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Directionality in FLP Protein-promoted Site-specific Recombination Is Mediated by DNA-DNA Pairing*

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The 2μ plasmid of the yeast *Saccharomyces cerevisiae* encodes a site-specific recombination system consisting of plasmid-encoded FLP protein and two recombination sites on the plasmid. The recombination site possesses a specific orientation, which is determined by an asymmetric 8-base pair spacer sequence separating two 13-base pair inverted repeats. The outcome or directionality of site-specific recombination is defined by the alignment of two sites in the same orientation during the reaction. Sites containing point mutations or 1-base pair insertions or deletions within the spacer generally undergo recombination with unaltered sites at reduced levels. In contrast, recombination between the two identical mutant sites (where homology is restored) proceeds efficiently in all cases. Sites containing spacer sequences of 10 base pairs or more are nonfunctional under all conditions. A recombination site in which 5 base pairs are changed to yield an entirely symmetrical spacer sequence again recombines efficiently, but only with an identical site. This reaction, in addition, produces a variety of new products which can only result from random alignment of the two sites undergoing recombination, *i.e.* the reaction no longer exhibits directionality. These and other results demonstrate that both the efficiency and directionality of site-specific recombination is dependent upon homology between spacer sequences of the two recombining sites. This further implies that critical DNA-DNA interactions between the spacer region of the two sites involved in the reaction occur at some stage during site-specific recombination in this system. The specific spacer sequence itself appears to be unimportant as long as homology is maintained; thus, these sequences are probably not involved in recognition by FLP protein.

The 2μ circle is an autonomously replicating plasmid present in many strains of yeast. The plasmid encodes replication functions which allow it to exist in high copy number. Additionally, the plasmid encodes a site-specific recombination system. *In vivo*, recombination occurs between two 599-base pair inverted repeats on the plasmid and inverts the unique sequences between them (1).

The gene encoding the recombinase, designated FLP, has been cloned and expressed in *Escherichia coli* (2). Using partially purified FLP protein from these cells, we have been able to establish an *in vitro* assay for FLP activity. Using this system, we have begun to define the features of the recombination site utilized by FLP in this reaction. Earlier work demonstrated that the recombination site consisted of no more than 65 base pairs within the 599-base pair repeats (3). This region contains many short repeated sequences. Most notable is the presence of three 13-base pair repeats, with the second and third separated by an 8-base pair spacer. An *Xba*I cleavage site included within the spacer was previously shown to be essential for recombination (3). Previous work demonstrated that at least *in vitro*, the minimal size of a fully functional recombination site is no more than 28 base pairs (4). This excludes one of the 13-base pair repeats and 3 base pairs from the outer ends of each of the other two repeats (Fig. 1). Cleavage mediated by the FLP protein occurs at the boundaries of the spacer sequence, resulting in an 8-base pair staggered cut ((4, 5) Fig. 1). The spacer region is, therefore, the region of crossover as well. In the recombination product, each of the DNA strands within the spacer are derived from different substrates. The protein becomes covalently attached to the 3′ phosphate on each strand of the duplex DNA at the site of cleavage, leaving a free 5′ hydroxyl. Furthermore, there is limited flexibility in the size of the spacer region with 1-base pair insertions or deletions resulting in reduced but not abolished recombination when such sites are reacted with unaltered ones (4). Other studies have indicated that the FLP protein binds to and specifically protects a 50-base pair region which includes the three 13-base pair repeats and the 8-base pair spacer (5). These experiments were performed using DNase as a probe for protein binding. In this work, the authors also demonstrated binding of FLP protein to single 13-base pair repeats (5). It was not clear from these studies whether the spacer region itself was bound specifically by FLP protein.

Studies with analogous systems have shown that homology within the crossover region is essential for recombination. Recent work using the bacteriophage λ site-specific recombination system and a large number of site affinity mutations within the attachment sites have demonstrated that homology between recombining partners is necessary for high level recombination *in vivo* and *in vitro* (6–8). Similarly, experiments with the bacteriophage P1 cre/lox site-specific recombination system have shown that sites containing mutations within the spacer region lose their ability to recombine with a wild type site (9). These data suggest that DNA-DNA pairing is important for strand exchange in these systems.

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FIG. 1. **Sequence of the yeast 2μ plasmid DNA within which recombination occurs.** The 13-base pair repeats are *shaded*. Cleavage sites are indicated with *arrows*. See text for details.

The recombination site, lox, is very similar in both structure and sequence to the site utilized in the FLP system. These similarities suggest that an analogous requirement for DNA homology may exist in FLP protein-promoted recombination.

Such pairing could have a second important function. The products of recombination promoted by site-specific recombination systems are determined by the relative positions and orientation of the two recombination sites. The recombination sites of all systems studied to date contain elements of asymmetry which define orientation. Reactions between two sites on the same DNA molecule always result in inversion if sites are in the opposite orientation and deletion if they are in the same orientation. The outcome of an intermolecular reaction can be predicted by assuming that sites must be aligned in the same orientation during recombination. In at least two systems, the FLP system and the bacteriophage P1 cre/lox system, the sole determinants of site orientation are asymmetric sequences within the 8-base pair spacer regions. It is possible that the outcome or directionality of recombination events in these systems could be mediated by DNA-DNA pairing involving these spacer sequences.

While 1-base pair insertions or deletions in the spacer region of the FLP recombination site reduced recombination efficiency when a mutant site was reacted with an unaltered site, reactions involving two identical mutant sites exhibited nearly normal levels of recombination (4). This suggested that pairing of sequences within the spacer regions of the sites undergoing recombination was an important feature of the reaction in this system. The goal of the present study was to examine in greater detail the role of the spacer sequences in FLP protein-promoted site-specific recombination. Utilizing the mutants described above, as well as new mutant sites which change the sequence of the spacer but not its length, we have shown that homology within this region is essential for high level recombination promoted by FLP protein. Furthermore, we have demonstrated that mutant sites with spacer sequences which are entirely symmetrical will undergo recombination efficiently with identical sites but that alignment of the sites during the reaction is random. These data imply that DNA-DNA pairing occurs at some point during recombination between sequences in the spacer regions of the two reacting sites.

MATERIALS AND METHODS

Purification of FLP Protein—Partially purified FLP protein was prepared essentially as described previously (10). All fractions used were free of detectable nuclease activity, and FLP protein represented approximately 3–10% of the total protein present. Assuming this level of purity, the amounts of FLP protein fractions employed in the assays described in this work provided approximately 1–12 monomers of FLP protein/recombination site in the reaction mixture.

Plasmid Constructions—Plasmids pJFS110, pJFS36, pJFS35r, pJFS37r, and pJFS37b were constructed as previously described (4). Plasmid pJFS38 was constructed by isolating the small fragment of a *Pst*I/*Xba*I double digest of pJFS37r and ligating it to the large fragment of the same digest of pJFS37b. The resulting recombination site contains a 2-base pair insert within the spacer region, creating a spacer which is 10 base pairs in length. pJFS39 was constructed by isolating the small fragment of a *Pst*I/*Xba*I double digest of pJFS35r and ligating it to the large fragment of the same digest of pJFS37b. The resulting plasmid contains a recombination site which has an 8-base pair spacer with a perfect dyad symmetry. The plasmids pJFS40, pJFS41, and pJFS42 were each constructed by isolating the large fragment of a *Xba*I/*Eco*RI double digest of the plasmid pJFS110 and ligating this to a short synthetic double-stranded DNA molecule containing the change of interest. All plasmids were purified from the strain HB101 (11) by banding twice in CsCl gradients in the presence of ethidium bromide. The sequence of all synthetic inserts was verified by recloning the fragments into the bacterophage M13 derivative, mp8 and sequencing (12). For *in vivo* recombination assays, all plasmids were linearized with either *Eco*RI or *Pst*I as shown in Fig. 2. Digests were extracted once with a phenol/$CHCl_3$/isoamyl alcohol mixture (25:24:1) and ethanol precipitated. Mutant FLP recombination site sequences used in this work are provided under "Results."

Enzymes and Chemicals—Restriction endonucleases were purchased from New England Biolabs or IBI, Inc. T_4 DNA ligase was purified as described (13). Klenow fragment was purified as described (14). Radionucleotides were purchased from New England Nuclear. Dideoxy sequencing reagents were from New England Biolabs.

In Vitro FLP Reactions—Reactions were carried out essentially as described previously (4). Reactions (20 μl) contained 25 mM Tris-HCl, 80% cation (pH 7.5), 5% (v/v) glycerol, and 200 mM NaCl. DNA (0.4 μg or 0.2 pmol of DNA molecules) was incubated with an appropriate amount of FLP protein-containing extract empirically determined to give an optimal reaction. Reactions were incubated at 30 °C for the times indicated and stopped by the addition of 2 μl of 10% sodium dodecyl sulfate and 6 μl of running buffer (50% (v/v) glycerol, 50 mM EDTA, 0.01% bromphenol blue). For reaction time courses, reaction volume was increased to 140 μl. Aliquots (20 μl) were removed at the times indicated and treated as described above. The reactions were analyzed by electrophoresis in a 1% horizontal agarose gel. All plasmids used in this work are nearly identical in size, usually differing only in the modifications made within the recombination site (see Figs. 2 and 3). Each set of reactions, therefore, contains two identical or nearly identical sets of plasmid molecules, one of which is linearized with *Pst*I and the other with *Eco*RI. A reaction is detected in this assay only if it occurs between DNA molecules cut with different restriction enzymes. Reaction between two DNA molecules cut with the same restriction enzyme is assumed to occur but is not detected because the products generated are identical to the substrates. Recombination between two populations of pJFS36 DNA molecules (approximately 2821 base pairs) prepared in this way results in the production of two new DNA species of 2066 and 3576 base pairs (Fig. 2). This reaction is employed as a standard positive control in these experiments.

Miscellaneous Procedures—Plasmid transformations, rapid plasmid DNA isolations, DNA ligations, restriction digests, and agarose gel electrophoresis were performed following published procedures (11, 13). Oligonucleotide synthesis was carried out at the Protein Sequence-DNA Synthesis Facility within the University of Wisconsin Biotechnology Center. Sequence determination was carried out by the method of Sanger (12). Where indicated, the relative efficiencies of two or more recombination reactions were estimated by densitometric scanning of photograph negatives of gels stained with ethidium bromide. In some cases, more accurate data was obtained by excising the ^{32}P end-labeled substrate and product bands from gels followed by Cerenkov counting. Scans were performed on a Zeineh Soft Laser scanning densitometer SL-504-XL from Biomed Instruments, Inc.

RESULTS

Homology in the Spacer Region of the Recombination Site Is Necessary for Efficient Recombination Mediated by FLP Protein—All of the reactions involving mutant recombination sites are compared to reactions employing pJFS36 which harbors a recombination site containing all sequences known to be required for recombination *in vitro* ((4) Figs. 2 and 3). The maximum extent of reaction observed with pJFS36 was approximately 50% conversion to recombination products, as determined in many experiments by ethidium bromide staining and densitometric scanning or by quantitation of end-labeled DNA in product and substrate bands. This extent of reaction was not exceeded for any substrate except pJFS39 (see below). It is not clear whether this represents an approach to dynamic equilibrium or whether each recombination site

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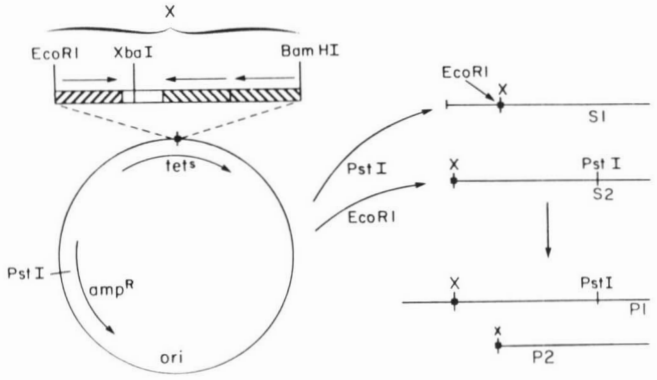


FIG. 2. **Cloning and assay of recombination site derivatives.** *Left*, structure of plasmids containing FLP recombination site derivatives. Plasmids are all derived from the plasmid pXf3 (11) and contain FLP recombination site inserts between *Eco*RI and *Bam*HI restriction sites of that plasmid. The region designated *X* corresponds to recombination site sequences and is the only region which varies from one plasmid to another. *Right*, schematic representation of recombination assay. The plasmids analyzed in this study contain unique recombination sites. Plasmids are linearized at restriction sites *Eco*RI or *Pst*I. *S* and *P* refer to substrates and products, respectively. Details of the assay are described under "Materials and Methods."



FIG. 3. **Summary of FLP recombination site spacer size mutants generated in this study.** Sequences shown correspond to region "*X*" in Fig. 2. Details are described in the text.

reacts only once. Each substrate molecule has an equal probability of reacting with either a molecule cut with a different restriction enzyme, yielding detectable products, or another molecule cut with the same restriction enzyme, yielding products indistinguishable from the substrates. Since only 50% of the recombination events yield detectable products, a system where only single turnovers are possible may yield the same result as a system in equilibrium. Experiments to distinguish between these possibilities are not a part of this study.

All of the experiments within a figure always use the same active fraction of FLP protein. Each of the experiments described was repeated several times. Some variability was noted in extents of recombination achieved in the standard 20-μl reactions, with the control reaction occasionally converting as little as 30–35% of the substrate to detectable products as determined by densitometric scanning. In the time course experiments involving larger reaction volumes, 45–50% conversion to products was always observed with pJFS36. Time courses were carried out for all of the mutants described. Quantitative comparisons reported below rely primarily on data obtained in these time courses which, in most cases, were repeated at least three times.

In several figures, a number of slowly migrating minor DNA bands are identified as "I." At least some of these are reaction intermediates (4). They have not been further characterized.

Spacer Size Mutations—It was previously demonstrated that mutant FLP recombination sites which contain single base pair insertions or deletions within the spacer region undergo recombination with unaltered sites at a reduced efficiency (4). In contrast to these results, when two recombination sites which contain identical alterations within the spacer region undergo recombination, the final extent of reaction is restored to levels comparable to wild type. Representative reactions are presented in Figs. 4 and 5. In Fig. 4, efficient recombination is observed in all reactions where the spacers are homologous. Based on many experiments, the variability evident in reactions B, D, F, and H is probably not significant. In contrast, a large drop in reaction efficiency is observed when plasmids containing mutant sites are reacted with pJFS36. This is evident with at least two of the mutants in the experiment presented in Fig. 4 (pJFS35r and pJFS37b). A more quantitative comparison was carried out in which the time course of each reaction was followed under conditions in which the FLP protein concentration was slightly suboptimal for the pJFS36 reaction. Reactions were quantitated by densitometric scanning. Time courses for the most reactive of the three mutants (pJFS37r) are presented in Fig. 5. In this case, the reaction involving heterologous recombination sites (37r x 36) is reduced approximately 15-fold in rate relative to the

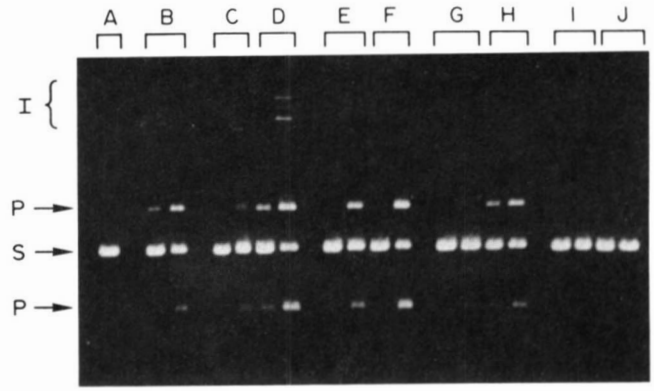


FIG. 4. **Effect of nonhomology in the spacer region on recombination.** Reaction conditions and gel electrophoresis are described under "Materials and Methods." *S* and *P* refer to substrate and product DNA bands, respectively (see Fig. 2). In each set of two reactions the same levels of FLP protein fraction are used (0.4 or 1.0 μl from left to right). Substrate plasmids are as described in the legends to Figs. 2 and 3. Digestion with *Eco*RI (R) or *Pst*I (P) is indicated in parentheses after the plasmid name below. The plasmids are in all cases 2821 ± 2 base pairs in length and the product bands are approximately 3576 and 2066 base pairs. Reactions are: *A*, 2821-base pair marker (0 FLP); *B*, pJFS36(R) x pJFS36(P); *C*, pJFS35r(R) x pJFS36(P); *D*, pJFS35r(R) x pJFS35r(P); *E*, pJFS37r(R) x pJFS36(P); *F*, pJFS37r(R) x pJFS37r(P); *G*, pJFS37b(R) x pJFS36(P); *H*, pJFS37b(R) x pJFS37b(P); *I*, pJFS38(R) x pJFS36(P); *J*, pJFS38(R) x pJFS38(P).

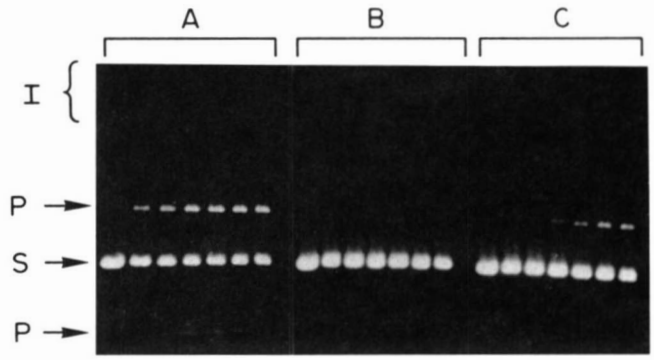


FIG. 5. **Spacer insertion mutants: reaction time course.** Reactions and symbols are as described under "Materials and Methods" and in the legend to Fig. 4. In each set of reactions, 1.0 μl of an active FLP protein fraction was employed. The incubation times for each set were: 0, 2, 5, 10, 30, 60, or 120 min from left to right. Reactions are: *A*, pJFS36(R) x pJFS36(P); *B*, pJFS36(R) x pJFS37r(P); *C*, pJFS37r(R) x pJFS37r(P).

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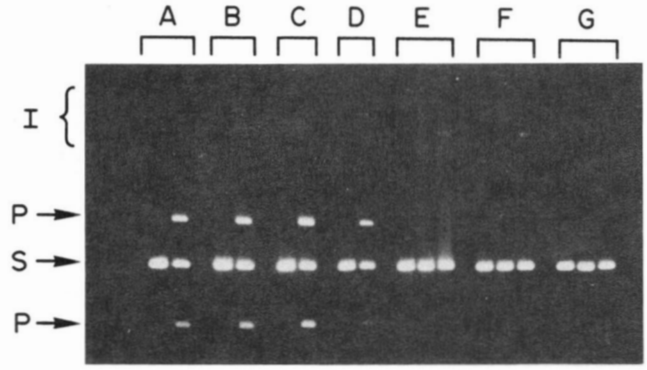


FIG. 6. **Recombination between spacer size mutants.** Reactions and symbols are as described under "Materials and Methods" and in the legend to Fig. 4. In each case the same amount of FLP-containing fraction (0 or 1 μl from left to right for *A*, *B*, *C*, and *D*, or 0, 1, or 2 μl for *E*, *F*, and *G*) was used. Reactions are: *A*, pJFS36(R) x pJFS36(P); *B*, pJFS35r(R) x pJFS35r(P); *C*, pJFS37r(R) x pJFS37r(P); *D*, pJFS37b(R) x pJFS37b(P); *E*, pJFS35r(R) x pJFS37r(P); *F*, pJFS35r(R) x pJFS37b(P); *G*, pJFS37r(R) x pJFS37(P).

control reaction (36 x 36). The rate is increased 3-fold with respect to the heterologous reaction when identical mutant sites are reacted (37r x 37r). A series of these experiments revealed that the order of reactivity of these mutants was pJFS37r > pJFS35r > pJFS37b in reactions with pJFS36. In homologous reactions, the order of reactivity is pJFS35r > pJFS37r > pJFS37b. In every case the rate of the reaction between two identical mutants was reduced relative to the pJFS36 reaction, and the rate was further reduced when the mutant was reacted with pJFS36. The extent of these changes in reactivity was a function of the concentration of FLP protein, ionic strength, and several other factors. A more detailed comparison will be reported elsewhere.

Factors other than homology may influence the reactivity of these various sites. If FLP protein recognizes and binds to the repeated sequences bordering the spacer, the size of the spacer could also be important since it should determine the distance between FLP protein bound to each repeat. In Fig. 4 it is demonstrated that a site containing a 10-base pair spacer, pJFS38, does not undergo recombination under any conditions. The flexibility of the spacer size is, therefore, limited. Footprinting experiments have shown that a site containing a 12-base pair spacer is protected less effectively than a normal site (5). This data may imply that a protein-protein interaction is affected when the spacer size is changed.

Another factor which may affect the efficiency of recombination is the structure of the product DNA molecules. Previous work has shown that the FLP protein cleaves the normal recombination site at the boundary of the spacer sequences, leaving 8-base pair staggered ends ((4, 5) Fig. 1). Recent experiments have shown that sites containing 7- or 9-base pair spacers are still cleaved at the spacer boundaries, leaving 7- or 9-base pair staggered ends.[1] Products formed from recombination between wild type and mutant sites should, therefore, contain an unpaired base. The formation of such a product may be thermodynamically unfavorable. The reduced reactivity of the mutant sites may be a consequence of improper protein alignment, lack of homology, unfavorable product formation, or any combination of these factors.

Partial restoration of reaction efficiency in the mutant x mutant reactions is an effect of spacer homology and not of spacer size (Fig. 6). In this experiment, the final extent of all homologous mutant x mutant reactions is comparable to the wild type of control (50 ± 6%). Little or no recombination is observed when two nonhomologous mutant sites are used in the reaction. Only recombination between pJFS35r and pJFS37r yields a detectable reaction. Since the spacers in the recombination sites of these two mutants share the most homology, this is consistent with the hypothesis that the extent of reactions observed is related to the degree of homology between the spacer regions. More importantly, recombination reactions between the plasmids pJFS37r and pJFS37b do not produce products. The spacers of these plasmids are identical in size (9 base pairs) but share little homology when the sites of cleavage are aligned. Thus, spacer size identity is not sufficient to permit FLP protein-mediated recombination *in vitro*.

Spacer Point Mutations—The mutations described above change both the sequence and size of the spacer region. To assess further the function of the spacer relative to the flanking repeats, as well as the role of spacer sequence, homology, and size, a series of point mutations were constructed. These mutations are illustrated in Fig. 7. Most significant are the changes in the plasmids pJFS41 and pJFS42. These changes bracket the FLP protein cleavage site, while all other mutations described above leave these base pairs intact.

The results obtained with the point mutations are illustrated in Fig. 8. A negligible (<10%) reaction is observed between pJFS36 and pJFS40. Restoring homology to the pJFS40 reaction results in an increase in product formation to levels comparable to those observed in the control reaction. In this case, time courses failed to indicate any decrease in reactivity of this plasmid in homologous crosses when compared to pJFS36 reactions under any set of conditions (data not shown). This indicates that the base pair change does not affect the inherent activity of this sequence. The reduced rate observed with the spacer size mutations in homologous reactions is thus an effect of spacer size, and not sequence.

A point mutation in the spacer adjacent to the cleavage site

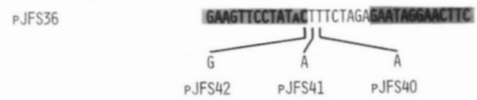


FIG. 7. *A*, summary of FLP recombination site point mutations generated in this study. Sequences shown correspond to the region *X* of the plasmid illustrated in Fig. 2. Construction of the mutant recombination sites is described under "Materials and Methods."

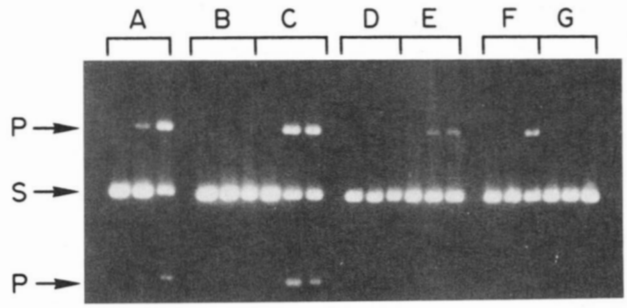


FIG. 8. **The effects of FLP recombination site point mutations on FLP protein-promoted recombination *in vitro*.** Reactions and symbols are as described under "Materials and Methods" and in the legend to Fig. 4. Plasmids are as described in the legends to Figs. 2 and 7. In each case the same amount of an FLP protein-containing fraction was added (0, 1, or 2 μl from left to right). Reactions are: *A*, pJFS36(R) x pJFS36(P); *B*, pJFS36(R) x pJFS40(P); *C*, pJFS40(R) x pJFS40(P); *D*, pJFS36(R) x pJFS41(P); *E*, pJFS41(R) x pJFS41(P); *F*, pJFS36(R) x pJFS42(P); *G*, pJFS42(R) x pJFS42(P).

[1] R. Bruckner, unpublished data.

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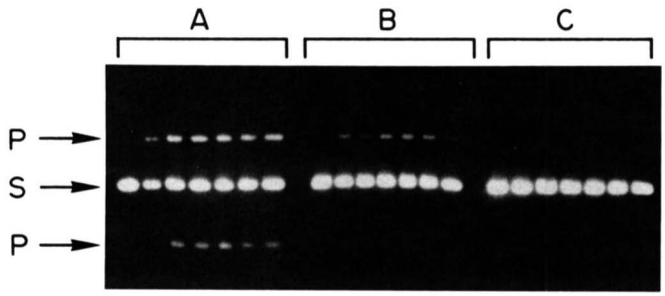


FIG. 9. **Rates of recombination with the plasmid pJFS42.** Reactions and symbols are described under "Materials and Methods" and in the legend to Fig. 4. Plasmids are as described in the legends to Figs. 2 and 7. In each reaction, 1 μl of an FLP protein-containing fraction was used. Each set of reactions corresponds to 0, 2, 5, 10, 30, 60, and 120 min from left to right. Reactions are: *A*, pJFS36(R) x pJFS36(P); *B*, pJFS36(R) x pJFS42(P); *C*, pJFS42(R) x pJFS42(P).

also shows homology-dependent reactivity. However, recombination between pJFS41 and pJFS36 yields no detectable products, while restoring homology yields a reaction which is still reduced at least 3-fold relative to the wild type reaction in this experiment. The reaction efficiency observed in pJFS41 x pJFS41 reactions is a direct function of the FLP protein concentration and approaches that observed in the pJFS36 reaction when sufficient FLP protein is provided. The requirement for excess FLP protein may reflect an adverse effect on FLP protein-promoted cleavage brought about by this sequence change.

A different pattern of reactivity occurs when the 1st base of the 13-base pair repeat immediately adjacent to the spacer is changed. The plasmid pJFS42 recombines with pJFS36 at reduced efficiency. The difference observed is again a function of FLP protein concentration. In this case, however, recombination between two molecules of pJFS42 is reduced further still, to less than 5% of the normal reaction. These results suggest an abrupt change of function at the boundary of the spacer region. First, the decreased level of reaction in pJFS42 x pJFS42 crosses as compared to pJFS36 x pJFS42 reactions indicates that homology is important only within the spacer sequence. Second, this decrease suggests that sequences within the flanking repeats are important in recognition and binding of FLP protein. A third possibility is that this mutation reduces the efficiency of the cleavage step.

The extent rather than the rate of recombination appears to be reduced in reactions involving pJFS42. The appearance of recombination products is reduced as described above, with little change after the first 2 min of the reaction (Fig. 9). In this case, the pJFS36 x pJFS42 and the pJFS42 x pJFS42 reactions are reduced at least 2- and 5-fold, respectively.

Mutations Generating a Symmetrical Spacer—The sequences within the spacer region are the sole determinants of site asymmetry and reaction directionality in the FLP system (4). Results described above indicate that DNA-DNA pairing occurs between spacer sequences during recombination but that the specific spacer sequence is relatively unimportant. If reaction directionality is mediated entirely by this same DNA-DNA pairing, a symmetrical spacer should permit an efficient reaction with an identical recombination site, but the reaction would proceed with loss of directionality. The more extensive alterations required to produce such a spacer would also provide a more rigorous test to the hypothesis that spacer sequence is unimportant as long as homology is maintained.

A symmetrical spacer was constructed as described under "Materials and Methods," and the plasmid containing the resulting recombination site was designated pJFS39. This recombination site is illustrated in Fig. 10. The spacer in the recombination site in pJFS39 has 5 base pair changes with respect to the normal site in pJFS36. None of these changes affect base pairs adjacent to the normal FLP cleavage sites. A summary of results obtained with pJFS39 is presented in Fig. 11. The normal products produced in this assay are observed with the pJFS36 x pJFS36 reaction (*A*). When pJFS36 molecules linearized with only one restriction enzyme are employed in the reaction, no products can be observed because the products are identical to the substrates (*B*). A reaction between pJFS36 and pJFS39 produces two new product DNA species in spite of the fact that there is little homology between the spacers of the respective recombination sites (*C*). These products, however, are not the products produced in the wild type reaction. In reaction series *D*, it is demonstrated that these products are not derived from a cross between pJFS36 and pJFS39, but are produced when pJFS39 linearized with *PstI* is the only DNA species in the reaction mixture. This result indicates that recombination between



FIG. 10. **Sequence of a recombination site with a symmetrical spacer.** Sequences shown correspond to the region *X* of the plasmid described in Fig. 2. Construction of pJFS39 is described under "Materials and Methods."

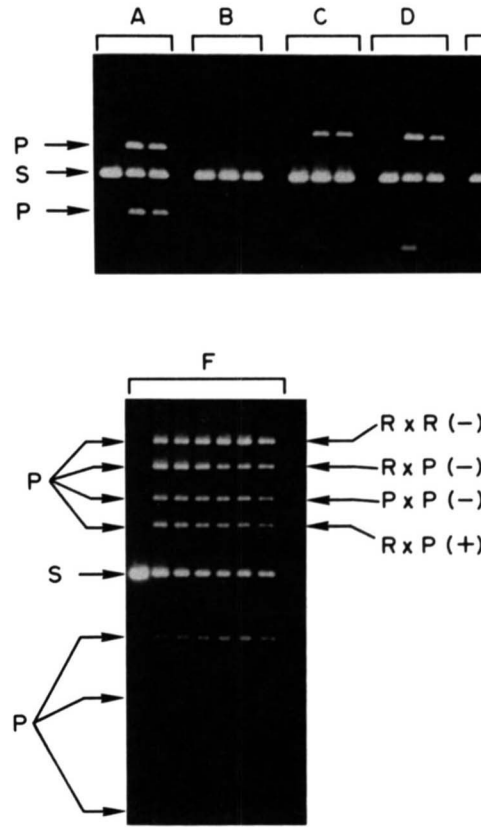


FIG. 11. **Symmetrical sequences within the spacer cause loss of site directionality.** Reactions and symbols are described under "Materials and Methods" and in the legend to Fig. 4. Plasmids are as described in Figs. 2 and 10. In each set of three reactions, the same amount of an active FLP protein-containing fraction (0, 1, or 2 μl) was added from left to right. Reactions are: *A*, pJFS36(R) x pJFS36(P); *B*, pJFS36(P); *C*, pJFS36(R) x pJFS39(P); *D*, pJFS39(P); *E*, pJFS39(R) x pJFS39(P); *F*, time course of reaction *E*, employing 1 μl of an active FLP protein-containing fraction. Reaction incubation times were 0, 2, 5, 10, 30, 60, and 120 min from left to right.

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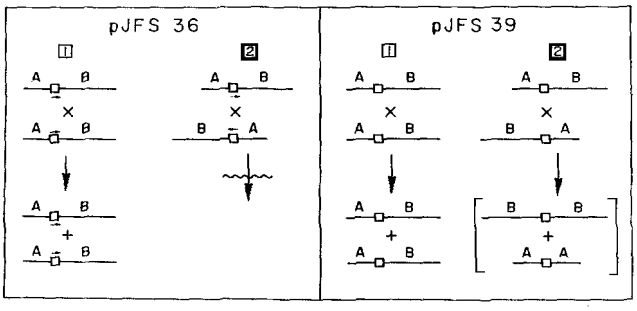


FIG. 12. **New products observed in a reaction employing a recombination site with a symmetrical spacer.** See text for details.

pJFS39 and pJFS36 does not occur. The origin of the new DNA products is a reaction involving two pJFS39 molecules with their recombination sites paired in the alignment which is opposite to the normal one and usually forbidden, as illustrated in Fig. 12. The reaction has thus lost directionality, with two different site alignments possible for any recombination event. This result is reinforced in reaction series *E*. A reaction mixture containing two different pJFS39 species (linearized with different restriction enzymes) produces a number of new products in addition to the normal ones. Designating the two species R (*Eco*RI cleaved) and P (*Pst*I cleaved), eight detectable products can be predicted, four larger and four smaller than the starting material. These are derived from the normal R x P reaction (+), an R x P reaction with the opposite (−) site alignment, and P x P and R x R reactions with the wrong (−) site alignments. The products observed correspond in size exactly with those predicted (only seven are large enough to be observed on this gel). Efficient recombination is, therefore, observed with pJFS39, but the directionality of the recombination event has been abolished by employing a symmetrical spacer.

The rate of the reaction with pJFS39 appears to be comparable to that observed with pJFS36 (Fig. 11*F*). Since many different reactions are possible here, approximately 75% of the DNA is converted to the various products in this experiment as determined by densitometric scanning. No conditions have been found in which the pJFS39 reaction is obviously slower than the pJFS36 reaction. The origin of the four large products is identified in Fig. 11*F*. DNA-DNA pairing involving spacer sequences, therefore, is important to both the efficiency and the outcome of the recombination event. Large changes in spacer sequence are well tolerated in this system as long as the two spacers involved in a given reaction are homologous.

DISCUSSION

Our principal conclusion is that at some step during FLP protein-promoted recombination, DNA-DNA pairing occurs between the spacer sequences of the two recombination sites involved. This pairing is essential for reaction efficiency and also determines the outcome or directionality of the reaction. The actual sequence of the spacer is relatively unimportant, as long as the spacers in the two recombining sites are homologous. Spacer sequences, therefore, are probably not involved in recognition or binding by FLP protein.

Several pieces of evidence support the notion that pairing between spacer sequences is a feature of FLP protein-promoted recombination. 1) For a series of spacer alterations including point mutations and one-base pair insertions or deletions, recombination is always more efficient in reactions involving homologous spacers than it is for reactions in which the spacers differ in one or more positions. 2) The increased efficiency of recombination observed when a site with a 9-base pair spacer is reacted with itself, relative to the reaction of the same mutant with a normal site, is not due to restoration of spacer size. Recombination between two sites with different 1-base pair insertions in the spacer does not occur at detectable levels. 3) A number of recombination reactions involving spacers which are not entirely homologous occur at reduced efficiency but are not abolished. While careful quantiation has not yet been attempted, the efficiency of recombination is approximately proportional to the extent of homology in the spacers involved. 4) Spacer sequences (8 base pairs in length) can be modified extensively with no detectable effect on reaction efficiency as long as homology is maintained. 5) The importance of maintaining homology is observed only for sequences within the spacer, with the characteristic properties of spacer mutations ceasing abruptly at the spacer boundary. Restoring homology in a recombination event involving a point mutation in a flanking repeat decreases, rather than increases, the efficiency of recombination.

The flexibility we have observed in the size of the spacer is limited. Recombination sites with 10-base pair spacers do not function in recombination. Recombination events involving two homologous 7- or 9-base pair spacers are somewhat reduced in rate relative to crosses involving homologous 8-base pair spacers, regardless of sequence. Binding studies have shown that FLP protein binds to both of the 13-base pair repeats flanking the spacer and that this binding is greatly reduced when one repeat is deleted (5). The present results provide additional evidence which suggests that important interactions occur between the FLP protein bound to each of the repeats flanking the spacer. An explanation for the differences in reactivity we have observed among the three active spacer size mutants must await a detailed analysis of protein-DNA and protein-protein interactions in this system.

Since FLP protein cleaves this recombination site at the spacer boundaries leaving 8-base pair staggered ends (4, 5), the 8 nucleotides of one strand of a spacer must, in the product, be paired with a complementary strand derived from the recombination site of the second substrate molecule. The homology requirement we observe for spacer sequences may, therefore, be due to the simple fact that nonhomologous spacers will generate an unstable product. In its simplest form this hypothesis would require that recombination sites are aligned entirely by protein-protein interactions or by the binding of one FLP protein monomer to two different sites. All steps preceding the pairing of sequences in the crossover region, including cleavage, would occur before spacer homology was detected. Even in a reaction between two normal recombination sites, 50% of the reactions should result in sites aligned in the wrong orientation, resulting in an aborted reaction. Simple abortive cleavages of recombination sites however, is rarely observed in this system (4, 5). This apparent precision suggests that pairing between spacer sequences occurs early in the reaction, prior to the cleavage event and may involve a 4-stranded DNA intermediate such as that described by Kikuchi and Nash (15). More work is required to test this hypothesis.

The results obtained with this set of mutations suggests that sequence flexibility and the effects of homology or lack of it will be most pronounced for the six central base pairs in the spacer. A mutation in one of the base pairs at the ends of the spacer reduces the efficiency of recombination somewhat, even when the recombination sites are completely homologous. These base pairs are adjacent to the cleavage site, and the reaction could be more sensitive to changes at these

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positions for a variety of reasons. Homology is clearly still essential at these positions, however.

The FLP system is closely related to the bacteriophage P1-lox/cre recombination system and exhibits many similarities to it (9, 10). These results may be applicable to this and other systems which have comparable recombination sites. In general these results suggest that the mechanisms by which the outcome of site-specific recombination events is regulated in the cell may be quite simple.

Note Added in Proof—Andrews and Sadowski ((1986) *Mol. Cell. Biol.*, in press) have studied a spacer mutation similar to that in pJFS40 with comparable results.

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