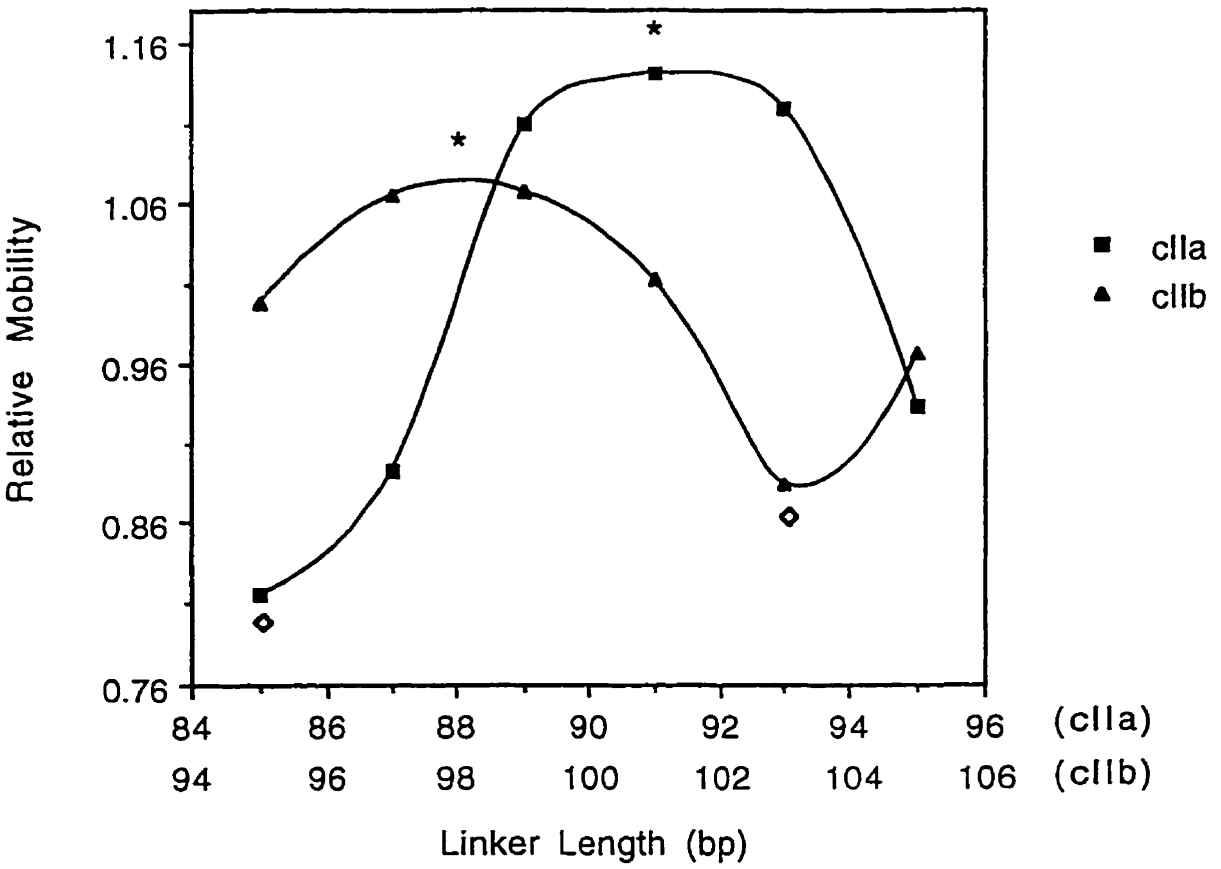
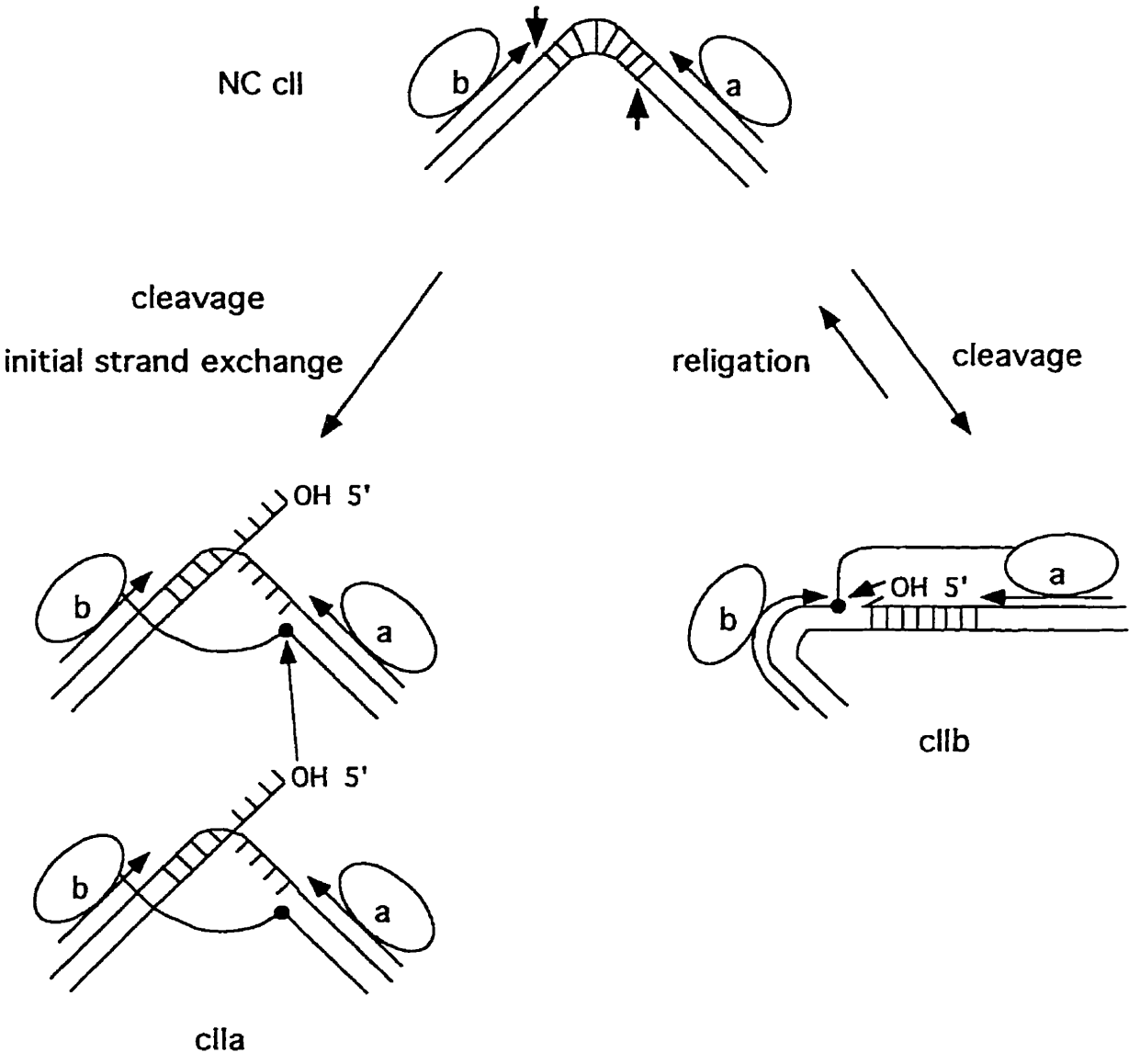

1.16
1.06
0.96
0.86
0.76
Relative Mobility
*
*
84
86
88
90
92
94
96
(cIIa)
94
96
98
100
102
104
106
(cIIb)
Linker Length (bp)
cIIa
cIIb

Figure 2-8. Model showing how the location of type II bend centres in nicked, covalently bound complex IIs dictates the fate of the complex in the recombination reaction. An uncleaved, noncovalently bound complex II (NC cII) is shown at the top. Symmetry elements *a* and *b* are indicated by the arrows to which protein monomers (ovals) are bound. Short lines perpendicular to two parallel lines (DNA strands) indicate the eight base pairs of the core. Flp cleavage sites are indicated by small vertical arrows. The bend centre in this complex is in the middle of the core as indicated. This complex may be cleaved in the bottom strand to yield a nicked complex IIa (cIIa, bottom left) in which one protein monomer is covalently bound (line and solid dot joining protein monomer to DNA strand) at the *a* symmetry element/core junction or it may be cleaved in the top strand to yield a nicked complex IIb (cIIb, bottom right) in which one monomer is covalently bound at the *b* symmetry element/core junction. Note the cleavages are shown in the *trans*-horizontal position according to the nomenclature of Chen et al. (1992) and Lee et al. (1994). The bend centre in complex IIa causes partial unwinding of the duplex core and facilitates strand exchange with a partner FRT site having a nick on the same strand. A complex IIb showing a bend centre in the *b* element retains an undistorted, duplex core which favors religation reverting this complex to the parental cII. Complex IIa forms the substrate for the initial strand exchange while complex IIb forms the substrate for the second strand exchange in the recombination reaction.


NC cII
b
a
cleavage
initial strand exchange
religation
cleavage
OH 5'
b
a
OH 5'
b
a
cIIa
OH 5'
b
a
cIIb

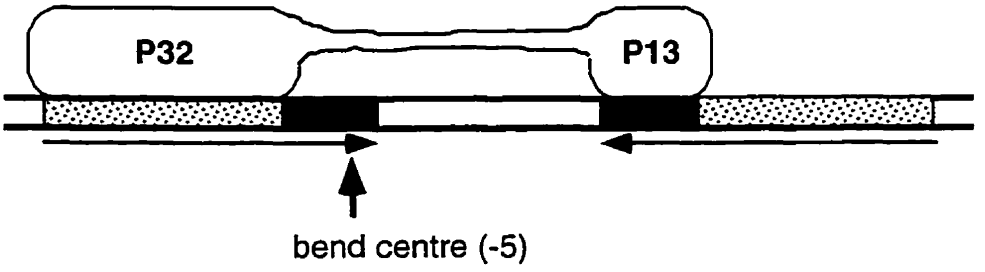


Figure 2-9. Model for binding of one Flp monomer to the FRT site. On a two-element FRT site, the P32 region binds to the core-distal 9 bp of the symmetry element (stippled boxes), but the NH_2-terminal region of Flp (P13) binds to the core-proximal 4 bp of the symmetry element (black boxes) on the other side of the core. This cross-core binding by P13 shifts the location of the type I bend centre on this substrate to position -5 (Fig. 2-1; Table 2-1, column b), as indicated by the vertical arrow.

CHAPTER 3.

DNA SEQUENCE DETERMINANT FOR FLP-INDUCED DNA BENDING

1. INTRODUCTION

Numerous studies indicate that the flexibility or bendability of DNA is dependent on its sequence (Kahn et al., 1994; Brukner et al., 1995; El Hassan and Calladine, 1996; Dlakic and Harrington, 1996). I have considered whether inherent flexibility in the core region of the FRT site may influence the Flp-induced bends. I have previously shown that the position of the type II bend was localized to the middle of the core region at the junction of two polypyrimidine tracts present in the FRT site (Fig. 3-1, base-pair position 0; chapter 2).

In this chapter I shifted the junction point of the polypyrimidine tracts in the core region and the effects on both the position of the type II bend and Flp activities were assayed. While I found no evidence to indicate that the junction of the polypyrimidine tracts in the core region influenced the type II bend, I found that the central AT base-pair in the core region was essential to positioning of the type II bend in the middle of the core region. When I replaced the central AT base-pair with a CG base-pair the DNA bend was positioned at the margin of the core region adjacent to the *a* symmetry element. This base-pair change was also associated with reduced recombination activity and apparently affected Flp-mediated cleavage activity suggesting that the functionality of the site is sensitive to the position of the DNA bend.

2. MATERIALS AND METHODS

(i) Oligonucleotide substrates

Oligonucleotides were synthesized at the Hospital for Sick Children/Pharmacia Biotechnology Service Centre, Banting Institute, University of Toronto. Oligonucleotides used in this study (symmetry element and core sequences of the wild-type FRT site are indicated in bold and in italics respectively):

(KL-12) 5' GCTCAGGTC**GAAGTTCCTATAC***TTTCTAGA***GAATAGGAAC TTC**ACGTGACAGTGA 3'

(KL-13) 5' TCACTGTCACGT**GAAGTTCCTATTC***TCTAGAAA***GTATAGG AACTTC**GACCTGAGC 3'

(HP-7) 5'T**GAAGTTCCTATTC***TCTAGAAA***GTATAGGAACTTC**GACCT 3'

(HP-31)5'GGTC**GAAGTTCCTATAC***TTTCTAGA***GAATAGGAACTTC**AC3'

(j4RTOP) 5' CTAG**GAAGTTCCTATTC***TCT*TTCT*A***GTATAGGAACTTC** 3'

(j4RBOT) 5'TCGA**GAAGTTCCTATAC***T*AGAA*AGA***GAATAGGAACTTC**3'

(j2RTOP) 5'CTAG**GAAGTTCCTATTC***TCT*CT*AAA***GTATAGGAACTTC** 3'

(j2RBOT) 5'TCGA**GAAGTTCCTATAC***TTT*AG*AGA***GAATAGGAACTTC** 3'

(j2LTOP) 5'CTAG**GAAGTTCCTATTC***T*AG*AGAAA***GTATAGGAACTTC** 3'

(j2LBOT) 5' TCGA**GAAGTTCCTATAC***TTTCT*CT*A***GAATAGGAACTTC** 3'

Oligonucleotides were 5' end-labeled with [γ-32P] ATP (NEN Dupont) using T4 polynucleotide kinase (New England Biolabs). Following extraction with phenol/chloroform and ethanol precipitation, the labeled oligonucleotide was annealed to the appropriate oligonucleotide(s) by heating and slow cooling in 100 mM NaCl and 5 mM $MgCl_2$. Annealed DNA substrates were purified on 10% polyacrylamide gels (acrylamide:bis-acrylamide (30:1), 1xTBE).

(ii) Plasmids and plasmid-derived substrates

Construction of the pB2Flp(ba) plasmid is described in chapter 2. The pB(j2L), pB(j4R) and pB(j2R) plasmids were constructed by ligating the annealed, complementary oligonucleotides j2LTOP and j2LBOT, j4RTOP and j4RBOT, j2RTOP and j2RBOT (sequences given above), each containing a FRT site with the *b* and *a* symmetry elements flanking a mutated core region, into the pBEND2 vector as described previously (Zwieb et al., 1989; chapter 2). Circularly permuted DNA substrates were obtained by digestion of pB(j2L), pB(j4R) or pB(j2R) as described in chapter 2. Enzymes were obtained from New England Biolabs.

(iii) Flp preparations

Flp and Flp R191K preparations are described in chapter 2. Flp Y343F was purified to about 30% purity by chromatography on Bio-Rex 70 followed by fast protein liquid chromatography using a Mono S column (Pan et al., 1991). The concentration of Flp was estimated by comparison with highly purified Flp standards on Coomassie blue-stained sodium dodecyl sulfate (SDS)-PAGE. The Bradford (1976) assay was used to determine the concentration of the homogeneous Flp standards.

(iv) Flp assays

Cleavage reaction mixtures containing 0.013 pmol of DNA substrate radioactively labeled at both 5' ends were incubated with 14 pmol Flp or 49 pmol Flp R191K in a 20 µl volume (50 mM Tris-HCl, pH 7.4, 33 mM NaCl, 1 mM EDTA, 100 µg calf thymus DNA/ml).

Recombination reaction mixtures contained, in addition to these constituents, 0.1 pmol of an unlabeled synthetic DNA substrate and were incubated with 14 pmol Flp. Cleavage and recombination reaction mixtures were incubated at 22°C for 120 minutes and subsequently treated with 20 µg proteinase K and 0.005% (w/v) SDS for 60 minutes at 37°C. Following phenol/chloroform extraction and ethanol precipitation, the DNA was analyzed on an 8% denaturing polyacrylamide gel.

(v) Quantitation

Dried gels were scanned using a Molecular Dynamics Phosphorimager and analyzed using Imagequant software.

Circular permutation assays were carried out and analyzed as described in chapter 2.

3. RESULTS

(i) Mutation of the central two base-pairs in the core region of the FRT site affects the position of the Flp-induced type II bend

To examine the possible influence of the junction of the polypyrimidine tracts in the core region of the FRT site on Flp-induced bends, the effects of shifting the position of the junction of the polypyrimidine tracts on the mobility of Flp:DNA complex IIs was studied. Substrates were constructed in which the junction of the polypyrimidine tracts was shifted two base-pairs to the left (j2L

FRT), four base-pairs to the right (j4R FRT) or two base-pairs to the right (j2R FRT) of the junction positioned between 0 and -1 in the core region of the wild-type FRT site (Fig. 3-2A). The j2L FRT site contained altered base-pairs at positions -1 and -2 of the core region, the j4R FRT site contained altered base-pairs at positions 0, +1, +2, +3 of the core region and the j2R FRT site contained altered base-pairs at positions 0 and +1 of the core region (shaded boxes, Fig. 3-2A). In making these changes the AT/GC content of the core region was not altered. The location of the Flp-induced DNA bend centres was determined using circular permutation substrates that contained the j2L, j4R or j2R FRT sites as described in chapter 2.

Circular permutation assays in which Flp was bound to substrates which contained the j2L, j4R or j2R FRT sites are shown in Figure 3-2B (i), (ii) and (iii). Substrates containing the j2R, but not the j2L or j4R, FRT sites affected the position of the type II bend. The type II bend was positioned in the middle of the core region when the substrate contained a j2L, j4R or wild-type FRT site (Fig. 3-1, Table 3-1, bp positions +1, +1, 0) but was positioned at the end of the core region immediately adjacent to the *a* element when the substrate contained a j2R FRT site (Fig. 3-1, Table 3-1, bp position +4). Since one of the two base-pair changes in the j2R FRT site (Fig. 3-2A, GC→TA at bp position +1) was also contained in the j4R FRT site but had no effect, I concluded that the AT→CG change at base-pair position 0 in the j2R FRT site influenced the position of the type II bend (Fig. 3-2A). The j4R FRT site contained an AT→TA change at base-pair position 0 but it did not alter the position of the type II bend. This indicates that an AT base-pair, regardless of its

orientation, is important for positioning of the type II bend at base-pair position 0. A CG at base-pair position 0, however, altered the position of the type II bend. Since changed base-pairs at positions -1 and -2 in the j2L FRT site and changed base-pairs at positions 0, +1, +2 and +3 in the j4R FRT site had no detectable effect on the position of the type II bend, this indicated that the sequence context of the AT base-pair at position 0 was of secondary importance to positioning of the type II bend. Thus while the position of the junction of the polypyrimidine tracts did not appear to influence the type II bend, the AT base-pair at position 0 in the core region was essential to positioning of the type II bend.

(ii) The effect of the mutations to the central two base-pairs of the core region on the position of the type II bend is not dependent on Flp-mediated cleavage activity

To examine whether the position of the type II bend formed when Flp was bound to the j2R FRT site was dependent on cleavage and covalent attachment of Flp to the DNA, I carried out a circular permutation assay in which the cleavage-defective Flp Y343F protein was bound to substrates containing j2R FRT sites (Fig. 3-2B(iv)). This experiment indicated that the type II bend was positioned at the right end of the core region immediately adjacent to the *a* element when the Flp Y343F or wild-type Flp proteins were used (Fig. 3-1, Table 3-1, bp position +4, +4). Thus the altered position of the type II bend when substrates containing j2R FRT sites were used was not dependent on cleavage activity.

(iii) The position of the bend in covalent Flp R191K:DNA complexes is not affected by changes to the core sequence

The Flp R191K protein is defective in ligation but is capable of cleaving DNA and thus covalent intermediates in which the protein is covalently bound at one of the two cleavage sites accumulate (Friesen and Sadowski, 1992; Pan et al., 1993a). Cleavage of the bottom strand of the FRT site (at the *a* symmetry element/core junction) by Flp R191K yields a complex (cIIa) in which one of the bound monomers is covalently attached at the nick in the bottom strand. Flp R191K-mediated cleavage of the top strand of the FRT site (at the *b* symmetry element/core junction) leads to formation of a similar complex (cIIb). Complex IIa and complex IIb have distinct mobilities on a native polyacrylamide gel and can be distinguished from each other by the relative intensity of the respective bands since Flp R191K cleaves the bottom strand of the FRT site about twice as efficiently as the top strand (Friesen and Sadowski, 1992).

To determine the relative efficiency of cleavage of the top *versus* bottom strands of mutated FRT sites the products of Flp R191K-mediated cleavage of the j2L, j2R and j4R FRT sites were analyzed (Fig. 3-4A, lanes 9-11). The result indicated that Flp R191K cleaved the bottom strand of the j2L and j2R FRT sites more efficiently than the top strand but cleaved the top strand of the j4R FRT site more efficiently than the bottom strand (quantitation not shown). This result enabled me to identify and distinguish complex IIa and complex IIb generated by Flp-mediated cleavage of the j2L, j4R and j2R FRT sites on a native gel.

Circular permutation assays in which Flp R191K was bound to substrates containing the j2L, j4R or j2R FRT sites were carried out to determine whether these core mutations also affected the type II bend in covalent complexes (Fig. 3-3A (i), (ii), (iii)). The j2L, j4R and j2R FRT sites did not affect the position of the type II bend in complex IIa and complex IIb. The type II bend in complex IIa was positioned in the right half of the core region when the substrate contained a j2L, j4R, j2R or wild-type FRT site (Fig. 3-1, Table 3-2, bp positions +4, +3, +3, +2). In complex IIb the type II bend was positioned in the core-proximal end of the *b* element when the substrate contained a j2L, j4R or wild-type FRT site (Fig. 3-1, Table 3-2, bp positions -7, -8, -8). I conclude that the effect of cleavage and formation of covalent complexes outweighs the influence of the central AT base-pair in the core region on the positioning of the type II bend.

(iv) The position of the type II bend influences the recombination proficiency of the site

To determine whether the AT->CG change at position 0 in the core region of the j2R FRT site which affected the position of the Flp-induced type II bend also affected Flp-mediated recombination activity, an *in vitro* recombination assay was carried out to determine the effects of the core mutations in the j2R, as well as, the j2L and j4R FRT sites on Flp-mediated recombination activity. The products of recombination resulting from incubation of Flp with two linear substrates which had the same core sequence but which differed in length were analyzed (Fig. 3-4B). The products of Flp-

mediated recombination resulting from strand exchange between two cleavage sites in the bottom strand and from strand exchange between two cleavage sites in the top strand were quantitated and the values obtained are shown in Table 3-3.

The core mutations in the j2R FRT site reduced the efficiency of recombination by 79% while the j2L and j4R FRT sites both reduced the efficiency of recombination by 42% as compared to the wild-type FRT site (Fig. 3-4B, lanes 13, 14, 15 *vs* 12; Table 3-3). Since the AT→CG base-pair change at position 0 in the j2R FRT site affected the position of the type II bend and the j2R FRT site had the most deleterious effect on the efficiency of recombination, this suggests that the structure of the core DNA is critical to the functionality of the site.

(v) The position of the type II bend may influence Flp-mediated cleavage of the top and bottom strands of the FRT site

As described above, alterations to the central two base-pairs of the core contained in the j2R FRT site affected the position of the type II bend and had a negative effect on Flp-mediated recombination. Which particular steps in the Flp-mediated recombination reaction might be affected by altering the position of the type II bend was investigated further. Thus Flp-mediated cleavage of the j2R, as well as, the j2L, j4R and wild-type FRT sites was assayed (Fig. 3-4A, lanes 4-7). The products of Flp-mediated cleavage of the top and bottom strands of the FRT sites were quantitated and the values obtained are shown in Table 3-3.

The cleavage assay showed that Flp-mediated cleavage of the top strand of the j2R FRT site was reduced by about 75% and cleavage of the bottom strand was increased by 2.5-fold as compared to cleavage of the wild-type FRT site (Fig. 3-4A, lanes 6 *vs* 4; Table 3-3). This result suggests that the position of the type II bend relative to the cleavage site may influence the efficiency of cleavage of the site.

Since base-pair changes in the j4R FRT site (Fig. 3-2A) had no effect on the position of the type II bend but appeared to have a significant effect on Flp-mediated cleavage (Fig. 3-4A, lanes 7 *vs* 4; Table 3-3), it is possible that the altered position of the type II bend resulting from base-pair changes in the j2R FRT site is independent of the effect base-pair changes in the j2R FRT site have on Flp-mediated cleavage. However as the j2R FRT site had the most deleterious effect on the efficiency of Flp-mediated recombination and the strongest effect on strand cleavage as compared to the j4R, j2L and wild-type FRT sites, this interpretation seems less likely (Fig. 3-4A, lanes 4-7; Table 3-3). My observations that the Flp R308K, Flp G328E and Flp H345L proteins effect the position of the type II bend (data not shown) and are defective in strand cleavage (Parsons et al., 1988, 1990; Schwartz and Sadowski, 1989; Kulpa et al., 1993) may also support the conclusion that the position of the type II bend influences the efficiency of cleavage of the site.

Flp-mediated cleavage of the top strand of the j4R FRT site was increased slightly (1.6-fold) and cleavage of the bottom strand was reduced by 50% as compared to cleavage of the wild-type FRT site (Fig. 3-4A, lanes 7 *vs* 4; Table 3-3). Since base-pair changes at

positions 0, +1, +2 and +3 in the j4R FRT site (Fig. 3-2A) had no affect on the position of the type II bend this result suggests that these changes may disrupt the recognition sequence for the cleavage site in the bottom strand.

Flp-mediated cleavage of the top and bottom strands of the j2L FRT site was essentially unaffected as compared to cleavage of the wild-type FRT site (Fig. 3-4A, lanes 5 *vs* 4; Table 3-3). Since the j2L FRT site contains base-pair changes at positions -1 and -2 (Fig. 3-2A), close to the cleavage site in the top strand, one may have expected top strand cleavage to be affected. This result together with our finding that base-pair changes at positions 0 and +1 in the j2R FRT site (Fig. 3-2A) decreased Flp-mediated cleavage of the top strand by about 75% suggests that recognition of the cleavage site in the top strand may rely on the structure rather than the sequence of the site.

In conclusion, it appears that the position of the type II bend and thus the structure of the DNA in the core region influence Flp-mediated cleavage of the top and bottom strands of the FRT site. Cleavage of the bottom strand but not cleavage of the top strand is also dependent upon recognition of the core sequence adjacent to the site of cleavage.

4. DISCUSSION

(i) The central AT base-pair in the core region of the FRT site is important to the positioning of the Flp-induced type II bend

A circular permutation experiment described in chapter 2 indicated that the type II bend was localized at base-pair position 0 in the core region. Data described in this chapter indicated that the central AT base-pair at position 0 in the core region was critical to positioning of the type II bend in a noncovalent complex in which Flp or Flp Y343F was bound to the FRT site. The sequence context of the AT base-pair had no detectable influence on the positioning of the type II bend. While inversion of the AT base-pair (AT->TA) had no effect, a substitution of the AT base-pair with a CG base-pair resulted in positioning of the type II bend at base-pair position +4 (Fig. 3-1). There are two possibilities which may account for the importance of the AT base-pair at position 0. The first is that Flp may make a base-specific contact(s) with the central AT base-pair. The second possibility is that the central AT base-pair may provide a point of flexure in the helix which is important to positioning of the type II bend.

Interestingly, the crystal structure of the Cre:*lox* synaptic complex shows that the central A residue in the core region of the Cre target site provides the nucleation point for the ~100° DNA bend induced by Cre (Guo et al.,1997). The Cre:*lox* structure showed that there were no contacts between Cre and the central four base-pairs of the core (Guo et al., 1997). A mobile alpha-helix of the cleaving

Cre monomer, however, makes three phosphate contacts in the concave surface of the DNA bend thereby utilizing a mechanism of charge neutralization at the phosphodiester backbone to induce the bend (Guo et al., 1997; Strauss and Maher, 1994; Strauss et al., 1996).

While both Flp and Cre position a severe DNA bend at a central A residue in the core region of their respective target sites, the mechanism whereby they induce this distortion may not be entirely the same. Phosphate ethylation interference studies of Flp-mediated recombination identified only Flp:phosphate contact points clustered opposite the cleavage sites and not in the central region of the core (Bruckner and Cox, 1986; Beatty and Sadowski, 1988). It is possible that these two contact areas at the margins of the core are sufficient to induce the bend positioned centrally in the core. Alternately, Flp may wedge an intercalating hydrophobic group into the helix to induce the type II bend, however, such a mechanism would require base-specific contacts (Werner et al., 1996).

Alterations to the central two base-pairs of the core region of the FRT site which included the AT->CG base-pair change at position 0 had the strongest negative effect on recombination (Table 3-3; Fig. 3-4B). Since these alterations also affected the position of the type II bend, this suggests that the position of the type II bend is critical to the function of the site. Similarly a AT->GC mutation in the centre of the core of the Cre target site abolished the recombination proficiency of the site (Hoess et al., 1986). The ~100° Cre-induced DNA bend is centred at this A residue (Guo et al., 1997). The Cre/*lox* structure suggests that the DNA bend facilitates strand exchange

following cleavage since three unpaired nucleotides between the DNA bend and the cleavage site are highly mobile and are moved toward the centre of the strand exchange cavity in the synapse (Guo et al., 1997). It is possible that the Flp-induced type II bend of >140° in the core region facilitates recombination in the same way. If this is the case, one can speculate that positioning of the bend at the margin of the core furthest from the site of cleavage may be too distant to facilitate unwinding of the core at the site of the nick.

(ii) The position of the bend in covalent Flp R191K:DNA complexes is not affected by changes to the core sequence

None of the mutations to the core region affected the type II bend in covalent complexes generated by Flp R191K (Fig. 3-3, Table 3-2). Surprisingly although we found that the AT->CG base-pair change at position 0 affected the position of the type II bend induced by Flp or Flp Y343F, this change did not affect the position of the type II bend in complex IIa in which a Flp R191K monomer is covalently bound at the cleavage site in the bottom strand.

This result indicated that the position of the type II bend in complex IIa differed from the position of the type II bend in a noncovalent complex II generated by Flp. Since the difference in the position of the type II bend in a noncovalent complex II generated by Flp (position 0, Fig. 3-1, Table 3-1) and in a covalent complex IIa generated by Flp R191K (position +2, Fig. 3-1, Table 3-2) was relatively small, I did not previously make a distinction between them (chapter 2). The differing position of the type II bend in complex IIa *versus* a noncovalent complex II suggests that a

conformational change in the structure of the complex takes place in conjunction with cleavage and covalent attachment of a Flp R191K monomer to the cleavage site in the bottom strand.

Interestingly, the AT->CG mutation at position 0 in the core of the j2R FRT site shifted the position of the type II bend generated by Flp (Fig. 3-1, Table 3-1, bp position +4) to about the same position as the position of the type II bend in complex IIa (Fig. 3-1, Table 3-2, bp position +2 to +4). This result may reflect the presence of a secondary site of flexure in the core region of the FRT site immediately adjacent to the *a* symmetry element.

(iii) DNA structure and sequence requirements for Flp-mediated cleavage of the top *versus* bottom strands may be different

I studied the effect of mutations in the core region of the FRT site on Flp-mediated cleavage. Base-pair changes at positions 0, +1, +2 and +3 (j4R FRT site, Fig. 3-4A, Table 3-3) which had no detectable effect on the position of the type II bend, nevertheless reduced the efficiency of cleavage of the bottom strand by 50%. This suggests that these base-pair changes disrupted the recognition sequence for the cleavage site in the bottom strand. Base-pair changes at positions -1 and -2 (j2L FRT site, Fig. 3-4A, Table 3-3), close to the cleavage site in the top strand, which also had no detectable effect on the position of the type II bend, had little effect on the efficiency of cleavage of either strand. A mutation to the core sequence which shifted the position of the type II bend from base-pair postion 0 to +4 also reduced Flp-mediated cleavage of the top strand by 75% and

increased cleavage of the bottom strand by 2.5-fold (j2R FRT site, Fig. 3-1, Fig. 3-4A, Table 3-3). The data appear to indicate that positioning of the type II bend closer to the site of cleavage in the bottom strand increased the efficiency of cleavage of that site while positioning of the type II bend further from the cleavage site in the top strand strongly decreased the efficiency of cleavage of that site. Thus, the structure of the DNA influences cleavage of both the top and bottom strands although cleavage of the bottom strand also appears to be dependent upon the sequence of the core region adjacent to the site.

The structure of the DNA upon which Flp acts during the initial *versus* the final cleavage events of the recombination pathway is different. For the initial cleavages, Flp acts upon duplex DNA which contains a Flp-induced bend positioned in the middle of the core region (Schwartz and Sadowski, 1989; chapter 2), while for the final cleavage events Flp acts upon a four-armed Holliday intermediate. It is possible that the variation in the sequence and DNA structure of the initial *versus* the final sites of cleavage enables Flp to recognize and distinguish between the two sites. Since the Holliday structure is a highly unusual DNA conformation, Flp may rely solely on the local DNA conformation for recognition of the second cleavage sites. Recognition of the initial cleavage sites, however, is likely to involve components of both sequence and structural specificity. Since cleavage of the bottom strand is apparently dependent upon both the sequence and structure of the DNA while cleavage of the top strand is apparently dependent only on structure, this may suggest that the initial cleavage events occur at the sites in the bottom

strands and the final cleavage events, leading to resolution of the Holliday intermediate, occur at the sites in the top strands.

Table 3-1. Location of type I and type II bend centres induced by Flp proteins

Substrate [a]	Flp protein	Type I bend centre [b]	Type II bend centre [b]
WT FRT (TCTAGAAA)	Flp	- 5 (-6, -4, -4) [c]	0 (-1, +1, -1) [c]
j2L FRT (TAGAGAAA)	Flp	- 6	+1
j4R FRT (TCTTTCTA)	Flp	- 3 (-3, -2)	+1 (0, +1)
j2R FRT (TCTCTAAA)	Flp	- 6 (-5, -7)	+4 (+4, +3)
j2R FRT (TCTCTAAA)	Y343F	- 6 (-5, -6)	+4 (+4, +4)

[a] The wild-type FRT (WT FRT) site or the modified FRT site that was used as a substrate to determine the bend centres. The mutated core sequences of the j2L, j4R and j2R FRT sites is shown in Fig. 3-2A.

[b] Numbers indicate the bp position of the bend centre, according to the scale of the FRT site shown in Fig. 3-1.

[c] Location of type I and type II bend centres induced by Flp bound to a wild-type FRT site (chapter 2). Numbers in parentheses indicate the results of individual experiments.

Table 3-2. Location of bend centres in covalent R191K:FRT complexes

Substrate [a]	cIIa bend centre[b]	cIIb bend centre[b]
WT FRT (TCTAGAAA)	+2 (+2, +2)[c]	-8 (-9, -7)[c]
j2L FRT (TAGAGAAA)	+4	- 7
j4R FRT (TCTTTCTA)	+3	- 8
j2R FRT (TCTCTAAA)	+3	-

[a] The wild-type FRT (WT FRT) site or the modified FRT site used as a substrate to determine bend centres. The mutated core sequences of the j2L, j4R and j2R FRT sites are shown in Fig. 3-2A.

[b] Numbers indicate the bp position of the bend centre, according to the scale of the FRT site shown in Fig. 3-1.

[c] Location of bend centres in complex IIa and complex IIb induced by Flp R191K bound to a wild-type FRT site (chapter 2). Numbers in parentheses indicate the results of individual experiments.

Table 3-3. Quantitation of the products of cleavage and recombination mediated by Flp.

[a] The wild-type FRT (WT FRT) site or the modified FRT site that was used as a substrate in Flp-mediated cleavage and recombination assays. The mutated core sequences of the j2L, j4R and j2R FRT sites are shown in Fig. 3-2A.

[b] Quantitation of the products of cleavage from Fig. 3-4A. The amount of product is expressed as a percentage of the total counts in the product and substrate bands.

[c] The amount of cleavage in the bottom strand at *a* was compared to the amount of cleavage in the top strand at *b* and was expressed as a ratio.

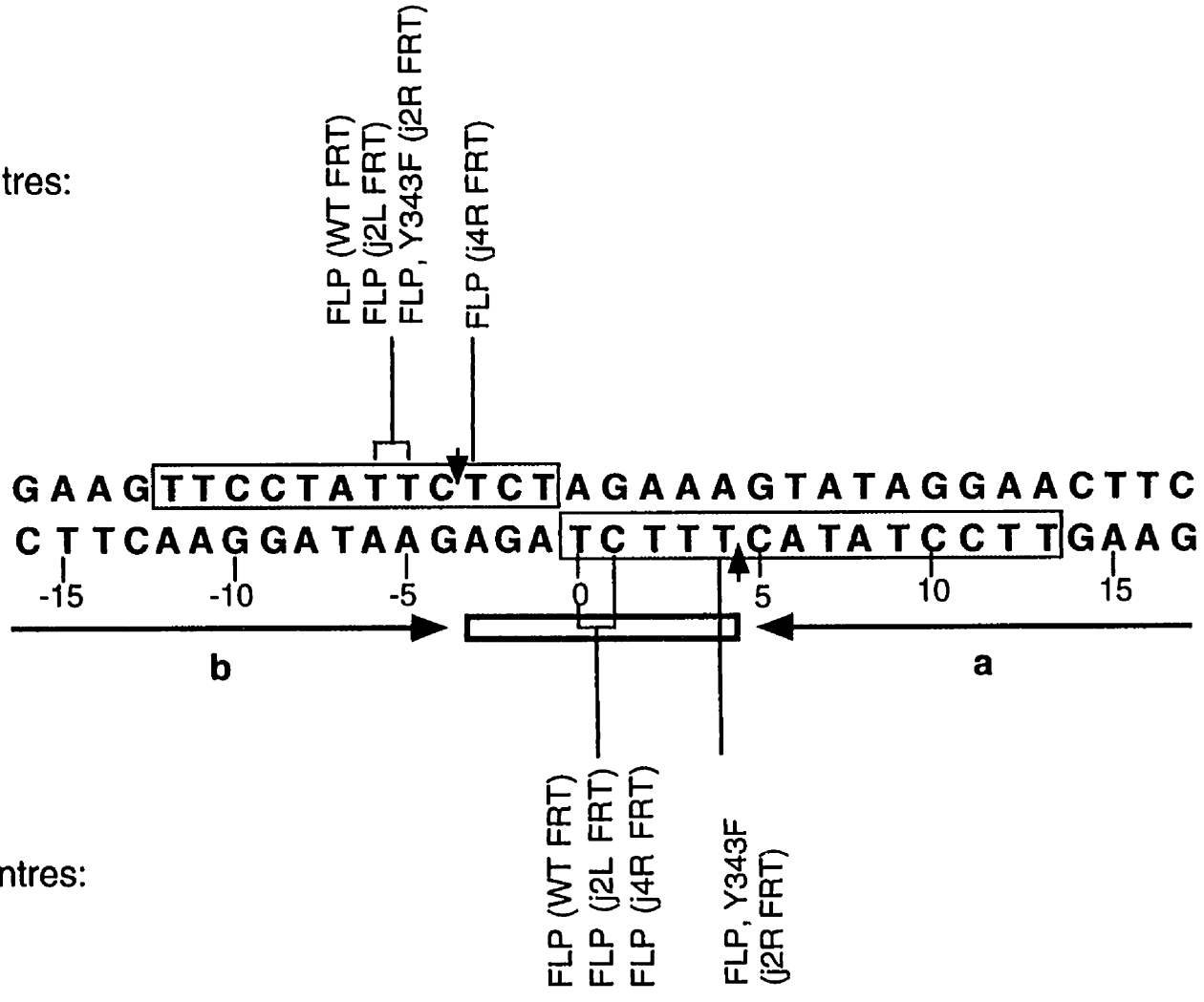
[d] Quantitation of the products of recombination from Fig. 3-4B. Recombination products formed using modified FRT sites were expressed as a percentage of recombination products formed using a wild-type FRT site which was assigned a value of 100%.

Table 3-3. Quantitation of the products of cleavage and recombination mediated by Flp

Substrate [a]	Products of cleavage at *a* (%) [b]	Products of cleavage at *b* (%) [b]	Ratio of *a* : *b* [c]	Total cleavage products (%) [b]	Total recombination products (%) [d]
WT FRT (TCTAGAAA)	1.1	1.1	1:1	2.2	100
j2L FRT (TAGAGAAA)	1.6	1.5	1.1:1	3.1	57.8
j4R FRT (TCTTTCTA)	0.5	1.8	1:3.6	2.3	57.6
j2R FRT (TCTCTAAA)	2.7	0.3	5.4:1	3.0	20.7

Figure 3-1. The position of Flp-induced DNA bends in FRT sites with mutated core sequences. The sequence of the minimal FRT site is shown. The 13 base-pair symmetry elements (*a* and *b*) are indicated by horizontal arrows and the 8 base-pair core, by an open rectangle. The vertical arrows indicate the sites of Flp cleavage in the top and bottom strands. The stippled boxes indicate the polypyrimidine tracts present in the FRT site. The locations of type I and type II bend centres determined using modified FRT sites (indicated in parentheses) are indicated according to the scale of the wild-type FRT site. The mutated core sequences of the j2L, j4R and j2R FRT sites is shown in Fig. 3-2A. The location of bend centres is based on the results of 1-3 experiments as indicated in Table 3-1.

Type I bend centres:

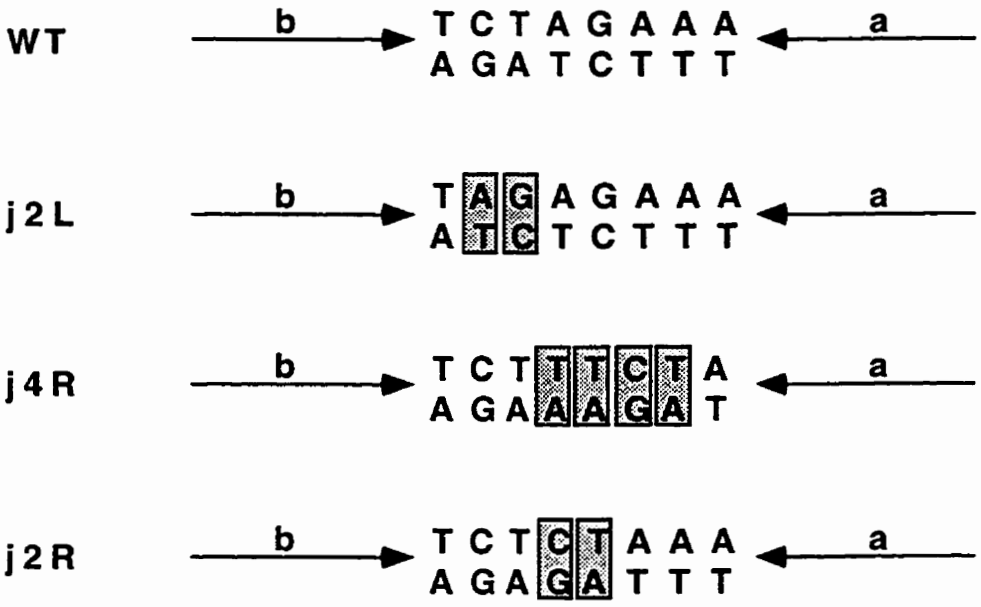


Type II bend centres:

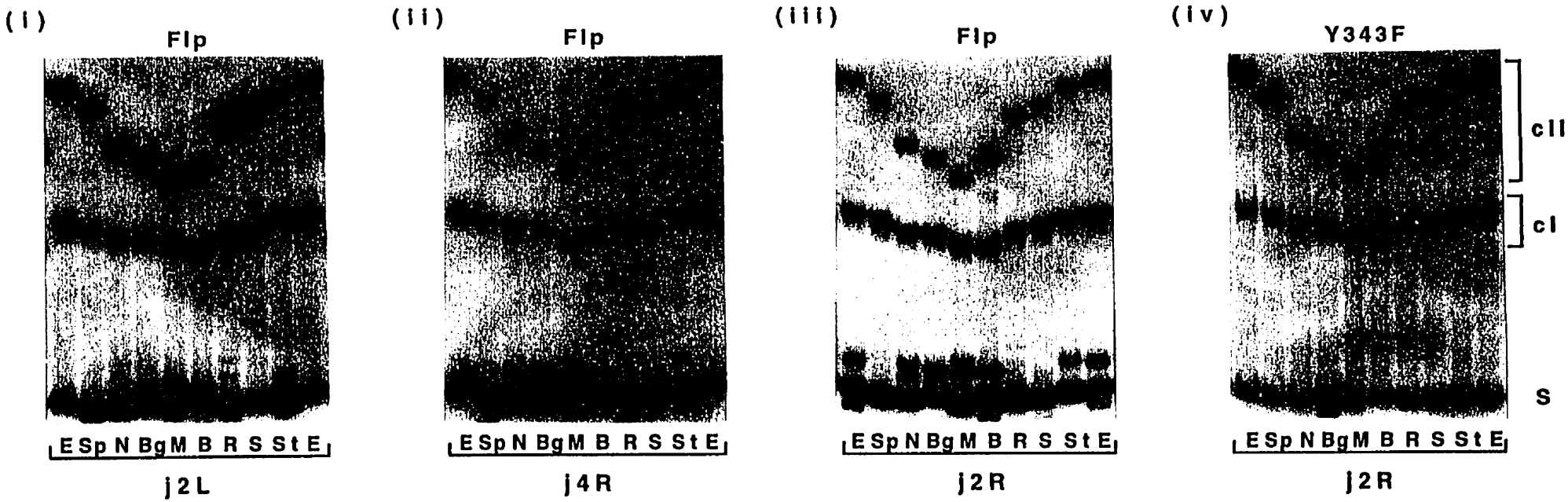
Figure 3-2. Positions of bend centres induced by Flp or Flp Y343F bound to FRT sites containing mutated core sequences. **A**. Sequence of the core region of the wild-type (WT) and modified FRT sites (j2L, j4R, j2R). The *a* and *b* symmetry elements flanking the core region are indicated by horizontal arrows. Base pairs which have been changed from the wild-type FRT core sequence are boxed. **B**. Gel mobility shift assays of circularly permuted substrates containing FRT sites with mutated core sequences bound by Flp or Flp Y343F. The pB(j2L), pB(j4R) or pB(j2R) plasmids contained the j2L FRT site (i), j4R FRT site (ii) or the j2R FRT site (iii) (iv) cloned into the pBEND2 plasmid that contained two tandemly repeated sets of restriction enzyme sites. The substrates (152 bp) were obtained by cleaving the pB(j2L), pB(j4R) or pB(j2R) plasmids at the different restriction sites. Each circularly permuted substrate is named according to the restriction enzyme used to obtain it and is indicated below the autoradiogram. E, *Eco*RV; Sp, *Spe*I; N, *Nhe*I; Bg, *Bgl*II ; M, *Mlu*I; B, *Bam*HI; R, *Rsa*I; S, *Ssp*I; St, *Stu*I. 5' end-labeled DNA substrates were incubated with Flp or Flp Y343F (indicated at the top) and complexes were separated on 5% native polyacrylamide gels. S, unbound substrate; cI, complex I; cII, complex II.
C. Determination of the bend centres in complex I and complex II. The relative mobilities of Flp:DNA complexes were plotted as a function of the position of the FRT site relative to the end of the substrate. The linear portions of the curve were extrapolated to identify the position at which the curve reached a minimum and the position of the bend centre with respect to the FRT site was estimated (chapter 2). The positions of the bend centres estimated

from the curves are indicated in Table 3-1.

A


WT
b
T C T A G A A A
A G A T C T T T
a
j2L
b
T A G A G A A A
A T C T C T T T
a
j4R
b
T C T T T C T A
A G A A A G A T
a
j2R
b
T C T C T A A A
A G A G A T T T
a

B


(i)
Flp
E Sp N Bg M B R S St E
j2L
(ii)
Flp
E Sp N Bg M B R S St E
j4R
(iii)
Flp
E Sp N Bg M B R S St E
j2R
(iv)
Y343F
cII
cI
S
E Sp N Bg M B R S St E
j2R

C

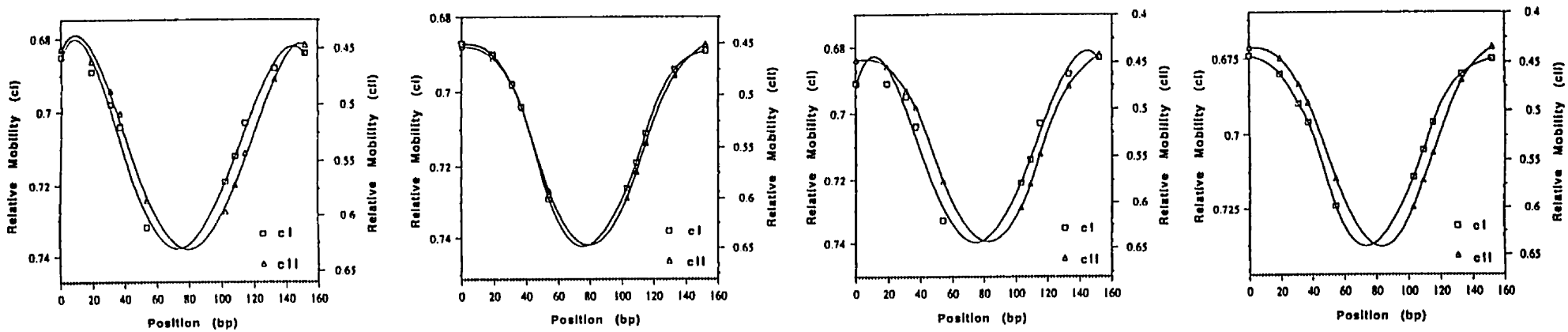
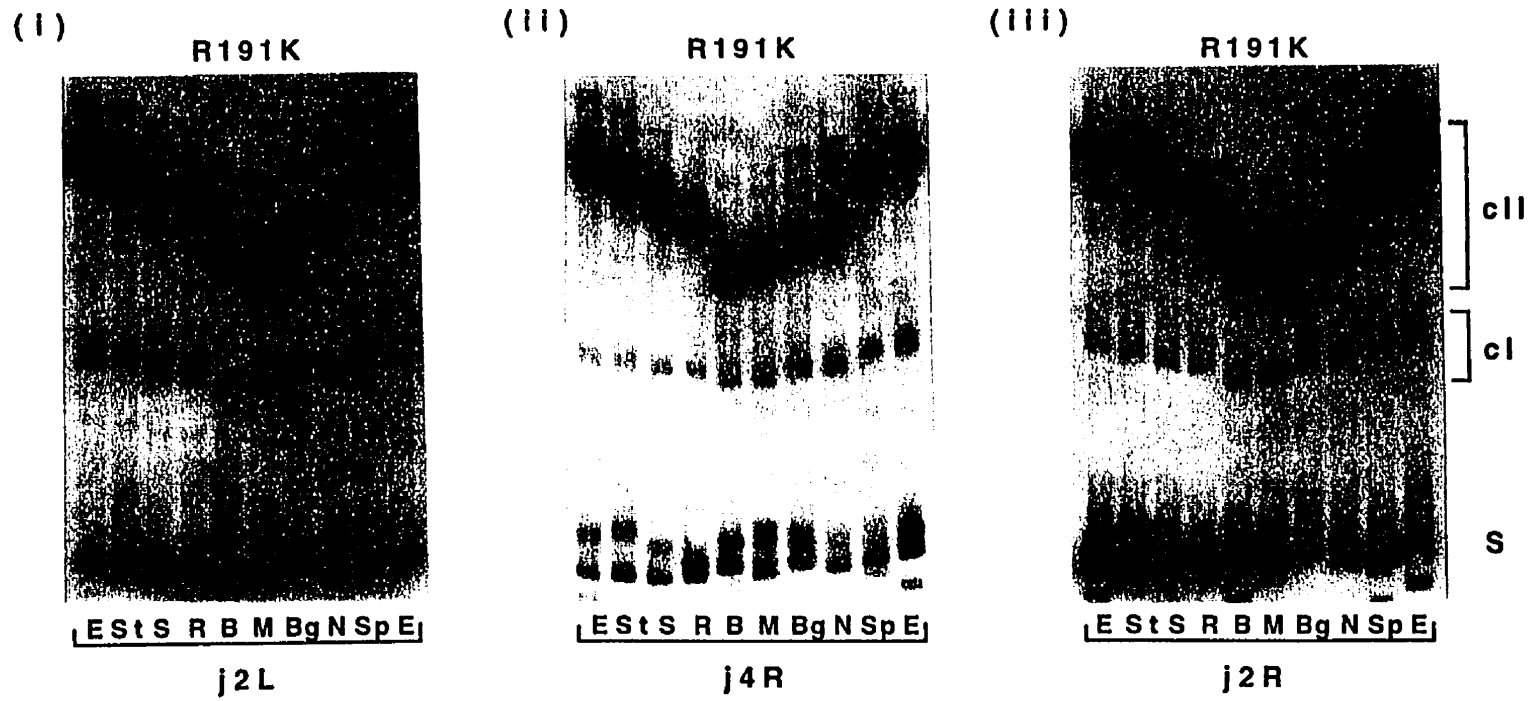

Relative Mobility (cI)
Relative Mobility (cII)
Position (bp)
cI
cII

Figure 3-3. Positions of bend centres induced by Flp R191K bound to FRT sites containing mutated core sequences. **A.** Gel mobility shift assays of circularly permuted substrates containing FRT sites with mutated core sequences bound by Flp R191K. DNA substrates (152 bp) contained the j2L FRT site (i), j4R FRT site (ii) or j2R FRT site (iii) and were obtained as described in Figure 3-2B. Each circularly permuted substrate is named according to the restriction enzyme used to obtain it and is indicated below the autoradiogram and as in Figure 3-2. 5' end-labeled DNA substrates were incubated with Flp R191K and complexes were separated on 5% native polyacrylamide gels. S, unbound substrate; cI, complex I; cII, complex II. **B.** Determination of the bend centres in complex IIa and complex IIb. The relative mobilities of complex IIs were plotted as a function of the position of the FRT site relative to the end of the substrate. The positions of the bend centres with respect to the FRT site were estimated from the curves as described in Figure 3-2C and are indicated in Table 3-2. Note that the position of the bend centre in complex IIb where the substrate contained a j2R FRT site could not be determined because of the inefficiency with which Flp R191K cleaved the top strand of the j2R FRT site.

A


(i)
R191K
(ii)
R191K
(iii)
R191K
cII
cI
S
E St S R B M Bg N Sp E
E St S R B M Bg N Sp E
E St S R B M Bg N Sp E
j2L
j4R
j2R

B

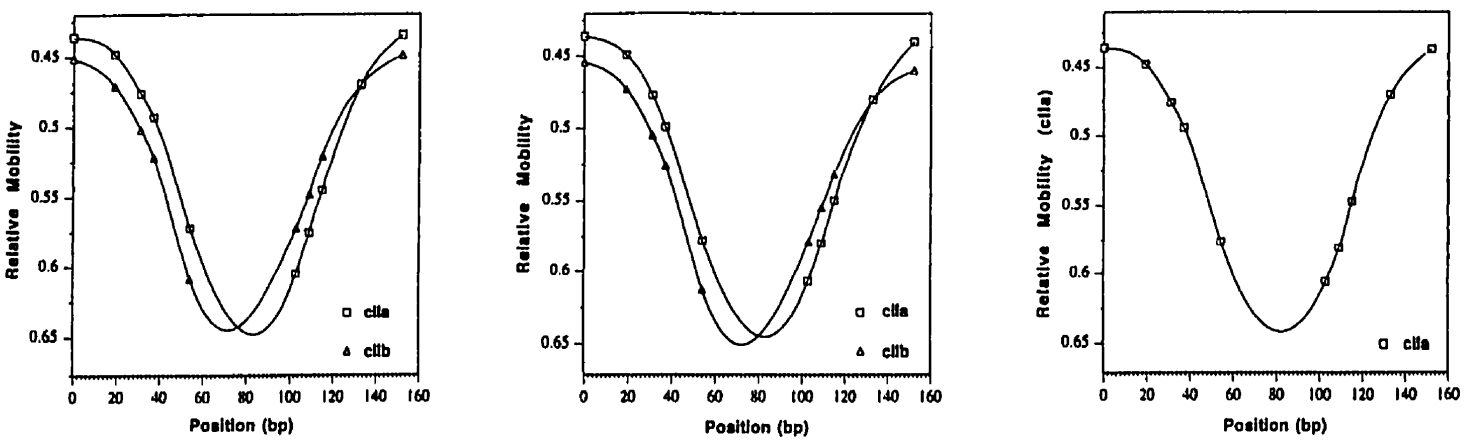
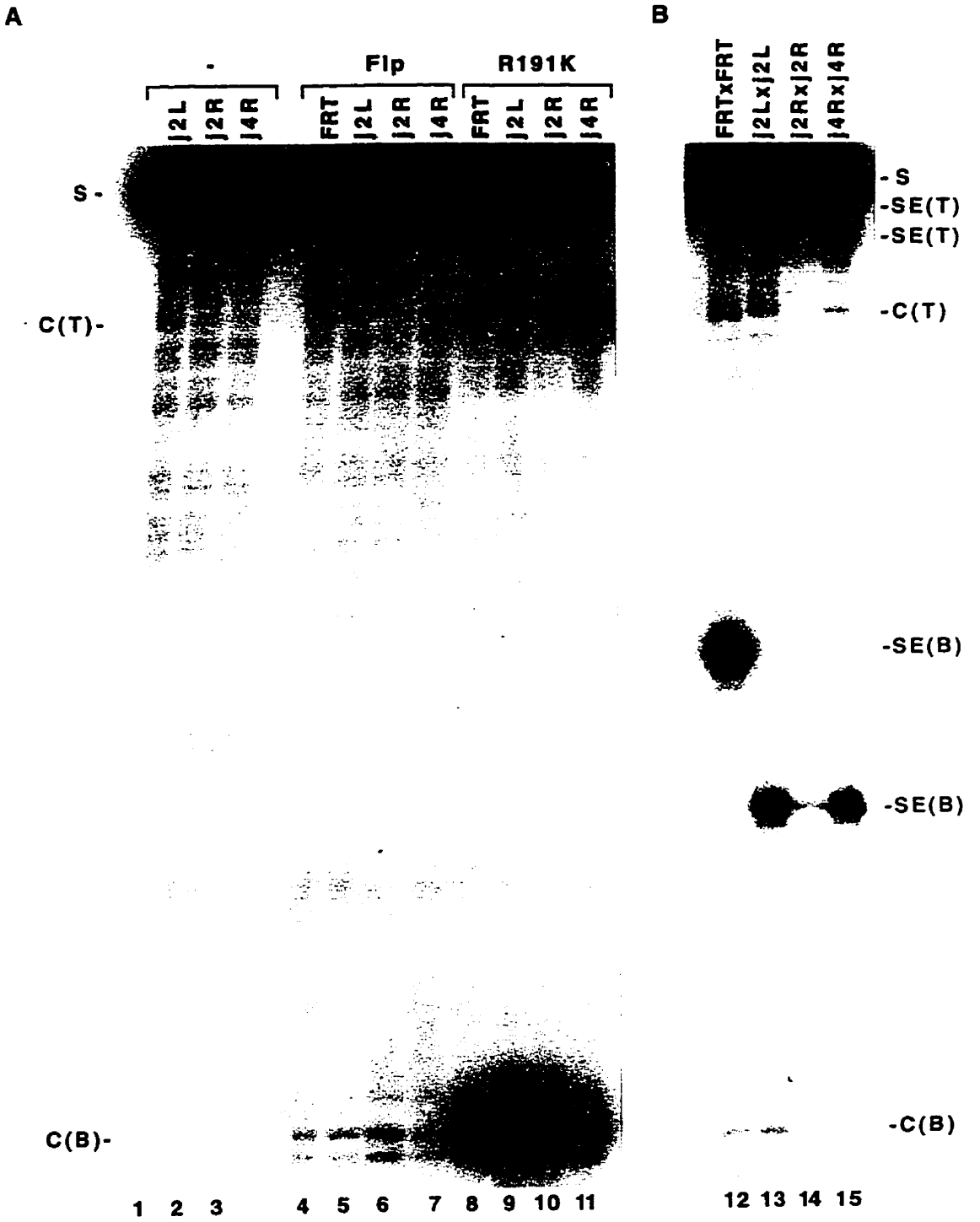

Relative Mobility
0.45
0.5
0.55
0.6
0.65
0 20 40 60 80 100 120 140 160
Position (bp)
cIIa
cIIb
Relative Mobility
0.45
0.5
0.55
0.6
0.65
0 20 40 60 80 100 120 140 160
Position (bp)
cIIa
cIIb
Relative Mobility (cIIa)
0.45
0.5
0.55
0.6
0.65
0 20 40 60 80 100 120 140 160
Position (bp)
cIIa

Figure 3-4. Flp-mediated cleavage and recombination assays with FRT sites containing mutated core regions. **A**. Cleavage assay of FRT sites containing mutated core regions. Flp substrates were prepared by 5' end-labeling fragments obtained by digestion of the pB2Flp(ba), pB(j4R), pB(j2L) and pB(j2R) plasmids with *Nhe*I. The DNA substrates (indicated at the top of the autoradiogram) contained a j2L FRT site (j2L), a j2R FRT site (j2R), a j4R FRT site (j4R) or a wild-type FRT site (FRT). The sequences of the wild-type, j4R, j2R and j2L FRT sites is given in Figure 3-2A. Substrates were incubated with no protein (-), Flp or Flp R191K (as indicated at the top) for 120 minutes at 22°C. The reaction mixtures were treated with proteinase K and SDS, and the DNA was extracted with phenol/chloroform, precipitated with ethanol and analyzed on an 8% denaturing polyacrylamide gel. Substrates (S), top strand cleavage products (C(T)) and bottom strand cleavage products (C(B)) are indicated at the left. **B**. Recombination assay of FRT sites containing mutated core regions. Radio-labeled substrates (152 bp) were prepared by 5' end-labeling fragments obtained by digestion of the pB2Flp(ba), pB(j4R), pB(j2L) and pB(j2R) plasmids with *Nhe*I. Unlabeled synthetic DNA substrates were prepared by annealing the following oligonucleotides: KL-12 and KL-13 (55 bp, lane 12), j2LTOP and j2LBOT (34 bp, lane 13), j2RTOP and j2RBOT (34 bp, lane 14), j4RTOP and j4RBOT (34 bp, lane 15). A radio-labeled substrate and an unlabeled substrate containing the same FRT site were incubated with Flp for 120 minutes at 22°C and reactions were subsequently treated as described in A. Radio-labeled substrates (S), top strand cleavage products (C(T)), bottom strand cleavage products

(C(B)), top strand exchange products (SE(T)), and bottom strand exchange products (SE(B)) are indicated at the right of the autoradiogram. **C.** Schematic diagram of the recombination assay.


A
B
-
Flp
R191K
J2L
J2R
J4R
FRT
J2L
J2R
J4R
FRT
J2L
J2R
J4R
S -
C(T)-
C(B)-
1 2 3 4 5 6 7 8 9 10 11
FRTxFRT
J2LxJ2L
J2RxJ2R
J4RxJ4R
- S
-SE(T)
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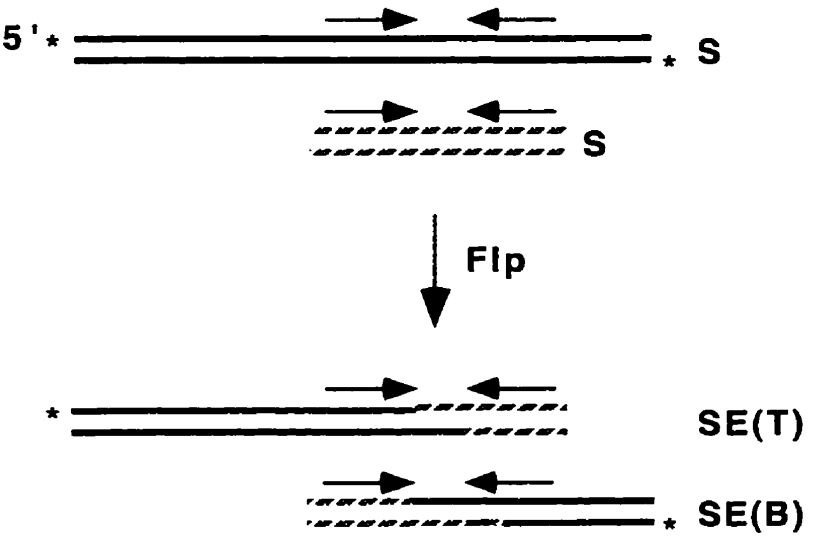
-SE(B)
-C(B)
12 13 14 15

C



CHAPTER 4.

ASYMMETRY IN FLP-MEDIATED CLEAVAGE

Material in this chapter has been published in Luetke, K.H., Zhao, B. and Sadowski, P.D. (1997). *Nucleic Acids Res.* **25**, 4240-4249.
All the experiments were carried out by myself. Dr. B. Zhao prepared the oligonucleotides which contained a phosphorothioate substitution.

1. INTRODUCTION

Flp-mediated cleavage of a DNA strand in the FRT site generates an intermediary complex in which one of the two bound Flp monomers is covalently attached to the 3'-terminus of the nick. The observation that the position and direction of DNA bends in complexes where one strand was cleaved by Flp differed depending on which strand was cleaved (chapter 2), provided the impetus for further investigation of covalent Flp:DNA complexes. Formation of these complexes precedes strand exchange events in the recombination pathway which are not well understood.

I have used FRT sites in which the phosphate at one cleavage site was substituted with a phosphorothioate. Since cleavage of the phosphorothioate modification generates a 5'-sulfhydryl group at the nick which is inefficient in the subsequent ligation reaction (Burgin et al., 1995), covalent intermediates which are cleaved in either the top or bottom strand of the FRT site were accumulated. Probing of these intermediates revealed that an adenine residue opposite the site of cleavage was specifically protected from methylation within the cleaved covalently bound complexes. Cleavage was also associated with changes in the interaction between the binding site and the noncovalently bound Flp monomer in the cleavage complex as revealed by methylation interference experiments. A comparison of Flp-mediated cleavage of the top and bottom strands of the FRT site indicated that the two cleavage events involve differences in the contacts of Flp with bases opposite each cleavage site.

2. MATERIALS AND METHODS

(i) Oligonucleotide substrates

Unmodified oligonucleotides were synthesized at the Hospital for Sick Children/Pharmacia Biotechnology Service Centre, Banting Institute, University of Toronto. Deprotected oligonucleotides used for methylation protection and interference experiments were purified by denaturing polyacrylamide gel electrophoresis (15% polyacrylamide, acrylamide:bis-acrylamide (19:1), 8 M urea, 1xTBE (90 mM boric acid, 90 mM Tris, 2 mM EDTA)).

Oligonucleotides used in this study (**S** indicates the position of the 5'-bridging phosphorothioate; sequences of the symmetry elements of the FRT site are indicated in bold; the core sequence of the FRT site is indicated in italics):

(KL-7) 5' T**GAAGTTCCTATTC***TCTAGAAA***GTATAGGAACTTC**GACCT 3'

(KL-31) 5' TTTCCAGGTC**GAAGTTCCTATAC***TTTCTAGA***GAATAGGA ACTTC**ACTC 3'

(KL-7M(S)) 5' TTGT**GAAGTTCCTATTCS***TCTAGAAA***GTATAGGAACT TC**GA 3'

(KL-31M(S)) 5' TTTCCAGGTC**GAAGTTCCTATACS***TTTCTAGA***GAATA GGAACTTC** 3'

(HP31) 5' GGTC**GAAGTTCCTATAC***TTTCTAGA***GAATAGGAACTTC**AC 3'

(KL-7V) 5'TTGT**GAAGTTCCTATTC***TCTAGAAA***GTATAGGAACTTC**GA3'

(HP-33V) 5' TTGT**GAAGTTCCTATTC** 3'

(HP-14V) 5' *TCTAGAAA***GTATAGGAACTTC**GA 3'

(HP-2) 5' *TTTCTAGA***GAATAGGAACTTC**A 3'

(HP-5) 5' AGGTC**GAAGTTCCTATAC** 3'

(HP-33) 5' T**GAAGTTCCTATTC** 3'

(HP-14) 5' *TCTAGAAA***GTATAGGAACTTC**GACCTGATC 3'

Oligonucleotides were 5' end-labeled with [γ-^{32}P]ATP (NEN DuPont) using T4 polynucleotide kinase (New England Biolabs). Following extraction with phenol/chloroform and ethanol precipitation, the radioactively labeled oligonucleotide was annealed to the appropriate oligonucleotide(s) by heating and slow cooling in 100 mM NaCl and 5 mM $MgCl_2$. Annealed DNA substrates were purified on 10% polyacrylamide gels (acrylamide:bis-acrylamide (30:1), 1x TBE). Alternatively, annealed DNA substrates were 3' end-labeled with [α-^{32}P]dCTP (NEN DuPont) using AMV reverse transcriptase (Life Sciences) and purified as described above.

(ii) Preparation of 5'-bridging thiooligonucleotides

The thiooligonucleotides were synthesized on the ABI 394 DNA/RNA synthesizer and the standard protocol for oligonucleotide synthesis on controlled pore glass support was followed except for the step in which the sulfur linkage was formed. For the latter linkage, the 5'-phosphorothioate link was created using 5'-(S-trityl)-mercapto-5'-deoxythymidine-3'-O-(2-cyanomethyl)-N,N-isopropylphosphoramidite prepared as described previously (Mag et al., 1991) and as modified by Burgin et al. (1995).

We also extended the time for detritylation of the sulfur by $AgNO_3$ from 10 to 20 min and the coupling time of the phosphoramidite containing the 5'-mercapto group with the next phosphoramidite from 5 to 10 min. The thiooligonucleotides were

deprotected by the standard procedure using ammonium hydroxide and purified by gel electrophoresis.

(iii) Flp assays

Cleavage reaction mixtures containing 0.01 pmol of DNA substrate that was radioactively labeled at both 5'-ends were incubated with 1.4, 2.8 or 14.1 pmol Flp or 58.7 pmol Flp R191K in a 20 µl volume containing 50 mM Tris-HCl (pH 7.4), 33 mM NaCl, 1 mM EDTA, 100 µl calf thymus DNA/ml. Reaction mixtures were incubated at 22°C for 90 minutes and subsequently treated with 10 µg proteinase K and 0.005% (w/v) SDS for 60 minutes at 37°C. Following phenol/chloroform extraction and precipitation with ethanol, the DNA was analyzed on a 15% denaturing polyacrylamide gel.

Binding reaction mixtures containing 0.03 pmol of radioactively labeled DNA substrate were incubated with 3.5 pmol Flp protein in a 20 µl volume containing 50 mM Tris-HCl (pH 7.4), 33 mM NaCl, 1 mM EDTA, 100 µg calf thymus DNA/ml. Reaction mixtures were incubated at 22°C for 60 minutes. Some mixtures were shifted to higher temperatures for an additional 10 minutes (as indicated in the figure legend). A 2.7 µl quantity of dye mixture (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 100 µg bovine serum albumin/ml, 20% glycerol) was added and reactions were subjected to electrophoresis on 5% polyacrylamide gels (1xTBE) at 4°C. Dried gels were subjected to autoradiography.

(iv) DMS methylation protection

DNA substrates (1.23-1.43 pmol), 5' end-labeled on the top or bottom strands, were incubated with 38 pmol Flp protein for 90 minutes at 22°C, in a 20 µl volume containing 50 mM Na cacodylate (pH 8.0), 5 mM Tris-HCl (pH 7.6), 12.5 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 100 µg calf thymus DNA/ml [pH 7.7]. Then, DMS was added to a final concentration of 24.5 mM and after one minute at 22°C, β-mercaptoethanol was added to 200 mM. After five minutes at 22°C, reaction mixtures in which Flp was bound to phosphorothioate-containing FRT sites were placed at 49°C for 15 minutes. Subsequently 3 µl of a dye mixture was added and samples were analyzed by electrophoresis on a 5% native polyacrylamide gel at 4°C. Both Flp-bound and unbound fractions of DNA were visualized by autoradiography, excised and eluted. The DNA was depurinated at 90°C for 10 minutes and cleaved by heating with NaOH at 90°C for 5 minutes (Panigrahi et al., 1992). The DNA was precipitated with ethanol. In order to obtain DNA free of most of the protein, the precipitated pellets were washed 4-5x with 70% ethanol, the washes were pooled and reprecipitated with 1.5 µg carrier DNA. Typically 6-12% of the DNA present in the Flp:DNA complexes was recovered by this method. The recovered DNA was analyzed on a 15% denaturing polyacrylamide gel.

(v) Methylation interference

DNA substrates (8.8-11.1 pmol), 5' end-labeled on the top or bottom strands, were methylated with DMS (30 mM final concentration) in a 50 µl volume as described above, except that Flp was omitted. The

methylation reaction was terminated by addition of 12.5 µl of a stop solution (1.5 M Na acetate (pH 7.0), 1.0 M β-mercaptoethanol) and the DNA was precipitated with ethanol. Methylated DNA substrate (1.23 pmol) was incubated with Flp, as described above, in a reaction containing 50 mM Tris-HCl (pH 7.4), 33 mM NaCl, 1 mM EDTA and 100 µg calf thymus DNA/ml. Thereafter complexes and DNA were treated in the same way as described for the methylation protection protocol.

(vi) Quantitation

Dried gels or autoradiograms were scanned using a Molecular Dynamics Phosphorimager and analyzed using Imagequant software.

Flp preparations are described in chapter 2.

3. RESULTS

(i) Accumulation of covalent Flp:DNA complexes

Flp mediates strand cleavage of the FRT site through a transesterification reaction which results in the covalent linkage of the protein to the 3'-phosphoryl terminus through the active-site tyrosine residue. The otherwise transient covalent intermediates resulting from Flp-mediated cleavage may be trapped by the use of ligation-defective mutant proteins (Friesen and Sadowski, 1992; Parsons et al., 1988; Pan et al., 1993) or by the use of suicide substrates containing nicks that are a few nucleotides away from the site of strand cleavage (Nunes-Düby et al., 1987). Recently, Burgin et

al. (1995) used a novel suicide substrate containing a 5'-bridging phosphorothioate linkage at the site of enzyme-mediated strand cleavage. Covalent enzyme:DNA intermediates accumulated after cleavage of the phosphorothioate linkage since the 5'-sulfhydryl group which was liberated was incompetent for the subsequent ligation reaction.

To determine the effect of a 5'-bridging phosphorothioate linkage on Flp-mediated cleavage activity, strand cleavage was assayed on a denaturing gel (Fig. 4-1). Flp was incubated with an unmodified FRT site (Fig. 4-1, lanes 2-4) or FRT sites containing single phosphorothioate modifications at the top (Fig. 4-1, lanes 7-9) or bottom strand cleavage sites (Fig. 4-1, lanes 12-14). Incubation of Flp with FRT sites containing phosphorothioate modifications results in an accumulation of cleavage product at the site of modification (Fig. 4-1, compare lanes 7-9, 12-14 and 2-4), consistent with published results for calf thymus topoisomerase I and lambda integrase (Burgin et al., 1995). Incubation of Flp with an unmodified FRT site yields very little cleavage product (Andrews et al., 1985; Fig. 4-1, lanes 2-4). This is presumably due to the transient nature of the covalent Flp:DNA intermediate which is quickly lost due to the rejoining of the phosphodiester bond *via* the adjacent 5'-OH group. Cleavage of the phosphorothioate linkage by wild-type Flp was as efficient as by the ligation-defective protein Flp R191K (Fig. 4-1, compare lanes 9 and 10, 14 and 15), as one would expect if the phosphorothioate substrate is inhibiting Flp-mediated ligation. Cleavage of an unmodified FRT site by Flp R191K was also comparable in efficiency to the cleavage of a phosphorothioate

linkage by Flp (Fig. 4-1, compare lanes 5 and 9, 5 and 14). The presence of the phosphorothioate in one strand also seemed to inhibit the cleavage of the non-substituted strand by Flp R191K (Fig. 4-1, compare lanes 5, 10, 15).

Since the phosphorothioate substrate appears to inhibit Flp-mediated ligation, it was predicted that the phosphorothioate-substituted FRT site would be unable to engage in Flp-mediated strand exchange. Therefore the phosphorothioate-substituted FRT substrate was incubated with an unmodified FRT site and assayed for strand exchange on a denaturing polyacrylamide gel. As expected the phosphorothioate substitution inhibited the formation of the strand exchange products (data not shown). This result is consistent with the interpretation that the phosphorothioate substrate inhibits Flp-mediated ligation.

One molecule of Flp binds to each of the symmetry elements of the FRT forming a cleavage-competent complex (complex II) that can be separated on an acrylamide gel. I sought to isolate the population of covalent Flp:DNA complexes from a reaction in which Flp was bound to a phosphorothioate-containing FRT site. The efficiency of Flp-mediated cleavage of a phosphorothioate linkage was compared to the cleavage of an unmodified site after isolating complexes from preparative reactions. Before heating, 32% of the phosphorothioate-containing strand was cleaved in complex IIs in which Flp was bound to a phosphorothioate-containing FRT site, while in complex IIs in which Flp was bound to an unmodified FRT site, 10% of the analogous strand was cleaved (data not shown).

Friesen and Sadowski (1992) have shown that complex IIs formed with Flp R191K show an increased stability to heat when compared to FRT sites complexed with wild-type Flp. I therefore tested whether heating could be used to isolate covalent Flp:DNA complexes formed with phosphorothioate-containing substrates. A significant proportion of complex IIs formed with a phosphorothioate substrate were stable to temperatures of 46-55°C (Fig. 4-2A, lanes 6, 7, 8). In contrast, the majority of complex IIs formed on unmodified FRT sites were dissociated at 46°C (Fig. 4-2A, lane 14). Analysis of the DNA from phosphorothioate substrates complexed with Flp that had been heated at 49°C for 15 minutes before isolation indicated that 98% of the phosphorothioate-containing strand was cleaved in these complexes (data not shown). Thus increased stability of complex IIs formed with a phosphorothioate substrate is likely due to the accumulation of covalent Flp:DNA complexes with this substrate.

It was important that the cleavage not have arisen during the preparation of the covalent complex IIs, notably during the heating step. Comparison of the extent of cleavage of the phosphorothioate-substituted strand of the DNA used in Fig. 4-2 before and after heating revealed that about 27% of the phosphorothioate-containing strand was cleaved before heating whereas no further cleavage occurred after heating. Furthermore, examination of the cleavage of the unsubstituted strands of phosphorothioate-substituted substrates also revealed that the extent of cleavage was the same before heating at 49°C as after (data not shown).

(ii) Methylation protection in Flp:DNA complexes

DMS methylates double-stranded DNA at the N7 position of guanine in the major groove and the N3 position of adenine in the minor groove. Protection from methylation by DMS can be used to probe the proximity of a protein to DNA (Siebenlist and Gilbert, 1980; Wissmann and Hillen, 1991).

Flp has been shown to confer significant protection of G and A residues in both strands of the symmetry elements (Panigrahi et al., 1992; Bruckner and Cox, 1986; Beatty and Sadowski, 1988). However, in DNA isolated from complex IIs, protection of bases in the core region was not observed (Panigrahi et al., 1992; Beatty and Sadowski, 1988). To determine whether Flp-mediated cleavage activity was associated with a detectable change in the interaction of Flp with the FRT site, the G>A specific cleavage reaction of methylated bases was used.

In order to accumulate covalent Flp:DNA complexes that were cleaved in only one strand, FRT sites containing a 5'-bridging phosphorothioate at one or other of the Flp cleavage sites were used. To enable us to isolate the covalent Flp:DNA complexes heat was used to destroy the noncovalent Flp:DNA complexes. Flp was incubated with DNA substrates, the mixture was treated with DMS and the methylation reaction was terminated by the addition of β-mercaptoethanol. A control reaction in which Flp:DNA complexes were treated with β-mercaptoethanol in the absence of DMS indicated that the Flp:DNA complexes were stable in 200 mM β-mercaptoethanol (data not shown). A reaction in which Flp was bound to a phosphorothioate-containing FRT site was incubated at

49°C for 15 minutes to obtain a homogeneous population of covalent Flp:DNA complexes. Control reactions were also run in which Flp was bound to a phosphorothioate-containing FRT site, a wild-type FRT site or a FRT site with a nick at one cleavage site but were not shifted to 49°C. A FRT site with a nick at one cleavage site was complexed with Flp as a control for the effect of a break in the DNA strand on the pattern of methylation protection. The complex IIs, the substrate that was not bound by Flp and the untreated substrate DNAs were then separated and isolated from a preparative polyacrylamide gel. The DNAs were depurinated at modified G and A residues, cleaved with alkali and analyzed on a denaturing polyacrylamide gel (Figure 4-3A, 4-4A). The results are summarized in Figure 4-3B and 4-4B. DNA obtained from covalent Flp:DNA complexes was compared to substrate DNA, since the unbound fraction from heat-treated reactions consisted of DNA derived from multiple species. In these experiments we have examined the DNA strand opposite the site of Flp-mediated cleavage.

DNA isolated from the covalent Flp:DNA complexes showed several modifications that were not seen in DNA isolated from complex IIs that were not enriched for covalent complexes. First, DNA from complex IIs in which Flp was covalently attached to the bottom strand, showed significant protection of the first adenine residue (+4A) of the core region opposite the cleavage site immediately adjacent to the *a* symmetry element (+4A; Fig. 4-3A, compare lanes 1 and 2). Similarly, DNA from covalent complex IIs that were cleaved in the top strand, showed significant protection of the first adenine residue (-4A) of the core region opposite the cleavage site

immediately adjacent to the *b* symmetry element (-4A, Fig. 4-4A, compare lanes 1 and 2). In agreement with the results of Panigrahi et al. (1992), no protection of residues in the core region of DNA from complex IIs generated by Flp binding predominantly noncovalently to a wild-type FRT site was observed (Fig. 4-3A, compare lanes 5 and 7; Fig. 4-4A, lanes 5 and 7). DNA from complex IIs generated with phosphorothioate-containing sites which were not heated prior to isolation, showed only partial protection of the A residues at +4 and -4 (+4A, Fig. 4-3A, compare lanes 3 and 2; -4A, Fig. 4-4A, compare lanes 3 and 2, 4). This is presumably because the DNA was derived from a mixture of covalently-bound and non-covalently-bound complex IIs. DNA from complex IIs generated by binding of Flp to a DNA substrate containing a nick at the bottom or top strand cleavage site showed no protection of residues in the core region (Fig. 4-3A, compare lanes 8 and 10). Since a substrate with a nick at the cleavage site did not show protection of the first adenine residue of the core region when complexed with Flp, I conclude that this protection is not attributable to a change in the DNA structure caused by a break in the DNA strand at the cleavage site. Therefore, the protection of the first adenine residue of the core region is attributed to covalent attachment of Flp to the 3' terminus at the cleavage site.

The core residues (+4A, +3A, +2A, +1G, -1A) in the unbound fraction of the DNA labeled in the top strand were protected when compared to the same residues present in the substrate not exposed to protein (Fig. 4-3A, compare lanes 4 and 2; 6 and 7). This may have arisen due to association of Flp with these residues in a complex

that was dissociated during electrophoresis. These observations do not affect the interpretations of the data since the DNA isolated from the complex IIs was compared to the substrate DNA which was not exposed to Flp.

I also observed enhanced methylation of the +1G residue in the top strand of the core region in DNA from covalent complex IIs that were cleaved in the bottom strand (+1G; Fig. 4-3A, compare lanes 1 and 2), in the DNA from complex IIs generated by the binding of Flp to a wild-type FRT site (+1G; Fig. 4-3A, compare lanes 5 and 7), and in the DNA from complex IIs generated by the binding of Flp to a FRT site containing a nick in the bottom strand (+1G; Fig. 4-3A, compare lanes 8 and 10). Hypermethylation of the +1G residue in a complex II formed on a wild-type FRT site has been reported previously (Panigrahi et al., 1992; Beatty and Sadowski, 1988). Schwartz and Sadowski (1989) have associated hypermethylation of the G residue in the bottom strand of the core with Flp-induced bending of the FRT site. The observation of the enhanced methylation of the +1G residue in DNA from covalent complex IIs that were cleaved in the bottom strand, suggests that the Flp-induced DNA bend is maintained in the core region of these complexes consistent with previous localization of the centre of the Flp-induced DNA bend in covalent complex IIs to the core region using circular permutation analysis (chapter 2). Thus I conclude that hypermethylation of the +1G residue of the top strand is unaffected by cleavage of the bottom strand.

I also observed enhanced methylation of the -3G residue in the bottom strand of the core in DNA from complex IIs in which Flp was bound to a wild-type FRT site (-3G, Fig. 4-4A, compare lanes 5 and 6,

7), consistent with previous reports (Bruckner and Cox, 1986; Beatty and Sadowski, 1988; Schwartz and Sadowski, 1989). However this hypermethylation of the -3G residue is not observed in DNA from covalent complex IIs that were cleaved in the top strand (-3G; Fig. 4-4A, compare lanes 1 and 2), or in DNA from complex IIs generated by Flp binding to a FRT site containing a nick at the top strand cleavage site (-3G; Fig. 4-4A, compare lanes 8 and 9, 10). This would suggest that within a Flp:DNA complex II, there is a localized change in the DNA structure which is associated with breakage of the phosphodiester bond at the top strand cleavage site. Thus hypermethylation of the -3G residue in the bottom strand is absent when the top strand is cleaved.

Flp caused strong protection of guanine (+5, +10, +11; -5, -10, -11) and adenine (+7, +9; -6, -7, -9) residues in the top and bottom strands of the symmetry elements in the DNAs from the complex IIs of all three substrates (Fig. 4-3A, lanes 1, 5 and 8; Fig. 4-4A, lanes 1, 5 and 8). DNA from complex IIs in which Flp was bound to phosphorothioate-containing FRT sites, which were not heated at 49°C prior to gel isolation, however, showed a comparatively weaker protection of several G (+10, +11; -10, -11) and A (+7, +9; -7, -9) residues in the binding elements (Fig. 4-3A, compare lanes 3 and 1; Fig. 4-4A, lanes 3 and 1). Since DNA from covalent complex IIs (Fig. 4-3A, compare lanes 1 and 2; Fig. 4-4A, lane 1 and 2) showed strong protection of these residues, I deduced that the weaker protection of residues in the symmetry elements was due to the noncovalent interaction of Flp with phosphorothioate-containing FRT sites. The phosphorothioate modification appears to weaken the interaction of

Flp with the binding element, *cis* to the modification, prior to cleavage of the phosphorothioate linkage (see discussion for explanation).

(iii) Methylation interference in Flp-mediated cleavage activity

Methylation protection provides information on the accessibility of DMS to G and A residues in a protein:DNA complex. A methylation interference experiment determines whether methylation of specific bases interferes with protein function. I was interested in determining whether methylation of G and A residues interferes with Flp-mediated cleavage and the formation of covalent Flp:DNA complexes.

The experimental approach was similar to that used for the methylation protection experiments except that DNA substrates were treated with DMS prior to incubation with Flp. A reaction in which Flp was bound to a phosphorothioate-containing FRT site was placed at 49°C for 15 minutes to obtain a homogeneous population of covalent Flp:DNA complexes that were cleaved in one strand. Control reactions were also done in which Flp was bound to a phosphorothioate-containing or a wild-type FRT site but the reactions were not shifted to 49°C. In order to examine the DNA strand opposite the site of Flp-mediated cleavage, DNA from isolated complex IIs was analyzed on a denaturing gel to determine the pattern of methylation in the top (Fig. 4-5A) or bottom strand of the FRT site (Fig. 4-6A).

Methylated residues may interfere with the formation of complex IIs by inhibiting the binding of Flp to the DNA. However, the methylated residues may also block the formation of covalent complex IIs by interfering with cleavage. To identify residues whose methylation interfered with the formation of complex IIs, the interference for each nucleotide was quantitated, as described in the legend to Fig. 4-5B. Where methylation of a residue interfered with the formation of complex IIs, the value for the intensity of a band from the substrate divided by the value for the same band from the complex II was greater than 1. The interference was considered to be significant when this ratio exceeded 1.6. 1.6 was chosen as the cutoff because the ratios for residues (+12A, +13A, -12A, -13A) that are outside the minimal length of the symmetry elements needed for recombination were less than 1.6 (Gronostajski and Sadowski, 1985; Proteau et al., 1986). To identify residues whose methylation interfered with Flp-mediated cleavage, the interference obtained with noncovalent complex IIs was then compared to that obtained with covalent complex IIs and I plotted the results on a histogram (Fig. 4-5B, 4-6B).

Methylation of the core residues -1A, +2A, +3A, +4A (but not +1G), in the top strand, strongly interfered with formation of both a complex II and a covalent complex that was cleaved in the bottom strand (Fig. 4-5A, compare lanes 7 and 8, lanes 1 and 2; Fig 4-5B). However, the methylation of residues +2A, +3A and +4A interfered more with the formation of covalent complex IIs than with the formation of uncleaved complex IIs (+2A, +3A, +4A, hatched *vs* solid bars, Fig. 4-5B). This suggests that these residues are important for

bottom strand cleavage and formation of the associated covalent complex IIs. It was also noted that methylation of the -1A residue interfered particularly strongly with the formation of both complex II and covalent complex II. This suggests that the -1A residue may be critical to the bendability of the core DNA and thus of importance to the formation of complex II (see Discussion). Formation of complex II is accompanied by induction of a DNA bend of greater than 140° (Schwartz and Sadowski, 1990).

Methylation of the -2A, -3G and -4A residues of the bottom strand (adjacent to the *b* symmetry element), interfered with formation of complex II (Fig. 4-6A, compare lanes 7 and 8; Fig. 4-6B). The magnitude of the interference was comparable to the interference by methylation of the +2A, +3A and +4A residues (adjacent to the *a* element in the top strand) with the formation of complex II (-2A, -3G, -4A, Fig. 4-6B, solid bars and +2A, +3A, +4A, Fig. 4-5B, solid bars). However after correction for inequivalency of samples loaded in lanes 1 and 2 (Fig. 4-6A), no detectable interference by methylation of the -2A, -3G and -4A residues in the formation of covalent complex IIs, cleaved in the top strand, was apparent. This was in contrast to the enhanced interference by methylated core residues *cis* to the *a* element (top strand) in the formation of covalent complex IIs (cleaved in the bottom strand). This suggests that the -2A, -3G and -4A residues are not important for Flp-mediated cleavage of and covalent attachment to the top strand of the FRT site. I suggest that the top and bottom strand cleavage sites comprise two sequences which are recognized differently by Flp.

Whereas methylation of the +7A residue in the top strand of the *a* symmetry element significantly interfered with the formation of covalent complex that was cleaved in the bottom strand, no interference by the methylation of this residue in the formation of complex IIs was apparent (+7A, Fig. 4-5A, compare lanes 1 and 2, lanes 7 and 8; +7A, Fig. 4-5B, hatched *vs* solid bars). Thus the +7A residue does not appear to be essential for binding of Flp and formation of complex II, but it is likely important for Flp-mediated cleavage of the bottom strand and formation of the associated covalent complex II.

In the bottom strand, methylation of the -6A and -7A residues of the *b* element interfered with the formation of covalent complex IIs, cleaved in the top strand, but there was negligible interference with the formation of complex IIs (-6A, -7A, Fig. 4-6A, compare lanes 1 and 2, lanes 7 and 8; -6A, -7A, Fig. 4-6B, hatched *vs* solid bars). Although the -6A and -7A contacts do not seem to be essential for binding of Flp, they do appear to be important for Flp-mediated cleavage of the top strand. This result is consistent with the enhanced interference shown by the methylated +7A residue of the top strand in formation of covalent complex IIs that are cleaved in the bottom strand. This data support a change in the interaction of Flp with the symmetry element, *cis* to the cleavage site, associated with cleavage and formation of covalent complex IIs. The remaining methylated G and A residues in the bottom strand of the *b* symmetry element (-5G, -9A, -10G, -11G, -12A, -13A), showed comparable interference in the formation of both covalent complex IIs, cleaved in the top strand, and complex IIs.

In summary, methylation protection experiments indicate that protection of the first adenine residue of the core, opposite each of the cleavage sites, is associated with Flp-mediated cleavage. Secondly, the absence of hypermethylation of the -3G residue of the core, when the top strand contains a nick at the cleavage site, may be indicative of a change in DNA structure associated with cleavage of the top strand. Methylation interference experiments indicate that Flp recognizes the top and bottom strand cleavage sites differently. Formation of covalent Flp:DNA complexes appears to be associated with changes in the interaction of Flp with the symmetry elements.

4. DISCUSSION

(i) Phosphorothioate-substituted FRT sites trap covalent intermediates of Flp

Flp-mediated cleavage of an FRT site containing a 5'-bridging phosphorothioate modification results in an accumulation of cleavage product at the site of modification (Fig. 4-1), consistent with the results reported for calf thymus topoisomerase I and lambda integrase (Burgin et al., 1995). Incorporation of a 5'-bridging phosphorothioate linkage at the Flp cleavage site in the FRT site has enabled the accumulation of covalent Flp:DNA intermediates because the 5'-sulfhydryl which is liberated is inefficient for subsequent ligation reactions (Burgin et al., 1995). Use of phosphorothioate modifications, as opposed to ligation-defective mutants or nicked suicide substrates (Nunes-Düby et al., 1987), more closely mimics a

wild-type reaction since covalent intermediates are accumulated by cleavage of an intact DNA strand using wild-type protein.

Surprisingly, methylation protection studies indicate that the phosphorothioate modification impedes the binding of Flp and formation of a noncovalent complex II. Methylation protection experiments (Fig. 4-3 and 4-4) suggested that the phosphorothioate substitution weakens the affinity of Flp for the symmetry element adjacent to the substitution. Covalent complex IIs generated on a phosphorothioate-containing substrate, however, showed strong protection of the symmetry element. I suggest that the phosphorothioate modification may impose a structural restraint on the flexibility of the DNA and may thereby inhibit Flp-induced DNA bending. This would weaken the interaction of Flp with the binding element *cis* to the modification leading to decreased protection of the FRT site. That this restraint is released by cleavage of the phosphorothioate linkage is demonstrated by the finding that protection of the FRT site is equally efficient in covalent complex IIs as in noncovalent complex IIs formed with an unsubstituted FRT site. Recent evidence shows that increased DNA flexibility due to tandem mismatches or due to replacement of thymine with 5-hydroxymethyluracil gives rise to tighter DNA binding by several DNA-bending proteins (Grove et al., 1996a, 1996b). Other evidence suggests that the core region of the FRT site contains a sequence-directed bend or point of flexure in the DNA (chapter 3). Bailly et al. (1995) suggest that decreased flexibility of DNA due to replacement of adenine with 2,6-diaminopurine weakens the interaction of the DNA bending protein, Fis, with its site. Phosphorothioate-containing

sites are thus likely to be more effective suicide substrates for DNA binding proteins which mediate phosphoryl transfer reactions in the absence of DNA bending.

Cleavage of a phosphorothioate linkage by Flp is comparable in efficiency to the cleavage of an unmodified FRT site by the ligation-defective Flp R191K (Fig. 4-1; Friesen and Sadowski, 1992; Pan et al., 1993). This supports the postulate that the phosphorothioate substitution blocks ligation (Burgin et al., 1995).

(ii) Flp-mediated cleavage causes changes in conformation of the Flp:FRT complex

I found that cleavage was associated with methylation protection of an adenine residue opposite each of the cleavage sites (+4A, -4A; Fig. 4-3 and 4-4). These protections were not observed using unmodified or nicked substrates. They could be due to a conformational change in the Flp protein that occurs upon covalent attachment. This change could result in closer approximation of the protein to the A residues. Alternatively, they could be due to a conformational change in the FRT site itself, for example a Flp-induced compression of the minor groove.

The possibility that protection of the adenine residues opposite the cleavage site is a result of the procedure used to isolate the covalent complexes was considered. However partial protection of these A residues in complex IIs generated by binding Flp to phosphorothioate-containing FRT sites which were not heated prior to electrophoresis was also observed. These complexes are thus a mixture of covalent and noncovalent complexes. In addition, a similar

protection was seen by Bruckner and Cox (1986) using a FRT site with a symmetrical core. Protection of these adenines may be indicative of a protein-controlled mechanism that prevents resealing of the nick and promotes intermolecular ligation required for strand exchange.

(iii) A change of DNA structure associated with cleavage of the top strand

I observed that the -3G residue is not hypermethylated in complex IIs that are nicked on the top strand. Hypermethylation of the -3G residue has previously been associated with a severe Flp-induced DNA bend (>140°) localized in the core (Schwartz and Sadowski, 1989, 1990). The difference in reactivity of the -3G residue may be attributed to a localized change in DNA structure, resulting from breakage of the phosphodiester bond at the top strand cleavage site within the context of a Flp:DNA complex II. It is possible that the localized change in DNA structure extends to the neighboring -4A/T base pair at the site of the nick. It has been demonstrated that formation of the covalent vaccinia topoisomerase I:DNA intermediate results in unpairing of the T/A base pair 5' of the cleavage site (Sekiguchi and Shuman, 1996). Type I DNA topoisomerases employ a similar cleavage mechanism to that of recombinases of the integrase family, involving transesterification by an active site tyrosine to create a covalent 3'-DNA intermediate. In addition, the recent report of the crystal structure of the covalent Cre/*lox*A complex shows that the first three nucleotides of the core region, adjacent to the site of cleavage, are single-stranded (Guo et al., 1997). The structure of the

Cre/*lox*A complex also shows that a mobile alpha-helix of the cleaving Cre monomer is positioned to make three phosphate contacts in the concave surface of the ~100° DNA bend in the core region (Guo et al., 1997). This suggests that the change in the DNA structure which is associated with breakage of the DNA strand at the cleavage site in Flp:DNA complexes may be indicative of unpairing of bases in covalent Flp:DNA complexes.

Although an absence of hypermethylation of the -3G residue was observed, the +1G residue did not show a similar effect upon nicking of the bottom strand. This G remained hypermethylated in complex IIs generated on phosphorothioate-substituted, nicked and unmodified FRT sites. This may mean that the structural change associated with cleavage is localized near the cleavage site and does not extend into the middle of the core. This conclusion is supported by the structure of the covalent Cre/*lox*A complex which shows that unpairing of the core region is limited to the three base-pairs of the six base-pair core region, adjacent to the site of cleavage (Guo et al., 1997).

(iv) Flp contacts the two cleavage sites differently

Interference of methylated residues with Flp-mediated cleavage activity was examined. These studies showed asymmetrical interference of methylated core residues in Flp-mediated cleavage of the top versus bottom strands. Whereas methylation of the +2A, +3A and +4A residues in the top strand interfered with Flp-mediated cleavage of the bottom strand, methylation of the -2A, -3G and -4A residues in the bottom strand did not interfere with Flp-mediated cleavage of the top strand (Fig. 4-5 and 4-6). The adenine contacts

are in the minor groove whereas methylation of the -G3 residue disrupts a potential contact in the major groove. This suggests that asymmetrical recognition of the cleavage sites may occur as a function of the asymmetry in the core sequence and possibly also the single base-pair mismatch between the *a* and *b* elements. This suggestion is supported by the effect of several core mutations on Flp-mediated cleavage (chapter 3). These experiments showed that mutation of the -2 and -3 base pairs had no effect on the cleavage of the top or bottom strands whereas mutation of the -1, +1, +2 and +3 base pairs reduced the cleavage of the bottom strand by 50%.

The asymmetrical recognition of the cleavage sites may represent one regulatory step leading to a bias in cleavage of the top *vs* bottom strands of the FRT site. It should follow that the subsequent sequence of strand exchanges would reflect this bias. However to date there is no data to support a preferred order to the initiation of strand exchange by Flp.

(v) Covalent attachment is associated with changes in contacts of Flp with the symmetry elements

Cleavage by Flp is reported to proceed by a *trans*-horizontal mechanism (Lee et al., 1994). In a dimeric complex, the Flp monomer which contributes the nucleophilic tyrosine and becomes covalently attached to the DNA is bound to the symmetry element on the other side of the core from the cleavage site. Thus the Flp monomer bound to the symmetry element *cis* to the site of cleavage is associated noncovalently with the DNA.

The data show that methylation of the -7A, -6A and +7A bases interfered specifically with the formation of covalent complex IIs. Thus, the -7A, -6A and +7A residues do not appear to be essential for binding, but they do seem to be important for Flp-mediated cleavage. These data support a change in the interaction of the Flp monomer bound noncovalently to the symmetry element, *cis* to the site of cleavage, which is associated with cleavage and covalent attachment. Consistent with my data, Beatty and Sadowski (1988) observed that methylation of -6A and -7A interfered with Flp-mediated recombination. This observation renders unlikely the possibility that the change in the interaction of Flp with the symmetry element *cis* to the site of cleavage in covalent complex IIs arises as an artifact due to the temperature shift used to isolate covalent complex IIs. Changes in the interaction of Flp with the FRT site upon formation of covalent complex IIs may contribute to the enhanced stability of covalent Flp:DNA complexes to temperatures of 46-55°C, as compared to noncovalently bound Flp complex IIs (Fig. 4-2).

The crystal structure of Cre complexed with the *lox*A site reveals a difference in the interaction of the non-cleaving monomer and the cleaving monomer with the DNA of each symmetry element (Guo et al., 1997). Guo et al. (1997) suggest this difference may arise due to the conformational changes associated with cleavage of the substrate by one of the two Cre monomers. These data are consistent with the finding that covalent attachment is accompanied by a change in the interaction of the Flp monomer with the symmetry element *cis* to the site of cleavage.

Figure 4-1. Flp-mediated cleavage of unmodified and phosphorothioate-containing FRT sites. Flp substrates were prepared by 5' end-labeling and annealing the following oligonucleotides: KL-7 and KL-31 (lanes 1-5), KL-7M(S) and KL-31 (lanes 6-10), KL-7 and KL-31M(S) (lanes 11-15). The DNA substrates are indicated at the bottom of the autoradiogram. Horizontal arrows represent the *a* and *b* symmetry elements. The asterisks indicate the 5' radioactively labeled ends. *S* indicates the site of phosphorothioate modification. Substrates were incubated with no protein (-), 1.4 pmol (lanes 2, 7, 12), 2.8 pmol (lanes 3, 8, 13), 14.1 pmol (lanes 4, 9, 14) of Flp or 58.7 pmol Flp R191K (as indicated at the top of each lane) for 90 min at 22°C, reactions were treated with proteinase K and SDS, extracted with phenol/chloroform, precipitated with ethanol and analyzed on a 15% denaturing polyacrylamide gel. Substrate bands (S), top (b) and bottom strand (a) cleavage products, and their sizes in nucleotides (nt) are shown at the left and right.

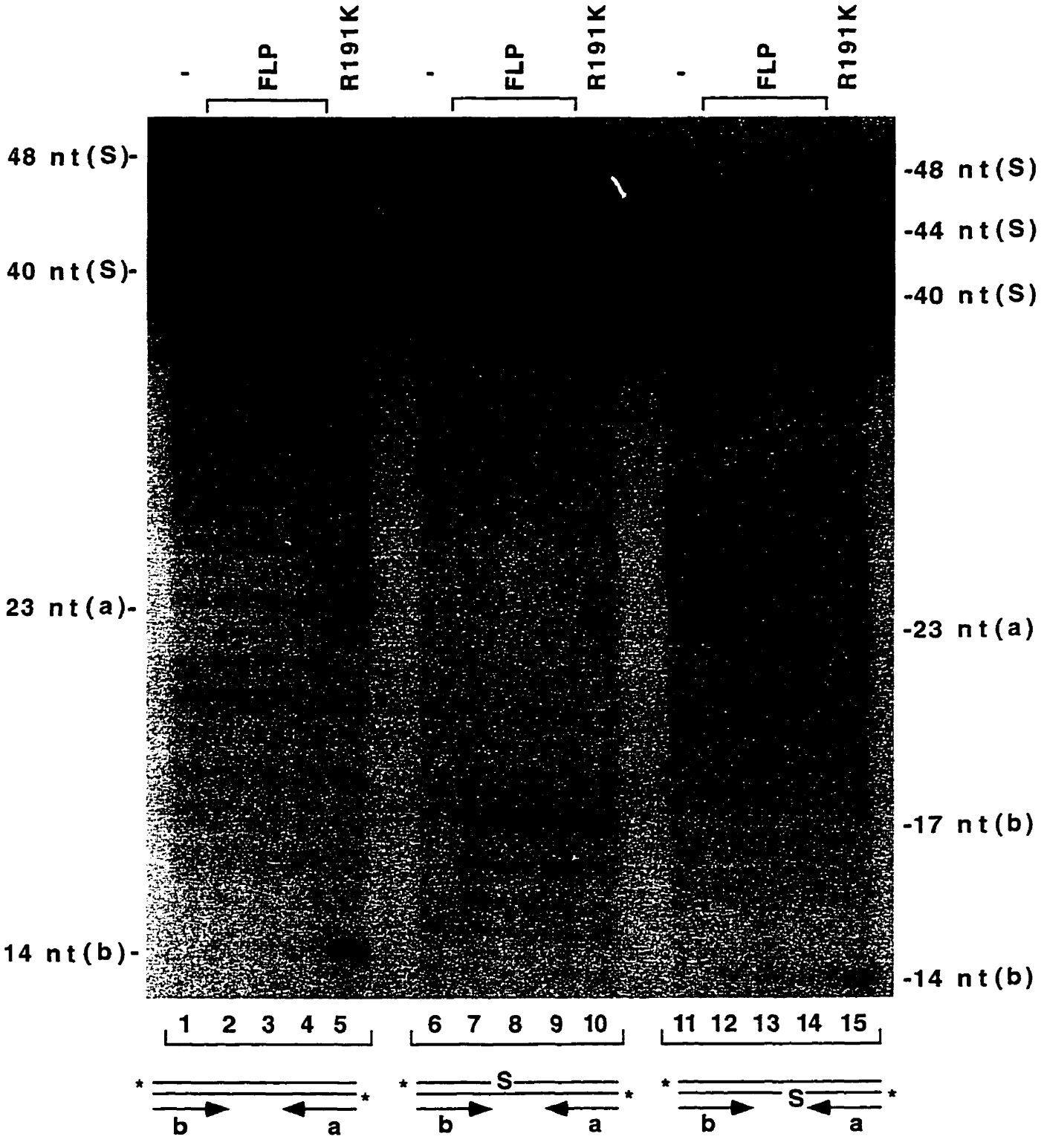
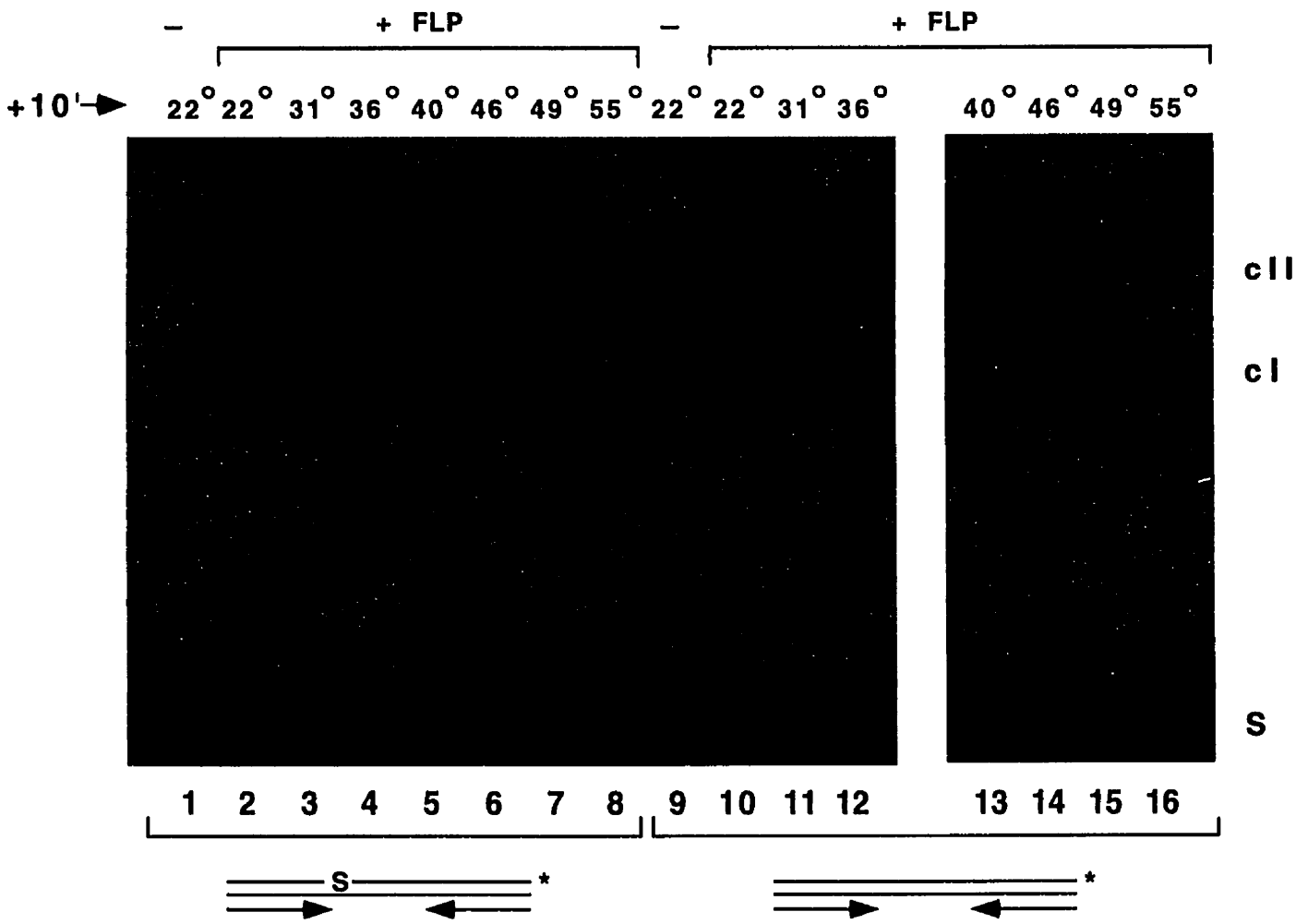

-
FLP
R191K
-
FLP
R191K
-
FLP
R191K
48 nt(S)-
40 nt(S)-
23 nt(a)-
14 nt(b)-
-48 nt(S)
-44 nt(S)
-40 nt(S)
-23 nt(a)
-17 nt(b)
-14 nt(b)
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
S
S
b
a
b
a
b
a

Figure 4-2. Stability of Flp:DNA complexes to heat. **A**. Gel mobility shift assay of Flp:DNA complexes subjected to different temperatures. Flp substrate was prepared by 3' end-labeling DNA generated by annealing oligonucleotides KL-7M(S) and HP-31 (lanes 1-8), KL-7V and HP-31 (lanes 9-16). DNA substrates (indicated at the bottom of the autoradiogram) were incubated with no protein (-) or Flp (as indicated at the top), for 60 min at 22°C and then were retained at 22°C or placed at 31°C, 36°C, 40°C, 46°C, 49°C or 55°C for 10 min (+10', as indicated at the top), before loading on a 5% native polyacrylamide gel. S, unbound substrate; cI, complex I containing a single molecule of Flp bound to the FRT site; cII, complex II containing two molecules of Flp bound to the FRT site. **B**. Quantitation of the % complex II retained at different temperatures. The fraction of species retained as complex II was quantified as described in materials and methods. Solid and hatched bars indicate % complex II retained on wild-type (WT) and phosphorothioate-containing ('S') FRT substrates respectively, following shift of reactions to different temperatures.

A


–
+ FLP
–
+ FLP
+10'
22° 22° 31° 36° 40° 46° 49° 55° 22° 22° 31° 36° 40° 46° 49° 55°
cII
cI
S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
S
*
*

B

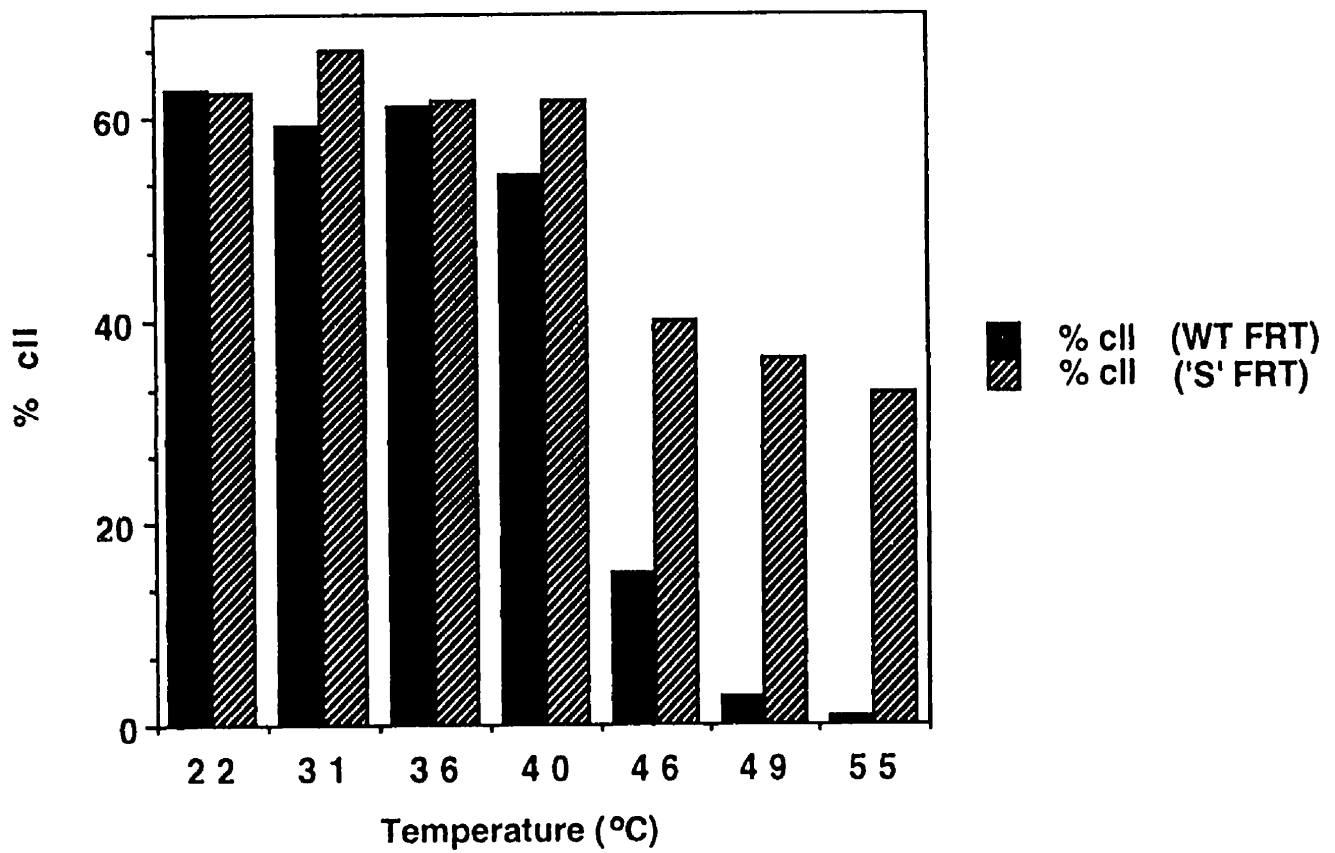
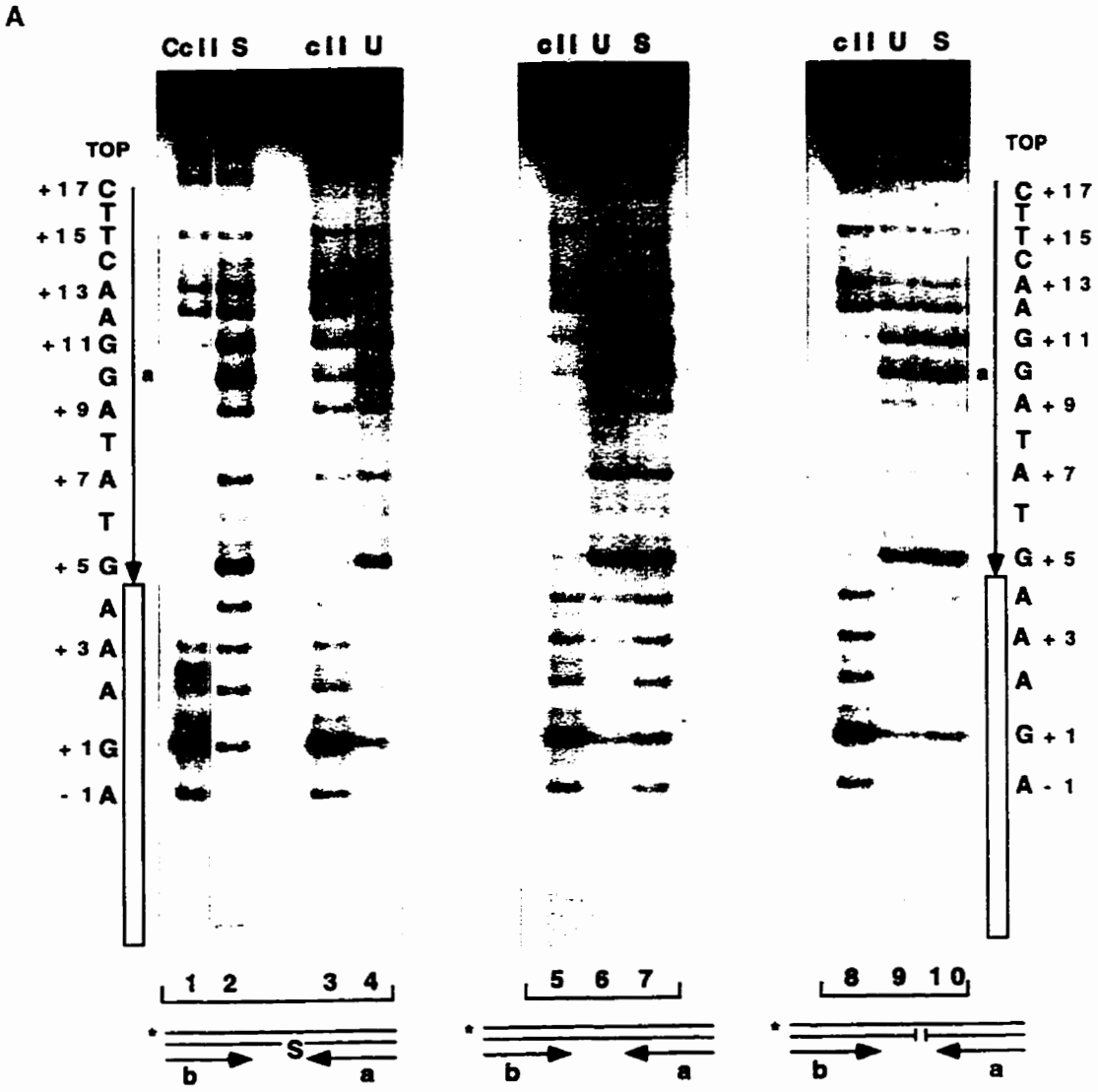

60
40
20
0
% cII
22
31
36
40
46
49
55
Temperature (°C)
% cII (WT FRT)
% cII ('S' FRT)

Figure 4-3. Methylation protection in Flp:DNA complexes. **A.** Flp substrates were prepared by 5' end-labeling (*) the top strand (TOP) and annealing the following oligonucleotides: KL-7 and KL-31M(S) (lanes 1-4), KL-7 and HP-31 (lanes 5-7), KL-7, HP-2 and HP-5 (lanes 8-10). The DNA substrates containing either a 5' bridging phosphorothioate, an unmodified FRT site, or a nick at the Flp cleavage site in the bottom strand (indicated at the bottom of the autoradiogram), were incubated with Flp for 90 min at 22°C, treated with DMS followed by β-mercaptoethanol. Complexes were isolated, depurinated, cleaved with alkali and analyzed on a 15% denaturing polyacrylamide gel. The reaction analyzed in lane 1 was placed at 49°C for 15 min to facilitate isolation of homogeneous covalent Flp:DNA complexes. The FRT sequence and nucleotide numbers are shown to the right and left. Symmetry element and core regions are indicated by arrows and open rectangles respectively. U, isolated unbound substrate; S, substrate in the absence of protein; cII, DNA isolated from complex II; CcII, DNA isolated from covalent complex II. **B.** Summary of methylation protection data in Flp:DNA complexes. The methylation protection data for covalent complex IIs generated on a FRT site containing a phosphorothioate linkage at the cleavage site in the bottom strand is shown at the top. The methylation protection data for complex IIs generated on an unmodified FRT site are shown at the bottom. The sequence of the radioactively labeled top strand of the FRT site is shown. Horizontal lines designate the unlabeled strand with the site of covalent attachment indicated by the solid dot. Protected bases are indicated by a circle. Enhanced methylation is indicated by a triangle.


A
Ccll S
cll U
cll U S
cll U S
TOP
+17 C
T
+15 T
C
+13 A
A
+11 G
G
a
+9 A
T
+7 A
T
+5 G
A
+3 A
A
+1 G
-1 A
TOP
C +17
T
T +15
C
A +13
A
G +11
G
a
A +9
T
A +7
T
G +5
A
A +3
A
G +1
A -1
1 2 3 4
5 6 7
8 9 10
S
b
a
b
a
b
a

B

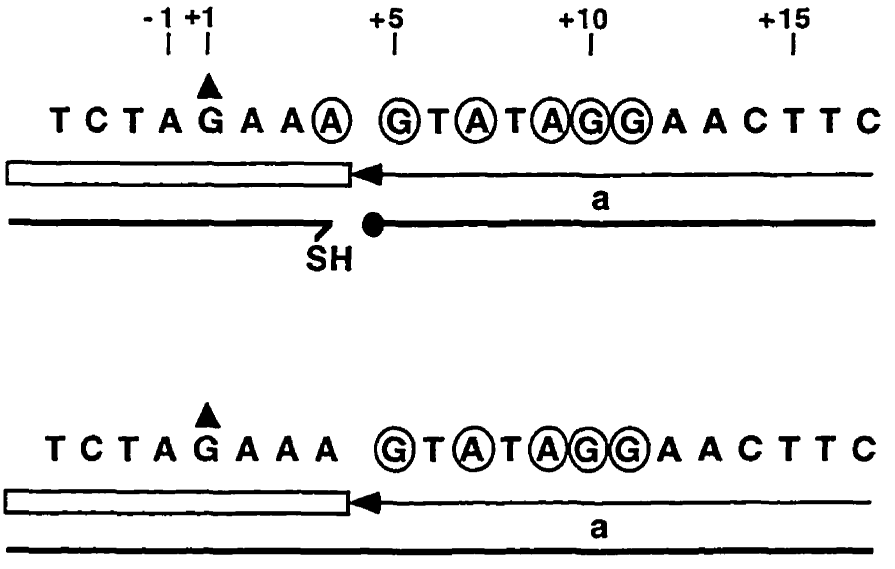
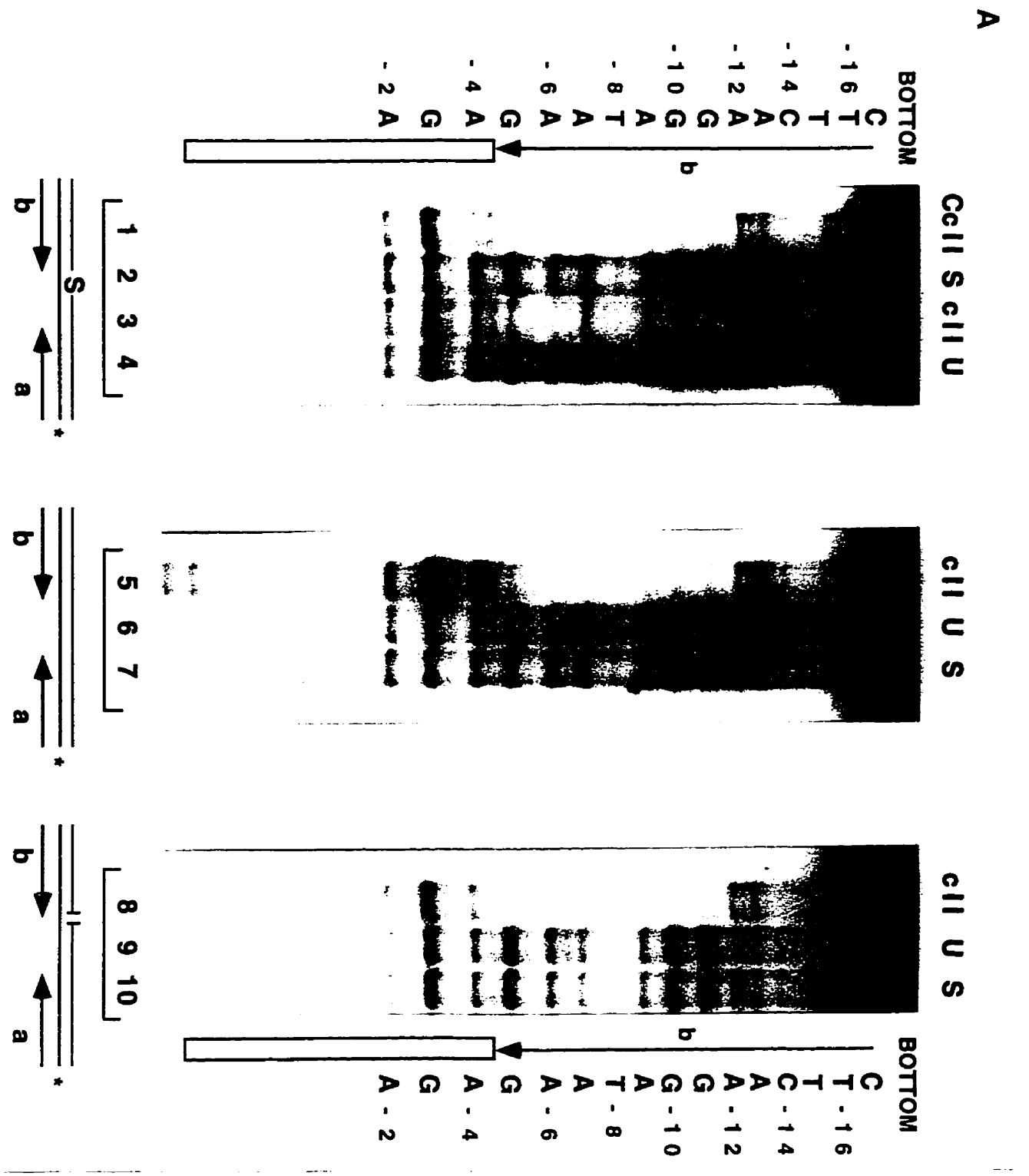

-1 +1
+5
+10
+15
T C T A G A A A G T A T A G G A A C T T C
a
SH
T C T A G A A A G T A T A G G A A C T T C
a

Figure 4-4. Methylation protection in Flp:DNA complexes. **A.** Flp substrates were prepared by 5' end-labeling (*) the bottom strand (BOTTOM) and annealing the following oligonucleotides: KL-7M(S) and HP-31 (lanes 1-4), KL-7 and HP-31 (lanes 5-7), HP-33, HP-14 and HP-31 (lanes 8-10). The DNA substrates contained either a 5' bridging phosphorothioate, an unmodified FRT site, or a nick at the Flp cleavage site in the top strand (indicated at the bottom of the autoradiogram). Experimental conditions were as described in Figure 4A. **B.** Summary of methylation protection data in Flp:DNA complexes. The methylation protection data for covalent complex IIs generated on a FRT site containing a phosphorothioate linkage at the cleavage site in the top strand are shown at the top. The methylation protection data for complex IIs generated on an unmodified FRT site are shown at the bottom. The sequence of the radioactively labeled bottom strand of the FRT site is shown. Horizontal lines designate the unlabeled strand with the site of covalent attachment indicated by the solid dot. Protected and partially protected bases are indicated by solid and stippled circles, respectively. Enhanced methylation is indicated by a triangle.


A
BOTTOM
- 16 T
- 14 C
- 12 A
- 10 G
- 8 T
- 6 A
- 4 A
- 2 A
G
G
A
A
T
A
G
G
C
T
b
CcII S cII U
cII U S
cII U S
1 2 3 4
5 6 7
8 9 10
S
a
b
*

B

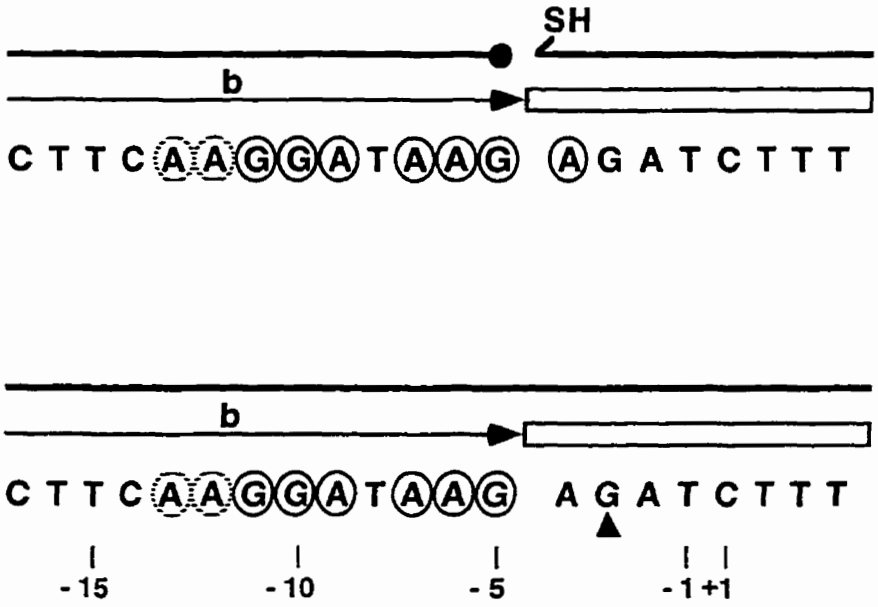
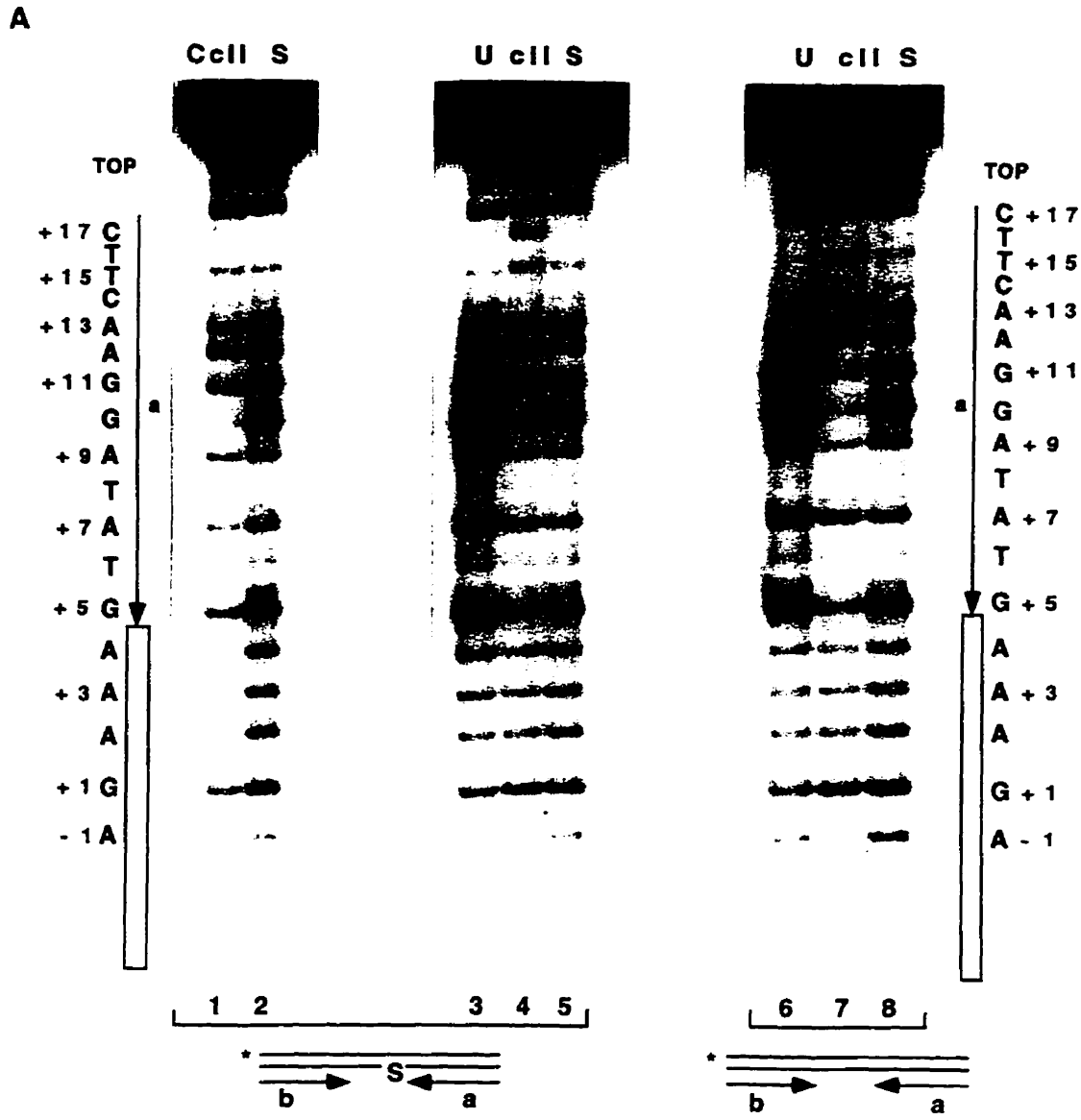

SH
b
C T T C A A G G A T A A G A G A T C T T T
b
C T T C A A G G A T A A G A G A T C T T T
-15
-10
-5
-1 +1

Figure 4-5. Methylation interference in Flp-mediated cleavage of the bottom strand. **A**. Flp substrates (indicated at the bottom of the autoradiogram) were 5' end-labeled (*) in the top strand (TOP) and are as described in Figure 4A. Experimental conditions were as described in Figure 4A except that DNA substrates were methylated with DMS prior to incubation with Flp. The reaction analyzed in lane 1 was placed at 49°C for 15 min to facilitate isolation of homogeneous covalent Flp:DNA complexes. **B**. Quantitation of interference data. Interference with cleavage of the bottom strand was quantified as described in materials and methods. Background values were initially subtracted from each band quantified. Subsequently all bands to be compared in substrate (S) and complex II (cII, CcII) lanes were normalized to correct for inequivalency in the amount of sample loaded in each lane. This was achieved by quantifying the background intensity of an equivalent area, which excluded any bands, in each of the substrate and complex II lanes to be compared (lanes 2 *vs* 1, 8 *vs* 7). A correction factor was obtained by dividing the larger of the two values by the second value. This correction factor was multiplied by the quantitated intensity of each band in the lane in which a lesser amount of sample had been loaded. The -fold interference (x interference) was then calculated for each nucleotide, as the quantitated value for the substrate band divided by the value for the complex II band. Solid bars indicate the -fold interference in a complex II formed on a wild-type (WT) FRT site; hatched bars indicate the -fold interference in a covalent complex II formed on a phosphorothioate-containing ('S') FRT site. The sequence of the radioactively labeled top strand of the FRT site is shown below the

histogram. The site of cleavage is indicated by a vertical arrow.


A
CcII S
U cII S
U cII S
TOP
+17 C
+15 TTC
+13 AA
+11 G
G
+9 A
T
+7 A
T
+5 G
A
+3 A
A
+1 G
-1 A
a
TOP
C +17
TTC +15
AA +13
G +11
G
A +9
T
A +7
T
G +5
A
A +3
A
G +1
A -1
a
1 2
3 4 5
6 7 8
*
S
b
a
*
b
a

B

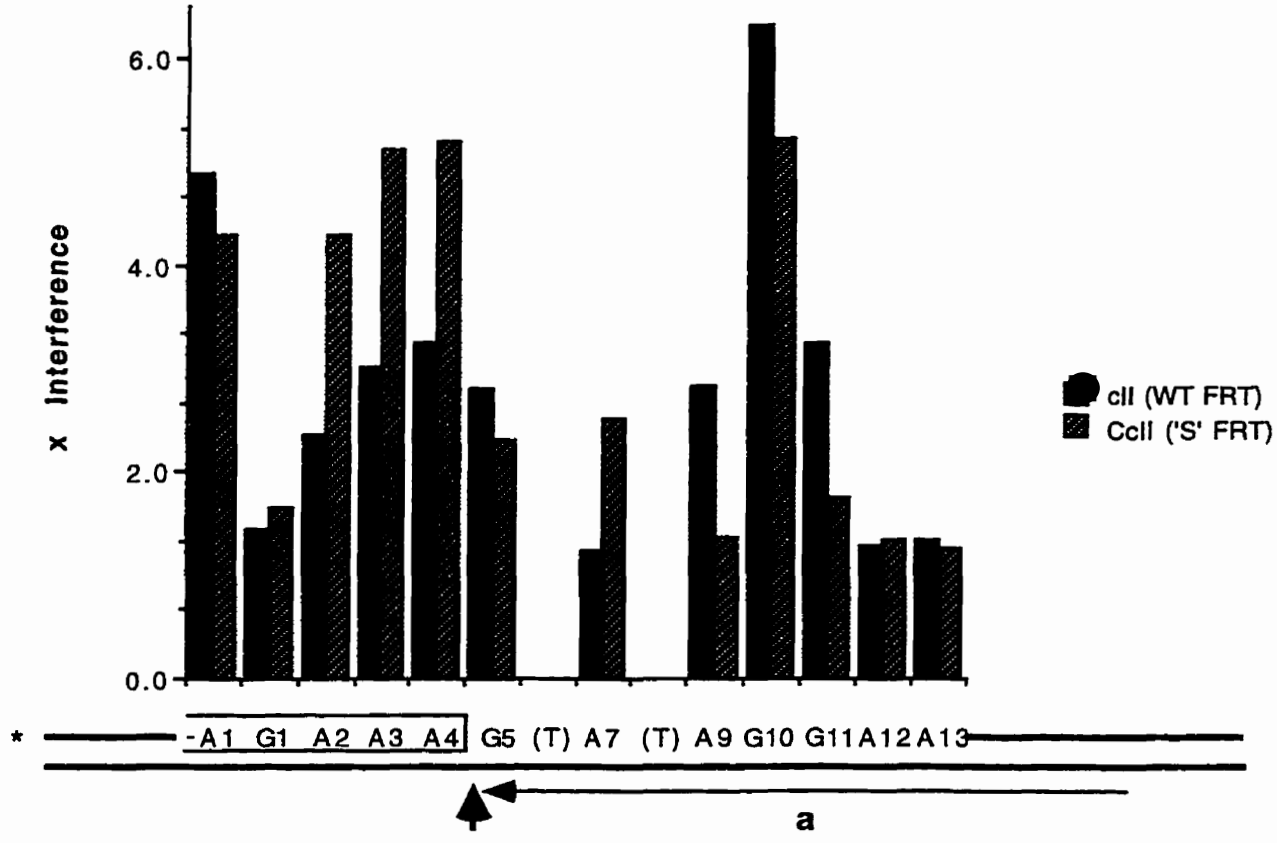
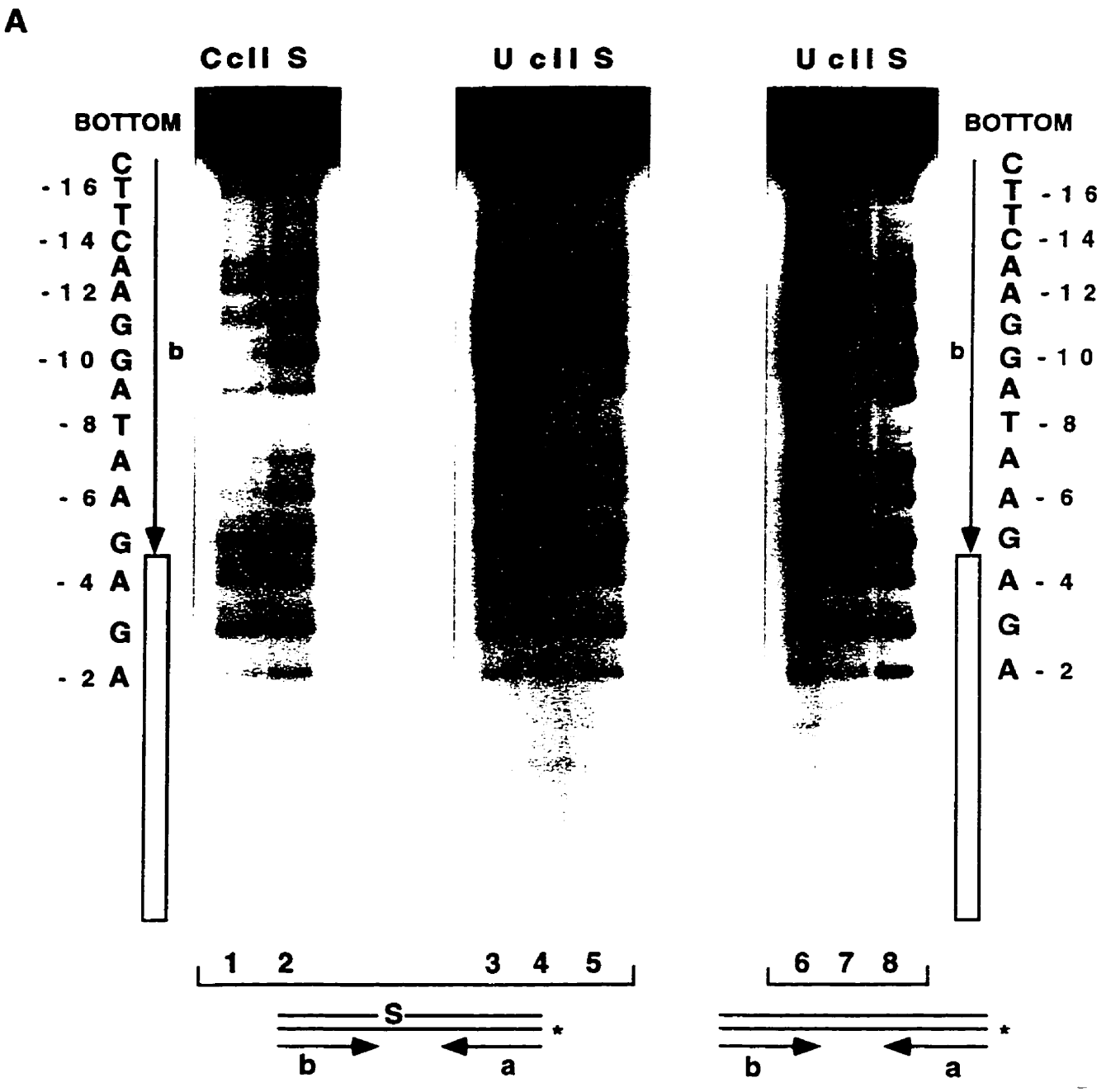
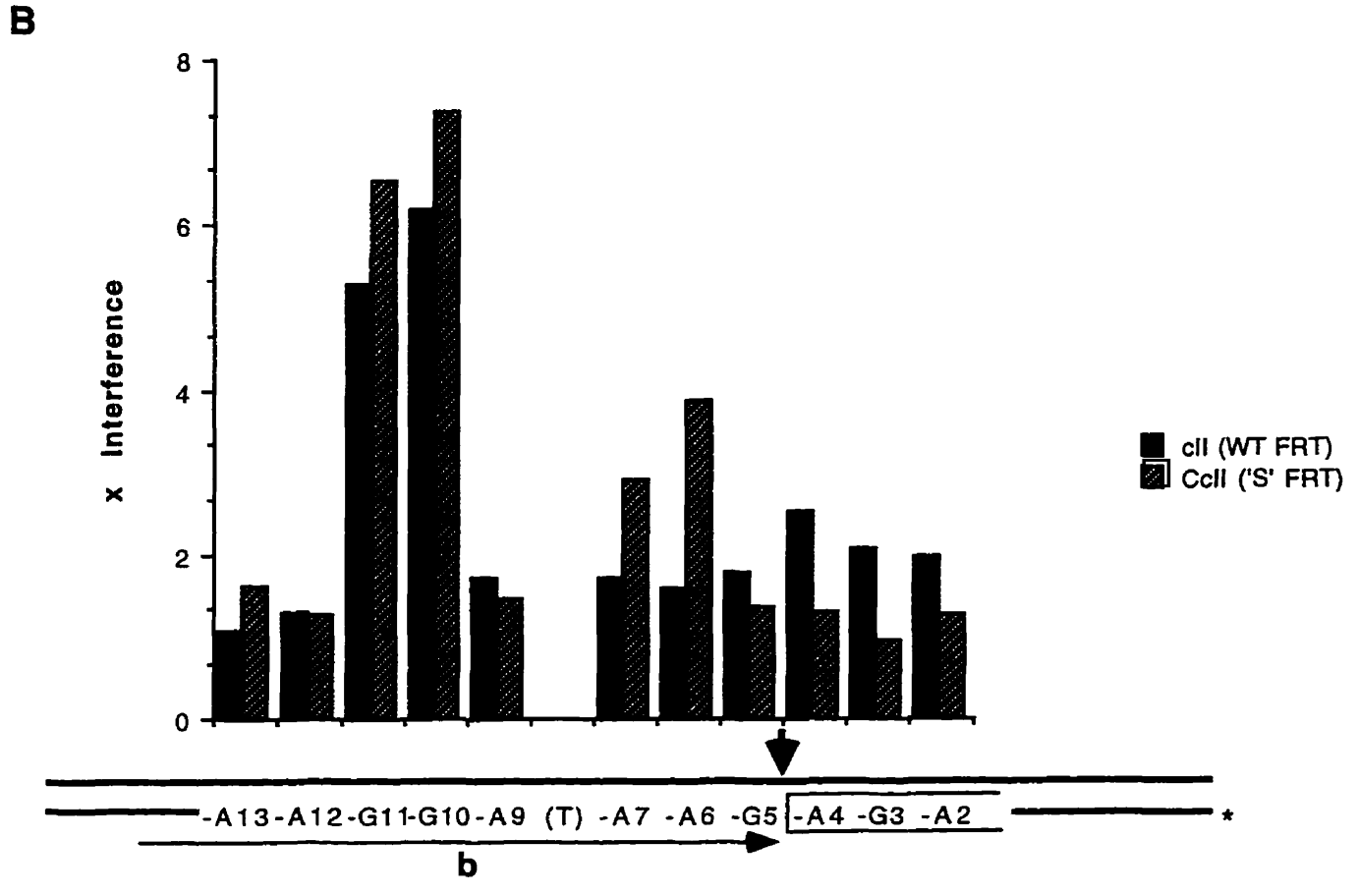

6.0
4.0
2.0
0.0
x Interference
cII (WT FRT)
CcII ('S' FRT)
*
-A1 G1 A2 A3 A4 G5 (T) A7 (T) A9 G10 G11 A12 A13
a

Figure 4-6. Methylation interference in Flp-mediated cleavage of the top strand. **A**. Flp substrates (indicated at the bottom of the autoradiogram) are 5' end-labeled (*) in the bottom strand (BOTTOM) and are as described in Figure 5A. Experimental conditions were as described in Figure 4A except that DNA substrates were methylated with DMS prior to incubation with Flp. The reaction analyzed in lane 1 was placed at 49°C for 15 min to facilitate isolation of homogeneous covalent Flp:DNA complexes. **B**. Quantitation of interference data. Interference with cleavage of the top strand was quantified as described in Figure 5B. The -fold interference (x interference) was calculated for each nucleotide, as the quantitated value for the band in the substrate lane divided by the value for the band in the complex II lane (lanes 2 *vs* 1, 8 *vs* 7). Solid bars indicate the -fold interference in a complex II formed on a wild-type (WT) FRT site; hatched bars indicate the -fold interference in a covalent complex II formed on a phosphorothioate-containing ('S') FRT site. The sequence of the radioactively labeled bottom strand of the FRT site is shown below the histogram. The site of cleavage is indicated by a vertical arrow.


A
CcII S
U cII S
U cII S
BOTTOM
BOTTOM
C
-16 T
T
-14 C
A
-12 A
G
-10 G
b
A
-8 T
A
-6 A
G
-4 A
G
-2 A
C
T -16
T
C -14
A
A -12
G
G -10
b
A
T -8
A
A -6
G
A -4
G
A -2
1 2 3 4 5
6 7 8
S
b
a
b
a


B
8
6
4
2
0
x Interference
cII (WT FRT)
CcII ('S' FRT)
-A13-A12-G11-G10-A9 (T) -A7 -A6 -G5 -A4 -G3 -A2
*
b

CHAPTER 5.

THESIS SUMMARY AND FUTURE EXPERIMENTS

1. THESIS SUMMARY

This thesis has dealt with the structure of DNA bound by Flp and Flp:FRT interactions preceding the initial strand exchange event.

Flp:DNA complexes were examined to determine the position and direction of DNA bends in these complexes. In complexes where two molecules of Flp or Flp Y343F protein were bound to the FRT site, the bend was positioned in the middle of the core region and directed toward the major groove. I found, however, that in complexes where one DNA strand was cleaved and bound covalently by one of two Flp molecules in the complex, the position and direction of bends differed depending on which strand was cleaved. When the bottom strand was cleaved, the DNA bend was also positioned approximately in the middle of the core region and directed toward the major groove. However, when the top strand was cleaved the bend was positioned in the core-proximal end of the *b* symmetry element and directed toward the minor groove.

I have proposed a model which associates the different structure of covalently bound Flp:DNA complexes cleaved in the top or bottom strand with a defined order of the two strand exchange events in the recombination reaction (chapter 2). This model is in part supported by the crystal structure of the synaptic Cre/*lox*A complex (Guo et al., 1997). The structure indicates that a bend-coupled strand exchange mechanism leads to formation of the Holliday intermediate. The Cre/*lox*A structure suggests that the position of the ~100° bend in the core region and its proximity to the nick may

be important for unpairing of the base pairs between the nick and the DNA bend.

Mutation of the central AT base-pair in the core region of the FRT site resulted in positioning of the bend at the margin of the core region adjacent to the *a* symmetry element and affected recombination and cleavage activities of Flp. The positioning of the bend in the middle of the core region is apparently important to the proficiency of Flp-mediated recombination.

λ Int and P1 Cre recombination proceed by an ordered sequence of strand exchanges (Nunes-Düby et al., 1987; Kitts and Nash, 1987, 1988a, 1988b; Hoess et al., 1987). As described above, the differing position and direction of bends in covalently bound Flp:DNA complexes cleaved in the top or bottom strand suggests that Flp recombination may also proceed by an ordered sequence of strand exchanges. It should follow that the preceding cleavage events would reflect this bias. Two ligation-defective Flp mutants are known to cleave the bottom strand twice as efficiently as the top strand of the FRT site (Friesen and Sadowski, 1992; Parsons et al., 1988). I have found that sequence changes close to the cleavage site in the top strand had no effect on the efficiency of cleavage of that strand, whereas sequence changes close to the bottom strand cleavage site reduced the efficiency of cleavage of that strand. In addition, Flp-mediated cleavage of the top and bottom strands were differentially interfered with by methylation of bases opposite each cleavage site. The asymmetrical recognition of the cleavage sites supports the postulate that a bias in cleavage of the top *versus* bottom strands of the FRT site exists.

I have found that cleavage and covalent attachment of Flp to DNA is associated with conformational changes in the structure of the complex providing an indication of the dynamic nature of the recombination reaction. The position and direction (top strand cleavage only) of the DNA bend changed following cleavage of the FRT site. In addition, an adenine residue opposite the site of cleavage was specifically protected from methylation within the covalently bound complexes. Changes in the interaction between the noncovalently-bound Flp monomer in the cleavage complex and the binding element were also associated with cleavage. These conformational changes are likely required for the initial strand exchange event.

These findings are supported by the crystal structure of Cre complexed with the *lox*A site. Conformational changes associated with cleavage are thought to give rise to the difference in the interaction of the non-cleaving monomer and the cleaving monomer with the DNA of each symmetry element and the core region of the *lox*A site (Guo et al., 1997).

The position of the bend induced by binding of a single Flp molecule to DNA varied depending upon whether the substrate contained one or two binding elements. I have disproved a model which postulated that a single Flp monomer interacted with both symmetry elements of a single FRT site. The position of the DNA bend is apparently influenced by the movement of a Flp monomer between the *a* and *b* elements of one FRT site during electrophoresis and by the DNA sequences flanking the site.

2. FUTURE EXPERIMENTS

(i) Investigation of Flp-mediated cleavage of χ structures

Asymmetry in the recognition of the cleavage sites was demonstrated by the differential interference of methylated bases opposite each cleavage site with cleavage of each strand (chapter 4). This finding was further supported by the observation that sequence changes adjacent to each site had different effects on the efficiency of cleavage of each site (chapter 3). Double-stranded DNA substrates were used for these experiments. It would be interesting to identify the Flp:DNA interactions required for Flp-mediated cleavage of a four-armed χ structure using methylation interference as described in chapter 4. Interactions identified by methylation interference could be confirmed by assaying the effect of specific base-pair changes on Flp-mediated cleavage of χ structures. These interactions could be compared with the interactions required for cleavage of a double-stranded substrate. Such experiments would reflect the effect of the structure of χ-form DNA on Flp cleavage activity.

Since a χ structure contains two pairs of cleavage sites, accumulation of cleaved χ structures covalently-bound by Flp requires χ substrates in which the phosphate at each of two identical cleavage sites has been substituted by a 5'-bridging phosphorothioate (chapter 4). A χ structure is reported to be a more sensitive substrate than double-stranded FRT sites for Flp activity which may obviate the need for the separation and isolation of

cleaved χ structures from uncleaved χ structures (Dixon, Ph.D. thesis).

(ii) Further investigation of the dynamic nature of Flp:DNA interactions during the recombination reaction

I described experiments which indicated that Flp-mediated cleavage is associated with conformational changes in the Flp:FRT complex (chapter 4). These changes are likely required for the subsequent strand exchange event. Additional conformational changes in the complex must take place to accommodate formation of the Holliday intermediate and resolution to duplex DNA molecules. The complex manipulation of DNA strands is likely to involve several changes in the interaction of Flp with DNA. The cleavage and ligation functions of Flp are known to be spatially separated in the protein (Pan et al., 1993b). It would thus be interesting to distinguish the interactions of Flp with the FRT site essential for ligation activity and for binding and resolution of χ structures. These interactions could be compared with the interactions known to be essential for binding and cleavage of duplex DNA substrates.

The active site tyrosine residue has been replaced by phenylalanine in the Flp Y343F protein (Evans et al., 1990). This mutant is unable to cleave DNA but otherwise appears to interact normally with the FRT site and is able to ligate activated FRT substrates containing a nick with 5'-hydroxyl and 3'-phosphotyrosyl termini (Pan et al., 1993a). Interactions of Flp with the FRT site required for ligation activity could be determined by examining the interference of methylated G and A residues (chapter 4) in the Flp

Y343F-mediated ligation of activated substrates. Base contacts which are important for ligation of the top and bottom strands of the FRT site could be determined by using activated substrates containing a nick in the top or bottom strands (Pan et al., 1993a).

Resolution is brought about by cleavage of the Holliday intermediate and ligation of exchanged strands to yield two recombinant DNA molecules. The resolution activity of Flp may be examined independent from the recombination reaction by incubation of Flp with a synthetic χ structure assembled from four oligonucleotides (Dixon and Sadowski, 1993). The interference of methylated G and A residues (chapter 4) in the resolution of synthetic χ structures by Flp may be used to identify essential Flp:DNA interactions required for resolution. In such an experiment the products of resolution may be isolated. The methylation interference pattern of resolution may be compared to the interference of methylated G and A residues in binding of the cleavage-defective Flp Y343F protein to synthetic χ structures. Similar experiments could be carried out with the Cre recombinase to enable a comparison between Flp and Cre-mediated binding and resolution of χ structures.

(iii) Investigating the contribution of protein:phosphate contacts to Flp- and Cre-induced DNA bending

It is feasible that the Flp-induced DNA bend enables the initial strand exchange following cleavage in the same way as is indicated by the crystal structure of the synaptic Cre/*lox*A complex. Some similarities between Flp- and Cre-induced DNA bending are evident.

In chapter 3, I showed that the central AT base-pair in the core region of the FRT site was important for both positioning of the Flp-induced bend (>140°) in the middle of the core region and recombination activity. The central AT base-pair in the *loxP* site is similarly essential to Cre-mediated recombination and is positioned at the middle of the ~100° Cre-induced bend (Hoess et al., 1986; Guo et al., 1997).

The Cre/*lox*A structure shows that the DNA bend is mediated in part by the interaction of a mobile alpha-helix of the cleaving Cre monomer with three phosphates in the middle of the core region (Guo et al., 1997). Phosphate charge neutralization thus contributes to Cre-induced DNA bending. An additional 33 contacts are made by two Cre monomers with phosphates at different positions in the *lox*A site (Guo et al., 1997). Flp:phosphate contacts, however, are only clustered opposite the cleavage sites at the margins of the core region as identified by phosphate ethylation interference studies of Flp-mediated binding and recombination (Bruckner and Cox, 1986; Beatty and Sadowski, 1988).

To investigate the contribution of protein:phosphate contacts to Flp- and Cre-induced DNA bending, it would be interesting to make substitutions of several phosphates at different positions in the FRT and *loxP* sites with neutral methylphosphonate analogs. This would disrupt any electrostatic interactions made by the protein with phosphates at these positions. It would be necessary to make substitutions of phosphates with neutral phosphate analogs at equivalent positions on each DNA strand to maintain a symmetrical distribution of charges along the phosphodiester backbone of DNA.

The effects of these substitutions on protein-induced DNA bending could be assayed using the circular permutation assay. The effects on other activities of the recombinase could be assayed as well.

In the case of Cre, I would expect that substitution of six phosphates (three on each strand) in the middle of the core region with neutral phosphate analogs would alter the position of the Cre-induced bend, affect cleavage and inhibit strand exchange. It is possible that phosphate substitutions in the middle of the core region of the FRT site may not affect the Flp-induced bend if the mechanism whereby Flp and Cre induce DNA bending is different.

(iv) Investigating the influence of flanking DNA on Flp-induced DNA bending

In appendix I, I concluded that the position of the Flp-induced type I bend may be influenced by DNA flanking the protein binding site. Since the type I bends are thought to contribute to the magnitude of the type II bend (Schwartz and Sadowski, 1990), it is likely that flanking DNA also influences the type II bend. The influence of sequences outside the symmetry element could be investigated further by generating several sets of circular permutation fragments in which the sequence adjacent to the binding site is varied between the different sets of fragments. It is possible that the influence of flanking DNA on the position of the Flp-induced type I bend is mediated by the phasing of a sequence-directed bend outside the site with the type I bend. It would thus be interesting to insert sequences of different bending propensity adjacent to the binding site as defined by crystallographic studies of oligomers and

DNase I digestion data (El Hassan and Calladine, 1996; Brukner et al., 1995). These FRT sites with modified flanking sequences would also be tested in assays for other activities of Flp.

The influence of flanking DNA on the position of the type I bend may also result from the direct interaction of Flp with DNA outside the binding site. Such interactions may aid in stabilizing the Flp-induced DNA bends. To investigate the possible interaction of Flp with sequences outside the site, Flp could be conjugated with the chemical nuclease 1,10-phenanthroline copper (Pan et al., 1994). The Flp conjugate would act as a site-specific nuclease. The pattern of DNA strand scission revealed following binding of the Flp conjugate to DNA would identify the sequences Flp interacts with at nucleotide resolution. Since it is not known what region of the Flp protein might interact with flanking DNA, several Flp conjugates in which 1,10-phenanthroline copper was coupled to a single cysteine residue at several different amino acid positions in Flp would need to be constructed and tested.

APPENDIX 1.

DETERMINANTS OF THE POSITION OF A FLP-INDUCED DNA BEND

1. INTRODUCTION

Flp interacts with the FRT site through two DNA binding domains that are present in the NH_2-terminal 13 kDa region (P13) and in the COOH-terminal 32 kDa region (P32; Pan et al., 1991; Pan and Sadowski, 1993). The NH_2-terminal P13 domain interacts with the core-proximal 4 bp of the symmetry element, while the COOH-terminal P32 domain interacts with the core-distal 9 bp of the symmetry element (Fig. 6-1B (i); Panigrahi and Sadowski, 1994).

Binding of a single monomer of Flp to one symmetry element results in the formation of a Flp:DNA complex (complex I) and induction of a DNA bend of 60° (type I bend; Schwartz and Sadowski, 1990). Further characterization of the type I bend, described in chapter 2, showed that it was positioned at the core-distal end of the *b* element when the substrate contained a single symmetry element but at the core-proximal end of the *b* element when the substrate contained a FRT site with two symmetry elements (Fig. 2-1).

These results suggested an explanation for the difference in bend centres: that a single Flp monomer associates with the two symmetry elements of a single FRT site simultaneously (Fig. 2-9). Here I considered this model as well as the possibility that a Flp monomer dissociates from a singly occupied FRT site and reassociates with the unbound element of another singly occupied FRT site during electrophoresis. I concluded that the apparent shift of the bend centre likely results from the movement of one Flp monomer between the *a* and *b* elements of one FRT site during

electrophoresis. Furthermore the position of the DNA bend resulting from the association of a Flp monomer with the FRT site is apparently influenced by the DNA flanking the site.

2. MATERIALS AND METHODS

(i) Oligonucleotide substrates

Unmodified oligonucleotides were synthesized at the Hospital for Sick Children/Pharmacia Biotechnology Service Centre, Banting Institute, University of Toronto. Oligonucleotides used in this study (symmetry element and core sequences of the wild-type FRT site are indicated in bold and in italics respectively):

(HP-7) 5'T**GAAGTTCCTATTC***TCTAGAAA***GTATAGGAACTTC**GACCT 3'

(HP-31)5'GGTC**GAAGTTCCTATAC***TTTCTAGA***GAATAGGAACTTC**AC3'

(KL-8) 5' CCCTC**GAAGTTCCTTAAG***TCTAGAAA***GTAT**GAAGGT 3'

(KL-9) 5' ACCTTC**ATAC***TTTCTAGA***CTTAAGGAACTTC**GAGGG 3'

(KL-18) 5' CCC**GAAGTTCCTATTC***TCTAGAAA***GTATAGG**GGA 3'

(KL-19) 5' TCC**CCTATAC***TTTCTAGA***GAATAGGAACTTC**GGG 3'

(KL-22) 5' CCC**GAAGTTCCTGAGG***TCTAGAAA***GTATAGG**GGA 3'

(KL-23) 5' TCC**CCTATAC***TTTCTAGA***CCTCAGGAACTTC**GGG 3'

Oligonucleotides were 5'-end-labeled with [γ-^{32}P]ATP (NEN Dupont) using T4 polynucleotide kinase (New England Biolabs). Following extraction with phenol/chloroform and precipitation with ethanol, the radioactively labeled oligonucleotide was annealed to the appropriate oligonucleotide(s) by heating and slow cooling in 100 mM NaCl and 5 mM $MgCl_2$. Annealed DNA substrates were

purified on 10% polyacrylamide gels (acrylamide:bis-acrylamide (30:1), 1xTBE).

(ii) Plasmids and plasmid-derived substrates

Construction of the pB2Flp(ba) and pB2Flp(b) plasmids is described in chapter 2. The pBsym plasmid was constructed by ligating the annealed, complementary oligonucleotides 5' CTAG**GAAGTTCCTATT C***TTCTAGAA***GAATAGGAACTTC** 3'/3'**CTTCAAGGATAAG***AAGATCTT* **CTTATCCTTGAAG**AGCT 5', containing a FRT site with two *b* symmetry elements (sequences indicated in bold) flanking a symmetrical core region (sequences indicated in italics), into *Xba*I and *Sal*I digested pBEND2 (Zwieb et al., 1989). Five of eight core base pairs were altered to generate the symmetrical core sequence. The pB(b-b) plasmid was constructed by ligating the annealed, complementary oligonucleotides 5' CTAG**GAAGTTCCTATTC***TCT AGAAA***GAATAGGAACTTC** 3'/3' **CTTCAAGGATAAG***AGATCTTT***C TTATCCTTGAAG**AGCT 5', containing a FRT site with two *b* symmetry elements (sequences indicated in bold) flanking a wild-type core region (sequences indicated in italics), into *Xba*I and *Sal*I digested pBEND2 (Zwieb et al., 1989). Circularly permuted DNA substrates were obtained by digestion of pBsym or pB(b-b) as described in chapter 2. Enzymes were obtained from New England Biolabs.

(iii) Electrophoresis time course

Binding reaction mixtures for the electrophoresis time course contained 0.007 pmol of radioactively labeled DNA substrate

incubated with 1.1 pmol Flp protein in a 20 µl volume (50 mM Tris-HCl, pH 7.4, 33 mM NaCl, 1 mM EDTA, 100 µg calf thymus DNA/ml). Each reaction was incubated at 22°C for 30 minutes and 2.7 µl of dye mixture was added. Reactions were subjected to electrophoresis (200 v, 4°C) on a 5% polyacrylamide gel (1xTBE) for different lengths of time (as indicated in the figure legend).

Flp preparations and circular permutation assays are described in chapter 2. DMS methylation interference experiments were carried out as described in chapter 4. Quantitation of data was performed as described in chapter 3.

3. RESULTS

(i) Substrate-dependent position of the DNA bend in Flp:DNA complex Is

In chapter 2, I showed that the position of the DNA bend induced by the binding of a single monomer of Flp to DNA depended upon whether the substrate contained one or two symmetry elements (type I bend). When the substrate contained a single symmetry element the type I bend was positioned at the core-distal end of the *b* element (Fig. 2-1, bp position -17) but was positioned at the core-proximal end of the *b* element (Fig. 2-1, bp position -6) when the substrate contained a two-element FRT site. A model in which the P32 and P13 regions of a single molecule of Flp (Pan et al., 1991; Pan and Sadowski, 1993) bound to different symmetry elements of the FRT site was proposed (Fig. 6-1A (II)). This would result in a shift in the bend

centre observed when a single Flp monomer was bound to a two-element substrate (chapter 2).

To test whether a single Flp monomer may simultaneously interact with two symmetry elements I examined the interference of methylated bases with the formation of Flp:DNA complex Is. I used DMS to methylate bases of DNA substrates prior to incubation with Flp and a G>A specific cleavage of methylated bases to analyze DNA from isolated complex Is.

Footprinting and cross-linking studies have shown that the COOH-terminal P32 domain interacts with the core-distal 9 bp of the symmetry element while the NH_2-terminal P13 domain interacts with the core-proximal 4 bp of the symmetry element (Fig. 6-1B (i), shaded and dotted boxes; Panigrahi and Sadowski, 1994). To prevent formation of a mixed population of complex Is in which Flp monomers were bound to either the *a* or the *b* element, substrates in which the P32-binding region of one symmetry element was absent were used. Thus a DNA substrate contained a partial FRT site comprised of the *b* element, the core and the core-proximal 7 bp (including the P13 binding region) of the *a* element (Fig. 6-1B (ii)). Since a single Flp monomer can bind to a single symmetry element, two other substrates that contained the core-distal 9 bp (P32 binding region) of the *b* element, the core and P13 binding region of the *a* element (Fig. 6-1B (iii), (iv)) but lacked the P13-binding region of the *b* element were also used. A two-element FRT site was used as a control for these experiments (Fig. 6-1B (i)). These substrates would allow me to distinguish between two models of binding (Fig. 6-1A). (i) Model I-Unipartite model. One molecule of Flp contacts a

single symmetry element. (ii) Model II-Bipartite model. One Flp molecule binds to two symmetry elements at once. In model I, the methylated bases (solid squares) that interfere with Flp binding would be confined to one symmetry element (Fig. 6-1C, left) whereas in model II, these methylated bases would be found in parts of two symmetry elements (Fig. 6-1C, right).

The results of methylation interference experiments designed to distinguish betwen the two models are shown in Figure 6-1. I found no interference by methylated residues in the formation of complex Is on a two-element FRT site consistent with the results of Beatty and Sadowski (1988) (Fig. 6-1D (i), lanes 3 *vs* 2 or 1, lanes 6 *vs* 5 or 4). The explanation for this result is the following. When a methylated base of one symmetry element weakens the affinity of Flp for that element, a Flp monomer then preferentially binds to the unmodified element of a two-element FRT site and forms a complex I.

When a substrate lacking part of the *a* symmetry element but containing the entire *b* element, the core and core-proximal 7 bp of the *a* element was used however, methylation of the -12A, -11G, -10G, -9A, -7A, -6A and -5G residues in the bottom strand of the *b* element did interfere with the formation of complex I (Fig. 6-1D (ii), lanes 12 *vs* 11 or 10; Fig. 6-1B (ii), solid dots). As expected, the intensities of the bands were enhanced in the DNA from the unbound fraction as compared to the untreated substrate DNA since Flp does not interact with DNA which is methylated at these positions (Fig. 6-1D, lanes 11 *vs* 10). Also methylation of the +5G and +7A residues in the top strand of the *a* element caused no interference

with the formation of complex I (Fig. 6-1D (ii), lanes 9 *vs* 8 or 7). These results mean that the contacts required by Flp for the formation of complex I on this substrate are all contained within the *b* element. This confirms the predictions of model I and renders model II less likely (Fig. 6-1A).

It was possible that a single molecule of Flp might interact with parts of two symmetry elements only if the P13-binding region of one of the symmetry elements were mutated. To test this idea, the P13-binding region of the *b* element was mutated (from -8A/T, -7T/A, -6T/A, -5C/G to -8T/A, -7A/T, -6A/T, -5G/C); the substrate contained the P32-binding region of the *b* element, the core and the P13-binding region of the *a* element (Fig. 6-1B (iii)). Using this substrate I found that methylation of the -12A, -11G, -10G and -9A residues interfered with the formation of complex I (Fig. 6-1D (iii), lanes 18 *vs* 17 or 16; Fig. 6-1B (iii), solid dots) consistent with the observations obtained when a substrate comprised of the *b* element, the core and the core-proximal 7 bp of the *a* element was used (Fig. 6-1B (ii)). Unexpectedly, methylation of the -8A (bottom strand), -7A, -6A and -5G (top strand) residues of the mutated P13-binding region also interfered with the formation of complex I (Fig. 6-1D (iii), lanes 18 *vs* 17 or 16, lanes 15 *vs* 14 or 13; Fig. 6-1B (iii), solid dots). This result suggests that there is some relaxed sequence specificity in the interaction of Flp with the P13 binding region, consistent with our previous findings that P13 has some nonspecific DNA binding activity (Pan and Sadowski, 1993).

In an attempt to abolish the interaction of P13 with the P13-binding region of the *b* element, the P13-binding region of the *b* element was mutated in a different way (changed -8A/T, -7T/A, -6T/A, -5C/G to -8G/C, -7A/T, -6G/C, -5G/C; Fig. 6-1B (iv)). I again found that methylation of the -12A, -11G, -10G and -9A residues of the P32-binding region in the *b* element interfered with the formation of complex I. However, methylation of residues in the mutated P13-binding region, the core and the core-proximal 7 bp of the *a* element did not interfere with the formation of complex I (Fig. 6-1D (iv), lanes 21 *vs* 20 or 19, lanes 24 *vs* 23 or 22; Fig. 6-1B (iv)). I conclude that these experiments do not support model II but favour model I (Fig. 6-1A and 6-1C).

(ii) The substrate-dependent position of the type I bend centre may result from the association of a single Flp monomer with the *a* and *b* elements of one FRT site

The use of a FRT site with two symmetry elements as a substrate for Flp binding may cause an artifact in which a Flp monomer may dissociate during electrophoresis from a singly occupied FRT site and reassociate with the unoccupied symmetry element of a complex I on another DNA molecule to form a complex II. This reaction pathway was described for Tet repressor-*tet* operator binding and results in the statistical disproportionation of the singly occupied complex in the gel (Kleinschmidt et al., 1991). This gives rise to smearing and loss of the band corresponding to complex I under conditions of relatively low binding affinity or with increased electrophoresis time. Since circular permutation analysis

involves measurement of the mobility of complexes, the smearing of the band corresponding to complex I may have introduced error in the determination of the position of the type I bend on a two-element FRT site. This may have accounted for the apparent shift in the bend centre observed when a single Flp monomer was bound to a two-element substrate.

To test whether disproportionation could be occurring in my experiments, I examined the stability of complex Is formed on a one-element and a two-element site during electrophoresis times of 1.3, 3, 6, 9, 12 and 20 hours (Fig. 6-2A). If disproportionation were occurring during electrophoresis, the band corresponding to complex I would have been expected to smear and then disappear as a function of time. Quantitation of the data shows that complex I formed on a two-element site was as stable as complex I formed on a one-element site during running times up to 20 hours (Fig. 6-2B). Since there was no loss of complex I on a two-element site, the data suggest that it is unlikely that disproportionation of complex I was occurring in the gel.

The use of a two-element FRT site may give rise to another reaction pathway involving the dissociation of a Flp monomer from one symmetry element and its reassociation with the other symmetry element on the *same* DNA fragment ('shuffling'; Kleinschmidt et al., 1991; note that the disproportionation phenomenon described above implies that a Flp molecule dissociates from one DNA molecule and reassociates with another DNA molecule). Shuffling may give rise to the merging of two bands with differing gel mobilities into one discrete band. Since binding of a Flp

monomer to a two-element site gives rise to a single band, shuffling offers a plausible explanation for the apparent shift in the type I bend (chapter 2; Fig. 6-2A).

(iii) Tests of asymmetry in the FRT site as determinants of the asymmetrical position of the type I bend on a two-element FRT site

If shuffling of a Flp monomer between the *a* and *b* elements of one substrate molecule was occurring, the type I bend resulting from the association of a Flp monomer with a two-element FRT site would nevertheless have been expected to be positioned in the middle of the core region. The type I bend was, however, positioned at the core-proximal end of the *b* element (chapter 2). There are two possible sources of asymmetry in the FRT sequence that might have contributed to this asymmetrical position of the type I bend on a two-element site. There is a one base-pair difference in the sequences of the two symmetry elements and the 8 base-pair core region is not symmetrical. This discrepancy in the position of the type I bend might be explained by a lower binding affinity of Flp for the *a* element *versus* the *b* element. When a Flp monomer shuffles between the two elements of one substrate molecule, the asymmetrical position of the type I bend could result from the fact that a single Flp monomer is associated with the *b* element more of the time than it is with the *a* element.

To test the possibility that a difference in the binding affinity of Flp for the *a* and *b* elements might influence the position of the type I bend I determined the location of the type I bend centre using

circular permutation substrates that contained FRT sites comprised of two *b* elements flanking a core region (Fig. 6-3 (i)). I found that the type I bend was positioned at the same location when the substrate contained a FRT site with two *b* elements (Fig. 2-1, bp position -7) as when the substrate contained a wild-type FRT site (Fig. 2-1, bp position -6). Thus the position of the type I bend resulting from the association of a Flp monomer with a two-element FRT site did not arise from sequence differences between the *a* and *b* symmetry elements.

The asymmetrical position of the type I bend on a two-element FRT site may have been due to asymmetry in the core sequence of the FRT site. For example, an asymmetrically-positioned sequence-directed DNA bend in the core region might influence the overall position of the Flp-induced type I bend formed due to the association of a Flp monomer with a two-element FRT site. To address this possibility a completely symmetrical FRT site comprised of two *b* elements flanking a symmetrical core region was used to determine the location of the type I bend (Fig. 6-3 (ii)). If the asymmetrical position of the type I bend on a two-element FRT site arose due to asymmetry in the core sequence, then the type I bend would have been expected to be positioned in the middle of the FRT site when a symmetrical FRT site was used. The position of the type I bend was, however, unaffected by the use of substrates containing a symmetrical FRT site. The type I bend was positioned at the core-proximal end of the *b* element (Fig. 2-1, bp position -5) irrespective of whether the substrate contained a symmetrical FRT site or a wild-type FRT site (Fig. 2-1, bp position -6). Thus the asymmetrical

positioning of the type I bend on a two-element FRT site does not arise due to asymmetries in the site.

4. DISCUSSION

In this study the basis for a substrate-dependent anomaly in the position of the Flp-induced type I bend was investigated. I have used methylation interference studies to test a model whereby a single Flp monomer interacts with both symmetry elements of a single FRT site. These methylation interference studies did not support the interaction of a single Flp monomer with both symmetry elements of one site. Another explanation must account for the substrate-dependent position of the type I bend.

I then examined whether the apparent shift in the position of the type I bend centre arose due to 'disproportionation', a phenomenon in which a Flp monomer dissociates from a singly occupied FRT site and reassociates with the unbound element of another singly occupied FRT site during electrophoresis to form a complex II. No evidence for disproportionation was found. I have also excluded different affinities of Flp for the two symmetry elements and asymmetry in the core sequence as factors which influence the position of the type I bend on a two-element FRT site.

Therefore, I conclude that a Flp monomer may continuously dissociate from one symmetry element and reassociate with the other symmetry element on the same DNA fragment during electrophoresis. This reaction mechanism is supported by the finding that binding of a Flp monomer to a two-element FRT site results in

one band. The observation that exonuclease stop points occur outside both of the *a* and *b* symmetry elements in the exonuclease footprint of a complex I formed on a two-element FRT site also supports this reaction mechanism (Beatty and Sadowski, 1988). Furthermore the position of the type I bend is apparently influenced by the DNA flanking the site.

The circular permutation assay was devised by Wu and Crothers (1984) as a method to determine the position of a bend in the DNA induced by the binding of a protein. The assay is based on the electrophoretic mobility of complexes formed by the binding of protein to a series of DNA fragments that differ from one another by the position of the protein binding site along the length of the fragment. The simplest interpretation of this assay is that the complex with the maximal relative migration contains a bend which is positioned near the end of the fragment while a complex with the minimal relative migration contains a bend which is positioned closest to the middle of the fragment. There are a number of factors other than DNA bending which may affect the mobility of the complexes. These include the aberrant shape of a protein or the extent of binding during the gel run (Gartenberg et al., 1990; Van der Vliet and Verrijzer, 1993; Hagerman, 1996). Thus determination of the position of a protein-induced DNA bend using the circular permutation method may not be straightforward.

Two potential complications of interpreting circular permutation assays were observed. The observation of only one band when a single monomer of Flp associated with a two-element FRT site suggested that a Flp monomer shuffled between the *a* and *b*

symmetry elements of a single FRT site during electrophoresis (chapter 2; Fig. 6-2B). The estimated position of the apparent bend would thus represent a composite of the positions of the DNA bends resulting from the association of Flp with each of the *a* and *b* elements. I conclude that the circular permutation assay cannot be used for the accurate determination of the location of a DNA bend induced by the binding of a single protein monomer to DNA when the DNA molecule contains more than one binding site.

Although the position of the type I bend on a two-element FRT site is apparently influenced by the continuous exchange of a Flp monomer between the *a* and *b* binding elements, this reaction does not account for the asymmetrical position of the type I bend on a two-element FRT site. I would expect the apparent position of a type I bend on a two-element FRT site to be in the middle of the core region rather than at the core-proximal end of the *b* binding element when both binding elements are the same. Since a FRT site with two identical binding elements or a completely symmetrical FRT site did not change the eccentric position of the type I bend, I conclude that the type I bend resulting from the association of a Flp monomer with a two-element FRT site is influenced by the flanking DNA. Flanking DNA may also have contributed to the positioning of the type I bend at the extreme outside end of the *b* element (Fig. 2-1, bp position -17) when the substrate contained a single element (chapter 2). The position of the type I bend on a two-element FRT site is however also likely to be influenced by the continuous exchange of a Flp monomer between the *a* and *b* binding elements as was described.

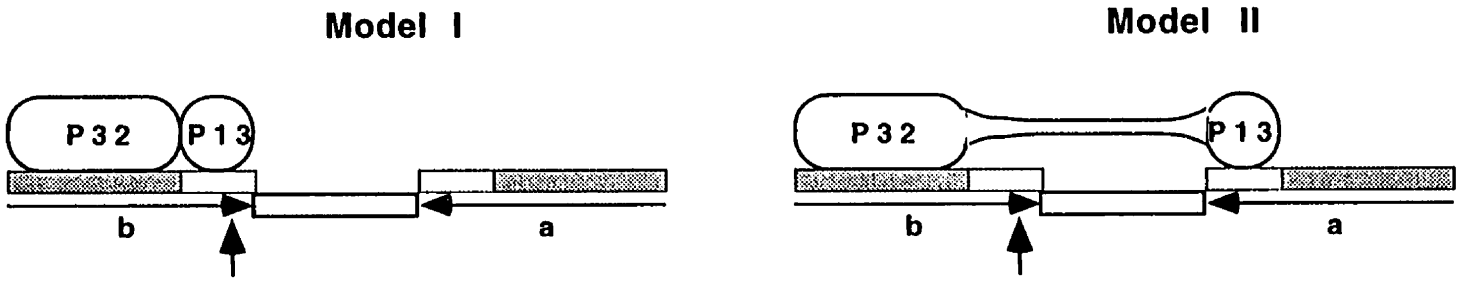
There are several possible ways in which flanking DNA may influence the position of the Flp-induced type I bend. The centre of the bend induced by Tn5 transposase also maps to the extreme outside end of the consensus sequence for Tn5 transposase, a finding which has been attributed to additional upstream nucleotide contacts that are required for optimal binding (York and Reznikoff, 1997). A second possibility is that the DNA flanking the site contains a sequence-directed bend which phases with the Flp-induced type I bend thus affecting the observed macroscopic position of the type I bend. A third possibility is that the DNA flanking the site may wrap around Flp forming non-specific contacts which may affect the position of the type I bend as in the case of the CAP and Fis proteins (Gartenberg and Crothers, 1988; Pan et al., 1996).

A possible way to distinguish among these possibilities would be to invert the Flp binding site in the circularly permuted fragments. If the position of the type I bend is influenced by the interaction of Flp with flanking DNA, then I would expect that inversion of the binding site in the fragments would alter the position of the type I bend with respect to the ends of the fragment. If, however, it is a sequence-directed bend in the flanking DNA which influences the position of the type I bend, then inversion of the binding site in the fragments may not alter the position of the type I bend with respect to the ends of the fragment provided that the spacing and thus phasing between the sequence-directed bend and the Flp-induced type I bend is maintained by inversion of the binding site.

Figure 6-1. Methylation interference in formation of complex I. **A.** Models of the binding of one Flp molecule to the FRT site. The core region of the FRT site is indicated by an open rectangle and the *a* and *b* symmetry elements by horizontal arrows. The binding region of the P32 domain of Flp is indicated by a dark shaded box and the binding region of the P13 domain by a light shaded box. The vertical arrow indicates the location of the type I bend centre. (I) Unipartite model. A single molecule of Flp binds to one symmetry element. (II) Bipartite model. A single molecule of Flp binds to two symmetry elements. **B.** The DNA substrates and summary of the interference data. Unmodified base-pairs of the symmetry elements are indicated by solid lines and solid arrows. Base-pairs of the *b* symmetry element which have been mutated are indicated by broken arrows. Modified guanine and adenine residues which interfered with the formation of complex Is are indicated by solid circles. **C.** Predicted interference data according to models I and II shown in A. The sequences of Flp substrates designated as (i), (ii), (iii) or (iv) are shown in B. Methylated bases (solid squares) that interfere with Flp binding to substrates (ii)-(iv), according to model I, are limited to a single symmetry element as shown to the left. Methylated bases that interfere with Flp binding, according to model II, are present in parts of two symmetry elements as shown to the right. **D.** Flp substrates were prepared by 5' end-labeling the top or bottom strand and annealing the following oligonucleotides: HP-7 and HP-31 (lanes 1-6), KL-18 and KL-19 (lanes 7-12), KL-8 and KL-9 (lanes 13-18), KL-22 and KL-23 (lanes 19-24). The DNA substrates were methylated with DMS and incubated with Flp for 30 minutes at 22°C.

Complex Is were isolated and the DNA was depurinated, cleaved with alkali and analyzed on a 15% denaturing polyacrylamide gel. The DNA sequences and nucleotide numbers are shown beside each panel. S, substrate in the absence of protein; U, isolated unbound substrate; cI, DNA isolated from complex I.

A



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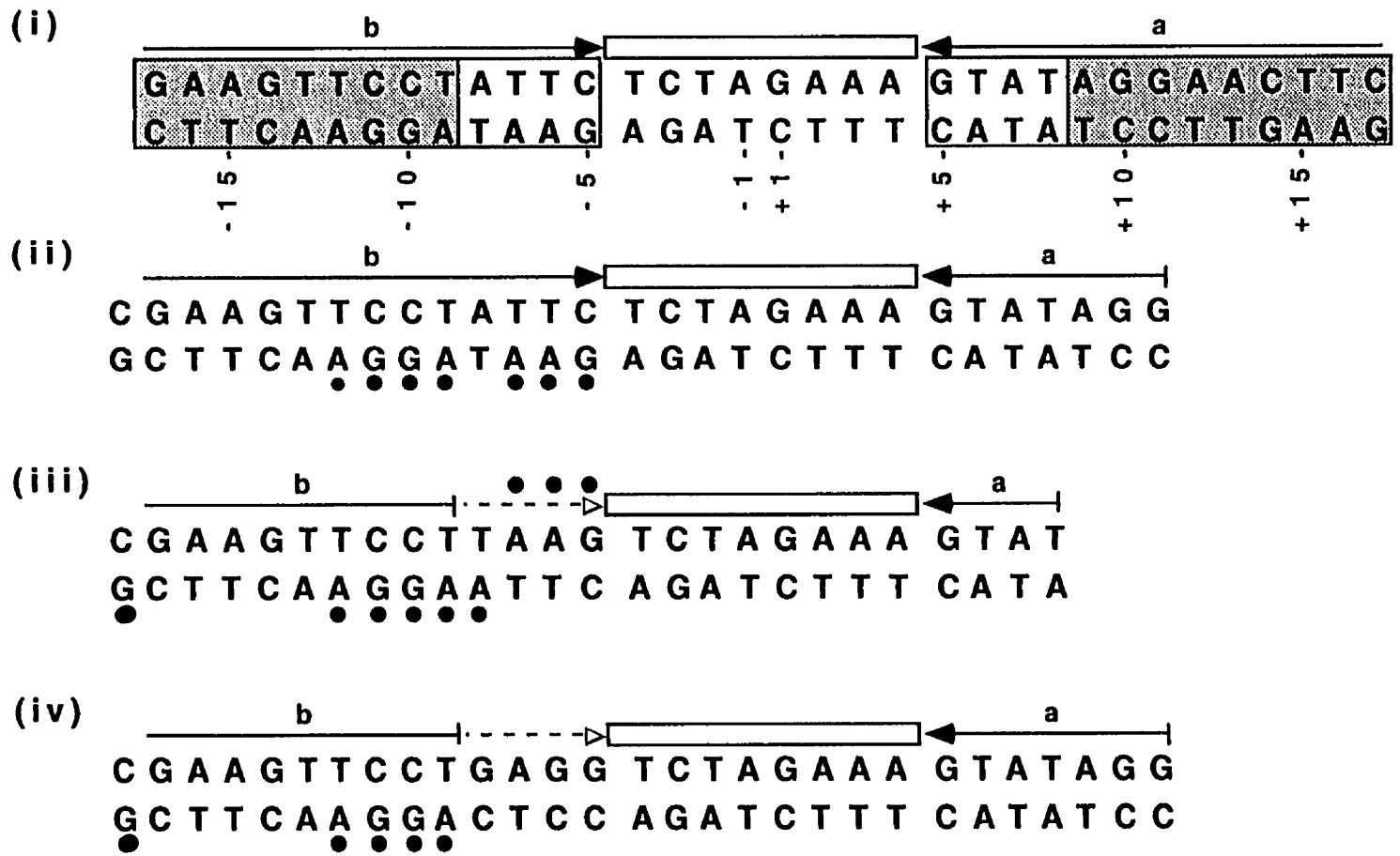
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(ii)
b
a
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GCTTCAAGGATAAG AGATCTTT CATATCC
(iii)
b
a
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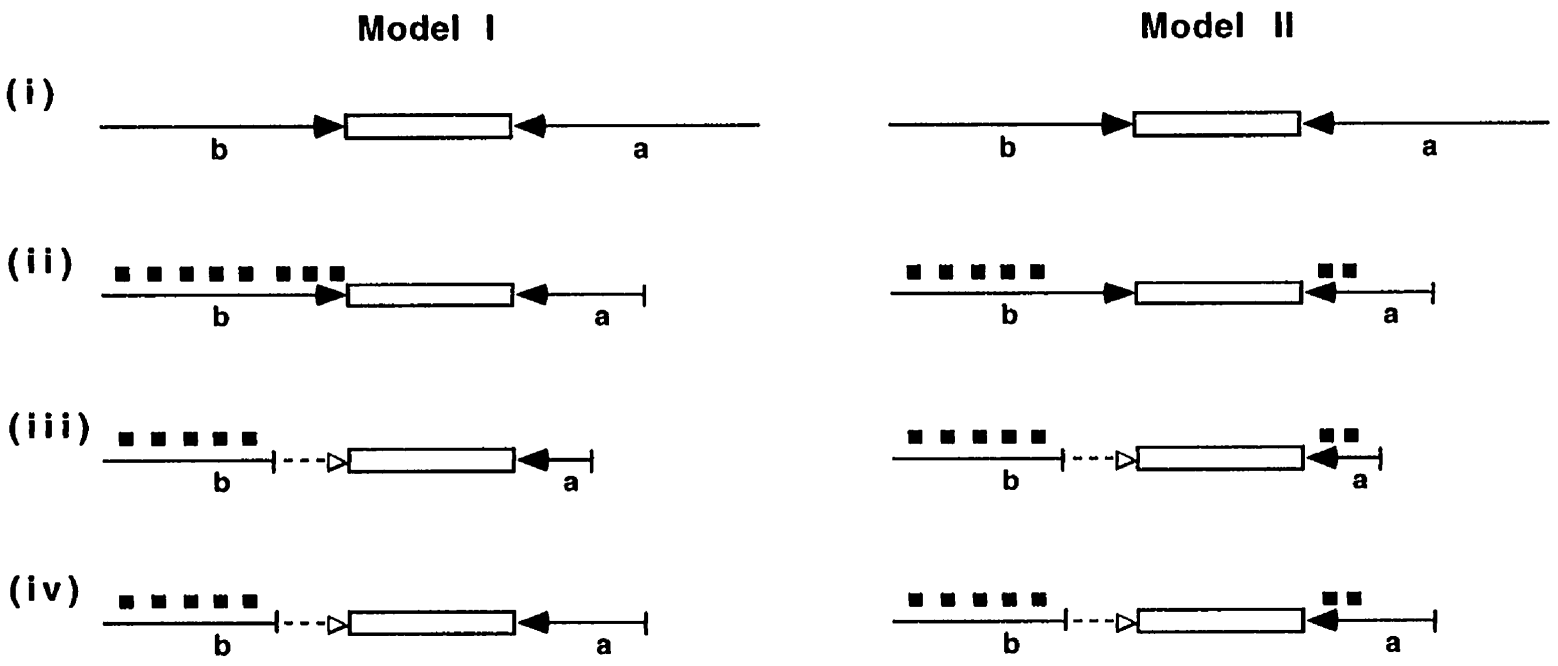
C



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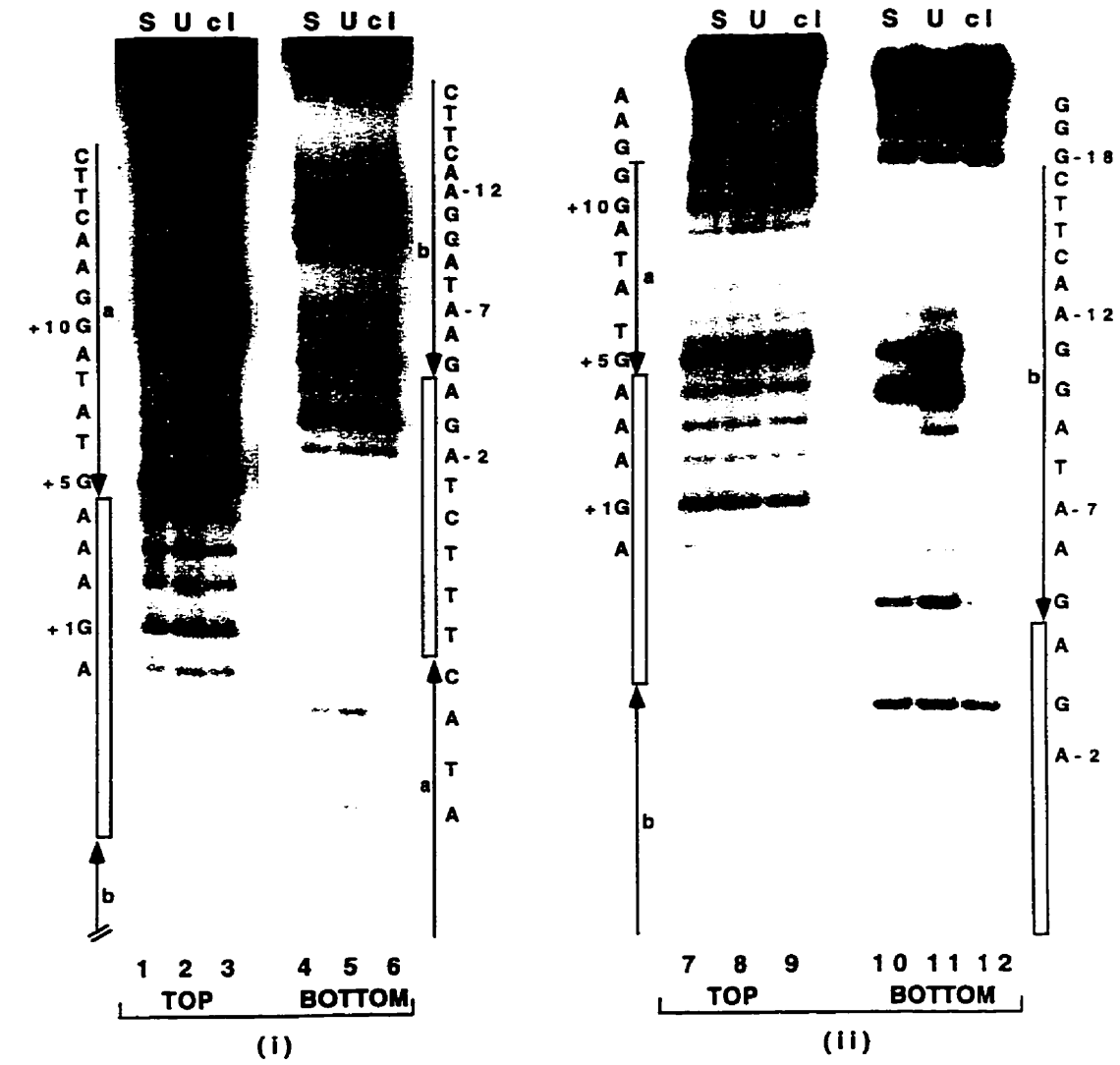
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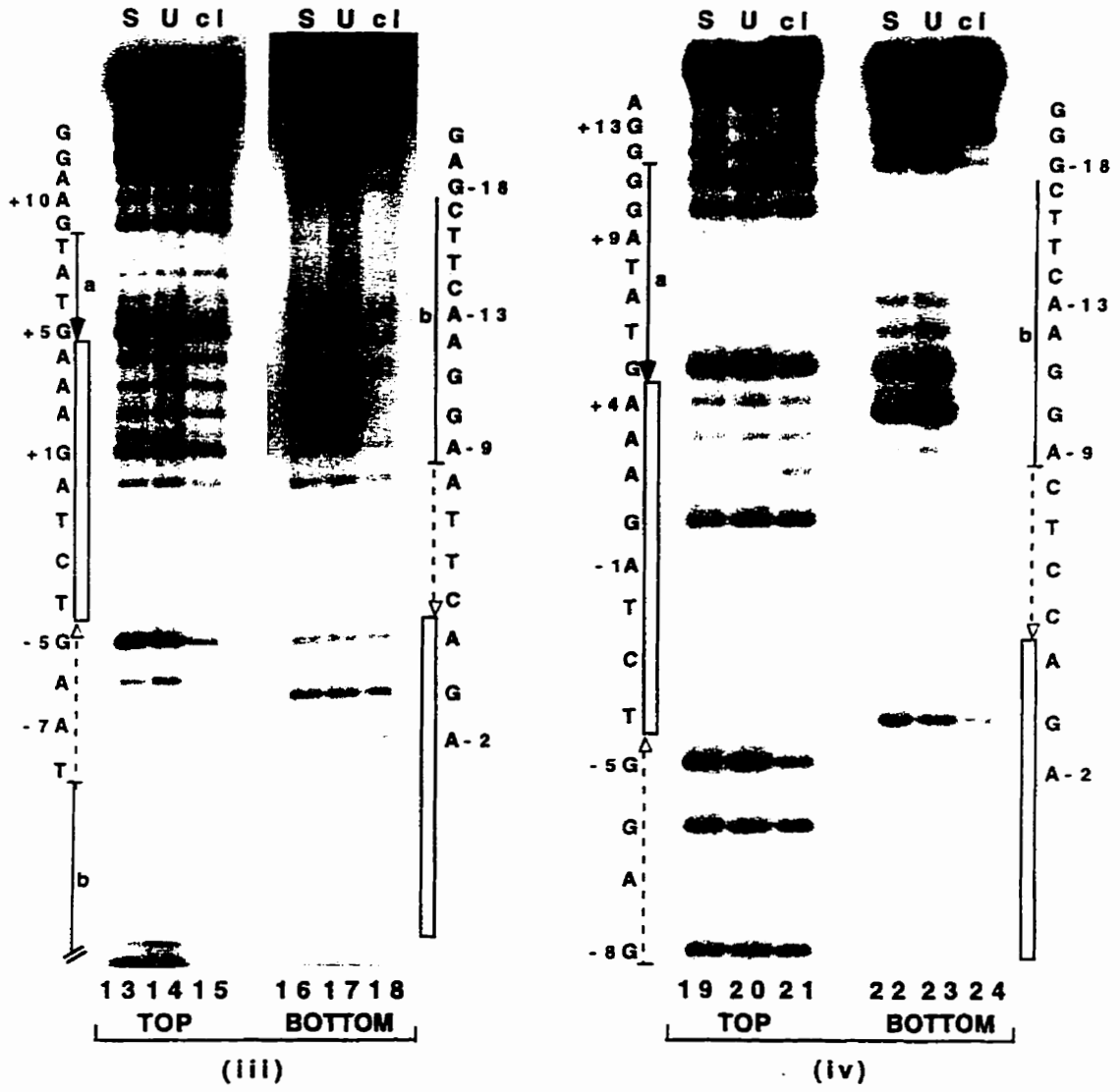
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(iii)
(iv)

Figure 6-2. Time course of electrophoresis of Flp:DNA complexes. **A**. Gel mobility shift assay of Flp:DNA complexes electrophoresed for different lengths of time. Flp substrates containing either a two-element FRT site (152 bp; lanes 1-6) or the *b* symmetry element (131 bp; lanes 7-12) were isolated from the pB2Flp(ba) and pB2Flp(b) plasmids (chapter 2) respectively and 5' end-labeled (*). DNA substrates (indicated at the bottom of the autoradiogram) were incubated with Flp for 30 minutes at 22°C and loaded on a 5% native polyacrylamide gel at different times. Electrophoresis was carried out for 1.3 (lanes 1 and 7), 3 (lanes 2 and 8), 6 (lanes 3 and 9), 9 (lanes 4 and 10), 12 (lanes 5 and 11) or 20 hours (lanes 6 and 12). In each of lanes 1-6, the bands with the slowest, intermediate or fastest mobilities represent complex II (cII), complex I (cI) or unbound substrate (S). In each of lanes 7-12, the bands with the slowest and fastest mobilities represent complex I and unbound substrate respectively. **B**. Quantitation of the stability of Flp:DNA complexes as a function of the time of electrophoresis. The fraction of complex I or complex II was quantitated for reactions in which Flp was incubated with DNA substrates containing a single binding element or a two-element FRT site respectively, as described in the materials and methods. Solid and hatched bars indicate % complex I or % complex II retained on substrates containing a single binding element or a two-element FRT site after electrophoresis for different lengths of time.


A
(hrs) 1.3 3 6 9 12 20 1.3 3 6 9 12 20
(lane 1)
cII
cI
S
- cI
- S
(lane 7)
1 2 3 4 5 6
7 8 9 10 11 12

B

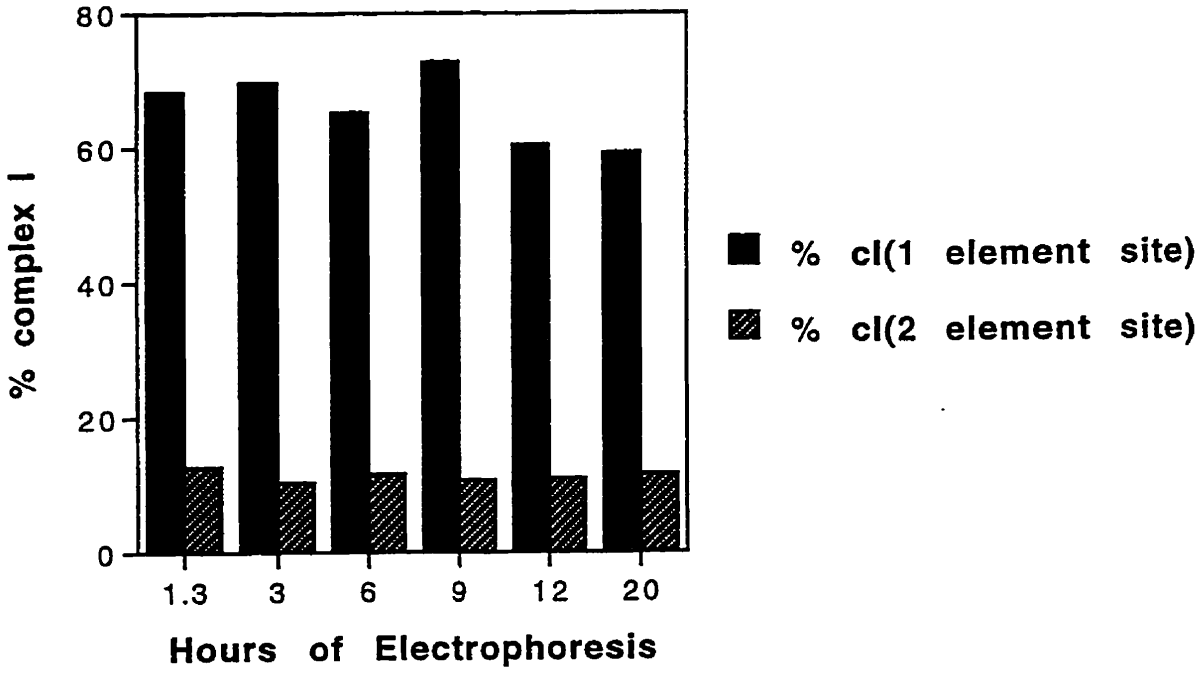
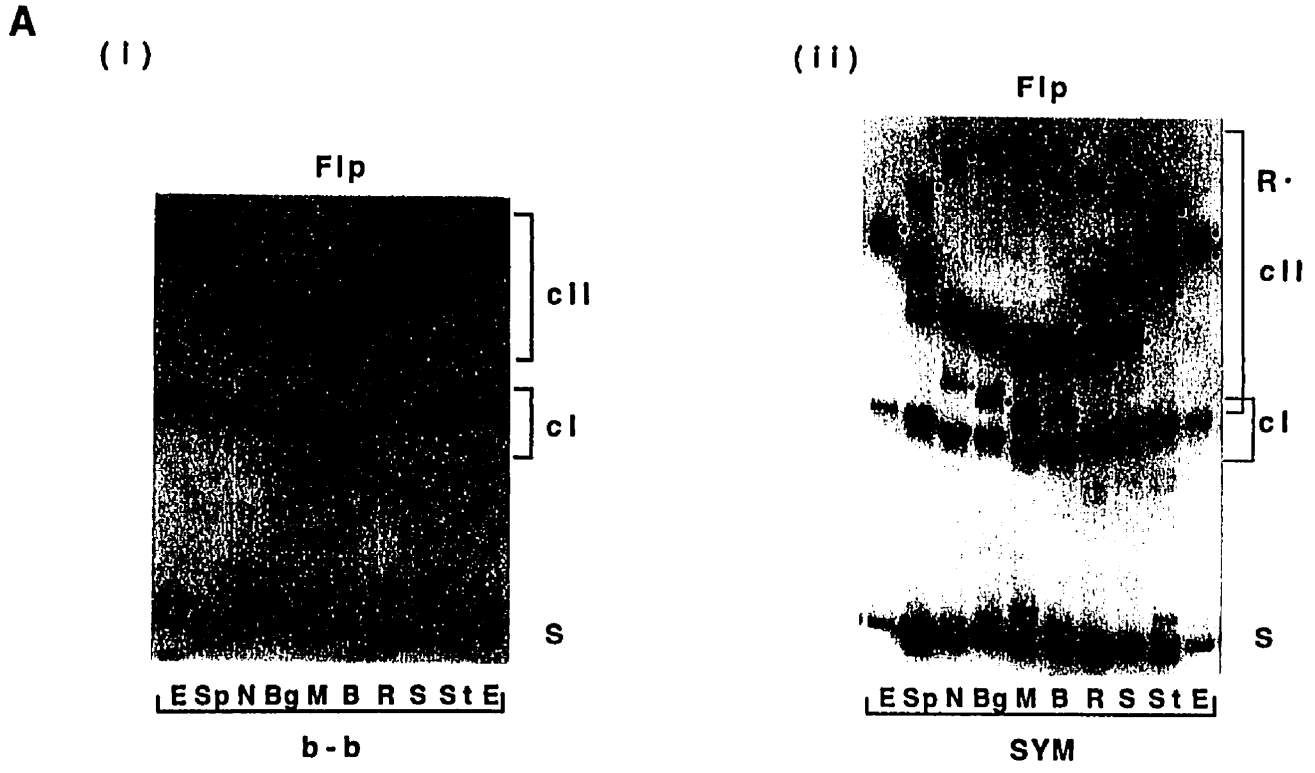

80
60
40
20
0
% complex I
1.3
3
6
9
12
20
Hours of Electrophoresis
% cI(1 element site)
% cI(2 element site)

Figure 6-3. Positions of bend centres induced by Flp bound to modified FRT sites with two *b* symmetry elements or to symmetrical FRT sites. **A**. Gel mobility shift assays of circularly permuted substrates bound by Flp. DNA substrates (152 bp) were obtained by cleavage of the pB(b-b) plasmid, containing a FRT site with two *b* symmetry elements (i), or by cleavage of the pBsym plasmid, containing a symmetrical FRT site (ii), at the different restriction sites flanking each FRT site in tandem. Each circularly permuted substrate is named according to the restriction enzyme used to obtain it and is indicated below the autoradiograms. E, *Eco*RV; Sp, *Spe*I; N, *Nhe*I; Bg, *Bgl*II; M, *Mlu*I; B, *Bam*HI; R, *Rsa*I; S, *Ssp*I; St, *Stu*I. 5' end-labeled DNA substrates were incubated with Flp (indicated at the top) and complexes were separated on 5% native polyacrylamide gels. Flp-mediated recombination between two symmetrical FRT sites which have aligned in an antiparallel manner generates two products which are longer and shorter than the parental DNA molecules. Complexes which arise due to the association of Flp with the products of antiparallel recombination are indicated by solid dots. S, unbound substrate; cI, complex I; cII, complex II; R, recombinant products (solid dot). **B**. Determination of the bend centres in complex I and complex II. The positions of the bend centres were estimated from the curves as described in chapter 2.


A
(i)
Flp
cII
cI
S
E Sp N Bg M B R S St E
b-b
(ii)
Flp
R·
cII
cI
S
E Sp N Bg M B R S St E
SYM
B
Relative Mobility (cI)
Relative Mobility (cII)
0.68
0.7
0.72
0.74
0.45
0.5
0.55
0.6
0.65
cI
cII
0 20 40 60 80 100 120 140 160
Position (bp)

REFERENCES

Abremski, K. and Hoess, R.H. (1992). Evidence for a second conserved arginine residue in the integrase family of recombination proteins. *Protein Eng.* **5**, 87-91.

Abremski, K., Hoess, R. and Sternberg, N. (1983). Studies on the properties of P1 site-specific recombination: evidence of topologically unlinked products following recombination. *Cell* **32**, 1301-1311.

Adams, D.E., Bliska, J.B. and Cozzarelli, N.R. (1992). Cre-*lox* recombination in *Escherichia coli* cells: mechanistic differences from the *in vitro* reaction. *J. Mol. Biol.* **226**, 661-673.

Amin, A.A. and Sadowski, P.D. (1989). Synthesis of an enzymatically active FLP recombinase *in vitro*: search for a DNA-binding domain. *Mol. Cell. Biol.* **9**, 1987-1995.

Amin, A.A., Beatty, L.G. and Sadowski, P.D. (1990). Synaptic intermediates promoted by the FLP recombinase. *J. Mol. Biol.* **214**, 55-72.

Andrews, B.J., Beatty, L.G. and Sadowski, P.D. (1987). Isolation of intermediates in the binding of the Flp recombinase of the yeast plasmid 2-micron circle to its target sequence. *J. Mol. Biol.* **193**, 345-358.

Andrews, B.J., Proteau, G.A., Beatty, L.G. and Sadowski, P.D. (1985). The FLP recombinase of the 2 μ circle DNA of yeast: interaction with its target sequences. *Cell* **40**, 795-803.

Ansari, A.Z., Bradner, J.E. and O'Halloran, T.V. (1995). DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374**, 371-375.

Ansari, A.Z., Chael, M.L. and O'Halloran, T.V. (1992). Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature* **355**, 87-89.

Arciszewska, L.K. and Sherratt, D.J. (1995). Xer site-specific recombination *in vitro*. *EMBO J.* **14**, 2112-2120.

Argos, P., Landy, A., Abremski, K., Egan, J.B., Haggard-Ljundquist, E., Hoess, R.H., Kahn, M.L., Kalionis, B., Narayana, S.V.L., Pierson, L.S., Sternberg, N. and Leong, J.M. (1986). The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO J.* **5**, 433-440.

Austin, S., Ziese, M. and Sternberg, N. (1981). A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell* **25**, 729-736.

Bailly, C., Waring, M.J. and Travers, A.A. (1995). Effects of base substitutions on the binding of a DNA-bending protein. *J. Mol. Biol.* **253**, 1-7.

Beatty, L.G. and Sadowski, P.D. (1988). The mechanism of loading of the FLP recombinase onto its DNA target sequence. *J. Mol. Biol.* **204**, 283-294.

Blakely, G.W. and Sherratt, D.J. (1996a). Determinants of selectivity in Xer site-specific recombination. *Genes Dev.* **10**, 762-773.

Blakely, G.W. and Sherratt, D.J. (1996b). *Cis* and *trans* in site-specific recombination. *Mol. Microbiol.* **20**, 234-237.

Blakely, G.W., Colloms, S.D., May, G. and Sherratt, D.J. (1991). *Escherichia coli* XerC recombinase is required for chromosome segregation at cell division. *New Biol.* **3**, 789-798.

Blakely, G.W., May, G., McCulloch, R., Arciszewska, L.K., Burke, M., Lovett, S.T. and Sherratt, D.J. (1993). Two related recombinases are required for site-specific recombination at *dif* and *cer* in *E. coli* K12. *Cell* **75**, 351-361.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Bracco, L., Kotlartz, P., Kolb, A., Diekmann, S. and Buc, H. (1989). Synthetic curved DNA sequences can act as transcriptional activators in *Escherichia coli*. *EMBO J.* **8**, 4289-4296.

Bruckner, R.C. and Cox, M.M. (1986). Specific contacts between the FLP protein of the yeast 2-micron plasmid and its recombination site. *J. Biol. Chem.* **261**, 11798-11807.

Brukner, I., Sánchez, R., Suck, D. and Pongor, S. (1995). Sequence-dependent bending propensity of DNA as revealed by DNase I: parameters for trinucleotides. *EMBO J.* **14**, 1812-1818.

Burgin, A.B., Huizenga, B.N. and Nash, H.A. (1995). A novel suicide substrate for DNA topoisomerases and site-specific recombinases. *Nucleic Acids Res.* **23**, 2973-2979.

Campbell, A.M. (1962). Episomes. *Adv. Genet.* **11**, 101-145.

Campbell, A. (1981). Some general questions about movable elements and their implications. *Cold Spring Harbor Symp. Quant. Biol.* **45**, 1-9.

Chen, J.-W., Evans, B.R., Zheng, L. and Jayaram, M. (1991). Tyr60 variants of Flp recombinase generate conformationally altered protein-DNA complexes. *J. Mol. Biol.* **218**, 107-118.

Chen, J.-W., Evans, B., Rosenfeldt, H. and Jayaram, M. (1992a). Bending-incompetent variants of Flp recombinase mediate strand transfer in half-site recombinations: role of DNA bending in recombination. *Gene* **119**, 37-48.

Chen, J.-W., Lee, J. and Jayaram, M. (1992b). DNA cleavage in *trans* by the active site tyrosine during Flp recombination: switching protein partners before exchanging strands. *Cell* **69**, 647-658.

Cornet, F., Mortier, I., Patte, J. and Louarn, J.-M. (1994). Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. *J. Bacteriol.* **176**, 3188-3195.

Crothers, D.M., Drak, J., Kahn, J.D. and Levene, S.D. (1992). DNA bending, flexibility, and helical repeat by cyclization kinetics. *Methods Enzymol.* **212**, 3-29.

Craig, N.L. (1988). The mechanisms of conservative site-specific recombination. *Ann. Rev. Genetics.* **22**, 77-105.

Dixon, J.E. (1994). Resolution of synthetic chi structures by the site-specific recombinase, Flp. Ph.D. thesis, University of Toronto, Toronto, ON.

Dixon, J.E. and Sadowski, P.D. (1993). Resolution of synthetic χ structures by the Flp site-specific recombinase. *J. Mol. Biol.* **234**, 522-533.

Dixon, J.E. and Sadowski, P.D. (1994). Resolution of immobile χ structures by the Flp recombinase of 2 μm plasmid. *J. Mol. Biol.* **243**, 199-207.

Dixon, J.E., Shaikh, A.C. and Sadowski, P.D. (1995). The Flp recombinase cleaves Holliday junctions in *trans*. *Mol. Microbiol.* **18**, 449-458.

Dlakic, M. and Harrington, R.E. (1996). The effects of sequence context on DNA curvature. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3847-3852.

El Hassan, M.A. and Calladine, C.R. (1996). Propeller-twisting of base-pairs and the conformational mobility of dinucleotide steps in DNA. *J. Mol. Biol.* **259**, 95-103.

Erie, D.A., Yang, G., Schultz, H.C. and Bustamante, C. (1994). DNA bending by Cro protein in specific and nonspecific complexes: implications for protein site recognition and specificity. *Science* **266**, 1562-1566.

Esposito, D. and Scocca, J.J. (1997). The integrase family of tyrosine recombinases: evolution of a conserved active site domain. *Nucleic Acids Res.* **25**, 3605-3614.

Evans, B.R., Chen, J.-W., Parsons, R.L., Bauer, T.K., Teplow, D.B. and Jayaram, M. (1990). Identification of the active site tyrosine of FLP recombinase. *J. Biol. Chem.* **265**, 18504-18510.

Frantz, B. and O'Halloran, T.V. (1990). DNA distortion accompanies transcriptional activation by the metal-responsive gene-regulatory protein MerR. *Biochemistry* **29**, 4747-4751.

Friesen, H. and Sadowski, P.D. (1992). Mutagenesis of a conserved region of the gene encoding the FLP recombinase of *Saccharomyces cerevisiae.* A role for arginine 191 in binding and ligation. *J. Mol. Biol.* **225**, 313-326.

Futcher, A.B. (1986). Copy number amplification of the 2 μm circle plasmid of *Saccharomyces cerevisiae. J. Theor. Biol.* **119**, 197-204.

Gartenberg, M.R., Ampe, C., Steitz, T.A. and Crothers, D.M. (1990). Molecular characterization of the GCN4-DNA complex. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6034-6038.

Gartenberg, M.R. and Crothers, D.M. (1988). DNA sequence determinants of CAP-induced bending and protein binding affinity. *Nature* **333**, 824-829.

Gartenberg, M.R. and Crothers, D.M. (1991). Synthetic DNA bending sequences increase the rate of *in vitro* transcription initiation at the *Escherichia coli* lac promoter. *J. Mol. Biol.* **219**, 217-230.

Glasgow, A.C., Hughes, K.T. and Simon, M.I. (1989). Bacterial DNA Inversion Systems. In *Mobile DNA*, Berg, D.E. and Howe, M.M. (eds.). American Society for Microbiology, Washington D.C., pp. 637-659.

Goodman, S.D. and Nash, H.A. (1989). Functional replacement of a protein-induced bend in a recombination site. *Nature* **341**, 251-254.

Gronenborn, A.M., Nermut, M.V., Eason, P. and Clore, G.M. (1984). Visualization of cAMP receptor protein-induced DNA kinking by electron microscopy. *J. Mol. Biol.* **179**, 751-757.

Gronostajski, R.M. and Sadowki, P.D. (1985a). Determination of DNA sequences essential for FLP-mediated recombination by a novel method. *J. Biol. Chem.* **260**, 12320-12327.

Gronostajski, R.M. and Sadowski, P.D. (1985b). The FLP recombinase of *Saccharomyces cerevisiae* 2 μm plasmid attaches covalently to DNA *via* a phosphotyrosyl linkage. *Mol. Cell. Biol.* **5**, 3274-3279.

Grove, A., Galeone, A., Mayol, L. and Geiduschek, E.P. (1996a). Localized DNA flexibility contributes to target site selection by DNA-bending proteins. *J. Mol. Biol.* **260**, 120-125.

Grove, A., Galeone, A., Mayol, L. and Geiduschek, E.P. (1996b). On the connection between inherent DNA flexure and preferred binding of hydroxymethyluracil-containing DNA by the DNA-binding protein TF1. *J. Mol. Biol.* **260**, 196-206.

Guo, F., Gopaul, D.N. and Van Duyne, G.D. (1997). Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. *Nature* **389**, 40-46.

Hagemann, A.T. and Craig, N.L. (1993). Tn7 transposition creates a hotspot for homologous recombination at the transposon donor site. *Genetics* **133**, 9-16.

Hagerman, P.J. (1996). Do basic region-leucine zipper proteins bend their DNA targets...does it matter? *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9993-9996.

Heltzel, A., Lee, I.W., Totis, P.A. and Summers, A.O. (1990). Activator-dependent preinduction binding of o-70 RNA polymerase at the metal-regulated *mer* promoter. *Biochemistry* **29**, 9572-9584.

Hickman, A.B., Waninger, S., Scocca, J.J. and Dyda, F. (1997). Molecular organization in site-specific recombination: the catalytic domain of bacteriophage HP1 integrase at 2.7 Å resolution. *Cell* **89**, 227-237.

Hoess, R.H., Wierzbicki, A. and Abremski, K. (1986). The role of the *loxP* spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14**, 2287-2300.

Hoess, R., Wierzbicki, A. and Abremski, K. (1987). Isolation and characterization of intermediates in site-specific recombination. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6840-6844.

Holliday, R. (1964). A mechanism for gene conversion in fungi. *Genet. Res.* **5**, 282-304.

Hunter, C.A. (1993). Sequence-dependent DNA structure: the role of base stacking interactions. *J. Mol. Biol.* **230**, 1025-1054.

Ivanov, V.I., Minchenkova, L.E., Chernov, B.K., McPhie, P., Ryu, S., Garges, S., Barber, A.M., Zhurkin, V.B. and Adhya, S. (1995). CRP-DNA complexes: inducing the A-like form in the binding sites with an extended central spacer. *J. Mol. Biol.* **245**, 228-240.

Jayaram, M. (1985). Two-micrometer circle site-specific recombination: the minimal substrate and the possible role of flanking sequences. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5875-5879.

Jayaram, M. (1994). Phosphoryl transfer in Flp recombination: a template for strand transfer mechanisms. *Trends Biochem. Sci.* **19**, 78-82.

Jayaram, M. (1997). The *cis-trans* paradox of integrase. *Science* **276**, 49-51.

Jayaram, M., Crain, L.L., Parsons, R.L. and Harshey, R.M. (1988). Holliday junctions in Flp recombination: resolution by step-arrest mutants of Flp protein. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7902-7906.

Johnson, R.C. (1991). Mechanism of site-specific DNA inversion in bacteria. *Curr. Opin. Genet. Dev.* **1**, 404-411.

Kahn, J.D., Yun, E. and Crothers, D.M. (1994). Detection of localized DNA flexibility. *Nature* **368**, 163-166.

Kerppola, T.K. and Curran, T. (1991). Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity. *Cell* **66**, 317-326.

Kim, J., Zwieb, C., Wu, C. and Adhya, S. (1989). Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. *Gene* **85**, 15-23.

Kim, J.L., Nikolov, D.B. and Burley, S.K. (1993). Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* **365**, 520-527.

Kim, Y., Geiger, J.H., Hahn, S. and Sigler, P.B. (1993). Crystal structure of a yeast TBP/TATA-box complex. *Nature* **365**, 512-520.

Kimball, A.S. (1990). Conformationally-defined Holliday structures and Flp-mediated recombination. Ph.D. thesis, Johns Hopkins University, Baltimore, MD.

Kitts, P.A. and Nash, H.A. (1987). Homology-dependent interactions in phage λ site-specific recombination. *Nature* **329**, 346-348.

Kitts, P.A. and Nash, H.A. (1988a). Bacteriophage lambda site-specific recombination proceeds with a defined order of strand exchanges. *J. Mol. Biol.* **204**, 95-107.

Kitts, P.A. and Nash, H.A. (1988b). An intermediate in the phage λ site-specific recombination reaction is revealed by phosphorothioate substitution in DNA. *Nucleic Acids Res.* **16**, 6839-6855.

Kleinschmidt, C., Tovar, K. and Hillen, W. (1991). Computer simulations and experimental studies of gel mobility patterns for weak and strong non-cooperative protein binding to two targets on the same DNA: applications to binding of Tet repressor variants to multiple and single *tet* operator sites. *Nucleic Acids Res.* **19**, 1021-1028.

Kotlarz, D., Fritsch, A. and Buc, H. (1986). Variations of intramolecular ligation rates allow the detection of protein-induced bends in DNA. *EMBO J.* **5**, 799-803.

Kulpa, J., Dixon, J.E., Pan, G. and Sadowski, P.D. (1993). Mutations of FLP recombinase gene that cause a deficiency in DNA bending and strand exchange. *J. Biol. Chem.* **268**, 1101-1108.

Kwon, H.J., Tirumalai, R., Landy, A. and Ellenberger, T. (1997). Flexibility in DNA recombination: structure of the lambda integrase catalytic core. *Science* **276**, 126-131.

Landy, A. (1993). Mechanistic and structural complexity in the site-specific recombination pathways of Int and FLP. *Curr. Opin. Gen. Dev.* **3**, 699-707.

Lee, J. and Jayaram, M. (1993). Mechanism of site-specific recombination: logic of assembling recombinase catalytic site from fractional active sites. *J. Biol. Chem.* **268**, 17564-17570.

Lee, J. and Jayaram, M. (1995). Functional roles of individual recombinase monomers in strand breakage and strand union during site-specific DNA recombination. *J. Biol. Chem.* **270**, 23203-23211.

Lee, J., Whang, I., Lee, J. and Jayaram, M. (1994). Directed protein replacement in recombination full sites reveals *trans*-horizontal DNA cleavage by Flp recombinase. *EMBO J.* **13**, 5346-5354.

Lerman, L.S. and Frisch, H.L. (1982). Why does the electrophoretic mobility of DNA in gels vary with the length of the molecule? *Biopolymers* **21**, 995-997.

Lu, F. and Churchward, G. (1994). Conjugative transposition: Tn916 integrase contains two independent DNA binding domains that recognize different DNA sequences. *EMBO J.* **13**, 1541-1548.

Lumpkin, O.J. and Zimm, D.H. (1982). Mobility of DNA in gel electrophoresis. *Biopolymers* **21**, 2315-2316.

Mag, M., Luking, S. and Engels, J.W. (1991). Synthesis and selective cleavage of an oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage. *Nucleic Acids Res.* **19**, 1437-1441.

McClintock, B. (1951). Chromosome organisation and genetic expression. *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13-47.

Meselson, M.S. and Radding, C.M. (1975). A general model for genetic recombination. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 358-361.

Meyer-Leon, L., Huang, L.-C., Umlauf, S.W., Cox, M.M. and Inman, R.B. (1988). Holliday intermediates and reaction by-products in Flp protein-promoted site-specific recombination. *Mol. Cell. Biol.* **8**, 3784-3796.

Meyer-Leon, L., Inman, R.B. and Cox, M.M. (1990). Characterization of Holliday structures in Flp protein-promoted site-specific recombination. *Mol. Cell. Biol.* **10**, 235-242.

Mirzabekov, A.D. and Rich, A. (1979). Asymmetric lateral distribution of unshielded phosphate groups in nucleosomal DNA and its role in DNA bending. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1118-1121.

Moitoso de Vargas, L., Kim, S. and Landy, A. (1989). DNA looping generated by DNA bending protein IHF and the two domains of lambda integrase. *Science* **244**, 1457-1461.

Moitoso de Vargas, L., Pargellis, C.A., Hasan, N.M., Bushman, E.W. and Landy, A. (1988). Autonomous DNA binding domains of lambda integrase recognize two different sequence families. *Cell* **54**, 923-929.

Morgan, T.H. (1911). An attempt to analyse the constitution of the chromosomes on the basis of sex-limited inheritance in *Drosophila*. *J. Exp. Zool.* **11**, 365-414.

Nunes-Düby, S.E., Matsumoto, L. and Landy, A. (1987). Site-specific recombination intermediates trapped with suicide substrates. *Cell* **50**, 779-788.

Nunes-Düby, S.E., Tirumalai, R.S., Dorgai, L., Yagil, E., Weisberg, R.A. and Landy, A. (1994). Lambda integrase cleaves DNA in *cis*. *EMBO J.* **13**, 4421-4430.

O'Halloran, T.V., Frantz, B., Shin, M.K., Ralston, D.M. and Wright, J.G. (1989). The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* **56**, 119-129.

Pan, C.Q., Finkel, S.E., Cramton, S.E., Feng, J., Sigman, D.S. and Johnson, R.C. (1996). Variable structures of Fis-DNA complexes determined by flanking DNA-protein contacts. *J. Mol. Biol.* **264**, 675-695.

Pan, C.Q., Landgraf, R. and Sigman, D.S. (1994). DNA-binding proteins as site-specific nucleases. *Mol. Microbiol.* **12**, 335-342.

Pan, G. and Sadowski, P.D. (1993). Identification of the functional domains of the FLP recombinase. *J. Biol. Chem.* **268**, 22546-22551.

Pan, H., Clary, D. and Sadowski, P.D. (1991). Identification of the DNA-binding domain of the FLP recombinase. *J. Biol. Chem.* **266**, 11347-11354.

Pan, G., Luetke, K., Juby, C.D., Brousseau, R. and Sadowski, P.D. (1993a). Ligation of synthetic activated DNA substrates by site-specific recombinases and topoisomerase I. *J. Biol. Chem.* **268**, 3683-3689.

Pan, G., Luetke, K. and Sadowski, P.D. (1993b). Mechanism of cleavage and ligation by Flp recombinase: classification of mutations in Flp protein by *in vitro* complementation analysis. *Mol. Cell. Biol.* **13**, 3167-3175.

Panigrahi, G.B. and Sadowski, P.D. (1994). Interaction of the NH_2- and COOH-terminal domains of the FLP recombinase with the FLP recognition target sequence.

J. Biol. Chem. **269**, 10940-10945.

Panigrahi, G.B., Beatty, L.G. and Sadowski, P.D. (1992). The Flp protein contacts both major and minor grooves of its recognition target sequence. *Nucleic Acids Res.* **20**, 5927-5935.

Parkinson, G., Wilson, C., Gunasekera, A., Ebright, Y.W., Ebright, R.E. and Berman, H.M. (1996). Structure of the CAP-DNA complex at 2.5 Å resolution: a complete picture of the protein-DNA interface. *J. Mol. Biol.* **260**, 395-408.

Parsons, R.L., Prasad, P.V., Harshey, R.M. and Jayaram, M. (1988). Step-arrest mutants of FLP recombinase: implications for the catalytic mechanism of DNA recombination. *Mol. Cell. Biol.* **8**, 3303-3310.

Parsons, R.L., Evans, B.R., Zheng, L. and Jayaram, M. (1990). Functional analysis of arg-308 mutants of Flp recombinase: possible role of arg-308 in coupling substrate binding to catalysis. *J. Biol. Chem.* **265**, 4527-4533.

Peck, L.J. and Wang, J.C. (1981). Sequence dependence of the helical repeat of DNA in solution. *Nature* **292**, 375-378.

Prasad, P.V., Young, L.-J. and Jayaram, M. (1987). Mutations in the 2-micron circle site-specific recombinase that abolish recombination without affecting substrate recognition. *Biochemistry* **84**, 2189-2193.

Proteau, G., Sidenberg, G. and Sadowski, P.D. (1986). The minimal duplex DNA sequence required for site-specific recombination promoted by the FLP protein in yeast *in vitro*. *Nucleic Acids Res.* **14**, 4787-4802.

Qian, X.-H., Inman, R.B. and Cox, M.M. (1990). Protein-based asymmetry and protein-protein interactions in FLP recombinase-mediated site-specific recombination. *J. Biol. Chem.* **265**, 21779-21788.

Rajagopal, P. and Feigon, J. (1989). Triple-strand formation in the homopurine:homopyrimidine DNA oligonucleotides $d(G\text{-}A)_4$ and $d(T\text{-}C)_4$. *Nature* **339**, 637-640.

Reed, R.R. (1981). Transposon-mediated site-specific recombination: a defined *in vitro* system. *Cell* **25**, 713-719.

Reynolds, A.E., Murray, A.W. and Szostak, J.W. (1987). Roles of the 2 μm gene products in stable maintenance of the 2 μm plasmid of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**, 3566-3573.

Rhodes, D. and Klug, A. (1980). Helical periodicity of DNA determined by enzyme digestion. *Nature* **286**, 573-578.

Rice, P.A., Yang, S.-W., Mizuuchi, K. and Nash, H.A. (1996). Crystal structure of an IHF-DNA complex: a protein-induced U-turn. *Cell* **87**, 1295-1306.

Sadowski, P.D. (1986). Site-specific recombinases: changing partners and doing the twist. *J. Bacteriol.* **165**, 341-347.

Sadowski, P.D. (1993). Site-specific genetic recombination: hops, flips, and flops. *FASEB J.* **7**, 760-767.

Schultz, S.C., Shields, G.C. and Steitz, T.A. (1991). Crystal structure of a CAP-DNA complex: the DNA is bent by 90°. *Science* **253**, 1001-1007.

Schwartz, C.J.E. and Sadowski, P.D. (1989). FLP recombinase of the 2 μm circle plasmid of *Saccharomyces cerevisiae* bends its DNA target. *J. Mol. Biol.* **205**, 647-658.

Schwartz, C.J.E. and Sadowski, P.D. (1990). FLP recombinase of 2 μ circle plasmid of yeast induces multiple bends in the FLP recognition target site. *J. Mol. Biol.* **216**, 289-298.

Sekiguchi, J. and Shuman, S. (1996). Covalent DNA binding by Vaccinia Topoisomerase results in unpairing of the thymine base 5' of the scissile bond. *J. Biol. Chem.* **271**, 19436-19442.

Senecoff, J.F. and Cox, M.M. (1986). Directionality in FLP protein-promoted site-specific recombination is mediated by DNA-DNA pairing. *J. Biol. Chem.* **261**, 7380-7386.

Shaikh, A.C. and Sadowski, P.D. (1997). The Cre recombinase cleaves the *lox* site in *trans*. *J. Biol. Chem.* **272**, 5695-5702.

Shi, Q., Thresher, R., Sancar, A. and Griffith, J. (1992). Electron microscopic study of (A)BC excinuclease: DNA is sharply bent in the UvrB-DNA complex. *J. Mol. Biol.* **226**, 425-432.

Siebenlist, U. and Gilbert, W. (1980). Contacts between *Escherichia coli* RNA polymerase and early promoter of phage T7. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 122-126.

Sobell, H.M. (1974). Concerning the stereochemistry of strand equivalence in genetic recombination. In *Molecular Mechanisms in Genetic Recombination*, Oak Ridge Symposium, Grell, R.F. (ed.). Plenum, New York, pp. 433-438.

Stark, W.M., Boocock, M.R. and Sherratt, D.J. (1992). Catalysis by site-specific recombinases. *Trends Genet.* **8**, 432-439.

Strauss, J.K. and Maher, L.J. (1994). DNA bending by asymmetric phosphate neutralization. *Science* **266**, 1829-1834.

Strauss, J.K., Roberts, C., Nelson, M.G., Switzer, C. and Maher, L.J. (1996). DNA bending by hexamethylene-tethered ammonium ions. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9515-9520.

Subramanya, H.S., Arciszewska, L.K., Baker, R.A., Bird, L.E., Sherratt, D.J. and Wigley, D.B. (1997). Crystal structure of the site-specific recombinase, XerD. *EMBO J.* **16**, 5178-5187.

Summers, D.K. and Sherratt, D.J. (1984). Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability. *Cell* **36**, 1097-1103.

Thompson, J.F. and Landy, A. (1988). Empirical estimation of protein-induced DNA bending angles: applications to λ site-specific recombination complexes. *Nucleic Acids Res.* **16**, 9687-9705.

Travers, A. (1997). DNA-protein interactions: IHF-the master bender. *Curr. Biol.* **7**, 252-254.

Umlauf, S.W. and Cox, M.M. (1988). The functional significance of DNA sequence structure in a site-specific genetic recombination reaction. *EMBO J.* **7**, 1845-1852.

Van der Vliet, P.C. and Verrijzer, C.P. (1993). Bending of DNA by transcription factors. *Bioessays* **15**, 25-32.

Vetter, D., Andrews, B.A., Roberts-Beatty, L. and Sadowski, P.D. (1983). Site-specific recombination of yeast 2-micron DNA *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7284-7288.

Volkert, F.C. and Broach, J.R. (1986). Site-specific recombination promotes plasmid amplification in yeast. *Cell* **46**, 541-550.

Voziyanov, Y., Lee, J., Whang, I., Lee, J. and Jayaram, M. (1996). Analyses of the first chemical step in Flp site-specific recombination: synapsis may not be a pre-requisite for strand cleavage. *J. Mol. Biol.* **256**, 720-735.

Wells, R.D., Collier, D.A., Hanvey, J.C., Shimizu, M. and Wohlrab, F. (1988). The chemistry and biology of unusual DNA structures adopted by oligopurine:oligopyrimidine sequences. *FASEB J.* **2**, 2939-2950.

Werner, M.H., Gronenborn, A.M. and Clore, G.M. (1996). Intercalation, DNA kinking and the control of transcription. *Science* **271**, 778-784.

Wissmann, A. and Hillen, W. (1991). DNA contacts probed by modificaton protection and interference studies. *Methods Enzymol.* **208**, 365-379.

Wu, H.M. and Crothers, D.M. (1984). The locus of sequence-directed and protein-induced DNA bending. *Nature* **308**, 509-513.

York, D. and Reznikoff, W.S. (1997). DNA binding and phasing analyses of Tn5 transposase and a monomeric variant. *Nucleic Acids Res.* **25**, 2153-2160.

Zinkel, S.S. and Crothers, D.M. (1987). DNA bend direction by phase sensitive detection. *Nature* **328**, 178-181.

Zinkel, S.S. and Crothers, D.M. (1990). Comparative gel electrophoresis measurement of the DNA bend angle induced by the catabolite activator protein. *Biopolymers* **29**, 29-38.

Zinkel, S.S. and Crothers, D.M. (1991). Catabolite activator protein-induced bending in transcription initiation. *J. Mol. Biol.* **219**, 201-215.

Zwieb, C., Kim, J. and Adhya, S. (1989). DNA bending by negative regulatory proteins: Gal and Lac repressors. *Genes Dev.* **3**, 606-611.

IMAGE EVALUATION
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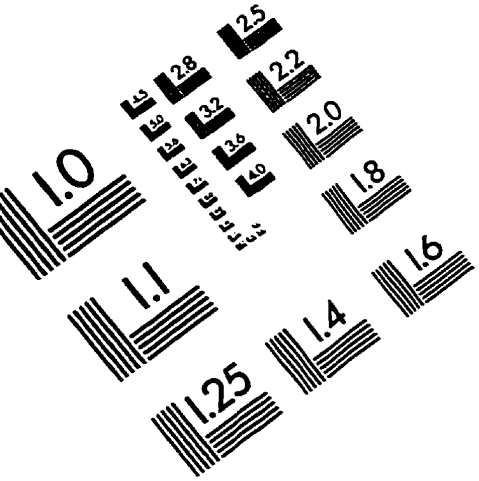

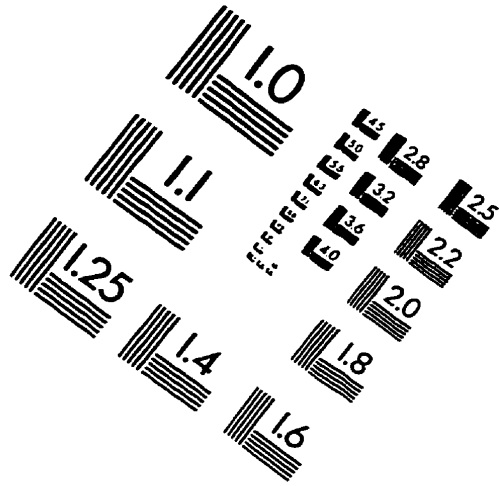

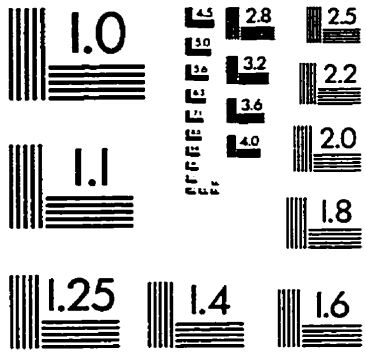

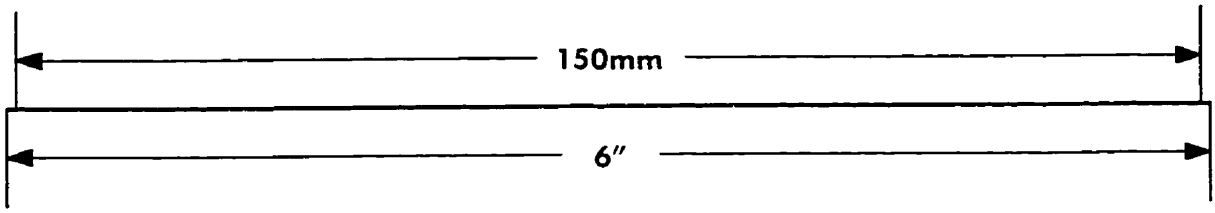

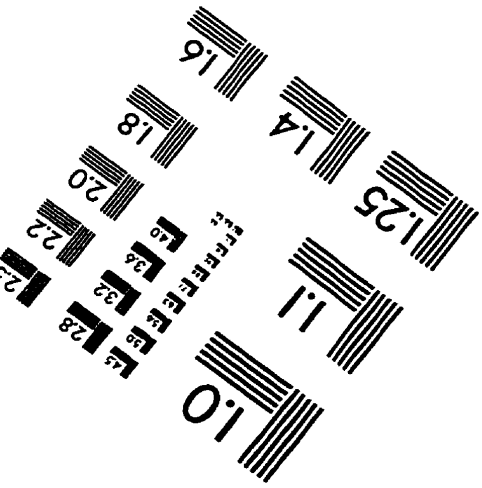

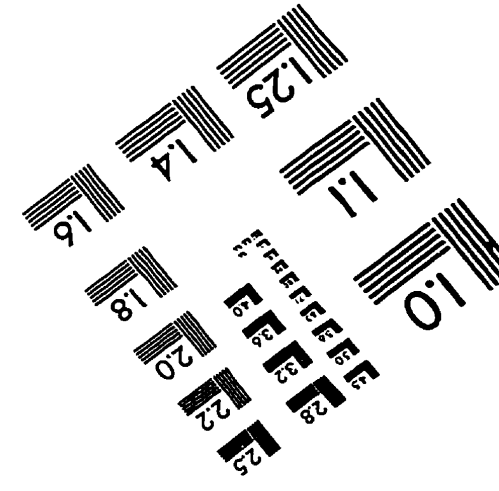

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