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The FLP recombinase of the yeast 2-μm plasmid: Characterization of its recombination site

(site-specific recombination)

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ABSTRACT **The minimal size of the recombination site required for efficient FLP recombinase-catalyzed recombination *in vitro* is no more than 28 base pairs, which includes parts of two 13-base-pair inverted repeats and all of an 8-base-pair spacer. The FLP recombinase cleaves the DNA at the boundaries of the spacer, becomes covalently linked to the spacer DNA via a 3′ phosphate, and leaves a free 5′ hydroxyl at the other end of the 8-base-pair spacer. The efficiency of recombination is reduced if the size of the spacer in a recombinant site is increased or decreased by 1 base pair, while the spacer in the second site is unaltered. Recombination between two sites with identical 1-base-pair additions or deletions in the spacer, however, is relatively unaffected. This result suggests that pairing of sequences in the spacer region is important in FLP-promoted recombination events. The sequence asymmetry utilized by the recombinase to determine the orientation of the site is located uniquely within the spacer region.**

The 2-μm plasmid is a 6318-base-pair (bp) circular DNA molecule common to many yeast strains (1). The plasmid encodes a site-specific recombination system that inverts unique sequences separating a pair of 599-bp repeats. The availability of the plasmid sequence (2) and ease of manipulation in both *Escherichia coli* and yeast have facilitated analysis of this eukaryotic recombination system. *In vivo* studies in yeast have demonstrated that fewer than 65 bp of the 599-bp repeat are required for a functional recombination site (3). A protein product of the plasmid, designated FLP, was also shown to be required for recombination (4). Studies in *E. coli* demonstrated that the FLP gene product was the only yeast protein required for this recombination event (5). The establishment of *in vitro* systems for FLP-promoted recombination has revealed that the reaction has very simple requirements and characteristics that are both reminiscent of the bacteriophage P1 *cre–lox* recombination system and consistent with results obtained *in vivo* in yeast (6–8).

Much of our recent effort has been directed at characterizing the recombination site for this reaction *in vitro*. The most prominent structural features of this site are shown in Fig. 1. A series of three 13-bp repeats is evident. The second and third are inverted relative to one another and separated by an 8-bp spacer. The first copy of this repeat is immediately adjacent to and in the same orientation as the second, as shown in Fig. 1. The third repeat contains a 1-bp mismatch relative to repeats 1 and 2 (t in sequence, Fig. 1). An *Xba* I restriction site accounts for 6 of the 8 bp in the spacer. Destruction of this restriction site by using methods that add or delete 4 bp has been shown to abolish this recombination event *in vivo* in yeast (3) and *E. coli* (5) and *in vitro* (7, 8).

It has been demonstrated that FLP protein binds to all 3 of the 13-bp repeats and cleaves the site at the boundaries of the spacer (9). The third repeat was shown to be unnecessary for cleavage (9).

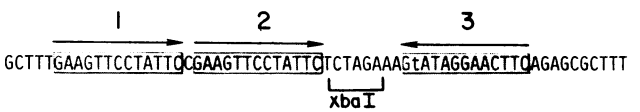


FIG. 1. Sequences of the yeast 2-μm plasmid within which FLP-mediated recombination occurs.

This work extends these results. The boundaries of the minimal site required for recombination *in vitro* are defined, and sequences that determine functional site asymmetry are localized to the spacer region. Modest flexibility in the size of the spacer region is demonstrated. In addition, the location of the cleavage site is confirmed. Some of these results have been described in a preliminary report (10).

MATERIALS AND METHODS

Bacterial Strains, Enzymes, and Reagents. Plasmids were maintained in the *E. coli* strain HB101 (ref. 11, p. 504). Bacteriophage M13 mp8 and its derivatives were maintained in the strain JM101 (12). FLP protein was partially purified as described (8). FLP protein fractions used were free of detectable nuclease. The large fragment of T4 DNA ligase and *E. coli* DNA polymerase I were purified by using strains and procedures described in ref. 13, p. 196 and ref. 14. Restriction enzymes, polynucleotide kinase, BAL-31 nuclease, dideoxy sequencing reagents, and DNA linkers were purchased from New England Biolabs. Restriction enzymes were also purchased from Bethesda Research Laboratories and International Biotechnologies (New Haven, CT). Calf intestine alkaline phosphatase and RNase A were purchased from Sigma. The [γ-^{32}P]ATP (≥5000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham, and [α-^{32}P]dATP and [α-^{32}P]dGTP (both ≥600 Ci/mmol) were purchased from New England Nuclear. Oligonucleotides were synthesized at the DNA Synthesis Facility in the University of Wisconsin Biotechnology Center.

DNA Manipulation. DNA fragments were isolated from polyacrylamide gels as described (15). Isolation of DNA from agarose gels, restriction digests, ligations, plasmid transformations, and 3′ DNA end labeling were performed by published procedures (ref. 11, pp. 156, 250, and 113). Rapid plasmid isolation was carried out by a modification of a procedure described in ref. 13, p. 120. Plasmid DNA utilized in FLP recombination assays *in vitro* was in all cases purified by banding twice in cesium chloride gradients in the presence of ethidium bromide. Agarose gel electrophoresis was carried out in a Tris/acetate/EDTA buffer system (ref. 11, p. 156). Polyacrylamide gel electrophoresis was carried out in either Tris/acetate/EDTA or Tris/borate/EDTA (ref. 11, p. 156).

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Abbreviation: bp, base pair(s).

Electrophoresis under denaturing conditions was performed by resuspending appropriately treated DNA samples in 98% formamide/0.1% xylene cyanol/0.1% bromphenol blue/5 mM EDTA. After denaturation at 90°C for 2 min the samples were cooled in an ice bath and electrophoresed in an 8.3 M urea/12% polyacrylamide gel using a Tris/borate/EDTA electrophoresis buffer. After electrophoresis gels were transferred to Whatman 3M paper, dried, and autoradiographed at room temperature. Dideoxy and chemical sequencing reactions were carried out following modifications of published procedures (15, 16).

Plasmid Constructions. The plasmid pJFS166 (3326 bp) was constructed by isolating a 166-bp fragment of 2-μm plasmid DNA, inserting it between synthetic DNA linkers containing *Hin*dIII restriction sites, and ligating this segment into the plasmid pXf3 (ref. 11, p. 6) cut with *Hin*dIII. This 166-bp fragment contains the 65-bp regions of the 2-μm repeat necessary for FLP-mediated recombination plus at least 40 bp on either side of this region. pJFS166 was cleaved with *Eco*RI, which cuts 29 bp away from the 2-μm DNA insert. Deletions were generated from this point by partial digestion of the linearized DNA with exonuclease BAL-31 followed by the addition of *Bam*HI linkers and then ligation. The resulting molecules were cut with *Bam*HI and *Hin*dIII, and fragments containing shortened recombination sites of appropriate size were isolated from polyacrylamide gels. These fragments were ligated to the large fragment produced by a *Bam*HI/*Hin*dIII double digestion of the plasmid pXf3. Transformants were isolated that contained plasmids with inserts of appropriate size that could be cleaved with *Xba* I (the *Xba* I site is unique to the 2-μm DNA insertion). Fragments containing deletions of interest were reinserted into bacteriophage M13 mp8 DNA and sequenced. Plasmids pJFS122 (2936 bp), pJFS110 (2924 bp), pJFS105 (2919 bp), and pJFS100 (2914 bp) were generated in this way. One deletion, pJFS110, was used for further experiments. The *Xba* I/*Eco*RI fragment of this plasmid was removed and replaced by a series of synthetic double-stranded DNA molecules that contained various sequence changes. The plasmids pJFS36 (2821 bp), pJFS37r (2822 bp), pJFS35r (2820 bp), pJFS36sym (2821 bp), pJFS33r (2818 bp), and pJFS31r (2816 bp) were all generated in this manner. The plasmid pJFS37b (2822 bp) was constructed by replacing the small *Bam*HI/*Xba* I fragment of pJFS36 with a synthetic double-stranded DNA molecule with the appropriate change(s). A sequence summary of these plasmid constructions is diagrammed in Fig. 2. All of these plasmids contain nearly identical sequences derived from pXf3 except for the short inserts illustrated. The identity of all altered recombination sites was confirmed by sequencing.

Recombination Assays. The reaction mixture (20 μl) for standard recombination assays was 25 mM Tris·HCl (pH 7.5)/200 mM NaCl/5% (vol/vol) glycerol. Linear DNA substrate (0.2 pmol, measured in total recombination sites) was incubated with an amount of FLP protein fraction, empirically determined, to give the maximal reaction for 60 min at 30°C. Reactions were initiated by the addition of FLP protein fractions and were terminated by the addition of $NaDodSO_4$ to a final concentration of 1%. Reaction products were analyzed by agarose gel electrophoresis. In all cases, recombination reactions were performed on linear DNA substrates containing only one recombination site. The resultant size(s) of the product DNA molecule(s) was a consequence of the position of the recombination site with respect to the ends of the DNA. In all cases the restriction site *Eco*RI was used to linearize one substrate and *Pst* I to linearize the other. The predicted products of intermolecular recombination between the two different substrates were a pair of hybrid linear DNA molecules, one larger and one smaller than the substrates. Recombination between two molecules of the same substrate DNA generated product DNA molecules that were identical to the starting material; this reaction was not, therefore, directly measured. The recombination assay has been described in detail (8) and is illustrated schematically in Fig. 3.

Determination of the FLP Recombination Cleavage Site. These experiments utilized uniquely 3′-end-labeled restriction fragments derived from pJFS122. In the experiments illustrated (see Fig. 6 *A* and *B*) the fragments were 126 and 157 bp in length, respectively, and contained a single recombination site. Fragments (≈0.2 pmol total recombination sites) were incubated with an aliquot of a FLP protein fraction (empirically determined to produce maximal cleavage, ≤0.3 μg of total protein) in 100 μl of cleavage buffer [200 mM NaCl/30 mM glycine (pH 9)/5% glycerol/5 mM EDTA] for 30 min at 0°C. Reaction was stopped by addition of $NaDodSO_4$ to 0.5% and proteinase K to 1 mg/ml followed by incubation at 37°C for 1 hr. Calf thymus DNA (100 μl of 100 μg of DNA per ml) was added, and the mixture was extracted with phenol/chloroform (1:1, vol/vol) and chloroform. The aqueous layer was precipitated twice with ethanol in the presence of 0.3 M sodium acetate, then the pellet was washed several times with 70% ethanol and vacuum dried. Identical cleavage products were generated at somewhat lower efficiency under standard *in vitro* recombination conditions. In

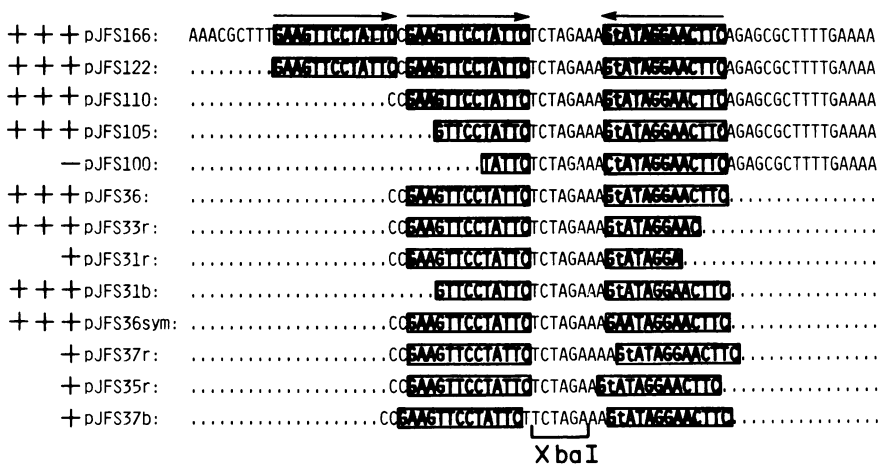


FIG. 2. Altered FLP recombination sites generated in this study. The + and − signs on the left indicate the relative extent of reaction of each site illustrated with an unaltered FLP recombination site.

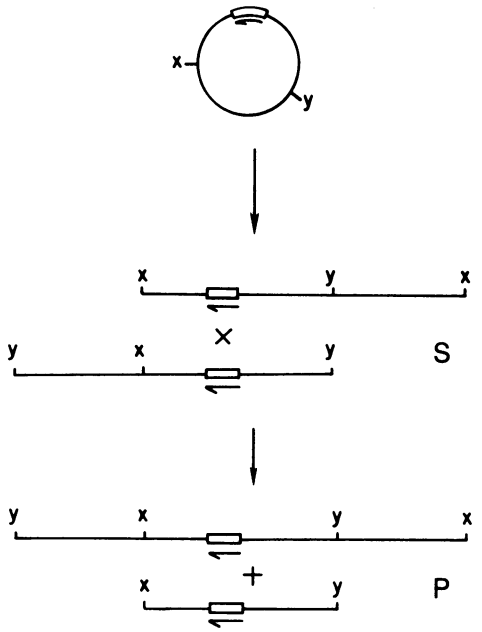


FIG. 3. Schematic representation of FLP intermolecular recombination assay. The illustrated plasmid contains a single recombination site (box). x and y represent two unique restriction enzyme cleavage sites, e.g., one for *Eco*RI and the other for *Pst* I. S and P, substrate and product.

addition, *in vitro* recombination is observed under the cleavage conditions described above.

Kinase and Phosphatase Experiments. The cleavage reaction described in the previous section was scaled up 4 times and treated as described except that the calf thymus DNA was omitted. The dried pellet was dissolved in 140 μl of denaturation buffer [20 mM Tris·HCl (pH 9.5)/1 mM spermidine/0.1 mM EDTA], heated to 90°C for 2 min, and cooled in an ice/water bath (17). Kinase buffer [40 μl of 250 mM Tris·HCl (pH 9.5)/50 mM $MgCl_2$/25 mM dithiothreitol/25% (vol/vol) glycerol] and 20 μl of 100 μM ATP were added, and the sample was divided into two 50-μl portions and one 100-μl portion. Various aliquots were subjected to the following treatments. (*i*) A 50-μl aliquot received no treatment. (*ii*) A 50-μl aliquot received 1 unit of calf intestinal alkaline phosphatase followed by incubation at 37°C for 30 min. (*iii*) A 100-μl aliquot received 10 units of polynucleotide kinase followed by incubation at 37°C for 30 min, then the kinase was inactivated by heating at 70°C for 10 min, and the sample was divided into two portions. The first received no further treatment, and the second received 1 unit calf intestine alkaline phosphatase followed by incubation at 37°C for 30 min (17).

The DNA in each sample was extracted, precipitated, and dried as described above, then resuspended in an appropriate buffer for electrophoresis.

RESULTS

The Minimal FLP Recombination Site. A series of deletions was generated to determine what part of the sequence presented in Fig. 1 represented the minimal site required for FLP protein-promoted recombination *in vitro*. These deletions are illustrated in Fig. 2. In each case the altered site has been inserted into approximately the same position in the plasmid pXf3, so that flanking sequences are constant, and the overall size of each substrate DNA molecule never differs by more than 200 bp. *In vitro* recombination assays were carried out between two plasmids linearized with different restriction enzymes. The first plasmid had the deleted sites, and the second plasmid, pJFS122, had a recombination site shown to be fully functional *in vitro* (8). Site-specific recombination between the two different plasmids produces one larger and one smaller DNA molecule detectable by agarose gel electrophoresis (Fig. 3). Recombination between two molecules of the same plasmid cannot be observed because the products are indistinguishable from the substrates. The results of a series of 60-min assays are presented in Fig. 4.

These reactions show that a deletion removing the third repeat (pJFS110) has no apparent effect on the efficiency of recombination. Three base pairs can also be removed from the repeats flanking the spacer region, on the ends distal to the spacer [pJFS105 (data not shown), -33r, and -31b), again with no apparent effect on the efficiency of recombination. We have also determined that a site lacking 3 bp on the ends of both repeats flanking the spacer is fully functional, in recombination both with itself and with an unaltered site (data not shown). Removal of 5 bp from one of these repeats (pJFS31r) results in a reduced level of recombination, while removal of 8 bp (pJFS100) abolishes recombination. The minimal recombination site for FLP protein-promoted recombination *in vitro* is therefore no greater than 28 bp, incorporating the spacer region, and 10 bp of both repeats flanking the spacer.

Recombination Site Asymmetry. The FLP protein detects asymmetry in the recombination site. This asymmetry determines the outcome of recombination events. Recombination between two sites on the same DNA molecule will result in inversion or deletion of intervening sequences, depending on whether the two sites are inverted or in the same orientation with respect to each other (8). Within the sequence illustrated in Fig. 1, several potential elements of asymmetry are evident. These include the third 13-bp repeat (already shown to be unnecessary for recombination *in vitro*), a 1-bp nonhomology between repeats 2 and 3, and the spacer sequence itself. An altered recombination site was constructed (pJFS36sym, see Fig. 2) in which the repeats flanking the spacer are symmetrical. The spacer sequence is unchanged. As shown in an *in vitro* assay (Fig. 5), recombination efficiency is unaffected by this modification in sequence. More important, the products are unchanged. If sequences in the repeats flanking the spacers were required as determinants of asymmetry, making the repeats identical should permit incorrect (inverted) alignment of sites during recombination. New products would be produced in these reactions, but such products are not observed in our assays. This

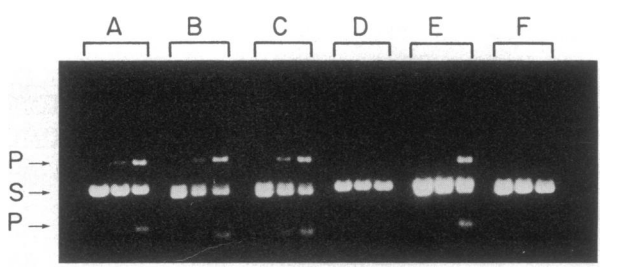


FIG. 4. Determination of the minimal size site required for FLP-mediated recombination *in vitro*. (S) and (P) identify substrate and product DNA bands, respectively. In each set of three reactions the same, empirically determined, levels of FLP protein were employed (from left to right: 0, 1, or 2 μl of an active FLP protein fraction). Substrate plasmids were linearized with the restriction enzyme *Eco*RI(*R*) or *Pst* I(*T*) as indicated in parentheses after the plasmid name. The linearized plasmids are in all cases 2880 ± 64 bp in length. Observed products are approximately 3600 bp and 2090 bp. Recombination reactions: A, pJFS122(*T*) × pJFS122(*R*); B, pJFS122(*T*) × pJFS36(*R*); C, pJFS122(*T*) × pJFS31b(*R*); D, pJFS122(*T*) × pJFS100(*R*); E, pJFS122(*R*) × pJFS33r(*T*); F, pJFS122(*T*) × pJFS31r(*R*).

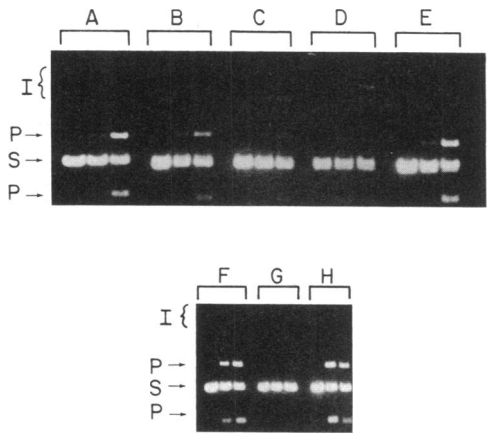


FIG. 5. Effect of sequence alterations in the spacer region. Symbols are described in the legend to Fig. 4. I identifies possible reaction intermediates described in the text. Recombination reactions: A, pJFS122(*T*) × pJFS122(*R*); B, pJFS122(*T*) × pJFS37r(*R*); C, pJFS122(*T*) × pJFS37b(*R*); D, pJFS122(*T*) × pJFS35r(*R*); E, pJFS122(*R*) × pJFS36sym(*T*); F, pJFS36(*T*) × pJFS36(*R*); G, pJFS36(*R*) × pJFS37b(*T*); H, pJFS37b(*R*) × pJFS37b(*T*).

demonstrates that the orientation of the recombination site is determined entirely by asymmetry within the 8-bp spacer.

Alterations Within the Spacer Region. Previous work has demonstrated that alteration of the *Xba* I site within the spacer abolishes FLP-promoted recombination. The effect of these alterations is the addition or deletion of 4 bp in the spacer region. In an effort to determine whether the size of the spacer was rigidly defined in this system, a series of recombination sites was constructed in which 1 bp was added or deleted in the spacer region. The alterations were designed so that the base pairs flanking the likely cleavage site (see below) were unaltered. As the site is presented in Fig. 2, single base pairs were added (pJFS37r) or deleted (pJFS35r) on the right side of the spacer, or added (pJFS37b) to the left side of the spacer. In each case, the alteration resulted in a reduced efficiency of recombination in reaction between altered and normal sites (Fig. 5). Recombination was not abolished, however, demonstrating that some flexibility in the size of the spacer region is tolerated. In Fig. 5, the reaction of pJFS37r appears to be more efficient than the reaction observed with the other two. We have observed some variability in the efficiency of recombination observed with all three of these spacer size mutants, and it is therefore unclear whether this difference is significant. A reduction in the efficiency of recombination between sites with altered spacers and sites with normal spacers is reproducibly observed.

While recombination between a site with an altered spacer and a site with an unaltered spacer is reduced, recombination between two sites with identical 1-bp additions or deletions is relatively unaffected (Fig. 5, reactions F–H). The result shown is for pJFS37b; very similar results were obtained for pJFS37r and pJFS35r (not shown). In this case the spacer size has been changed, but homology between the two sites undergoing recombination has been restored. Recombination between two sites with different 1-bp insertions (pJFS37r × pJFS37b) does not occur (data not shown), so that it is the restoration of spacer homology rather than spacer size which is important. This result suggests that pairing between spacer sequences in the two sites undergoing recombination may be a feature of FLP-promoted recombination. Recombination was abolished in reactions involving a recombination site in which the insertions in pJFS37r and -37b were combined to yield a 10-bp spacer (not shown). This was true even for recombination between two sites with identical 2-bp insertions. The observed flexibility in the size of the spacer region is therefore limited to addition of 1 bp. We have not yet determined if 2 bp can be deleted from the spacer.

Specific Cleavage of the Recombination Site by FLP Protein. In many FLP protein-promoted recombination reactions *in vitro*, minor products are observed that migrate slowly on an agarose gel (e.g., see Fig. 5). In several cases, evidence has been obtained that these products are DNA molecules with one or more monomers of FLP protein covalently linked to them (unpublished results). No high-energy cofactor is required for FLP protein-promoted recombination. It is expected that cleavage of the four phosphodiester bonds required to carry out the recombination event be accompanied by covalent linkage of the recombinase to the DNA at the site of cleavage.

Experiments were carried out to determine the nature of this putative covalent recombination intermediate and the point of DNA cleavage. A short restriction fragment containing the recombination site from pJFS122 was uniquely labeled at either 3′ end. After incubation with FLP protein the FLP-mediated cleavage product was subjected to polyacrylamide gel electrophoresis under denaturing conditions. The cleavage point is located by determining the migration of an FLP protein-mediated cleavage product relative to Maxam–Gilbert C+T and/or A+G sequencing reactions and the *Xba* I cleavage product of the same fragment run in parallel (Fig. 6 *A* and *B*). The products of Maxam–Gilbert sequencing reactions have 5′-phosphate termini. A DNA fragment with a 5′-hydroxyl terminus should therefore migrate more slowly than a sequencing fragment of equal length and more rapidly than a sequencing fragment one nucleotide larger. As shown in Fig. 6*B*, the 3′-end-labeled FLP cleavage product band

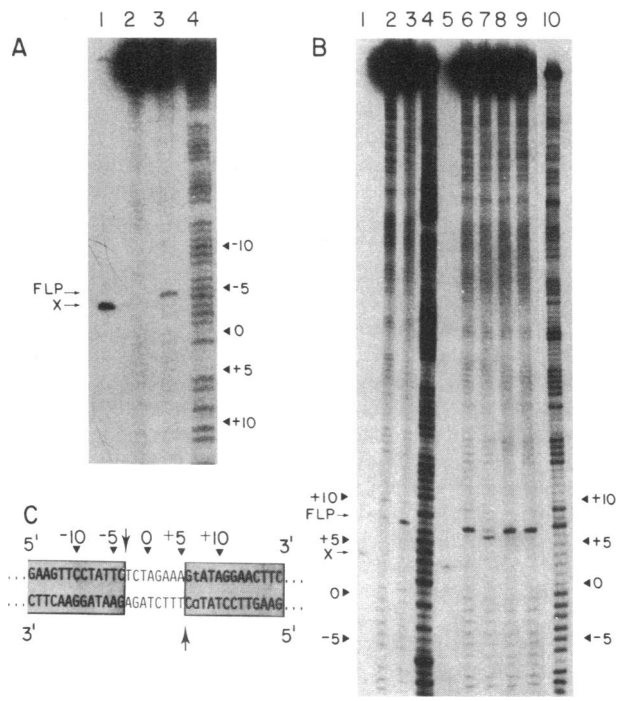


FIG. 6. Specific cleavage of the FLP recombination site by FLP protein. *A* and *B* identify points of cleavage in the upper and lower strands, respectively, of the FLP recombination site as depicted in *C*. X and FLP identify bands resulting from *Xba* I and FLP protein cleavage, respectively. (*A*) Lanes: 1, fragment produced by *Xba* I cleavage; 2, −FLP protein; 3, +FLP protein; 4, C+T sequencing. (*B*) Lanes: 1, fragment produced by *Xba* I cleavage; 2, −FLP protein; 3, +FLP protein; 4, C+T sequencing; 5, *Xba* I fragment; 6, +FLP protein; 7, +FLP protein, treated with kinase; 8, +FLP protein, treated with kinase followed by phosphatase; 9, +FLP protein, treated with phosphatase only; 10, A+G sequencing. In *C*, crossover points in FLP recombination site are identified with arrows.

migrates between two Maxam–Gilbert sequencing fragments, suggesting that the 5′ end terminates in a hydroxyl group. This is confirmed by treatment of this fragment with ATP and polynucleotide kinase, which causes the FLP cleavage band to migrate slightly faster and to comigrate with a Maxam–Gilbert sequencing fragment. Subsequent treatment with alkaline phosphatase restores the original mobility, while treatment with alkaline phosphatase alone has no effect (Fig. 6*B*). The points of cleavage defined in these experiments are shown in Fig. 6*C*. In similar experiments the FLP cleavage product obtained from a 5′-end-labeled substrate must be treated with proteinase K before electrophoresis to observe a discrete product band (not shown). This indicates that the FLP protein becomes covalently linked to the 3′ phosphate at the site of cleavage, leaving a free 5′ hydroxyl. The cleavage sites are at the boundary of the spacer, producing an 8-bp staggered cut with protruding ends terminating with 5′-hydroxyl groups. This conclusion is supported by additional published (9) and unpublished data.

DISCUSSION

This work defines several characteristics of the recombination site required for FLP protein-mediated recombination *in vitro*. The minimal site is no larger than 28 bp. Since a less efficient recombination reaction can be observed with a site in which more than 3 bp have been deleted from one end, the boundaries of the minimal site do not appear to be sharply defined. A more complete characterization of the deletions defining the boundary of the minimal site will be published elsewhere. This minimal site includes the 8-bp spacer region encompassing the *Xba* I site, as well as 10 bp of each of the flanking 13-bp repeats. While a site thus defined appears to be fully functional in our *in vitro* system, additional sequences appear to enhance the efficiency of recombination *in vivo* both in yeast and *E. coli* (22). The third 13-bp repeat is unnecessary *in vitro*, although it has been demonstrated that it is noncovalently bound by FLP protein (9). This repeat and other sequences within the 599-bp 2-μm plasmid large repeat may well play a role in recombination *in vivo* that is obviated *in vitro*.

The asymmetry that defines the orientation of the FLP recombination site is located entirely within the 8-bp spacer region. This represents another characteristic of this recombination system that identifies it with the *cre–lox* system of bacteriophage P1 (18). As described below, the positioning of two sites during recombination, and thus their relative orientation, may be mediated by direct pairing between the sequences within the spacer regions of the two sites.

Some flexibility is evident in the size of the spacer region. Addition or deletion of 1 bp reduces, but does not abolish, recombination. This result holds for recombination between two sites, only one of which is altered. The point at which cleavage occurs in sites with spacers of altered size has not yet been determined. The product of this recombination event may be a DNA molecule with an unpaired nucleotide in one strand. We have also demonstrated that recombination proceeds at normal or near-normal efficiency in reactions involving two recombination sites that both carry identical 1-bp additions or deletions in the spacer. This and other evidence (unpublished data) suggests that DNA–DNA pairing between spacer sequences occurs during FLP-mediated recombination. The spacer size flexibility we observe here appears to be limited to addition or deletion of 1 bp, since addition of 2 bp in the spacer abolishes recombination whether the second site is unaltered or contains an identical 2-bp insertion. The properties of these spacer size alterations collectively represent a feature of the FLP system that may provide important information about the mechanism by which this recombination event occurs. No flexibility is observed in the size of the crossover region in the bacteriophage λ integration system (19).

The site of FLP protein-mediated cleavage defined in this study agrees with published results (9). The covalent linkage of FLP protein to the DNA via a 3′ phosphate is a characteristic of this system that is identical in two other site-specific recombination systems, bacteriophage λ integration (17) and *cre–lox* recombination in bacteriophage P1 (20). In the FLP system no evidence is yet available that demonstrates that the observed FLP-mediated cleavage reflects an intermediate step in recombination, rather than an abortive side reaction. While it is reasonable to expect that the cleavage site and the normal crossover point in recombination are identical based on results from other systems (20, 21), further work is required for confirmation.

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