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DNA Recognition by the FLP Recombinase of the Yeast 2 μ Plasmid

A Mutational Analysis of the FLP Binding Site

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The 2 μ plasmid of the yeast *Saccharomyces cerevisiae* encodes a site-specific recombination system consisting of the FLP protein and two inverted recombination sites on the plasmid. The minimal fully functional substrate for *in-vitro* recombination in this system consists of two FLP protein binding sites separated by an eight base-pair spacer sequence. We have used site-directed mutagenesis to generate every possible mutation (36 in all) within 11 base-pairs of one FLP protein binding site and the base-pair immediately flanking it. The base-pairs within the binding site can be separated into three classes on the basis of these results. Thirty of the 36 sequence changes, including all three at seven different positions (class I) produce a negligible or modest effect on FLP protein-promoted recombination. In particular, most transition mutations are well-tolerated in this system. In only one case do all three possible mutations produce large effects (class II). At three positions, clustered near the site at which DNA is cleaved by FLP protein, one of the two possible transversions produces a large effect on recombination, while the other two changes produce modest effects (class III). For seven mutants for which FLP protein binding was measured, a direct correlation between decreases in recombination activity and in binding was observed. Positive effects on the reaction potential of mutant sites are observed when the other FLP binding site in a single recombination site is unaltered or when the second recombination site in a reaction is wild-type. This suggests a functional interaction between FLP binding sites both *in cis* and *in trans*. When two mutant recombination sites (each with 1 altered FLP binding site) are recombined, the relative orientation of the mutations (parallel or antiparallel) has no effect on the result. These results provide an extensive substrate catalog to complement future studies in this system.

1. Introduction

The 2 μ plasmid of the yeast *Saccharomyces cerevisiae* is a 6318 base-pair autonomously replicating DNA molecule present in 60 to 100 copies per haploid nucleus. The complete DNA sequence of the 2 μ plasmid has been determined (Hartley & Donelson, 1980). The sequence includes a pair of 599 base-pair inverted repeats. Several open reading frames are present. Three protein products have been identified to be involved in the replications and segregation of the plasmid. One of these, designated FLP, catalyzes a site-specific recombination reaction that is involved in amplifying the copy number of the 2 μ plasmid during DNA replication (Futcher, 1986; Volkert & Broach, 1986). The recombination sites for the FLP system are located within the 599 base-pair repeats.

Several laboratories have succeeded in cloning and expressing the FLP protein in *Escherichia coli* and in establishing *in-vitro* recombination assays for FLP activity (Cox, 1983; Meyer-Leon *et al.*, 1984; Sadowski *et al.*, 1984; Prasad *et al.*, 1986). The requirements of the *in-vitro* reaction are very simple; low concentrations of a variety of buffers between pH 5 and 9 and ionic strength conditions between 50 and 350 mM are tolerated. To date, the analysis of the reaction has been performed using partially purified FLP protein fractions derived from *E. coli* strains containing recombinant plasmid vectors that express FLP protein. The FLP protein has been purified to near-homogeneity in this

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laboratory (Gates *et al.*, 1987; Meyer-Leon *et al.*, 1987).

Rapid progress has been made in the identification and characterization of the specific sequences required for recombination. Broach and co-workers genetically identified a 65 base-pair region within the 2 μ repeat that functions well *in vivo* (Broach *et al.*, 1982). This region includes three 13 base-pairs repeats, with the third inverted relative to the other two and separated from them by an eight base-pair spacer as illustrated in Figure 1. This site has been designated FRT by Broach and co-workers (McLeod *et al.*, 1986). Deletion analysis revealed that the minimal fully functional active site consists of the 28 base-pairs (Senecoff *et al.*, 1985; Gronostajski & Sadowski, (1985) delimited in Figure 1. *In-vivo* studies have provided similar results (Jayaram, 1985). This substrate includes ten base-pairs from each of two 13 base-pair repeats and the eight base-pair spacer sequence between them. The eight base-pair spacer sequence that separates these two binding sites is involved in DNA–DNA pairing during strand exchange in this reaction (Senecoff & Cox, 1986). The central six base-pairs within this sequence are not contacted by the FLP protein during recombination. Multiple base-pair changes are tolerated within this sequence. There is, however, a requirement that the two recombining molecules contain the same alterations in this region. The outcome of a recombination event depends on a unique alignment of two recombination sites. This alignment is determined entirely by the asymmetry within the spacer sequence and is mediated by DNA–DNA interactions. Recombination sites containing a fully symmetrical spacer sequence show loss of functional site orientation, although recombination proceeds efficiently as long as spacer homology is maintained (Senecoff & Cox, 1986).

FLP protein binding sites have been identified by DNase I footprinting experiments (Andrews *et al.*, 1985) and methylation protection and interference experiments (Bruckner & Cox, 1986). The FLP protein makes specific purine contacts with at least eight base-pairs of the 13 base-pair inverted repeats proximal to the spacer and with the external base-pair in the spacer sequence (Fig. 1) These contacts define a 12 base-pair FLP recognition sequence that includes the external base-pair of the spacer and the first 11 base-pairs of the flanking repeat proximal to the spacer. The minimal recombination site as defined by identified purine contacts includes two FLP binding sites and is 30 base-pairs in size (2 base-pairs larger than the site defined in deletion analysis). Complementary mutational analyses have provided additional evidence that at least some of these contacts are absolutely required for recombination and binding *in vitro* (Prasad *et al.*, 1986; Andrews *et al.*, 1986). Only a small subset of possible mutations (11 of 36) have been generated and characterized in these studies. A limited mutational analysis conducted *in vivo*, which included single transition mutations at four positions, revealed that only one of the changes in this sample (guanine to adenine at position 11) reduced the efficiency of recombination (McLeod *et al.*, 1986).

We report here the construction and analysis of a complete set of FLP substrate mutations within 11 of the 12 base-pairs defining the FLP binding site. Only the position within the spacer is not examined. We have also generated all three changes at the position immediately flanking the binding site. This represents the first analysis of the substrate requirements for the FLP protein using a purified *in-vitro* system.

2. Materials and Methods

(a) *Purification of FLP protein*

FLP protein was purified to near-homogeneity using a modification of a procedure to be described elsewhere (Gates *et al.*, 1987). Protein obtained in this manner is greater than 85% homogeneous, as determined by SDS/polyacrylamide gel electrophoresis. Protein concentrations were determined using the method of Bradford (1976). Concentrations used in various experments are noted in the Figure legends.

(b) *Enzymes and chemicals*

Restriction enzymes were from New England Biolabs. Avian myeloblastosis virus (AMV) reverse transcriptase was from Life Sciences, Inc., or Pharmacia. Bacteriophage T4 DNA ligase was purified following published procedures (Davies *et al.*, 1980). Deoxy and dideoxy nucleotides were from Pharmacia. Agarose and acrylamide for gel electrophoresis and optical grade cesium chloride for DNA purifications were from Bethesda Research Laboratories. Ultrapure urea was from Schwarz-Mann. Radionucleotides were from Amersham ([γ-^{32}P]rATP) or New England Nuclear ([α-^{32}P]dATP). Bovine serum albumin (Pentax Fraction V) was from Miles Laboratories. Polyethylene glycol 6000 was from Sigma. Calf thymus DNA was from Cooper Biomedical. Buffers were from Research Organics or Boehringer-Mannheim. All other reagents were from Sigma or Malinckrodt.

(c) *Plasmids and strains*

All recombinations sites containing single point mutations were constructed in the plasmid pXf3 (Maniatis *et al.*, 1982; see below for constructions) and maintained in the *E. coli* strain HB101 (Maniatis *et al.*, 1982). The plasmid pUC119 was a generous gift from Vince Schulz of this department. This plasmid and its derivatives were maintained in the strain JM105 (Messing, 1983).

(d) *FLP recombination assay*

In-vitro recombinatin assays contained 25 mM-TAPS† (31% anion; pH 8·0 at 25°C), 1 mM-EDTA,

† Abbreviations used: TAPS, *N*-Tris(hydroxymethyl)-3-aminopropane sulfonic acid; PEG, polyethylene glycol; BSA, bovine serum albumin; DMS, dimethyl sulfate; DTT, dithiothreitol; FRT, FLP recombination target. FRT site and recombination site are used interchangeably.

200 mM-NaCl, 10% (w/v) PEG and 200 ng of linear substrate DNA (0·11 pmol recombination sites) in a total volume of 18 μl before addition of FLP protein. These reaction conditions are similar to those used in previous studies, with the exception of the polyethylene glycol. PEG has been shown to have a large stimulatory effect on intermolecular recombination in this purified system (C. Gates & J. Senecoff, unpublished results). Reactions were intiated by the addition of 2 μl of an appropriately diluted FLP protein fraction and were incubated for 60 min at 30°C. Then 8 μl of stopping solution (3·4% SDS, 50 mM-EDTA, 50% glycerol, 0·01% bromophenol blue, 0·1% xylene cyanol) was added and electrophoresis was carried out through 0·8% agarose gels in Tris-acetate/EDTA buffer (Maniatis *et al.*, 1982). Products were resolved and visualized by staining with ethidium bromide. All plasmids used in this work that contain a single mutation in the recombination site are identical in size and sequence except for the mutated position. The same enzymes are used to digest each substrate and the products generated are the same size in every experiment. Analysis involved mixing a *Pst*I digest of a mutant plasmid with an *Eco*RI digest of the same mutant or of the plasmid pJFS39 (which contains wild-type FLP binding sites; Senecoff & Cox, 1986), as described in Results.

(e) *Gel electrophoresis binding assay*

Gel retardation assays are based on the method of Fried & Crothers (1981). Reactions (20 μl) contained 25 mM-TAPS (pH 8·0 at 25°C; 31% anion), 1 mM-EDTA, 200 mM-NaCl, 100 μl sonicated calf thymus DNA/ml and 20 ng of end-labeled substrate DNA, digested with *Eco*RI and *Sal*I. Reactions were initiated with 2 μl of an appropriately diluted FLP fraction and incubated at 30°C for 30 min. Then 10 μl of loading buffer (100 μg BSA/ml, 50% glycerol, 50 mM-EDTA, 0·01% bromophenol blue, 0·01% xylene cyanol) was added and 20 μl of the final mix was loaded onto a 5% polyacrylamide gel in Tris-borate/EDTA buffer (Maniatis *et al.*, 1982). Where indicated, heparin was added to a final concentration of 5 μg/ml. BSA was required in the loading buffer for complex stability in the gel, and calf thymus DNA was required to prevent non-specific retention of labeled DNA fragments in the wells. The gels were run at 150 V for 2 to 3 h, dried and exposed to X-ray film overnight without a screen.

(f) *Dimethyl sulfate protection assay*

The protocol employed in these experiments is a modification of procedures described elsewhere (Ross & Landy, 1983; Bruckner & Cox, 1986). Approximately 0·05 pmol (16 ng) of a uniquely 3′ end-labeled DNA fragment, containing a FLP recombination site near the labeled end, was incubated for 15 min at 20°C with or without FLP protein. This was done in 200 μl of the standard reaction buffer including 2 μg of sonicated calf thymus DNA. Dimethyl sulfate (1 μl of a 10·7 M-solution) was added and the incubation was continued for an additional 2 to 5 min at 20°C. Next, 50 μl of stop solution (1 M-Tris-acetate (pH 7·5), 1 M-β-mercaptoethanol, 1·5 M-sodium acetate, 50 mM-$MgCl_2$, 1 mM-EDTA) and 300 μl of cold phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) was added. The reactions were mixed and centrifuged for 2 min. The aqueous phase was removed and precipitated with ethanol. The pellets were washed twice with 70% ethanol and dried under vacuum. The reactions were then resuspended in 100 μl of 1 M-piperidine and incubated for 30 min at 90°C. *n*-Butanol (1 ml) was added, the reactions were vortex mixed and spun for 15 min. The pellets were resuspended in 150 μl of 1% SDS and an additional 1 ml of *n*-butanol was added. After centrifugation, the pellets were washed 2 or 3 times with 70% ethanol and dried under vacuum. The DNA was then resuspended in 3 μl of double-distilled water and 3 μl of loading buffer (98% formamide, 0·37% EDTA, 0·03% bromphenol blue, 0·03% xylene cyanol) and electrophoresis was carried out through 8·3 M-urea/10% polyacrylamide gels in Tris-borate/EDTA buffer (Maniatis *et al.*, 1982) for 2·5 h. The gels were dried and subjected to autoradiography with or without an intensifying screen. As a control for methylation-independent cleavage, samples were reacted with FLP protein in the absence of DMS and treated as above.

(g) *DNA sequencing*

FLP substrates cloned in plasmid DNA were sequenced directly from rapid plasmid lysates. Briefly, following DNA isolation, 100 μl of 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA buffer containing 200 μg RNase A/ml was added to the pellets and the mixture incubated at 37°C for 15 min. The DNA was extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and then precipitated with ethanol for 5 min at room temperature. DNA was then collected by spining in an Eppendorf centrifuge for 5 min, washed once with 70% ethanol and dried. The DNA was resuspended in 20 μl of double-distilled water. Alkali denaturation was carried out by adding 2 μl of a 2 M-NaOH, 2 mM-EDTA solution and incubating for 5 min at room temperature. The reactions were neutralized by adding 10 μl of 0·3 M-sodium acetate and 75 μl of cold 100% ethanol. After spinning for 5 min, the pellets were dried and resuspended in 9 μl of hybridization buffer (60 mM-NaCl, 50 mM-Tris·HCl (pH 8·3), 10 mM-DTT) and 1 μl of end-labeled primer (0·8 μM-primer, 0·1 μM-plasmid DNA approx. final concn). Hybridization was carried out at 37°C for 15 min to 2 h. Then 2 μl of $MgCl_2$ solution (36 mM-$MgCl_2$, 60 mM-NaCl, 50 mM-Tris·HCl (pH 8·3), 10 mM-DTT) and 4 units of AMV reverse transcriptase were added to each hybridization mixture. Sequencing mixes contained each dNTP at 375 μM and the appropriate ddNTP at 50 μM in the above MgCl-containing buffer. A portion (2·5 μl) of the hybridization reaction containing reverse transcriptase was added to 3 μl of each of the sequencing mixes and the reactions were incubated at 37°C for 30 min. Reactions were terminated by the addition of 5 μl stop solution (98% formamide, 0·37% EDTA, 0·03% bromphenol blue, 0·03% xylene cyanol) and boiled for 3 min. Sequences were resolved on 8·3 M-urea/10% polyacrylamide gels run at 35 W for 2 to 3 h. Gels were dried and autoradiography was performed overnight with an intensifying screen. The use of an end-labeled primer reduces background sequences due to annealing of small oligoribonucleotides that remain following treatment with RNase. This technique was adapted from various protocols for sequencing DNA and RNA (Sanger *et al.*, 1980; Inoue & Cech, 1985; Zagursky *et al.*, 1985; Promega Biotech, 1985).

(h) *Contruction of recombination sites with single point mutations*

For each position, two DNA strands were synthesized containing an ambiguous nucleotide at the position of

interest so as to generate all possible base-pair changes. When annealed, the double-stranded molecule contains a single 13 base-pair repeat (half of a FLP recombination site) flanked by *Bam*HI and *Xba*I linkers. The unkinased cassette was then ligated to the large fragment of a *Xba*I–*Bam*HI digest of the plasmid pJFS35R (Senecoff & Cox, 1986). The oligonucleotides were not kinased to ensure that only single insertions would result. The plasmid pJFS35R contains a spacer sequence that is 1 nucleotide shorter than the wild-type sequence. This nucleotide is deleted on the side of the spacer adjacent to the *Eco*RI site in the vector. When the *Xba*I site from this molecule is ligated to the oligonucleotide cassette, the resulting spacer sequence is 8 base-pairs in length, the same as for the wild-type spacer. However, the sequence within the spacer region of the mutant recombination sites is entirely symmetrical and identical in sequence with the spacer in the plasmid pJFS39 (Senecoff & Cox, 1986). Mutants were identified and confirmed in every case by direct DNA sequencing as described above.

(i) *Construction of recombination sites with double point mutations*

For a subset of mutant recombination sites, double mutants were generated. These recombination sites contain an identical base-pair change in both FLP protein binding sites. The FLP protein itself was used as reagent to construct these double mutants, replacing restriction enzymes and DNA ligase. A mutant FLP substrate is digested with *Bam*HI and reacted with a large excess of FLP protein. The small product of this reaction (40 base-pairs) is a complete FLP recombination site (flanked by *Bam*HI linkers) in which both FLP binding sites contain an identical mutation (Fig. 2). This small fragment isolated in each case from a 5% polyacrylamide gel and ligated to a *Bam*HI digest of the pUC19 derivative pUC119. The resulting plasmids are identical except for the presence of different double mutations in the site. All are slightly larger in size than the plasmids carrying the single mutations. Experiments to assess the reactivity of these mutations in both mutant × mutant and mutant × wild-type recombination assays are carried out according to the protocol described above for the single mutants.

(j) *Miscellaneous procedures*

Isolation of RNA from agarose gels was performed as described (Dretzen *et al.*, 1981). Restriction digests, 5′ end-labeling and DNA ligations were carried out as described (Maniatis *et al.*, 1982). DNA fragments were 3′ end-labeled essentially as described, except that AMV reverse transcriptase was used in place of Klenow fragment (Maniatis *et al.*, 1982). Isolation of DNA from polyacrylamide gels was performed as described (Maxam & Gilbert, 1980). Rapid plasmid isolation was carried out using modifications of published procedures (Davis *et al.*, 1980). Plasmid DNA utilized in FLP recombination and binding assays was purified by banding twice in cesium chloride gradients in the presence of ethidium bromide. DNA was greater than 95% supercoiled, as judged by gel electrophoresis, and contained no visible RNA contamination, as determined by gel electrophoresis and spectrophotometric measurements. Oligonucleotide synthesis was performed at the Protein Sequence-DNA Synthesis Facility within the University of Wisconsin Biotechnology Center.

3. Results

(a) *Experimental design*

This study takes advantage of a special property of the spacer sequence of the FLP recombination site (FRT). There are no direct protein contacts identified within the central six base-pairs of this spacer. Many spacer sequence alterations have no effect on recombination efficiency. There is, however, a requirement for homology between the spacer of two reacting FRT sites (Senecoff & Cox, 1986). The spacer region also defines the alignment of two sites (parallel or anti-parallel relative to flanking sequences) during recombination and thus the outcome of the recombination reaction (Senecoff & Cox, 1986). Each of the mutant FRT sites studied here has the symmetrical spacer sequence of pJFS39 (Fig. 1). Sites that contain a symmetrical spacer of this sequence react as well as wild-type sites but do not exhibit a defined orientation. This increases the number of potential reaction products. Reactions involving single restriction digests of a non-symmetrical spacer substrate will only generate products identical with substrates (Fig. 3; and see Senecoff & Cox, 1986), and no discrete products will be observed on a gel. This is because the alignment of two sites in recombination is restricted to the parallel configuration. Our standard assay in this study, a reaction involving linear DNA substrates, all derived by a single cleavage of the plasmid by the same restriction enzyme, will result in visible product formation (Fig. 3). These new products result from the anti-parallel alignment of recombining sites permitted with a symmetrical spacer. If full-length linear substrates derived from two different restriction digests of the same plasmid are used in the assay, two discrete products will be generated with non-symmetrical spacer substrates, but eight products will be generated with a symmetrical spacer molecule (Fig. 3).

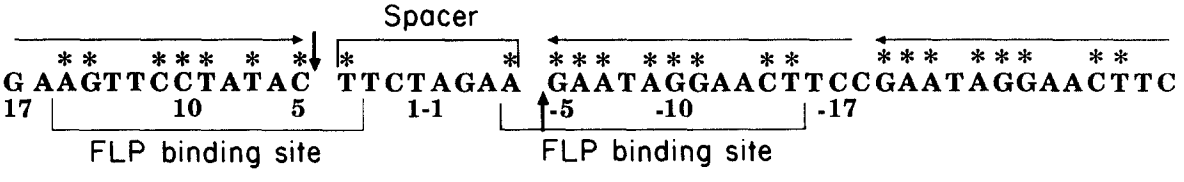


Figure 1. FLP recombination site. Arrows denote 13 base-pair inverted repeats. FLP binding sites, spacer and sites of cleavage are indicated. Numbering of base-pairs is the same as given by Bruckner & Cox (1986). Asterisks denote known purine contacts for the FLP protein.

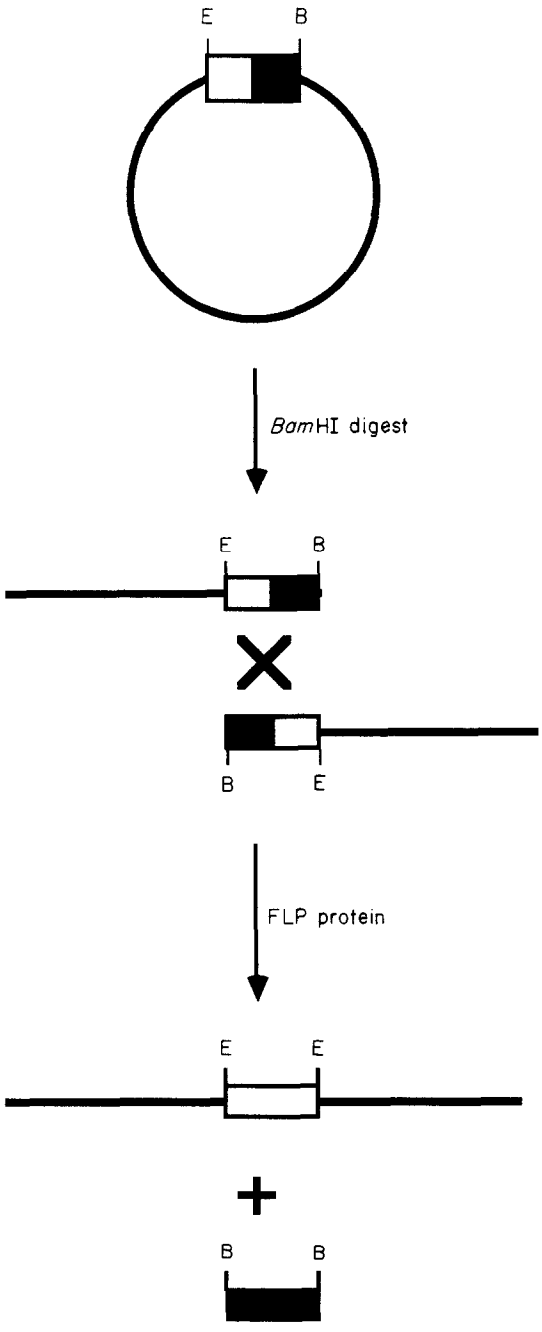


Figure 2. Cloning of recombination sites with double mutations. Double mutants were constructed by reacting single point mutants with FLP protein and isolating the small recombination product. This small DNA molecule is a complete recombination site, in which both repeats contain the mutation of interest. These small DNA molecules were isolated and cloned into the vector pUC119, which had been cleaved with *Bam*HI as described in Materials and Methods. Filled boxes represent mutant FLP binding sites, empty boxes represent wild-type binding sites.

The eight products generated in the last reaction described above result from four different reactions. This permits the analysis of several effects in a single experiment. To illustrate, we will designate full-length linear substrates produced from digestion of a substrate plasmid by either *Eco*RI or *Pst*I (Fig. 3) as R and P substrates, respectively. The four large products represent all four possible reactions. Starting with the largest, they result in order from R × R, R × P, P × P reactions (all in anti-parallel alignment), and finally a R × P reaction (in the parallel alignment). The R × R and P × P reactions are presumed to occur in the parallel alignments, but these will generate products identical with substrates and will not be observed here. If one of these substrates (e.g. R) contains an FLP binding site mutation and the other (e.g. P) does not, WT × WT, WT × mutants, and mutant × mutant reactions are readily compared internally in a single experiment. If both substrates contain mutations, the effect of orientation (parallel or anti-parallel) of mutations in the reacting complexes can be studied. These possibilities are outlined in Figure 3 and form the basis for our standard assays.

We have analyzed a complete set of 36 mutations, including all possible changes in the 11 base-pairs of the 13 base-pair repeat that were previously assigned to the FLP binding site (Bruckner & Cox, 1986), and all possible changes in the base-pair immediately flanking this binding site (distal to the spacer). Position 4, at the end of the spacer, is included within the defined FLP binding site (Bruckner & Cox, 1986). Altering this position would create an asymmetric spacer, and was therefore inaccessible with this experimental approach. Previous data for one change at this position is included below for comparison. All mutations were analyzed using the assay for recombination described above, in which mutant and wild-type substrates were combined in a single experiment. In each experiment, 2 to 2·5-fold serial dilutions of FLP protein were carried out under standard reaction conditions. Comparisons reflect the minimum level of FLP protein required to produce observable products. The numbers reported denote the factor by which the FLP protein concentration must be increased to produce this minimal reaction relative to the amount of FLP protein required for a minimal reaction between two wild-type sites in a reaction internal to each experiment. A larger number therefore corresponds to a less-reactive FRT site. In the reported data, the wild-type substrate pJFS39 was prepared by cleavage with *Eco*RI and the mutant substrate was cleaved with *Pst*I. Switching the two restriction enzymes used to prepare these substrates has no effect on the data. The results are accurate to within plus or minus one dilution, or plus or minus a factor of 2. Differences of a factor of 2 or less should not be considered significant.

The results of these titration assays are useful only to compare mutant effects in a relative sense and are offered without molecular interpretation. The information represents data from a series of experiments where the FLP protein concentration was varied and recombination was measured at a fixed time (60 min). This time-point was chosen arbitrarily and the results are virtually identical at

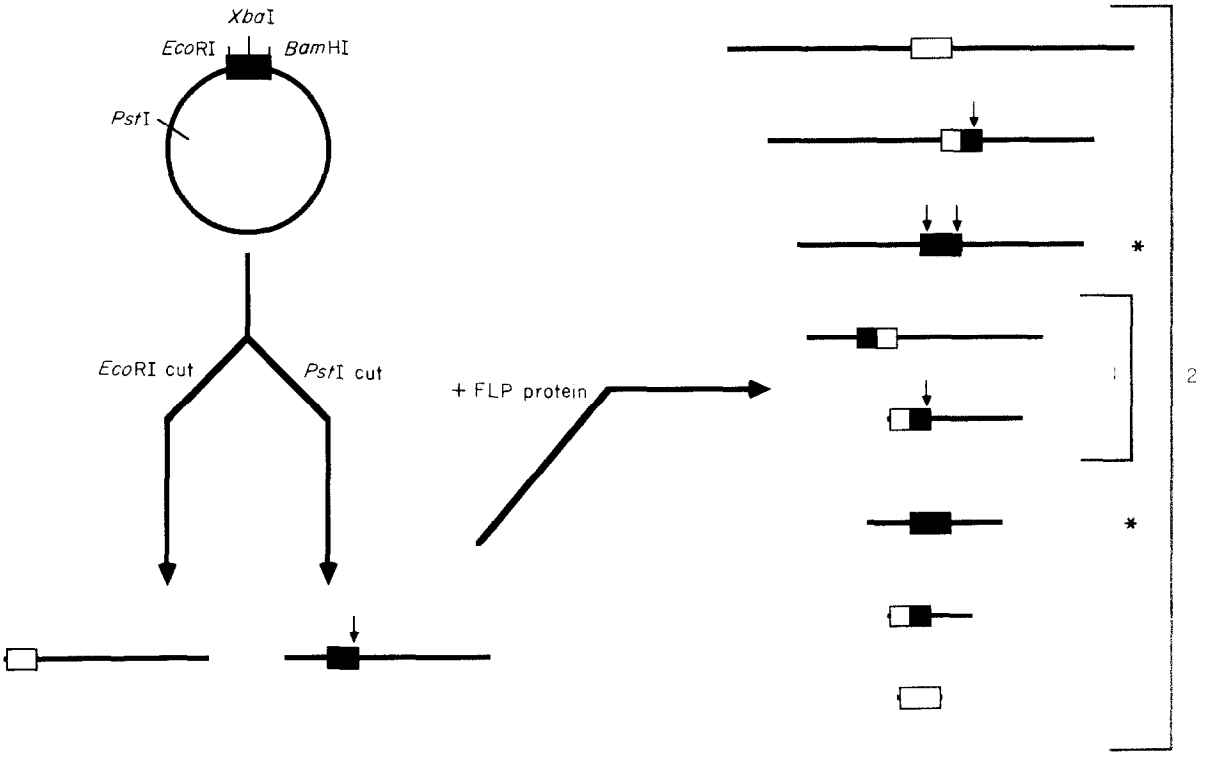


Figure 3. Products of intermolecular recombination. A standard recombination reaction as used in this study is illustrated, employing 2 linear substrates that differ only in the restriction enzyme used to linearize the parent plasmid. The bracket labeled 1 denotes products that result from recombination between both recombination substrates containing a non-symmetrical spacer sequence. In this case, the reaction of either substrate alone will result in products identical with substrates and will not be observed. Bracket 2 includes the new products formed from recombination between 2 substrates that contain symmetrical spacer sequences. The products denoted by an asterisk are those produced by a reaction of the *Pst*I-generated substrate alone with a symmetrical spacer. If 1 of the starting plasmids contains a mutated recombination site, denoted by an arrow, the distribution of mutations in the products is illustrated. The shading in products is indicative of the various possible combinations of reacting sites in the reactions which produce them. Note that for mutant sites with a mutation in only 1 of the 2 FLP binding sites, not all of the products resulting from reactions of the mutant sites will contain a mutation (see Figs 7 and 11). The product sizes are (from top to bottom) 5606, 4851, 4096, 3576, 2066, 1546, 791 and 36 base-pairs. The substrates are 2821 base-pairs. The 4 largest products are prominent in Figs 5, 6, 7 and 10, and are used in our analysis. See the text for details.

time-points as early as five minutes. We note that for mutants that exhibit greatly reduced levels of recombination, the extent of recombination remains low even when a large excess of FLP protein is used. The comparison used here tends to underestimate the effect of these mutants. A kinetic interpretation of these results will be provided elsewhere.

(b) *Effects in mutant × mutant reactions*

The effects of all mutations examined on FLP protein-promoted recombination are summarized in Figure 4. Several patterns are evident in the data for the reactions of mutants with identical mutants. The effects at different positions can be divided into three classes. Class I includes positions at which no mutation causes a greater than tenfold effect. Class II includes positions in which all changes produce large effects. Finally, class III includes positions in which one base change causes a large effect and the other two possible changes cause small effects. Examples of all three classes are presented in Figure 5.

Several general patterns deserve comment. First of all, with the exception of position 16 (previously determined to lie outside the FLP protein binding site as defined by identified purine contacts), at least one mutation at every position produces a significant decrease (5-fold or greater) in recombination. Interestingly, however, most positions fall into class I. Excluding position 11, this includes positions 8 through 16. In fact, the observed effects are modest, tenfold or less, with 31 or 37 total mutations. The small effects seen at positions distal to the spacer correlate well with previous observations that the effects of external deletions of this site increase gradually with the length of the deletion. Complete inactivation of the site required deletions of more than five base-pairs (positions 13 through 17; Senecoff *et al.*, 1985; Gronastajski & Sadowski, 1985*a*).

Only one position, 11, falls into class II. This was previously identified as a prominent purine contact in methylation protection and interference experiments (Bruckner & Cox, 1986). This again correlates well with the effects of deletion mutations. An eight base-pair deletion (which

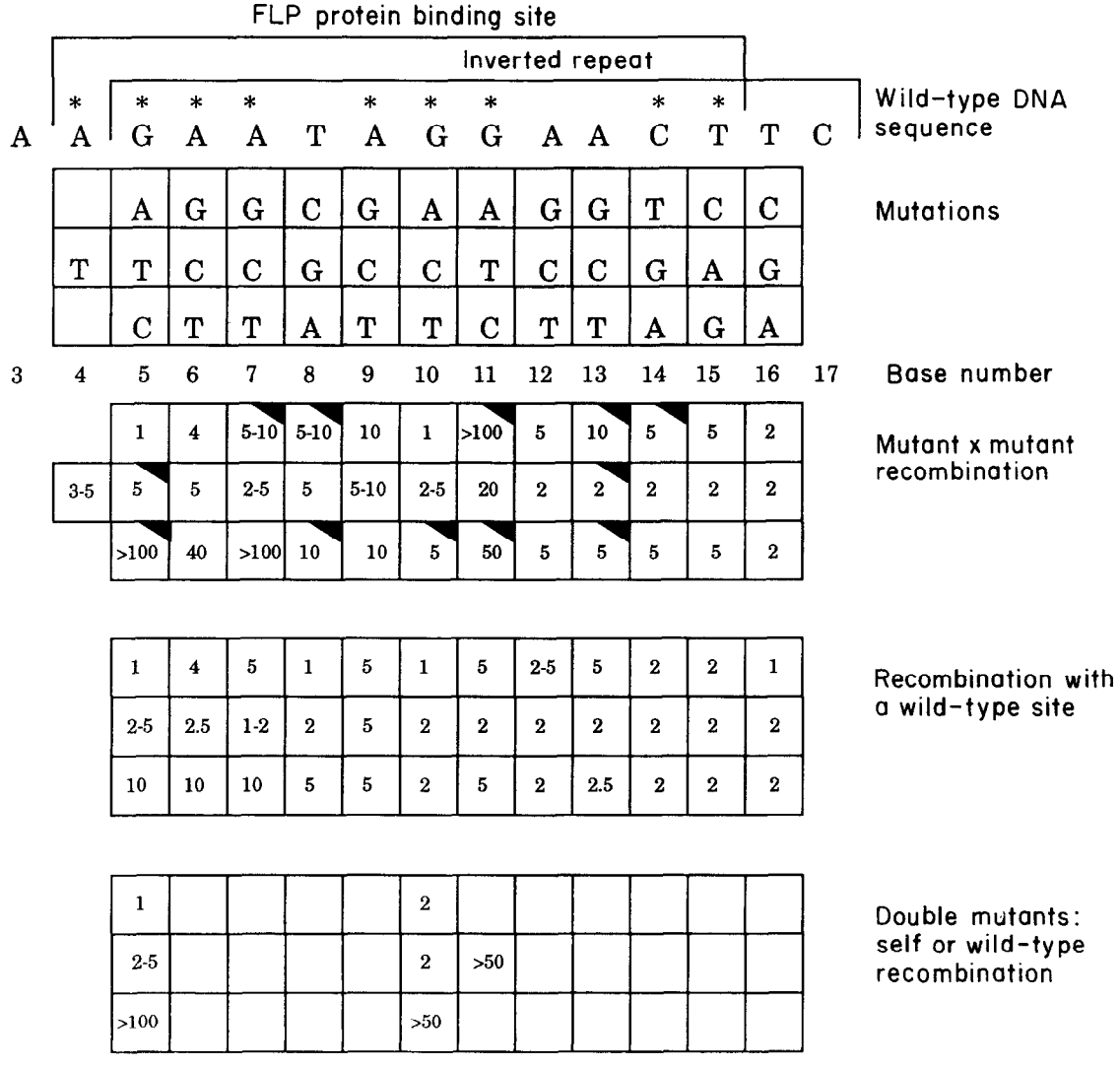


Figure 4. Summary of mutant effects. The wild-type sequence found in and immediately flanking an FLP binding site (a single 13 base-pair repeat) is indicated at the top. Quantitative effects of all mutations generated in this study are indicated. Transition mutations are arranged in the top row of the table for convenience. The numbers indicate the factor by which the FLP protein concentration must be increased in order for a detectable reaction to be observed. Higher numbers correspond to a less-reactive site. Data for the A to T transversion at position 4 are from Senecoff & Cox (1986). Mutations examined for orientation effects are denoted by shaded triangles in the upper right corner of the appropriate boxes. See the text for details.

changes position 11) inactivates the site, whilst a five base-pair deletion does not (Senecoff *et al.*, 1985).

The most interesting patterns are found at positions 5 through 7. In each case, one transversion mutation (a guanine to cytosine at position 5 or an adenine to thymine at positions 6 and 7) produces a large decrease in recombination efficiency, while the other two possible changes produce effects at least an order of magnitude less severe. The largest contrast is at position 5, with the guanine to cytosine transversion virtually inactivating the site, whilst the guanine to adenine transition has no detectable effect. None of the transversions that have the largest effects in this region has been characterized previously.

Position 6 is a special case. This is a point of asymmetry in the wild-type site (Fig. 1). A transversion on the other side of the spacer that places an adenine symmetrically at position −6 has been shown to have no detectable effect on recombination (Senecoff *et al.*, 1985). In contrast, we show here that the corresponding transversion at position 6, which leaves the site with symmetrical thymine residues at these positions, results in a very large decrease in recombination function. Results from all possible combinations of adenine and thymine at position 6 and −6 are presented in Figure 6. The site will evidently tolerate two adenine residues or one adenine and one thymine, at these positions with no decrease in recombination observed. Substrates with two thymine residues at these positions have substantially decreased recombination activity. It should be noted that there was thymine rather than adenine at position −6 for all of the mutant sites studied here. This

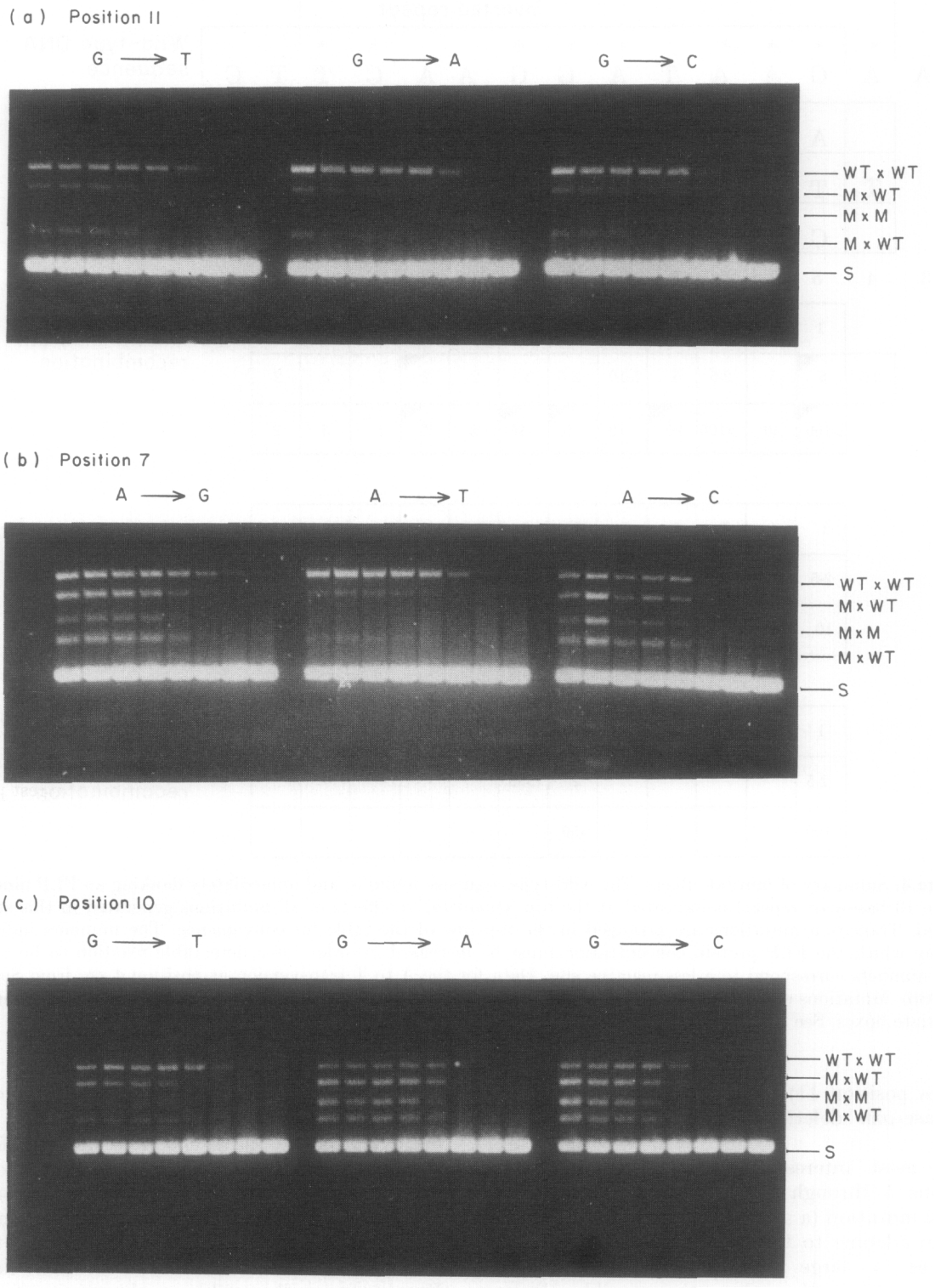


Figure 5. Illustration of effects of mutations at 3 positions on recombination. Examples of the standard comparative assay used to evaluate the effects of mutations on recombination are presented. Reactions were carried out as described in Materials and Methods. M and WT denote mutant and wild-type, respectively, and the origin of each of the product bands is indicated. S denotes the substrate band. Each set of 8 reactions is a series of FLP dilutions, using 1·0, 0·5, 0·25, 0·1, 0·05, 0·025, 0·01 and 0·005 μl of an active FLP fraction, from left to right. Final concentration of recombination sites in each reaction was 5·3 nM. The highest concentration of FLP (1·0 μl) represents a 32 : 1 ratio of FLP monomers to recombination sites and the lowest concentration (0·05 μl) a 0·2 : 1 ratio. The sizes of the 4 large products are listed in the legend to Fig. 3. Position numbers and base changes at that position are indicated.

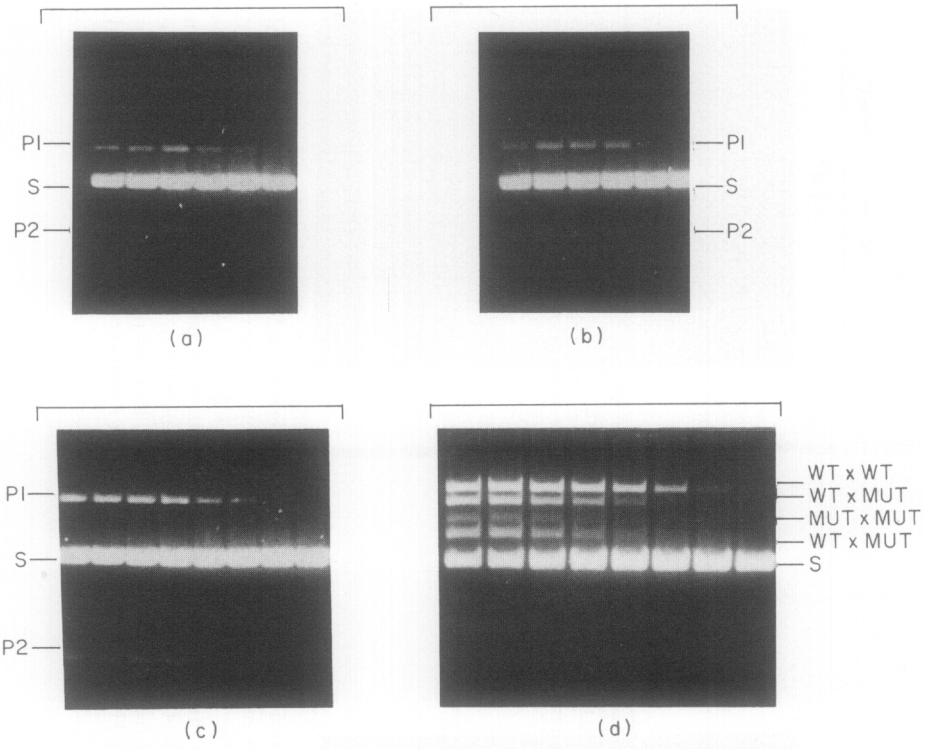


Figure 6. Effects of mutations at position 6 on recombination. Reactions were carried out as described in Materials and Methods. Results of recombination assays between substrates containing various combinations of adenine and thymine at positions 6 and −6. (a) pJFS36 × pJFS36 (T at −6 A at 6, non-symmetrical spacer); (b) pJFS36·6A × pJFS36·6A (A at 6 and −6, non-symmetrical spacer); (c) pJFS39 × pJFS39 (T at −6, A at 6, symmetrical spacer); (d) pJFS39 × pPJR39·6T (pJFS39·6T has T at 6 and −6, symmetrical spacer). For experiments with 2 different substrates, equal amounts of each were used to give a final concentration of FRT sites of 5·3 nM. Reaction products are noted in the diagram. S denotes the substrate band in each panel. The products from the reactions involving substrates with non-symmetrical spacer (a) and (b), P1 and P2, are 3576 and 2066 base-pairs. The products from the reaction involving a substrate with a symmetrical spacer in (c), P1 and P2, are 4096 and 1556 base-pairs. The 4 largest products in (d) are 5606, 4851, 4096 and 3576 base-pairs. The plasmid pJFS36·6A was previously called pJFS36sym (Senecoff *et al.*, 1985). Each set of 6 reactions in (a) and (b) is a series of FLP dilutions using 1·0, 0·5, 0·25, 0·1, 0·05 or 0·025 μl of an FLP protein preparation, from left to right. The highest concentration of FLP (1·0 μl) represents an 8 : 1 ratio of FLP monomers to recombination sites and the lowest (0·025 μl) a 0·2 : 1 ratio. Each set of 8 reactions in (c) and (d) is a series of FLP dilutions as described in the legend to Fig. 5.

may have increased the effects of some mutations (see Discussion).

Eleven of the 36 mutations presented in Figure 4 have been characterized either *in vivo* or *in vitro* (McLeod *et al.*, 1986; Prasad *et al.*, 1986; Andrews *et al.*, 1986). The only large effects noted in these studies occurred at position 11. The results presented in Figure 4 agree qualitatively with these studies.

(c) *Recombination between mutant and wild-type substrates*

A prominent pattern evident in Figure 4 is that the recombination activity of virtually all mutant sites is increased substantially when these sites are reacted with an unaltered recombination site. This property is common to all mutant classes, although it is much more pronounced for the more deleterious mutations. For all six mutations that exhibit strong effects in mutant × mutant recombination, the reaction is greatly improved when the recombining partner is wild-type. The reactions in Figures 5 and 6 illustrate this property of the reaction. In these cases, the extent of product formation in a mutant × wild-type reaction is generally less than that for two wild-type sites; however, it is generally increased relative to the reaction between two identical mutant sites.

(d) *Single point mutations show no orientation preference in mutant × mutant recombination*

A subset of the generated mutations was tested in mutant × mutant reactions for orientation effects, i.e. the effect on the reaction if the altered FLP protein binding sites are in the parallel (opposite one another) or anti-parallel orientations during recombination. The experimental design is described in section (a), above, and illustrated in Figure 3. The mutants tested included 12 separate mutations, as indicated in Figure 4. These include all three mutant classes and span the range of

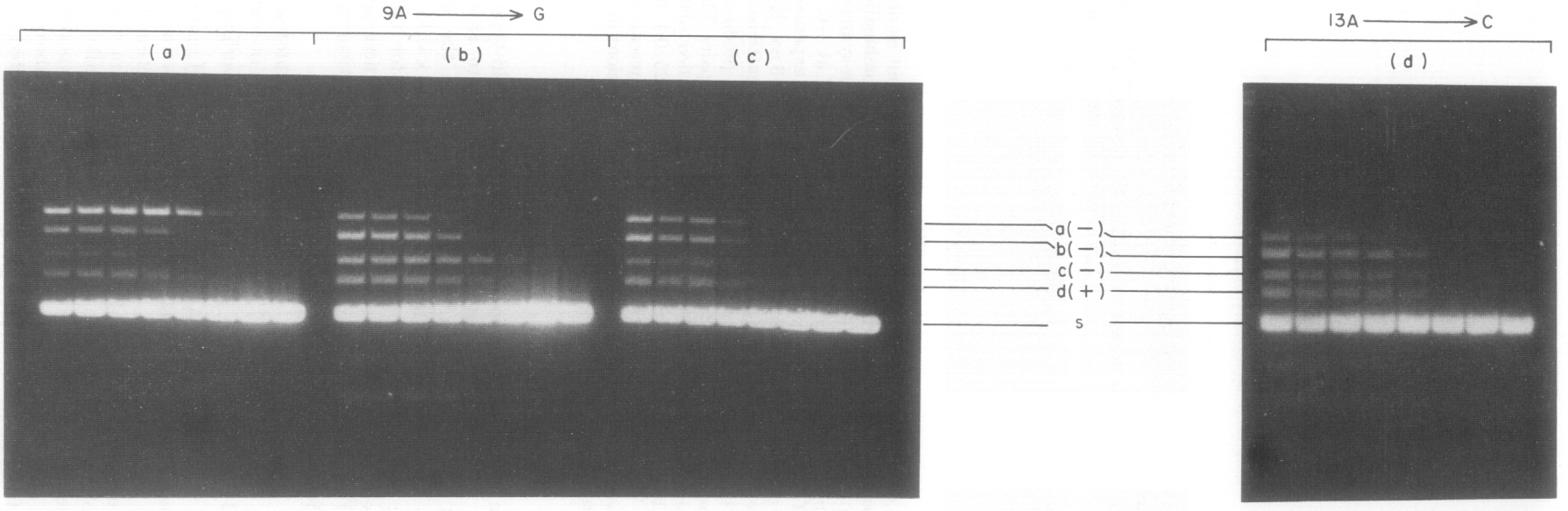


Figure 7. Effects of single point mutations: absence of orientation effects or position effects. Reactions were carried out as described in **Materials and Methods**. Final total concentration of FRT sites was 5·3 nM in all reactions. Again, each set of reactions is a series of FLP protein concentrations where the highest level (1st lane) of FLP represents a 35 : 1 ratio of FLP monomers to FRT sites and the lowest is 0·8 FLP monomers per recombination site (see the legend to Fig. 5). (a) and (b) Reactions in which one substrate is wild-type and the other is mutant. Restriction enzymes used to linearize the 2 substrates are reversed in these panels to determine if position effects influenced the results. (c) and (d) Example of experiments designed to detect orientation effects. Products resulting from parallel or antiparallel orientations are indicated by + and −, respectively. (a), (b) and (c) Reactions of the plasmid pJFS69, which contains an FRT site with an A to G at position 9. Reactions are: (a) pJFS39 (*Eco*RI) × pJFS69 (*Pst*I); (b) pJFS69 (*Eco*RI) × pJFS39 (*Pst*I); (c) pJFS69 (*Eco*RI) × pJFS69 (*Pst*I). (d) A mutant × mutant reaction with the plasmid pPJR13C, which has A to C transversion at position 13.

mutant effects observed. In none of these cases was evidence found for an orientation preference in the recombination complex. Representative data are presented in Figure 7.

(e) *Effects of double mutants on recombination*

For mutations in positions 5, 10 and 11, double mutants, with the same mutations occurring symmetrically in both FLP binding sites in a recombination site, were generated. Data are summarized in Figure 4. The comparisons are based on assays identical in concept to those described above, except that the DNA substrate containing the double mutant sites was slightly larger than pJFS39 (see Materials and Methods and Fig. 8). Results are again accurate to within a factor of 2. Reactions between double mutants at position 10 and wild-type sites are shown in Figure 8(a). In this case, since the mutations are in both FLP binding sites within the FRT site, there can be no orientation effect as described above. These experiments demonstrate that, for several mutations that have modest effects on recombination, the presence of the same mutation in both FLP binding sites does not greatly increase the effect. A G to T transversion at position 5 and a G to C transversion at position 10, as well as the transitions at both positions, exhibit the same effects whether present in both FLP binding sites or only one. Mutations that have larger effects as single mutants are at least as deleterious when the mutation is introduced into both repeats. An exception to these patterns is a single base change at position 10, which shows greatly decreased reactivity when present in both inverted repeats, relative to a modest effect when present in only one. This is the guanine to thymine transversion at position 10 (Fig. 8(b)). For all mutations tested, the activity of double mutants is not restored significantly in reactions with unaltered substrates.

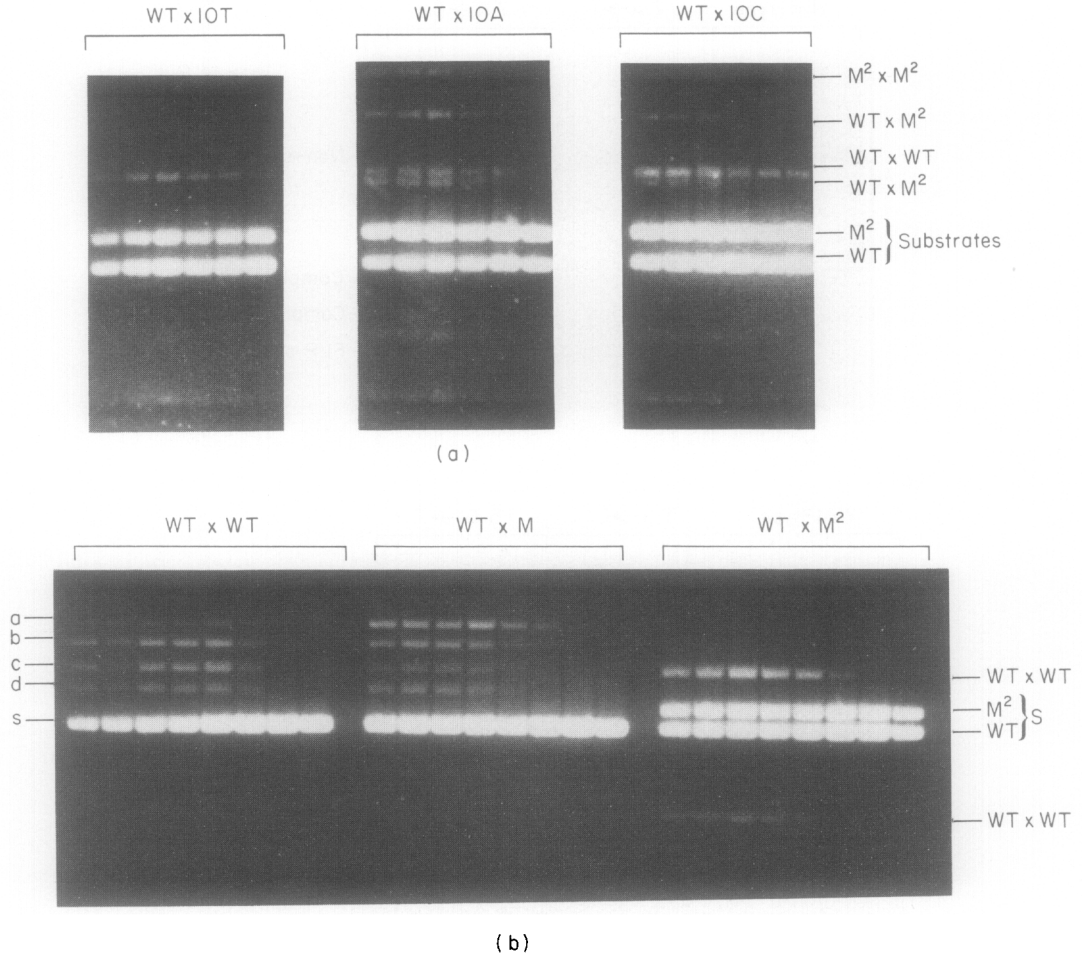


Figure 8. Effect of double point mutations on recombination. (a) Effect of double mutations at positions 10 and −10. Reactions are as described in the legends to Figs 6 and 7, and in Materials and Methods. M^2 and WT refer to double mutant and wild-type substrates, respectively. Reaction products are denoted similarly and indicated in the Figure. Product sizes are (from top to bottom) 6776, 5436, 4096 and 3791 base-pairs. (b) Difference in effect of single and double mutants (G to T) at position 10. Reactions are as described for Figs 6 and 7. Substrate and product molecules for the 3rd set of reactions are indicated in the Figure. Here, WT, M^2 and S denote wild-type, double mutant and substrate bands, respectively. For the 1st 2 sets of reactions, a, b, c and d are the 4 large products generated from a reaction such as that illustrated in Fig. 3. The sizes of these products are given in the legend to Fig. 3. S again denotes the substrate band.

(f) *Effect of single mutations on DNA binding*

In order to assess whether these mutations were indeed deficient in FLP protein binding, a gel retardation assay was developed based on the method of Fried & Crothers (1981). Similar assays have been used to assess FLP protein binding to FRT sites in other laboratories (Andrews *et al.*, 1987; Prasad *et al.*, 1986). The assay is based on the observation that DNA–protein complexes show altered mobility in polyacrylamide gels with respect to free DNA of the same molecular weight. A subset of the mutations was examined, including both transversions at positions 5 and 7 (Fig. 9(a), and (b), respectively), the G to T transversion at position 10, and the G to T and G to A changes at position 11. Data for the latter two changes were nearly identical with that for the G to C change at position 5, illustrated in Figure 9(b), while the G to T change at position 10 provided data similar to the G to T change at position 5. The data in Figure 9 therefore cover the range of effects observed. Although this sample is limited, it includes representatives from each mutation class. A good correlation is observed between the effects on recombination and the decrease in site-specific DNA binding. The wild-type DNA from pJFS39 promotes complex formation at lower protein concentrations than do the mutants that have decreased recombination activity. Furthermore, this DNA forms two discrete complexes of different mobilities. Most of the bound DNA is found in the slower migrating complex II. Presumably, these complexes represent binding of the FLP protein to either one or both of its binding sites (complex I or II, respectively). This result is substantiated by methylation protection experiments presented

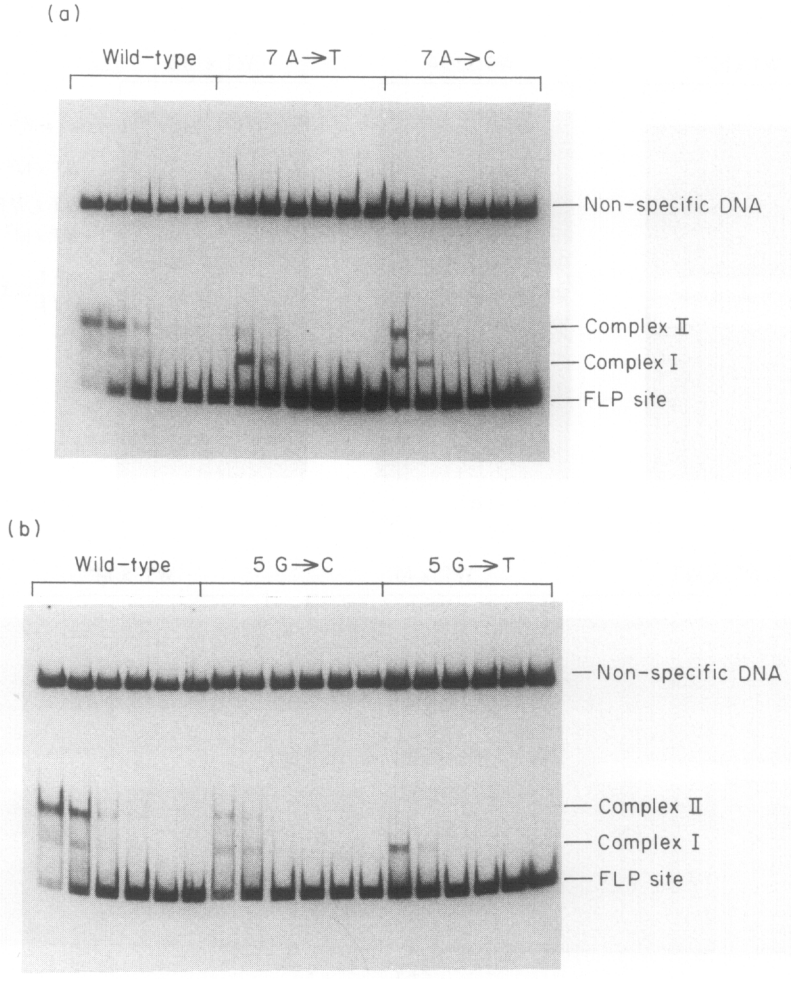


Figure 9. FLP protein binding to single point mutants. (a) Binding to substrates with mutations at position 7. Base changes in the substrate plasmids are indicated. The non-specific DNA is a 2505 base-pair fragment of the plasmid that does not contain the FLP recombination site. The 316 base-pair fragment containing the FLP site and FLP protein dependent complexes are indicated in the Figure. Each set of 6 reactions represents a dilution of FLP protein using 1, 0·5, 0·25, 0·1, 0·05 and 0·025 μl of an active FLP protein fraction from left to right. The final concentration of FRT sites was 0·5 nM in all cases. The highest protein concentration represents an approximate 300 : 1 ratio of FLP protein monomers to FRT sites, and the lowest an 8 : 1 ratio. (b) Binding to substrates with mutations at position 5. Base changes in the substrate plasmids are indicated. The fragments are labeled and the reactions were performed as described for (a). See the text for details.

below. These same complexes have been observed by Andrews *et al.* (1987). Substrates that have very low levels of recombination and contain a single altered binding site accumulate the more rapidly migrating complex I, with negligible production of complex II. This observation is consistent with the hypothesis that complex I represents binding to only one of the two FLP binding sites within the FRT site. Mutant sites that have decreased recombination activity would support efficient binding of the FLP protein only to the repeat that does not contain the mutation. Altered FRT sites that less severely affect recombination are competent in forming both protein complexes, although the relative fraction present as complex I is increased when compared to pJFS39.

To confirm this interpretation of the gel retardation experiments, we wished to determine directly if the formation of complex I in a mutant site reflected binding of FLP protein only to the non-mutant binding site. Methylation protection was used as a sequence-specific binding probe. Only one mutant (position −7, adenine to thymine) was treated and the results compared to pJFS39. The chosen mutant shows at least a 100-fold decrease in recombination activity and supports the formation only of complex I in the gel retardation assays described above. The results of these experiments

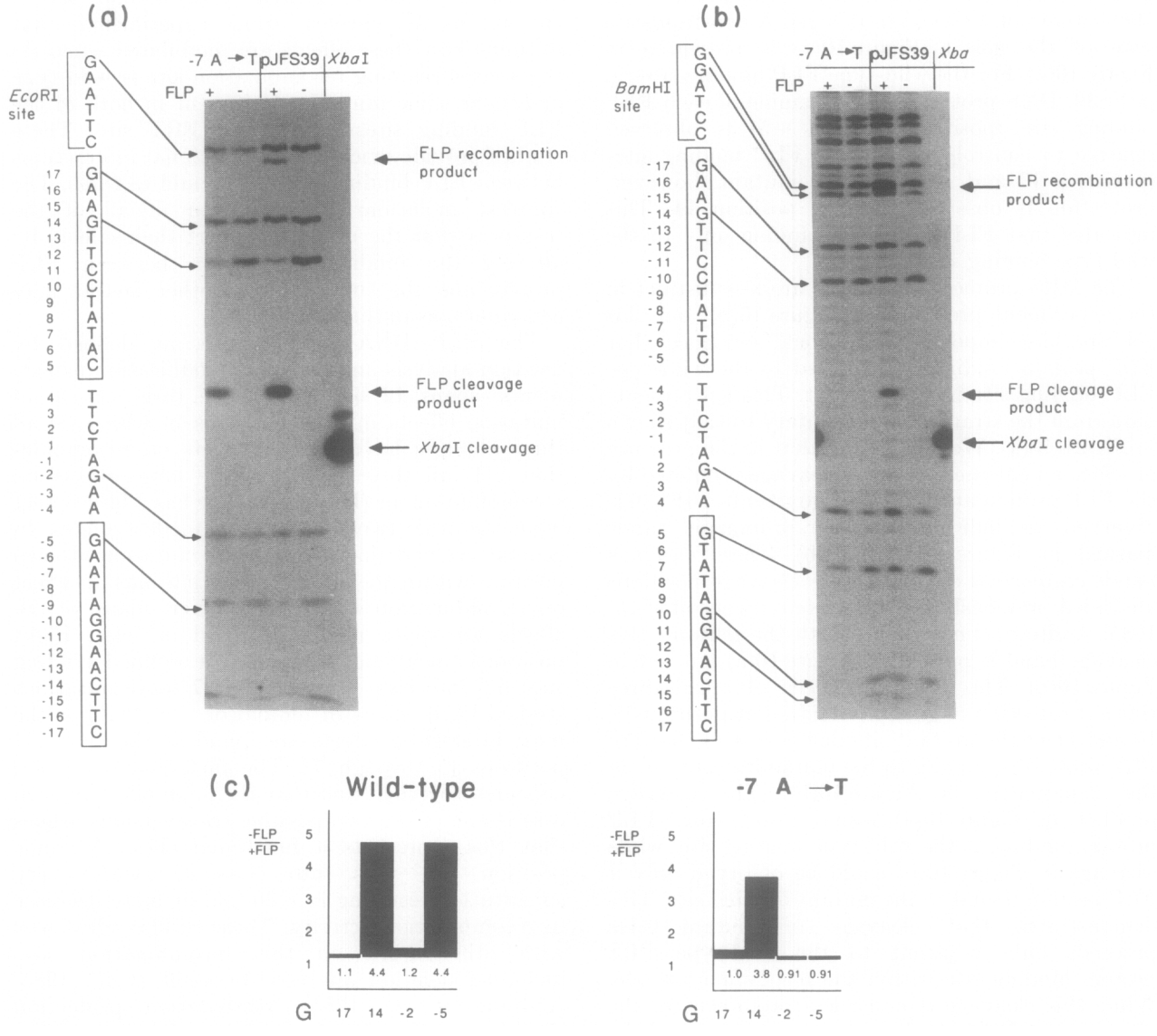


Figure 10. DMS protection assay for FLP protein binding to mutant recombination sites. Reactions were carried out as described in Materials and Methods. (a) Protection patterns for the strand illustrated in Fig. 1. Data for a wild-type site are compared to those for a mutant (A to T at position −7) that shows decreased recombination and gel retardation activity. Guanine residues are labeled in the diagram. Bands resulting from restriction enzyme cleavage by *Xba*I, cleavage by FLP protein and FLP-promoted recombination are noted. (b) Protection patterns for the complementary DNA strand. Labeling is as described for (a), above. (c) Quantification of protection as derived from densitometric scanning of autoradiographs. Scans are from the gel shown in (a). Band intensities were normalized to the guanine residue in the *Eco*RI site, which should be unchanged in the presence or absence of FLP. The numbers denote the relative intensities of the −FLP lanes to the +FLP lanes. Details are presented in Materials and Methods.

are shown in Figure 10. A *Xba*I digest of the substrate DNA is provided in each gel as an unambiguous marker for sequence orientation. The significant finding of these experiments was that a mutant site that promotes formation of only complex I in gel retardation experiments is protected from DMS methylation by the FLP protein only in the repeat that does not contain the altered sequence. In Figure 10 (a) and (c), we are looking at the protection pattern of four guanine residues within the recombination site, two of which (1 in each FLP binding site) are protected from DMS in the presence of FLP protein and two of which show no protection (Bruckner & Cox, 1986). Several other guanine residues that exhibited DMS protection in the earlier study (Bruckner & Cox, 1986) were not resolved in this gel. A densitometric scan of the gel in Figure 10(a) is presented in Figure 10(c). For the wild-type FLP binding sites in pJFS39, DMS protection of a guanine of each FLP binding site (position 14 and −5) is observed relative to controls outside the FLP binding sites (positions 17 and −2). For the mutant, however, protection is observed only at position 14. This indicates that FLP protein is binding only to the wild-type binding site.

The DMS protection patterns are less distinct in the experiment presented in Figure 10(b), but this gel provides important additional evidence that FLP protein binding is restricted to the wild-type FLP binding site in this mutant. This gel presents data from the strand complementary to that shown in Figure 10(a). We have found that in the presence of DMS, an enhanced level of cleavage promoted by the FLP protein at both cleavage sites in pJFS39 is observed, as indicated by the prominent bands marked in Figure 10(a) and (b). These cleavage bands correspond exactly to the cleavage positions identified previously for this system (Senecoff *et al.*, 1985; Andrews *et al.*, 1985). With the mutant, this cleavage band is present in Figure 10(a) but not in Figure 10(b). This result has been obtained three times. Protection of guanine residues at positions 5, 10 and 11 of the mutant site demonstrates that the FLP protein is binding to the non-mutant repeat in the experiment in Figure 10(b). The cleavage product in Figure 10(a) would result from FLP protein binding at the wild-type binding site, while cleavage in Figure 10(b) could be observed only if FLP protein bound to the mutant binding site. This demonstrates that cleavage of mutant sites proceeds only adjacent to the wild-type FLP protein binding site within the recombination site. While this cleavage is much less prominent in the absence of DMS (not shown), the specificity of the cleavage patterns provides strong evidence for FLP protein binding to the binding sites adjacent to each observed cleavage point. These data imply that complex I represents FLP protein bound to a single inverted repeat within the FRT site, and that complex II represents FLP bound to both repeats. No recombination products are observed in the protection experiments presented in Figure 10 for this mutant recombination site under these conditions, although recombination products are evident in the pJFS39 protection experiments.

4. Discussion

Two related sets of conclusions can be derived from this study. The first involves a general comparison of the effects of 36 FLP binding site mutations on recombination efficiency. The effects at 12 positions fall into three classes and provide clues that will help to guide continuing efforts to define protein–DNA interactions in this system. The results also provide a database that will facilitate further studies of this recombination site and the system in general. The second set of conclusions is derived from experiments that examine how these effects are modulated when (1) the second reacting recombination site is wild-type or (2) the same mutation is present in both of the FLP binding sites of a single FRT site. These studies provide evidence for functional interactions between FLP binding sites *in cis* and *in trans*. The simplest molecular interpretation of all of the results is that the mutations exert their effects by altering the binding interaction between FLP protein and the site, although other factors may contribute, as outlined below.

The FLP DNA binding site, as defined by deletion analysis and chemical modification experiments, spans 12 base-pairs. The three classes of mutation effects involve positions in which (I) all three changes have modest effects on recombination, (II) all three changes have large effects on recombination, or (III) one change has a large effect while the other two changes have modest effects. In general, we find that at least one mutation at each position within the FLP binding site has an effect on recombination *in vitro*. However, many of the effects are quite modest (10-fold or less). Eight positions (including 1 outside the defined binding site) fall into class I, and 31 of 37 total mutations studied have effects of tenfold or less. Perhaps the most interesting effects are found at the class III positions (5 through 7). The fact that these are clustered together, and that the large effect in each case is one of the two possible transversions argues that this represents a functional class. A single position, 11, falls into class II, with every substitution resulting in a 20-fold or more decrease in recombination activity. These results agree well with other work on this recombination site, including deletion analysis (Senecoff *et al.*, 1985; Andrews *et al.*, 1985), methylation protection (Bruckner & Cox, 1986), and other mutation studies (Andrews *et al.*, 1986; McLeod *et al.*, 1986; Prasad *et al.*, 1986). When combined with data on mutations in the spacer (Senecoff & Cox, 1986; Andrews *et al.*, 1986; S. Umlauf & M. Cox, unpublished results), it is evident that the recombination site, in general, exhibits a surprising degree of sequence flexibility.

Two of these positions, 6 and 10, require further comment. Positions 10 and 11 were both identified

as prominent purine contacts in methylation protection studies. The results of mutations at position 11 from several laboratories correspond well to this assignment. Position 10, in contrast, has been designated an incidental contact point on the basis of the negligible effect of a transition mutation (Prasad *et al.*, 1986). Our results suggest that this position plays a somewhat more important role in recognition, based on the effects of the transversions. The G to T transversion in particular has a modest but significant effect as a single mutant, and this effect is increased more than an order of magnitude when it is present in both FLP binding sites. Position 6 is a point of asymmetry in the wild-type recombination site. The A to T transversion that has the large effect here (40-fold) is the change that restores symmetry between positions 6 and −6. A T to A transversion at position −6 that also resotres symmetry, such that adenine is present in both positions, was previously shown to have no effect on the reaction (Senecoff *et al.*, 1985). Our results suggest that these transversions are analogous to the G to T transversion at position 10. The wild-type site can be thought of as having a mutation at position −6, which is tolerated because it has a negligible effect on recombination. The A to T transversion at position 6, however, generates what is effectively a symmetrical double mutant. The large effect observed would then be analogous to the effects observed for the double G to T trasversion at 10 and −10. We note that all of the single mutants generated in this study were linked to a "wild-type" FLP binding site containing thymine at position −6 rather than adenine. It is possible that this enhanced the deleterious effects of some mutants.

The greater than additive effects observed when some mutations are placed in both FLP binding sites within an FRT site rather than in one argue for a functional *in cis* interaction between FLP binding sites. This result is seen for the G to T and A to T transversions in positions 10 and 6, respectively, described above. Similar effects have been observed at position 9 (Prasad *et al.*, 1986). A functional interaction between FLP binding sites *in trans* is indicated by the fact that the reactivity of virtually all single mutants is improved when the reacting partner is a wild-type recombination site. Two additional results suggest that the *in cis* interaction is more important than the *in trans* interaction. First, we have observed no orientation effects in reactions between two recombination sites with single mutations. Any orientation effect observed would have to reflect an *in trans* interaction. Second, we do not observe the positive *in trans* effect of a wild-type recombination site when the mutant site is a double mutant.

All of the data in this study are consistent with the idea that the mutations exert their effects by altering the binding interaction with FLP protein. In particular, we note the strong correlation (in the 7 mutants tested) between the effects of mutations on recombination and the binding efficiency of FLP protein. For one deleterious mutation, A to T at position 7, we further demonstrate that FLP protein binding is largely restricted to the unaltered FLP binding site. The *in cis* and *in trans* interactions noted above are most easily explained by positive (co-operative) effects on binding mediated by protein–protein interactions within the recombination complex.

The resulting model for the reaction is illustrated in Figure 11. The minimal recombination complex contains four FLP protein binding sites in the two

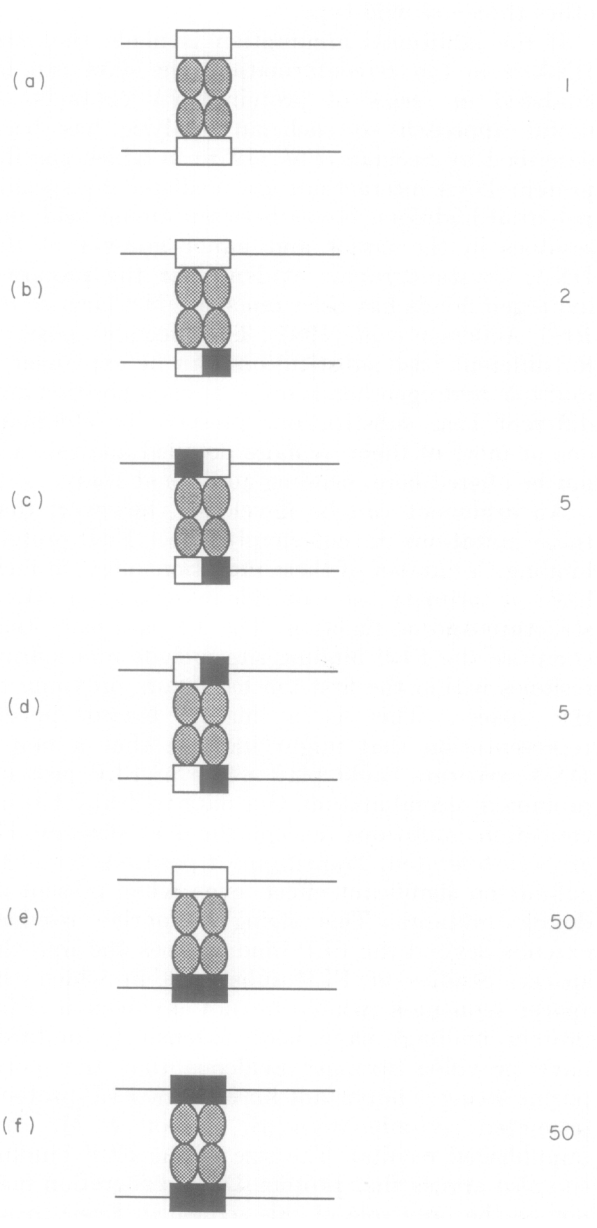


Figure 11. Suggested structure for a complex between 2 FLP : DNA substrates. Filled boxes represent a mutant FLP binding site, open boxes represent wild-type binding sites. Note that this representation is different from that in Fig. 3. Ovals represent active FLP protein monomers that bind to each inverted repeat within the recombination site. The numbers to the right are the relative amounts of FLP protein required for a minimum reaction with recombination sites containing a G to T change at position 10 or both 10 and −10.

aligned recombination sites. We hypothesize that an FLP monomer must be bound to each binding site for a productive reaction to occur. The effects of mutations at various positions in the complex are illustrated in Figure 11 for the G to T transversion at position 10. The relative importance of *cis* and *trans* interactions are evident here in the larger effects of two mutations when they are present *in cis* rather than *in trans*. The effect *in trans* is nevertheless evident in the finding that virtually any mutation can be tolerated in one binding site without abolishing recombination, as long as the other three are wild-type.

If the additional assumption is made that the DNA is in the *B* conformation, the data can be analysed in terms of protein–DNA contacts. A useful approach to such an analysis has been described by Seeman *et al.* (1976) in which specific protein–DNA interactions are mediated by specific potential hydrogen bonds between amino acid and residues in the major and minor grooves of the DNA. Crystallographic evidence for the proposed hydrogen bonds has been reported (McClarin *et al.*, 1986; Anderson *et al.*, 1987). The effects we observe for different base substitutions can be explained if multiple hydrogen bonds occur at each position and different base substitutions preserve or eliminate one or more of them. A more detailed analysis will not be offered here, pending additional study.

An argument can be developed, however, that these mutations do not simply affect FLP protein binding. A number of these mutations may, in fact, have a primary or coincident effect on DNA structure and/or function. The 12 base-pairs that comprise the FLP binding site include nine purine residues within the first ten base-pairs proximal to the spacer. This is a highly biased purine representation that might indicate that a non-*B* DNA structure could play a role in FLP protein-promoted recombination. Our data indicate that all transition mutations (except those at position 11) are well-tolerated. Transitions at positions 5 and 10 exhibit no significant effect, even when present as double mutants. This string of purine residues extends beyond the FLP binding sites and into the spacer. Studies of FLP substrates in which the spacer sequences (which are not involved in FLP protein binding) have been extensively mutated have provided stronger evidence that this poly-purine string is important for efficient FLP protein-promoted recombination (S. Umlauf & M. Cox, unpublished results). The size of the FLP binding site also argues that protein–DNA recognition may not be the only role of this sequence. Every base-pair in the FLP binding site provides seven potential interaction sites in the form of hydrogen bond donors or acceptors, four in the major groove and three in the minor (Seeman *et al.*, 1976; McClarin *et al.*, 1986). A high degree of specificity can be achieved with a sequence of four to six base-pairs (McClarin *et al.*, 1986). The functional significance of the sequence structure in the FRT site requires further study.

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