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Specific Contacts between the FLP Protein of the Yeast 2-Micron Plasmid and Its Recombination Site*

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Contact points between the FLP protein of the yeast 2-μm plasmid and its recombination site have been defined. Important features of the region previously defined as the minimal recombination site *in vitro* include a pair of 13-base pair inverted repeats separated by an 8-base pair spacer. The two FLP protein-binding sites within this region are 12 base pairs in length. In each case they include the internal 11 base pairs of one of the 13-base pair repeats, as well as the adjacent base pair within the spacer. The internal 6 base pairs within the spacer are not involved in binding or recognition by FLP protein. When the size of the spacer is increased or decreased by one base pair, the distance between the cleavage points is also increased or decreased correspondingly by one base pair. Points of cleavage are unaffected by changes in the spacer sequence. Specific contact points involving purine residues, identified by methylation protection and recombination interference experiments, are located in both the major and minor grooves of the DNA. Additional contact points between FLP protein and phosphate groups in the phosphate-deoxyribose backbone are clustered near the cleavage sites.

The 2-μm circle is an autonomously replicating plasmid which is found in many strains of yeast *Saccharomyces cerevisiae* (1). This 6318-base pair plasmid has been sequenced (2), and encodes a site-specific recombination system that serves to invert two sets of unique sequences on the plasmid. The recombination event occurs at defined sites within two 599-base pair repeated sequences which are arranged in inverted orientation (3).

The gene encoding the recombinase, designated FLP, has been cloned and expressed in *Escherichia coli* (4, 5). Using this source, efficient *in vitro* systems have been established for this recombination event (6, 7), and the protein has been extensively purified (8).[1] One primary focus of research to date has been the recombination site.

The minimal recombination site consists of parts of two 13-base pair sequences arranged in inverted orientation separated by an 8-base pair spacer. Optimal recombination requires only the 10 base pairs of each 13-base pair repeat that are adjacent to the spacer (9). A third 13-base pair repeat lies adjacent to the minimal site but is not required for *in vitro* recombination. This larger site is protected by FLP protein from DNase digestion (10). The function of the third repeat sequence is unknown.

Recent studies have shown that the spacer sequence is not directly recognized by FLP protein (11). A variety of changes in the spacer sequence are well tolerated by this recombination system. The spacers of two sites undergoing recombination, however, must be homologous for efficient recombination to occur (11). The homology dependence implies that DNA-DNA pairing occurs between spacer sequences during recombination in this system. This DNA-DNA pairing affects not only the efficiency of the reaction but the outcome as well. The asymmetry of the site is defined by the spacer sequence, and proper alignment of two sites in the same orientation determines the directionality (*e.g.* inversion *versus* deletion) of the recombination event. Sites with a symmetrical spacer are fully functional but can be aligned in either orientation during recombination and exhibit a complete loss of directionality (11). FLP protein cleaves this site at the boundaries of the spacer, leaving 8-base pair staggered cuts, and becomes covalently attached to the DNA via a 3′ phosphotyrosine linkage (9, 10, 12).

Since the spacer sequence is not recognized by FLP protein, it is likely that the flanking repeats represent the binding sites for FLP protein. Alteration of sequences within these repeats results in a large decrease in the efficiency of recombination at that site which is not affected by homology. Furthermore, the size of the spacer can be altered by ±1 base pair with modest loss of site function, whereas an increase of 2 base pairs in spacer size abolishes site function. These results suggest that FLP protein binds to both of the short repeats flanking the spacer and that important protein-protein interactions occur between the proteins bound to each repeat. The present work was undertaken to further define specific DNA contact points involved in recognition and binding by the FLP protein.

MATERIALS AND METHODS

Plasmids and Enzymes—Plasmids pJFS36, pJFS35r, pJFS37r, pJFS37b, pJFS122, and pJFS39 were constructed as described previously (9, 11). All plasmids were purified from the *E. coli* strain HB101 (13) by banding twice in CsCl gradients in the presence of ethidium bromide. DNA concentrations are given in terms of total DNA molecules or fragments. Since there is always one recombination site per molecule or fragment, 1 pmol of DNA fragments = 1 pmol of recombination sites. FLP protein used in this study was partially purified by a method which will be described in detail elsewhere.[1] The procedure involves cell lysis and fractionation of the cleared lysate using Bio-Rex 70 (Bio-Rad). In some cases fractions were also subjected to size fractionation using Fractogel TSK HW-50F (EM Reagents). FLP protein fractions used were in all cases approximately 5% pure. The band corresponding to FLP protein has been identified

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[1] L. Meyer-Leon, C. Gates, J. Attwood, and M. M. Cox, unpublished results.

by N-terminal sequencing.[1] Amounts of FLP protein reported in the text represent estimates calculated from determinations of purity (based on densitometric scanning) carried out on each fraction used in this study. The large fragment of *E. coli* DNA polymerase I was purchased from Boehringer Mannheim. Restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs, Bethesda Research Laboratories, and International Biotechnologies, Inc. (New Haven, CT). [α-^{32}P]dATP (≥600 Ci/mmol) was purchased from New England Nuclear. Dimethyl sulfate was purchased from Aldrich.

DNA Manipulations—Restriction digests were performed as described (13). DNA restriction fragments were 3′ end-labeled with α-^{32}P-labeled dATP and the large fragment of *E. coli* DNA polymerase I as described (13) except that *Bam*HI ends were labeled in the presence of 100 μM unlabeled dGTP. Labeled restriction fragments were isolated from polyacrylamide gels as described (14). Nondenaturing polyacrylamide gel electrophoresis was carried out in Tris/acetate/EDTA buffer (13). Chemical sequencing reactions were carried out following a modification of the published procedure (14, 15).

Electrophoresis under denaturing conditions was performed by resuspending appropriately treated DNA samples in the indicated volume of buffer X (80% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/w) xylene cyanol, 0.1% (w/w) bromphenol blue) (14). After denaturation at 90 °C for 2 min, the samples were cooled in ice water and electrophoresed in a 12% (w/v) polyacrylamide gel containing 8.3 M urea. In this case a Tris/borate/EDTA electrophoresis buffer (13) was employed.

Determination of Recombination Site Cleavage Points—A *Bam*HI-*Pst*I (792 base pairs) or an *Eco*RI-*Sph*I (316 base pairs) restriction fragment containing the altered FLP recombination site was uniquely 3′ end-labeled at either the *Bam*HI or *Eco*RI end, respectively. The sequences required for FLP protein-mediated site-specific recombination in all cases are located within 40 base pairs of the labeled end. Approximately 0.2 pmol of labeled DNA were incubated with an FLP protein fraction (0.5 μg of total protein, approximately 20 FLP protein monomers/recombination site) in 200 μl of buffer A (200 mM NaCl, 30 mM Tris-HCl, 80% cation (pH 7.5), 5% (v/v) glycerol, 1 mM EDTA) for 30 min at 30 °C. Reaction was stopped by addition of sodium dodecyl sulfate and proteinase K (to 0.1% and 1 mg/ml final, respectively) followed by incubation at 37 °C for 30 min. Following this incubation, 200 μl of 0.6 M sodium acetate were added and the mixture extracted sequentially with phenol/chloroform (1:1, v/v) and chloroform. The DNA was precipitated by addition of 1.0 ml of ethanol and washed twice with 70% ethanol and dried under vacuum. Controls were included in each experiment in which 0.1 pmol of labeled DNA in 100 μl of buffer A was treated in a similar fashion except that the FLP protein was omitted. The control samples were resuspended in 2 μl of buffer X and set aside.

The FLP protein-treated sample was resuspended in 70 μl of denaturation buffer (20 mM Tris-HCl, 4% cation (pH 9.5), 1 mM spermidine, 0.1 mM EDTA) (16), heated to 90 °C for 2 min, and cooled in ice water. Twenty μl of kinase buffer (250 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 25 mM dithiothreitol, 25% (v/v) glycerol) (16), and 10 μl of 200 μM ATP were added. The sample was divided into two 50-μl aliquots. T4 polynucleotide kinase (1 μl, 13 units total) was added to one sample, which was then incubated at 37 °C for 30 min. To this was added 50 μl of TE buffer (10 mM Tris-HCl, 80% cation (pH 7.5), 1 mM EDTA) and 100 μl of a solution containing 0.6 M sodium acetate and 50 μg/ml calf thymus DNA. The samples were precipitated and dried as described above and resuspended in 2 μl of buffer X. Additional samples of the same uniquely 3′ end-labeled DNA fragment were subjected to Maxam-Gilbert sequencing reactions or to digestion by the restriction enzyme *Xba*I. All samples were then electrophoresed on the same polyacrylamide gel under denaturing conditions. After electrophoresis, the gels were autoradiographed at −70 °C with an intensifying screen.

Methylation Protection—The protocol employed in these experiments is a modification of a procedure described elsewhere (17). Approximately 0.05 pmol of a uniquely 3′ end-labeled DNA containing an FLP recombination site near the labeled end was incubated for 5 min at 20 °C, with or without an aliquot of FLP protein (5 μl, approximately 0.25 μg of FLP protein assuming 5% purity, a ratio of approximately 100 FLP protein monomers/recombination site), in buffer B (50 mM sodium cacodylate (pH 8.0), 1 mM EDTA). Reaction volumes (200 μl) also included 200 mM NaCl, 5% (v/v) glycerol, and 2 μg of sonicated calf thymus DNA. Dimethyl sulfate (1 μl of a 10.7 M solution) was added and the incubation continued at 20 °C for 5 min. Next, 300 μl of ice-cold phenol and 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) were added. Samples were vortexed and centrifuged briefly in a microfuge. The aqueous phase was removed and precipitated with ethanol. The pellet was washed twice with 70% ethanol and dried under vacuum. The samples were then resuspended in 100 μl of 1.0 M piperidine and incubated as in the chemical sequencing procedures (14). To determine the level of methylation-independent DNA cleavage, 0.05 pmol of the 3′ end-labeled DNA was incubated under identical conditions for 10 min at 20 °C with the same amount of FLP protein but without addition of dimethyl sulfate. The sample was then treated as described above. Additional samples of the same 3′ end-labeled DNA were treated with *Xba*I to provide an additional marker, and all samples were electrophoresed as described above.

A similar experiment was performed to determine adenine contacts except that 0.1 pmol of 3′ end-labeled DNA and 2 μl of the dimethyl sulfate solution were used. These samples were then subjected to the Maxam and Gilbert G > A reaction (22). This procedure provided a low enough background of cleavage at purine positions (in the absence of dimethyl sulfate) to detect methylation protection at adenines. Determination of extents of protection of adenine residues was accomplished with the aid of densitometric scanning of autoradiograms.

Methylation Interference—This experiment is based on the method of Siebenlist and Gilbert (18). Approximately 2.5 pmol of a *Bam*HI-*Pst*I restriction fragment of pJFS39 DNA were uniquely 3′ end-labeled at the *Bam*HI end as described above. This treatment places the modified pJFS39 recombination site within 40 base pairs of the labeled end. This DNA was partially methylated at the purine positions with dimethyl sulfate. First, the labeled DNA was resuspended in 200 μl of buffer B containing 10 mM $MgCl_2$ (18, 19). Dimethyl sulfate (1 μl of a 10.7 M solution) was added, and the mixture was incubated at 20 °C for 3 min. Stop solution (50 μl) was added. The sample was then vortexed briefly, ethanol precipitated, washed with 70% ethanol 3 times, and dried under vacuum. After setting aside several aliquots for controls, the methylated DNA was employed as a substrate for an FLP-mediated site-specific recombination reaction. Reactions were carried out in 100 μl of buffer A for 30 min at 30 °C, employing an amount of FLP protein empirically determined to produce an optimal reaction.

The FLP recombination site in plasmid pJFS39 has an 8-base pair symmetrical spacer. This symmetry abolishes the polarity of the FLP recombination site (11). A small head to head recombination product (42 base pairs) resulting from recombination of two identical ends was produced. This small product was separated from the larger parent DNA molecule on a nondenaturing 10% polyacrylamide gel. It was then eluted from the gel (14), ethanol precipitated twice, washed with 70% ethanol, and dried under vacuum. The sample was resuspended in 100 μl of 1 M piperidine, incubated at 90 °C (which results in cleavage at methylated guanine residues (14)), and prepared for electrophoresis. Additional samples of the same end-labeled and methylated pJFS39 DNA were subjected directly to cleavage in piperidine or to cleavage by *Xba*I to provide markers. All samples were electrophoresed on the same denaturing polyacrylamide gel as described above.

Ethylation Interference—Approximately 2.0 pmol of a *Bam*HI-*Pst*I restriction fragment of pJFS39, uniquely 3′ end-labeled at the *Bam*HI end as described above, were partially ethylated at the phosphate positions by treatment with ethylnitrosourea. The labeled DNA was resuspended in 100 μl of buffer B. An equal volume of a saturated solution of ethylnitrosourea in 95% ethanol was added (18, 19). The mixture was incubated at 50 °C for 90 min. Stop solution (50 μl) was then added. The ethylated DNA was ethanol precipitated, washed 3 times with 70% ethanol, and dried under vacuum. This DNA was then resuspended in 100 μl of buffer A, and 0.25 μg of FLP protein were added. The mixture was incubated at 30 °C for 30 min. The resulting DNA was ethanol precipitated, dried, and resuspended in 20 μl of TE buffer. After the addition of 5 μl of a solution containing 30% glycerol, 0.25% xylene cyanol, 0.25% bromphenol blue, and 50 mM EDTA, samples were electrophoresed on a nondenaturing 10% polyacrylamide gel. Gel bands were visualized by autoradiography and eluted from the gel. The DNA was ethanol-precipitated twice, washed with 70% ethanol, and dried under vacuum. Both the 42-base pair head-to-head recombination product and the original labeled fragment were eluted from the gel. Each DNA sample was resuspended in 85 μl of a solution containing 20 mM sodium acetate and 1 mM EDTA. NaOH (15 μl of a 1.0 M solution) was added, and the mixture was incubated at 90 °C for 30 min. HCl (15 μl of a 1.0 M solution) and 50 μl of 20 mM Tris-HCl (pH 7.5) were added (19).

Samples were precipitated with butanol, resuspended in 150 μl 1% sodium dodecyl sulfate, reprecipitated with butanol, washed with 95% ethanol, and dried under vacuum (15). Samples were then resuspended in 2 μl of buffer X. Additional samples of the same labeled DNA fragment were subjected to the Maxam-Gilbert G reaction (14), or digestion by *Xba*I to provide markers. All samples were electrophoresed on the same polyacrylamide gel under denaturing conditions as described above.

RESULTS

Cleavage of Altered Recombination Sites—The FLP protein has been shown *in vitro* to cleave its recombination site at the junction of the spacer and the short inverted repeats. The FLP protein becomes covalently attached to a 3′ phosphate at the cleavage point and leaves a free 5′ hydroxyl group (9, 10). The cleavage points are staggered by 8 base pairs and have 5′ protruding termini.

A variety of altered recombination sites were constructed to examine the function of the spacer sequence (9, 11). Each of the alterations are within the spacer, and a number of those used in this study (Fig. 1) alter the size of the spacer. Each of these sites is functional in recombination, although with decreased efficiency in some cases (11). To provide further information concerning the function of sequences within the recombination site, the effect of these changes on the pattern of cleavage by FLP protein was examined.

During construction of these recombination sites, *Bam*HI and *Eco*RI restriction sites were placed immediately adjacent to each recombination site on the left and right side, respectively, as the sites are presented in Fig. 1. Restriction fragments containing the FLP site were uniquely 3′ end-labeled at either of these restriction sites. Incubation of this labeled DNA with an FLP protein fraction resulted in a small amount of a unique FLP cleavage product. The exact location of cleavage within the recombination site is determined by comparing the migration of the FLP cleavage product with the migration of DNA fragments produced by the Maxam-Gilbert sequencing reactions. These were performed on a separate (non-FLP protein-treated) sample of this end-labeled DNA and electrophoresed on the same denaturing polyacrylamide gel. A sample of the same DNA cleaved at the *Xba*I restriction site within the spacer was also run on the gel to provide an additional reference point.

Representative results are presented in Figs. 2 and 3 and a summary of all cleavage points is presented in Fig. 1. In each case cleavage is observed at the junction of the spacer and the flanking repeats, regardless of the spacer alteration involved. Each of the cleavage products also has a free 5′ hydroxy terminus as observed for the FLP cleavage product of the unaltered recombination site. This is demonstrated by treating the cleavage product with T4 polynucleotide kinase and ATP. This treatment will phosphorylate the DNA if a free 5′ hydroxyl is available. The phosphorylated cleavage product should migrate more rapidly through the polyacrylamide gel. This is shown in Fig. 2, *lane 4* and Fig. 3, *lane 5*. Two bands are present because the kinase reactions did not proceed to completion in these two experiments.

The Maxam-Gilbert sequencing reactions product DNA fragments with 5′ phosphate termini. The phosphorylated FLP cleavage product comigrates in each case with a Maxam-Gilbert sequencing fragment that corresponds to the cytidine in the recombination site sequence located on the repeat side of the repeat-spacer junction. Since the Maxam-Gilbert-sequencing reactions destroy the modified cytidine, this sequencing fragment actually terminates at the preceding thymine, which is on the spacer side of the junction. Thus, the FLP protein-mediated cleavage is always at the junction of the spacer and the 13-base pair repeats. The *Xba*I cleavage product included in each experiment provides an additional point of reference, confirming this assignment in every case.

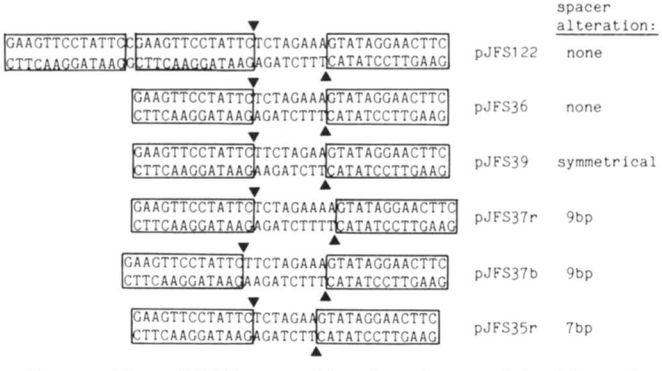


FIG. 1. **Altered FLP recombination sites used in this study.** Location of FLP protein cleavage points are indicated by ▲ symbols. An *Xba*I restriction site (recognition sequence T:CTAGA), present in all the spacer sequences, is utilized to provide size markers in many of these experiments. The *Bam*HI and *Eco*RI restriction sites used to label DNA fragments in this study lie immediately adjacent to the site on the *left* and *right* sides, respectively, as written (9). All plasmids used in this study are identical except for the site alterations illustrated. See "Materials and Methods" for details.

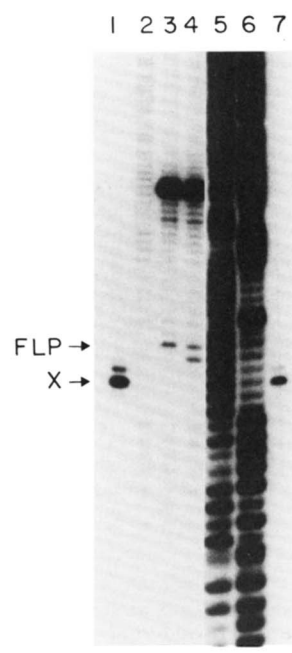


FIG. 2. (*left*). **Cleavage of the altered FLP recombination site of pJFS39 by FLP protein.** Reactions were carried out as described under "Materials and Methods," employing an *Eco*RI-*Sph*I restriction fragment of pJFS39, 3′ end-labeled at the *Eco*RI end. Lane identification: *lane 1*, marker fragment produced by an *Xba*I restriction digest; *lane 2*, −FLP protein; *lane 3*, +FLP protein; *lane 4*, +FLP protein, followed by T4 polynucleotide kinase and ATP; *lane 5*, C+T sequence markers; *lane 6*, A+G sequence markers; *lane 7*, *Xba*I digest marker as in *lane 1*. × and *FLP* denote positions of *Xba*I and FLP cleavage products, respectively. A prominent band which migrates approximately 16 positions above the FLP cleavage product in *lanes 3* and *4* is the head to head recombination product described in the text.

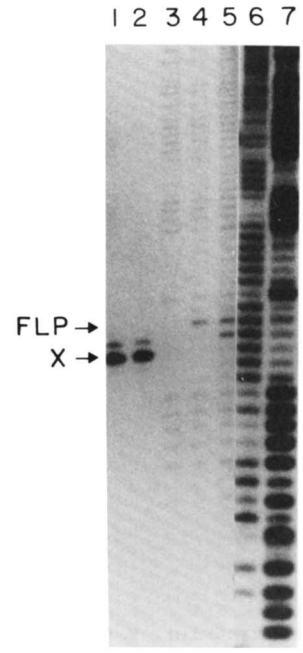


FIG. 3. (*right*). **Cleavage of the altered FLP recombination site of pJFS35r by FLP protein.** Reactions and symbols are described under "Materials and Methods" and the legend to Fig. 2. Lane identification: *lanes 1* and *2*, marker fragment produced by an *Xba*I restriction digest; *lane 3*, −FLP protein; *lane 4*, +FLP protein; *lane 5*, +FLP protein, followed by T4 polynucleotide kinase and ATP; *lane 6*, C+T sequence markers; *lane 7*, A+G sequence markers.

The minor band running above the *Xba*I cleavage product in Figs. 2 and 3 is a secondary product of the 3′ end-labeling reaction.

The recombination site in pJFS39 has an 8-base pair spacer with dyad symmetry which effectively eliminates the polarity of the entire site. This spacer matches the wild type spacer in pJFS36 at only 3 of 8 positions. The alterations have the effect of shifting the *Xba*I restriction site in the spacer to the right (as presented in Fig. 1) by one base pair without altering the size of the spacer or the sequence of the spacer-repeat junctions. This shift in spacer sequence has no effect on the point of cleavage by FLP protein. The cleavage point instead remains constant relative to the repeats. This indicates that the repeat sequences rather than the spacer serve as determinants of the FLP cleavage points and suggests that FLP protein recognizes and binds to the repeats.

This conclusion is strengthened by results derived from a series of mutations which alter the size of the spacer. These include two mutations which result in 9-base pair spacers and one which results in a 7-base pair spacer. The former, pJFS37b and pJFS37r, have an insertion of an A/T base pair at the left and right ends of the spacer, respectively, as illustrated in Fig. 1. The other mutant, pJFS35r, has a deletion of one base pair from the right end of the spacer. The sequence at the spacer-repeat junction is unaltered in each case. Again, the cleavage occurs at the spacer-repeat junction (Figs. 1 and 3). The cleavage points of pJFS37b and pJFS37r are staggered by 9 base pairs rather than 8 base pairs, and cleavage of pJFS35r is staggered by 7 base pairs. The cleavage points remain constant relative to the 13-base pair repeats and are unaffected by the spacer alterations.

The symmetrical spacer in pJFS39 eliminates the polarity of the recombination site and permits head-to-head alignment and recombination (11). A prominent band present in *lanes 3* and *4* in Fig. 2, in which the DNA has been treated with FLP protein, migrates in the position expected for a small recombination product resulting from this type of recombination. This recombination event does not occur in pJFS36. This band indicates that FLP protein-mediated recombination is occurring under these conditions.

The mutant recombination site of pJFS37b is equivalent to an insertion of an A/T base pair into the left end of the spacer of pJFS39. Eight of the nine base pairs in the pJFS37b spacer are equivalent to pJFS39. A minor band that migrates at a position that would correspond to a head-to-head recombination product is observed in the cleavage reaction for pJFS37b (data not shown). Head-to-head alignment in this case would result in two single mismatches located at either ends of the spacers. This reaction has not been observed in the standard *in vitro* recombination system, although a modest tolerance for spacer mismatches has been observed (11).

Methylation Protection—The FLP protein protects both inverted repeats and the spacer from digestion by DNase (10). A third 13-base pair repeat which is not required for recombination *in vitro* is also protected. The following experiments were carried out to begin to define specific contacts between the FLP protein and sequences within this recombination site.

To simplify discussion, a numbering system is presented in Fig. 4 and in subsequent figures to aid in identification of contact points. The system differs from systems used previously (9, 10) in that the origin is set at the center of the spacer rather than at the center of the *Xba*I site. This facilitates discussion of residues in the repeats, since corresponding positions in either repeat now have the same number. The repeats occupy positions 5 to 17 and −5 to −17 in this system,

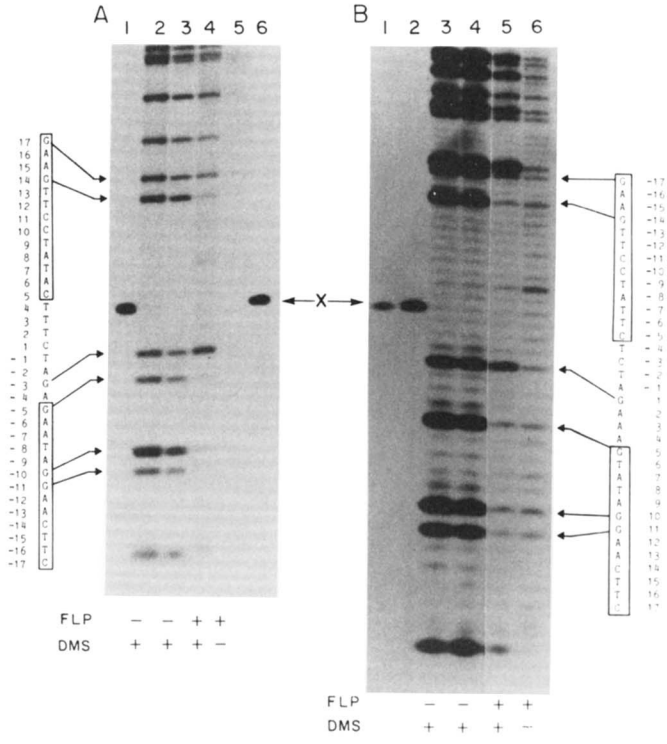


FIG. 4. **Dimethyl sulfate (*DMS*) methylation—protection of guanine residues in the FLP recombination site of pJFS36 by FLP protein.** Reactions were carried out as described under "Materials and Methods." × denotes the *Xba*I cleavage product. *A*, experiments employing a *Bam*HI-*Pst*I restriction fragment of pJFS36, 3′ end-labeled at the *Bam*HI site. Lane identification: *lane 1*, marker fragment produced by an *Xba*I restriction digest; *lane 2*, G sequence markers; *lane 3*, +DMS, −FLP protein; *lane 4*, +DMS, +FLP protein; *lane 5*, −DMS, +FLP protein; *lane 6*, *Xba*I digest as in *lane 1*. *B*, experiments employing an *Eco*RI-*Sph*I restriction fragment of pJFS36, 3′ end-labeled at the *Eco*RI site. Lane identification: *lanes 1* and *2*, marker fragment produced by an *Xba*I restriction digest; *lane 3*, G sequence markers; *lane 4*, +DMS, −FLP protein; *lane 5*, +DMS, +FLP protein; *lane 6*, −DMS, +FLP protein.

and the numbering proceeds from −17 at the left to 17 on the right in pJFS36 as the sequence is presented in Fig. 1. All plasmids are constructed (11) so that a *Bam*HI site immediately flanks the "−" repeat and an *Eco*RI site flanks the "+" repeat.

Protection of guanine residues within the recombination site from methylation to dimethyl sulfate was determined first. In these experiments, an aliquot of an FLP protein fraction is incubated with a 3′ end-labeled restriction fragment which has a recombination site near the labeled end. Dimethyl sulfate is then added. The N-7 position of guanine and the N-3 position of adenine will be methylated by dimethyl sulfate unless it is blocked by close contact with the FLP protein. Subsequent treatment breaks the DNA sugar-phosphate backbone at positions restricted to methylated guanines under the conditions employed in these experiments. A reduced amount of cleavage at a position indicates that the base is in close contact with FLP protein.

The plasmid pJFS36, which contains a site with an 8-base pair unaltered spacer, was treated with dimethyl sulfate in the presence of FLP protein (Fig. 4, *A* and *B*). Protection patterns were obtained for both DNA strands. Protection was observed for all guanines within a 10-base pair region of the 13-base pair repeats immediately adjacent to the spacer, positions 5–14 (−5 to −14). This 10-base pair sequence contains G/C base pairs at either end (one at the repeat-spacer junction) and a doublet of Gs approximately midway between.

Notably, little or no protection was observed for guanines located at the outer ends of the repeats, position 17 (−17). Neither of the guanines in the spacer, one on the top strand and one on the bottom strand (at positions 1 and −3), are protected. Methylation at position −3, in fact, is enhanced relative to methylation in the absence of FLP protein (Fig. 4*A*).

The protection pattern was also determined for a recombination site (plasmid pJFS122) which has all three 13-base pair repeats. The FLP protein protection pattern for the third repeat, which is not required for recombination, is similar to that of the other two repeats (Fig. 5). Guanines within the same 10-base pair sequence are protected in a manner analogous to the other two repeats. In contrast, the guanine which lies between the two direct repeats at position −18 is protected only slightly.

The situation in the symmetrical spacer mutant pJFS39 is very similar. The same guanine residues in the 10 base pairs of the 13-base pair repeats proximal to the spacer are protected from methylation (Fig. 6*A*). The guanines on either strand in the spacer are not. The guanines in the pJFS39 spacer occupy positions 2 and −2, whereas the guanines in the pJFS36 spacer occupy positions 1 and −3. In this case, neither appears to be enhanced. Changing the sequence of the spacer has no effect on the protection pattern within the repeats.

The experiments described above indicate that the interaction of FLP protein with the pJFS39 recombination site is identical to the interaction within the pJFS36 site in terms of cleavage points and methylation protection of guanine residues. The sequence of the repeat-spacer junctions is identical in both sites. The 5-base pair changes in pJFS39 are restricted to the internal six base pairs of the spacer. Previous results have shown that the efficiency of recombination observed *in vitro* with pJFS39 is comparable to that observed with an unaltered site as long as the reaction is restricted to sites with identical spacers (11). The remainder of the experiments in this study use pJFS39.

By altering conditions of the purine cleavage reactions as described under "Materials and Methods," methylation protection can be examined for both guanine and adenine residues (Fig. 6*B*). The data in Fig. 6*B* was quantitated by densitometric scanning. Several bands derived from nucleotides outside the recombination site (at the *top* of the figure as shown) were employed to normalize the band intensity data for the "−" and "+" FLP lanes (*lanes 2* and *3*, respectively). This procedure resulted in adjustment of recorded intensities for the "−" FLP lane by a factor of 0.62. The normalized band intensities for the "−" FLP lane were then divided by the intensities of the corresponding bands in the "+" FLP lane. Protection is indicated if the resulting ratio is greater than 1.0, and enhancement if it is less than 1.0. To allow for error in these determinations, a residue is described as protected in the discussion below only if this ratio exceeds an arbitrary cutoff of 1.5. Similarly, a cutoff of 0.7 was established for enhancement. The results are provided in histogram form in Fig. 6*C*.

Significant protection of adenine residues was observed at several positions, including −4, −6, −7, −9, −15, 4, 7, 9, and 15. The contacts at positions 4 (−4) and 15 (−15) extend the defined binding region by one base pair on either side, for a total of 12 base pairs. Methylation of adenines at position 16 (−16) is enhanced by the binding of FLP protein. Notably, adenines at positions 12 (−12) and 13 (−13) were not protected. Data at position 6 was obscured by a band resulting from FLP-mediated cleavage of the substrate DNA and no information about this position was obtained. Protection is not observed for any of the adenine residues in the internal six base pairs of the spacer, consistent with previous evidence that these positions are not recognized by FLP protein. The

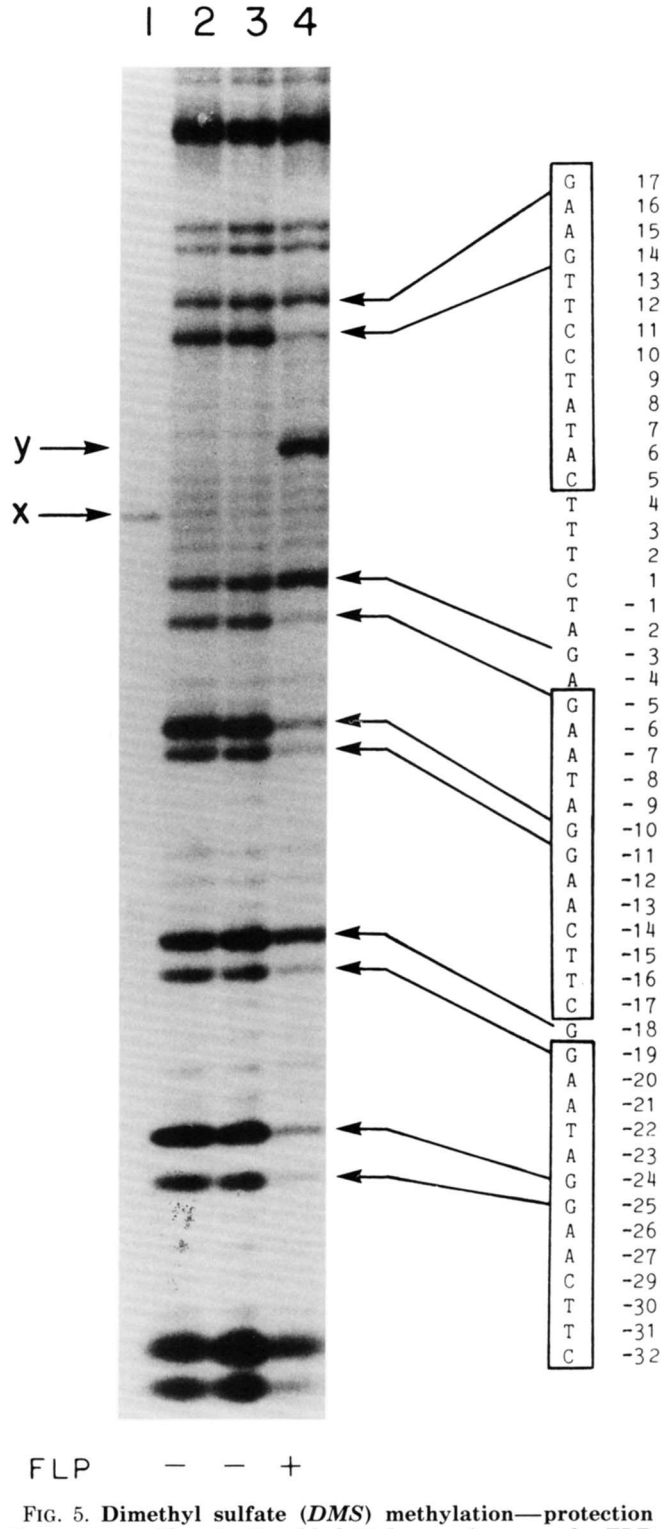


FIG. 5. **Dimethyl sulfate (*DMS*) methylation—protection of guanine residues in the third 13-base pair repeat by FLP protein.** Reactions were carried out as described under "Materials and Methods," employing a *Bam*HI-*Pst*I restriction fragment of pJFS122, 3′ end-labeled at the *Bam*HI site. Lane identification: *lane 1*, marker fragment produced by an *Xba*I restriction digest; *lane 2*, G sequence markers; *lane 3*, +DMS, −FLP protein; *lane 4*, +DMS, +FLP protein. × and *Y* denote *Xba*I and FLP cleavage products, respectively.

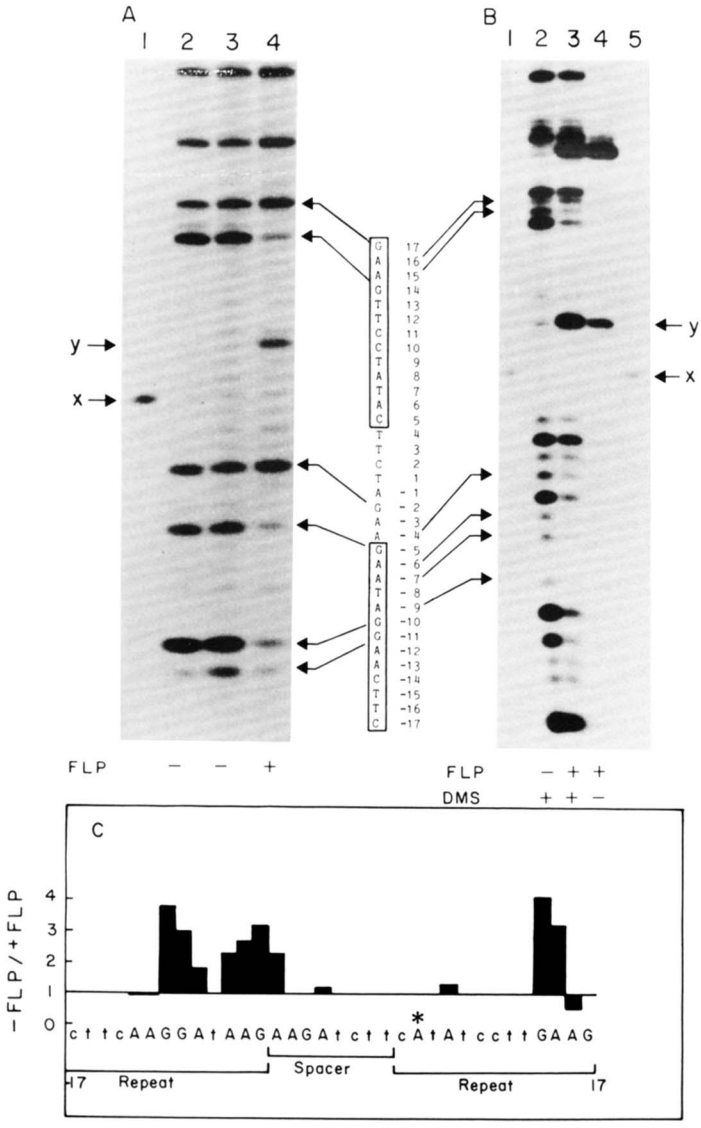


FIG. 6. **FLP protein protects guanine and adenine residues in the symmetrical FLP recombination site of pJFS39 against methylation by dimethyl sulfate (*DMS*).** Reactions were carried out as described under "Materials and Methods," employing a *Bam*HI-*Pst*I restriction fragment of pJFS39, 3′ end-labeled at the *Bam*HI end. × and *Y* denote *Xba*I and FLP cleavage products, respectively. *A*, samples subjected to the Maxam-Gilbert G cleavage reaction. Lane identification: *lane 1*, marker fragment produced by an *Xba*I restriction digest; *lane 2*, G sequence markers; *lane 3*, +DMS, −FLP protein; *lane 4*, +DMS, +FLP protein. *B*, samples subjected to the Maxam-Gilbert G > A cleavage reaction. Lane identification: *lane 1*, marker fragment produced by an *Xba*I digest; *lane 2*, +DMS, −FLP protein; *lane 3*, +DMS, +FLP protein; *lane 4*, −DMS, +FLP protein; *lane 5*, *Xba*I digest as in *lane 1*. *C*, quantitation of results in *B*. Ratio of band intensities for *lane 2* (−FLP) and *lane 3* (+FLP). * denotes position for which data is unavailable. See text for details.

sequences of pJFS36 and pJFS39 are identical in all positions where purines are contacted by FLP protein.

The methyl group on N-7 of guanine projects into the major groove, whereas the methyl group on N-3 of adenine projects into the minor groove. These results indicate that prominent interactions of FLP protein with its recombination site occur in both the major and minor grooves.

Methylation Interference—A complimentary experiment was carried out to determine if the guanine contacts identified in the methylation protection experiments were functionally significant. A restriction fragment of pJFS39 was 3′ end-labeled at the *Bam*HI site, again placing the recombination site at the labeled end and then was partially methylated with dimethyl sulfate. This modified DNA was incubated with FLP protein and a recombination product was isolated. If a methylated base normally comes into close contact with FLP protein during the reaction, the methyl group should interfere with either FLP protein binding or with a subsequent step in the reaction. DNA methylated at these positions should therefore be underrepresented in the isolated product.

As indicated above, the recombination site in the plasmid pJFS39 contains a symmetrical spacer which has the effect of abolishing the directionality of the reaction. The partially methylated DNA was incubated with FLP protein, and the 42-base pair recombination product isolated was that resulting from a head to head recombination event. The experiment is illustrated in Fig. 7. Whereas the parent site has a one-base pair mismatch in the repeats, at positions 6 and −6, this one-base pair mismatch has no effect on the polarity of the site (9). The head-to-head recombination product is completely symmetrical. Since both strands of this product are identical, information for both strands is obtained in a single experiment.

Results are presented in Fig. 8. The interference patterns observed correspond precisely with the methylation protection patterns observed above. Methylation of any of the guanine residues within the 10 base pairs of each repeat immediately flanking the spacer caused a pronounced inhibition of the recombination reaction. Methylation of guanines within the spacer had no apparent effect on the reaction. Again, the inhibitory effects of methylation of guanines within the repeats did not extend to the guanines located at the outer ends of each repeat, position 17 (−17). When the DNA is 3′ end-labeled at the *Eco*RI site, an identical interference pattern is observed (not shown).

Ethylation Interference—An experiment very similar to the methylation interference experiment described above was carried out, except that the phosphates were modified instead of the guanines. Ethylnitrosourea reacts with DNA and places an ethyl group on one of the phosphate oxygens. A *Bam*HI-*Pst*I restriction fragment of pJFS39 was uniquely 3′ end-labeled and prepared as described for the previous set of experiments. This DNA was partially ethylated with ethyl-

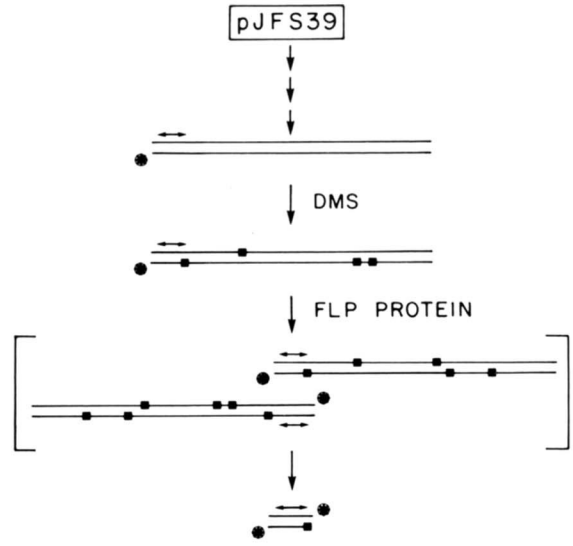


FIG. 7. **Outline of the recombination interference experiments.** Details are described in the text.

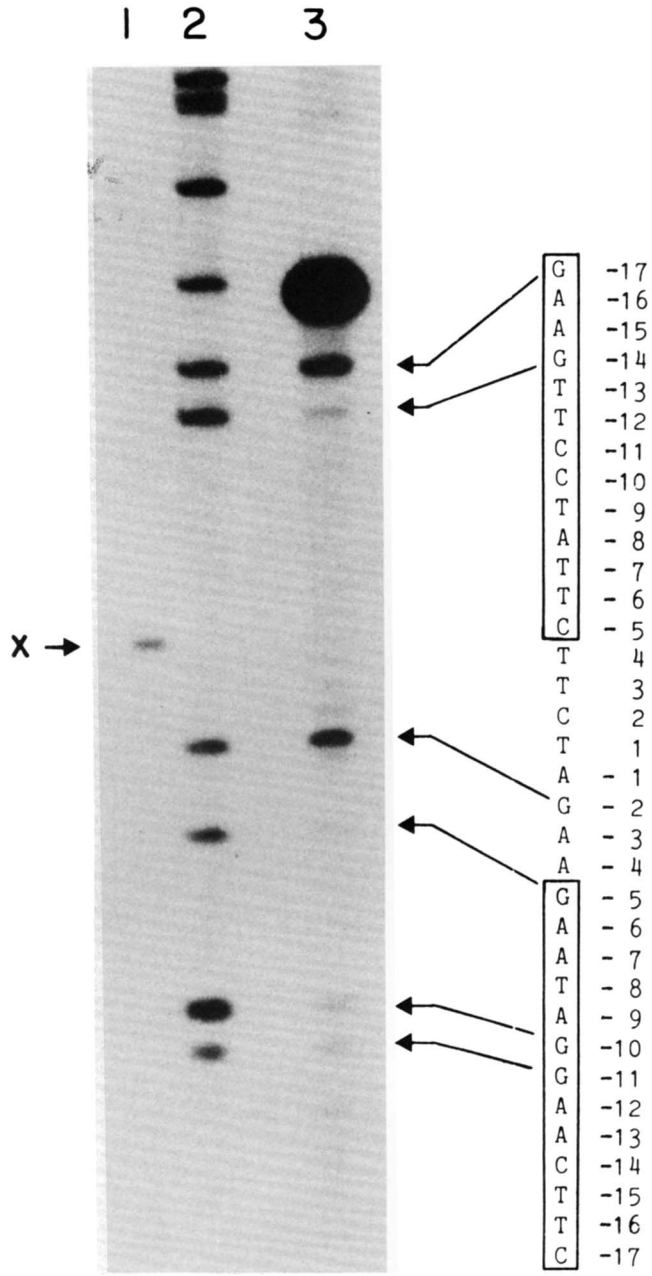


FIG. 8. **Methylation—interference of FLP protein-promoted recombination.** Reactions were carried out as described under "Materials and Methods," employing a *Bam*HI-*Pst*I restriction fragment of pJFS39, 3′ end-labeled at the *Bam*HI end. Lane identification: *lane 1*, marker fragment produced by an *Xba*I digest; *lane 2*, control reaction, with methylated DNA cleaved at guanine positions only; *lane 3*, head to head recombination product cleaved at methylated guanine positions. The numbering of the recombination product reflects the origin of each nucleotide in the head to head dimer sequence.

nitrosourea as described under "Materials and Methods." An aliquot of the ethylated DNA was cleaved with NaOH at ethylated phosphate positions as a control. The rest of the ethylated DNA was incubated with FLP protein, and the 42-base pair head-to-head recombination product was isolated.

To determine if the FLP protein treatment and subsequent gel elution increased the background level of cleavage, the unreacted *Bam*HI-*Pst*I fragment was also isolated. This DNA was cleaved at the ethylated phosphate positions by treatment with 150 mM NaOH. Samples were were then electrophoresed on a denaturing polyacrylamide gel along with the control sample (Fig. 9). Exposure to FLP protein and subsequent gel elution (Fig. 9, *lane 5*) does not appreciably change the cleavage pattern of the full-length DNA from that of the ethylated control sample (Fig. 9, *lane 4*).

Markers produced by the Maxam-Gilbert "G" sequencing reaction and *Xba*I digestion performed on nonethylated 3′ end-labeled DNA serve as markers for orientation within the sequence. A band resulting from cleavage at an ethylated phosphate position that migrates slightly above a Maxam-Gilbert G marker terminates at the phosphate that is 3′ to the guanine. Absence of a band in the isolated recombination product, or decreased intensity compared with that of unrecombined ethylated DNA, indicates that ethylation of that position somehow interfered with the recombination reaction.

Three regions of the recombination site are affected. A group of five consecutive phosphates that begins in each repeat and extends into the spacer, between positions 3 and 8 (−3 and −8), exhibits interference (Fig. 9, *lane 6*). In most experiments, ethylation at the phosphates between positions 7 and 8 (−7 and −8), as well as between 4 and 5 (−4 and −5),

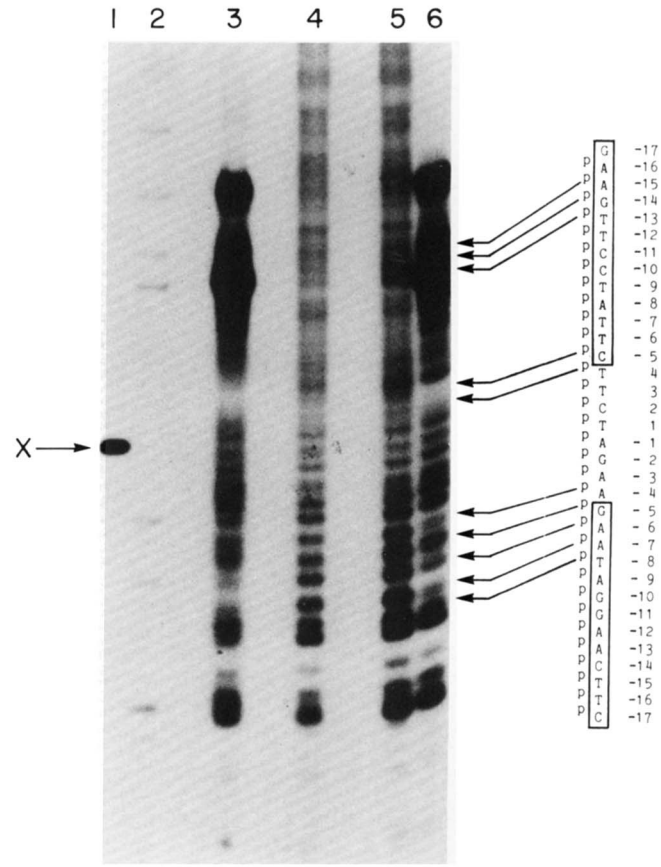


FIG. 9. **Phosphate ethylation—interference of FLP protein-promoted recombination.** Reactions were carried out as described under "Materials and Methods," employing a *Bam*HI-*Pst*I restriction fragment of pJFS39, 3′ end-labeled at the *Bam*HI end. Lane identification: *lane 1*, marker fragment produced by an *Xba*I restriction digest; *lane 2*, marker fragments produced by the Maxam-Gilbert G sequencing reaction; *lane 3*, isolated head to head recombination product cleaved at ethylated phosphate positions; *lane 4*, control, partially ethylated *Bam*HI-*Pst*I fragment cleaved at ethylated phosphate positions; *lane 5*, partially ethylated *Bam*HI-*Pst*I restriction fragment, isolated from the same polyacrylamide gel as the head to head recombination product before cleavage at the ethylated phosphate positions; *lane 6*, repeat of *lane 3*. The numbering of the recombination product reflects the origin of each nucleotide in the head-to-head dimer sequence. × denotes *Xba*I cleavage product.

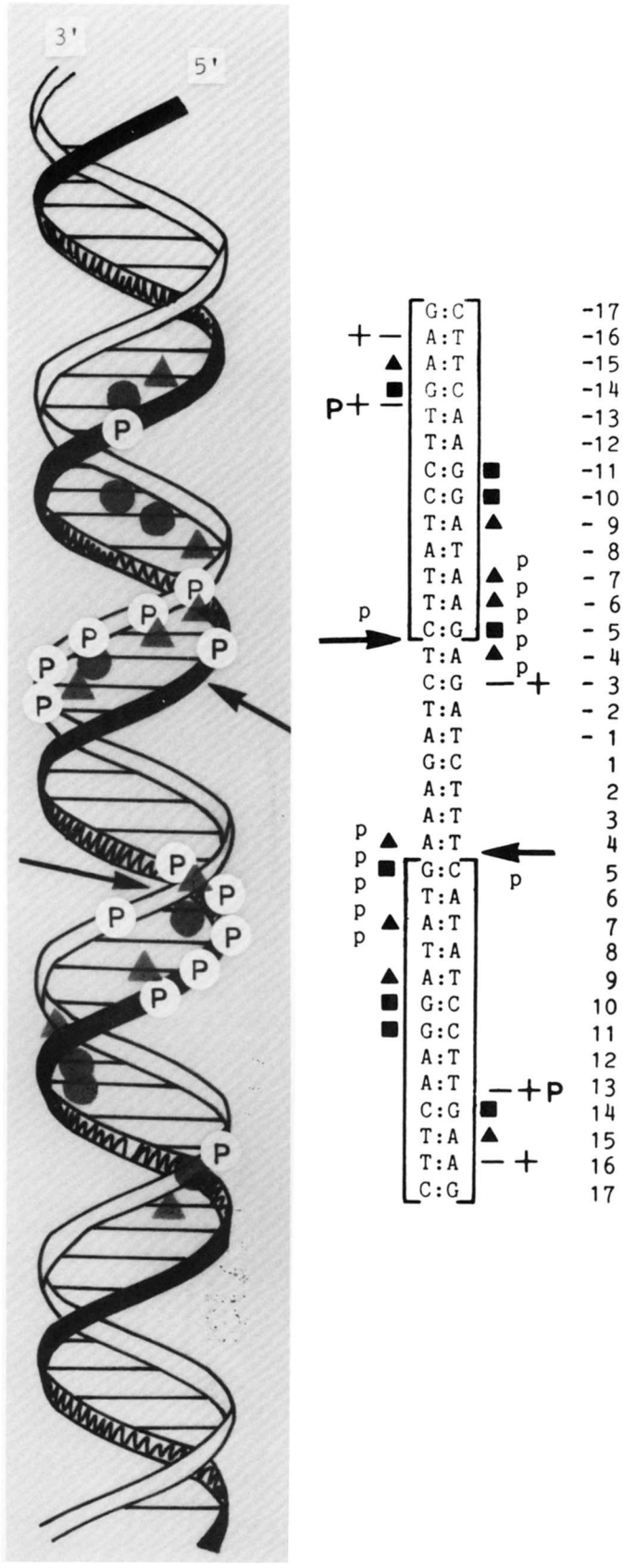


FIG. 10. **Summary of FLP protein—DNA contacts.** Contacts are shown on a helical representation of the DNA and within the sequence of the recombination site of pJFS36. Guanine and adenine contacts are located in the major and minor grooves, respectively. Symbol identification: ● or ■, guanine contacts; ▲, adenine contacts; *P*, phosphate contacts; +*P*, positions where ethylation of phosphates leads to an enhancement of recombination; +, positions where methylation of purines is enhanced by FLP protein binding. *Large arrows* indicate FLP protein cleavage points. The plasmids pJFS36 and pJFS39 are identical in sequence at all positions where purine contacts occur.

produced a relatively modest level of interference which is discernible in only one of the two experiments presented in Fig. 9. A second region, including the phosphate at the cleavage point and the adjacent phosphate (in the repeat), interacts with FLP protein. The bands corresponding to these phosphate residues are completely absent. Ethylation of the cleavage point could prevent attack by FLP protein, or the covalent protein-phosphotriester linkage could hydrolyze and abort the recombination reaction. The sequence of five phosphate contacts noted above encompass four protected purine residues and lie opposite the cleavage point on the strand that is not cleaved.

This observed interference could result from steric blocking of a close contact with FLP protein. Since either of two oxygens could be ethylated, this might be expected to have an effect only half of the time. Ethylation also eliminates the negative charge on the phosphate. It is more likely that disruption of a charge-charge interaction is responsible for interference (18).

Ethylation in a third region, near the end of the repeat distal to the spacer, produces a pronounced enhancement of recombination. This enhancement was observed in each of six experiments. Three consecutive phosphate residues are involved. The effect is modest in the case of the two outermost phosphates, between 14 and 16 (−14 and −16). A much shorter exposure of the gel in Fig. 9 (not shown) reveals a very pronounced enhancement of the interior phosphate, which lies between the guanine at position 14 (−14) of the repeat (identified above as an FLP contact point) and the thymine at position 13 (−13).

DISCUSSION

This work further defines the role of the two components of the FLP recombination site. Previous work (11) has shown that the spacer sequence is involved with the homologous alignment of two sites undergoing recombination via DNA-DNA pairing. This work provides direct evidence that the repeats flanking the spacer represent binding sites for FLP protein. FLP protein does not recognize or bind to the six central base pairs within the spacer. The base pair at either end of the spacer, adjacent to the cleavage site, appears to be important both for DNA-DNA pairing and in some sense for FLP protein recognition (11).

These conclusions are supported by several pieces of evidence. The site of cleavage by FLP protein remains constant relative to the repeat sequences regardless of mutations in the spacer which affect either spacer size or sequence. Specific contacts between FLP protein and the recombination site, identified in methylation protection experiments, span 11 base pairs of each repeat and the adjacent base pair in the spacer. The functional significance of the FLP protein-guanine contacts are confirmed in a recombination interference experiment which examines the effect of methylation at specific positions on the overall recombination reaction. Similar direct contact points are conspicuously absent in the six internal base pairs of the spacer, complementing previous evidence that FLP protein does not recognize sequences at these positions. The earlier study indicated that homology at these six positions was essential for recombination in this system, but the sequence itself could be altered without sig-

nificantly affecting the efficiency of the reaction (11). Results are summarized in Fig. 10.

In the presence of FLP protein, methylation of guanine residues between positions 5 and 14 does not occur. Methylation of the same guanine residues prior to recombination interferes with the reaction. No methylation protection of guanine residues in the spacer (for either pJFS36 or pJFS39), or for the guanine residue at the end of the repeats distal to the spacer is observed in the presence of FLP protein. Prior methylation of these guanine residues correspondingly does not interfere with recombination. These contacts, at N-7 of guanine, occur in the major groove.

Protection of adenine residues also occurs within this region, as well as at the single adenine base pairs flanking it on either side. These contacts, at N-3 of adenine occur in the minor groove. No information was obtained in this study about one position within the FLP-binding site (the adenine at position 6) because of interference from a band resulting from FLP-mediated cleavage of the DNA.

The third 13-base pair repeat sequence that lies outside of the minimal recombination site is also protected by FLP protein. The protection pattern is the same as for the other repeats. The guanine that lies between the two adjacent repeats, at position −18, is protected only weakly. Although this third repeat is not required for FLP protein-promoted recombination *in vitro*, evidence from experiments carried out *in vivo* suggests that sequences outside the minimal recombination site may play a role in this reaction (23).

FLP protein-phosphate contacts are clustered around the site of cleavage. There are no FLP protein-phosphate contacts at the five central phosphates of the spacer. The adenine at position 4 (−4) is flanked by phosphates contacted by FLP protein. This adenine defines the internal limit of the identified purine contacts. A mutation at this position can only partially be compensated for by reaction with a homologous site (11). This position appears to be a transition base pair between two regions of sequence with different functions. It is part of the sequence recognized by FLP protein and also participates in the DNA-DNA pairing which constitutes the primary function of the internal six base pairs of the spacer.

If the helical DNA representation of Fig. 10 is bisected by the *plane* of the figure, most of the contact points identified in this study lie on one face, within the 180° facing the reader. Exceptions are the guanine at position 5 (−5), and the phosphates between positions 3 (−3) and 5 (−5), which occur at positions just outside of the 180° defined by the other contacts. These contacts span two minor grooves and the major groove that separates them (Fig. 10).

Previous work has shown that three base pairs can be removed from the outer ends of the repeats without affecting the efficiency of recombination *in vitro*. However, removal of two additional base pairs significantly reduces the efficiency of recombination (9). This agrees well with results obtained in this study. On the end distal to the spacer, the repeats end in the sequence G-A-A-G at positions 14–17 (−14 to −17). The guanine at position 17 (−17) is not protected by FLP protein, and methylation at this position has no detectable effect on recombination. The guanine which lies at position 14 (−14), however, is fully protected by FLP protein, and methylation at this position has a very detrimental effect on the recombination reaction. Protection is also observed for the adenine at position 15 (−15), while methylation of the adenine at position 16 (−16) is enhanced. This confirms that deletion of more than three base pairs from the ends of the repeats affects interactions between the recombination site and the FLP protein which may be important in recognition. The contact at position 15 (−15) may be less essential since deletion of this position appears not to measureably affect the *in vitro* reaction (9).

An enhancement of methylation by dimethyl sulfate occurs at positions which flank the region bound by FLP protein. This could be due to a number of effects. FLP protein protects the spacer from digestion by DNase (10), indicating that FLP protein surrounds the spacer without binding tightly to it. The enhancement of methylation of the guanine at position −3 may reflect an increased dimethyl sulfate concentration in a hydrophobic protein pocket or local DNA helix unwinding (20, 21). Similar effects may apply to the adenine at position 16 (−16). The strong enhancement of recombination observed when the phosphates between positions 13 (−13) and 14 (−14) are ethylated, and the weaker enhancement seen when the phosphates between positions 14 (−14) and 16 (−16) of the repeat are ethylated, may be related to the resulting neutralization of the negative charge at these positions.

The 12-base pair-binding site described above is recognized by a protein which has a molecular weight of 48,000 (2). FLP protein will protect a single 13-base pair repeat from digestion by DNase although at a reduced level when compared to the whole site (10). If the spacer size is increased by four base pairs, DNase protection is still observed although the site is no longer competent in recombination (10). It is probable then that FLP protein binds to each repeat sequence but will mediate a recombination reaction only if the repeats are separated by seven to nine base pairs (9, 11). The restriction in spacer size is most easily explained by an essential protein-protein interaction between FLP protein monomers bound at each repeat. FLP protein might therefore span the spacer and protect it from DNase digestion without binding tightly to it.

The recombination site in the plasmid pJFS39 has a symmetrical spacer which differs from the normal spacer at 5 of 6 central positions. Sites with this spacer are fully competent in the recombination reaction if the reaction is restricted to sites with homologous spacers. This site has the interesting and useful property that the site no longer has an identifiable polarity. During recombination, sites can be aligned in either of two orientations, and recombination products resulting from alignment in both orientations are observed (11). This property was used in the design of the methylation and ethylation interference experiments. Since the two strands of a head-to-head recombination product are identical, it was possible to identify all important points of methylation interference with one experiment rather than carry out a separate experiment for each strand. These experiments assume that each repeat contributes to the interaction with FLP protein to an equal extent. Evidence presented here and elsewhere validates the use of this recombination site for experiments of this kind in the FLP system. This site appears to be identical to the normal recombination site in recombination efficiency (11), FLP protein cleavage patterns, and patterns of methylation protection by FLP protein. Within the limits of these measurements, the interaction of FLP protein with this site appears to be identical in every sense to that which occurs with the unaltered site. Use of this symmetrical site may simplify a variety of studies in this system.

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