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Site-specific Genetic Recombination Promoted by the FLP Protein of the Yeast 2-micron Plasmid In Vitro

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The yeast 2-micron plasmid is a 6318-bp circular DNA molecule present at 50–100 copies per cell in most yeast strains (Broach 1981). The plasmid has been sequenced (Hartley and Donelson 1980) and has been utilized as a cloning vector in yeast (Botstein and Davis 1982). A notable feature of the sequence of this plasmid is a 599-bp inverted repeat, which divides the plasmid into two roughly equivalent segments. Recombination between these repeats inverts one segment of the plasmid relative to the other (Broach et al. 1982). This results in two forms of the plasmid, A and B, which occur in equal concentrations in plasmid DNA isolated from yeast cells. In addition, 10–20% of the 2-micron plasmid DNA isolated from yeast is present in dimeric or other multimeric forms (Royer and Hollenberg 1977). These multimers could arise in vivo due to intramolecular recombination within a θ-structure replication intermediate or could be produced by intermolecular recombination. There are several open reading frames on the 2-micron plasmid. One of these, designated FLP, has been implicated in the yeast recombination reaction in vivo (Gerbaud et al. 1979; Broach and Hicks 1980). This gene, which should produce a protein product with a molecular weight of approximately 48,700, has been cloned and expressed in *Escherichia coli*. *E. coli* strains harboring a plasmid that expresses the FLP gene acquire the capability to carry out this site-specific recombination event in vivo. This has permitted an analysis of FLP-protein-mediated genetic recombination in the absence of any other yeast proteins or factors (Cox 1983). In *E. coli*, the FLP protein acts in *trans* to carry out 2-micron-specific recombination. All three possible types of site-specific recombination, which include inversions, deletions, and insertions, have been demonstrated in this in vivo system. This in vivo reaction is also completely dependent upon an intact FLP recombination site. Most important, this work demonstrates that no other yeast proteins are required for this recombination event (Cox 1983). In *E. coli*, the FLP protein promotes a site-specific recombination reaction that exhibits properties consistent with the properties of the same recombination event observed in yeast.

The other component of this system, the recombination site, has been partially defined. Efficient recombination does not require the entire 599-bp repeat. The site has been narrowed to a 56-bp site within this repeat, spanning an *Xba*I site (Broach et al. 1982; J.F. Senecoff, unpubl.). Deletion of 4 bp within the *Xba*I site abolishes the recombination reaction in yeast (Broach et al. 1982). Addition of 4 bp at the same site abolishes the recombination reaction in the *E. coli* in vivo system (Cox 1983). Clearly, the *Xba*I site is an essential part of the recombination site. An 8-bp spacer region, including the *Xba*I site, separates the first and second of a series of three repeated 13-bp sequences (Fig. 1). These repeats all lie within the 56-bp region described above. The effect of deletions or additions at the *Xba*I site is to alter the length of the spacer. This suggests that the position of at least the two 13-bp repeats that flank this spacer is an important feature of the recombination site. The 13-bp repeats also exhibit a considerable degree of homology with the *lox*P recombination site in the *cre/lox* site-specific recombination system of bacteriophage P1 (Abremski and Hoess 1984; Vetter et al. 1983).

In vitro, site-specific recombination systems have been developed for at least three prokaryotic recombination events. These include bacteriophage λ integration/excision mediated by the Int and Xis proteins (Nash 1981), resolution of cointegrate structures by the resolvase protein of the Tn*3* family transposable elements (Reed 1981), and the *cre/lox* system of bacteriophage P1 (Abremski et al. 1983). Each of these in vitro reactions exhibits at least one unique property. In the bacteriophage λ system, an additional host protein, integration host factor (IHF), is required. Resolvase will react only when two recombination sites are present in direct repeat on the same DNA molecule, generating a deletion (Krasnow and Cozzarelli 1983). The recombination event mediated by the Cre protein does not require a supercoiled substrate under any set of conditions.

In an effort to extend these studies to eukaryotes, an in vitro system for FLP-protein-promoted site-specific genetic recombination has been developed employing

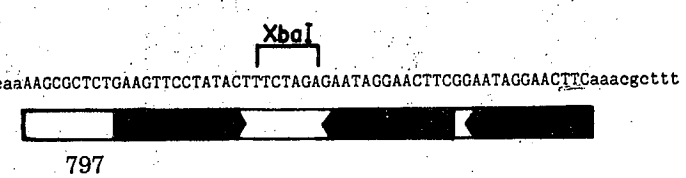


Figure 1. The FLP recombination site. Repeated sequences are indicated by arrows. The site has been narrowed to the 56 bp presented in capital letters.

yeast extracts (Vetter et al. 1983). Similarly, we have developed an in vitro system for this recombination event employing extracts of *E. coli* cells in which the FLP protein has been expressed. This report summarizes results obtained to date with the in vitro system derived from *E. coli*.

MATERIALS AND METHODS

Enzymes and plasmid DNA. Restriction endonucleases were purchased from New England Biolabs. Calf thymus type-1 topoisomerase was purchased from Bethesda Research Laboratories. Proteinase K was purchased from Beckman. Construction of the plasmids pMMC1, pMMC3, pMMC8, and pMMC10 (Fig. 2) is described elsewhere (Cox 1983). A 166-bp DNA fragment containing the FLP recombination site, excised from the 2-micron, 599-bp repeat and flanked with *HindIII* linkers, was generously provided to us by Richard Palmiter (University of Washington, Seattle). This fragment was inserted into the *Hin*dIII site of the plasmid pXf3 (Maniatis et al. 1982) to generate the plasmid pJFS2. Construction of the plasmid pLJC1, which incorporates those sequences from pMMC8 that are necessary to express the FLP protein, will be described elsewhere.

Strains. Plasmids described in this study were maintained in the *E. coli* strain HB101 (Maniatis et al. 1982).

Media and buffers. AZ broth has been described elsewhere (Cox et al. 1981). Buffer A contains 20 mM Tris-HCl, 80% cation (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 2 mM dithiothreitol (DTT), and 0.02 mM phenylmethylsulfonyl fluoride (PMSF). Varying amounts of NaCl were added to buffer A to give buffer A(X), where X is the concentration of NaCl. Buffer P(X) is identical to buffer A, except that the Tris-HCl is replaced by potassium phosphate, 80% dianion (pH 7.5); the symbol X refers to the concentration of potassium phosphate rather than to NaCl. TSE buffer contains 50 mM Tris-HCl, 50% cation (pH 8.1), 10% (w/v) sucrose, and 20 mM EDTA. Lysis buffer contains 50 mM Tris-HCl, 50% cation (pH 8.1), 300 mM spermidine, 2.0 M NaCl, and 10% (w/v) sucrose.

Cell growth and induction of FLP protein. *E. coli* strain HB101 harboring the plasmid pLJC1 was grown at 30°C in AZ broth containing 10 μg/ml of tetracycline in a 300-liter fermenter with continuous aeration. At a cell density corresponding to an OD_{595} of 1.0, the temperature was raised to 42°C for 20 min. Growth was continued at 37°C for an additional 3 hr. Cells were harvested in a Sharples continuous-flow centrifuge, resuspended in a volume of TSE buffer 5/8 the weight of the collected cells, frozen in liquid nitrogen, and stored at −70°C until use.

Partial purification of FLP protein. All of the following steps were carried out on ice or at 4°C. Frozen cell paste (1800*g*) was allowed to thaw overnight. To this was added 1800 ml of TSE buffer, 144 ml of a saturated solution of ammonium sulfate, 180 ml of lysis buffer, 3.6 ml of 1.0 M DTT, and 0.8 g of lysozyme. After 45 min on ice with occasional stirring, this mixture was centrifuged for 80 min at 13,000 rpm in a Beckman JA-14 rotor. The resulting supernatant is fraction I. A polyethylene glycol (PEG) solution (50% w/v) was added to fraction I to a final concentration

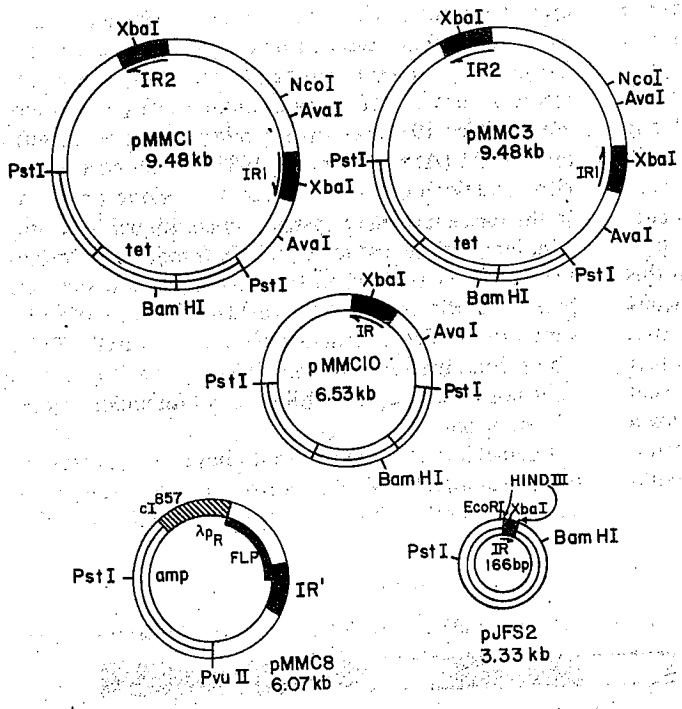


Figure 2. Plasmids utilized in this study. See text for details.

of 5.8%. This was stirred for 20 min and then centrifuged at 10,000 rpm in the JA-14 rotor for 25 min. The pellet (fraction II) was dissolved in buffer A (2.0 M) with the aid of a Waring Blendor. PEG was again added to a final concentration of 5.0%. The resulting mixture was again stirred for 20 min and recentrifuged at 10,000 rpm in a JA-14 rotor for 25 min. The supernatant (fraction III) was dialyzed in buffer A (0.15 M) for 8 hr. The resulting precipitate was collected by centrifugation at 9500 rpm in a Beckman JA-10 rotor for 25 min and resuspended in 60 ml of buffer A (1.0 M) to generate fraction IV. Fraction IV was mixed with 200 ml (settled bed volume) of Whatman P-11 phosphocellulose in buffer A (1.0 M) and dialyzed against buffer A (0.1 M) for 6 hr. The dialysis bags were inverted frequently during this period. After dialysis, a column was packed with the P-11/fraction-IV mixture and then washed in steps with 2.5 column volumes each of buffer A (0.1 M) and buffer A (0.4 M). FLP protein activity was then eluted with buffer A (1.0 M). Active fractions were pooled and dialyzed against 4 liters of buffer P (0.2 M) and loaded over a column containing 50 ml (bed volume) of hydroxylapatite (Bio-Gel HTP). This column was washed successively with 2.5 column volumes each of buffer P (0.2 M) and buffer P (0.22 M). FLP protein recombination activity was eluted with buffer P (0.3 M). Active fractions were pooled and designated fraction VI. After dialysis into buffer A (1.0 M), this fraction was used for most of the experiments described in this report. Where indicated, fractions were utilized that had undergone further purification by sedimentation through a glycerol gradient. Details of this procedure will be described elsewhere.

Miscellaneous methods. Isolation of supercoiled plasmid DNA and restriction digests were carried out as described elsewhere (Maniatis et al. 1982). Agarose gel electrophoresis was performed using 0.8% agarose in a Tris-acetate buffer system as described (Davis et al. 1980). Partial relaxation of supercoiled plasmid DNA with calf thymus type-1 topoisomerase was carried out under conditions similar to those used in the FLP protein assay. The length of incubation required to obtain complete relaxation was determined empirically.

Assay of FLP-protein-promoted recombination in vitro. Unless stated otherwise, reaction mixtures (20 μl) contained 25 mM Tris-HCl, 80% cation (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 5% (v/v) glycerol, 100 mM NaCl, 0.2 μg of substrate DNA, and an amount of FLP protein fraction VI, determined empirically, which produced an optimal reaction under these conditions. Larger reaction volumes were used where indicated. Incubations were carried out at 30°C for the indicated times. Reactions were stopped by the addition of 1/10 volume of 10% SDS, followed by the addition of 1/2 volume of a solution containing 50% (v/v) glycerol, 50 mM EDTA, and 0.05% bromphenol blue. Reaction products and substrates were separated by agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed as described previously (Maniatis et al. 1982).

RESULTS

Production and Fractionation of the FLP Protein

Total purification of the FLP protein has been hampered by two problems: (1) The protein is present at very low concentrations in our extracts, even after induction, and (2) the protein tends to precipitate at salt concentrations (NaCl) of less than 0.3 M. Although expression of the protein in *E. coli* was aided by the fortuitous placement of unique restriction sites (Cox 1983), expression of FLP protein in this construction is more than an order of magnitude less than that observed when the β-galactosidase gene of *E. coli* is similarly placed (R.C. Bruckner, unpubl.). In this construction, the FLP gene on the plasmid pMMC8 (Fig. 2) is located downstream from the λ rightward promoter and utilizes the λ*cro* gene ribosome-binding site. The initiation codon of the FLP gene is positioned exactly where the initiation codon of the *cro* gene is normally placed relative to its ribosome-binding site, an arrangement that has been confirmed directly by sequencing. Attempts to improve the level of expression of this protein have not been completed. We estimate that FLP protein represents less than 0.1% of the protein in fraction I. The second problem, low salt insolubility, is manifested after all of the nucleic acid is removed from the extract (fraction V). Precipitation at this stage is often accompanied by nearly total inactivation of the protein. The protein is therefore stored in buffers containing 1.0 M NaCl and is stable for several months under those conditions at 0°C.

The extent of purification achieved to date is difficult to assess because a protein band has not yet been unambiguously identified as FLP protein on an SDS-polyacrylamide gel. The most likely candidate is a band representing 3–5% of the protein in fraction VI. Further fractionation in glycerol gradients has produced fractions in which this putative FLP protein is 10–30% pure. Approximately 0.5 mg of this protein is present in fraction VI, generated from 1800*g* of cell paste. Fraction VI is free from contaminating nuclease activity. The level of recombination activity in this fraction is typically sufficient to carry out 100,000 of the standard assays described in Materials and Methods.

Characteristics of and Requirements for FLP-protein-promoted Recombination In Vitro

The substrate used for the standard assay is the plasmid pMMC3 (Fig. 2), which is linearized by digestion with the restriction endonuclease *Bam*HI. This plasmid contains two FLP recombination sites in direct orientation. As illustrated in Figure 3, this substrate can participate in both intramolecular and intermolecular site-specific recombination. Intramolecular recombination results in a deletion reaction. As illus-

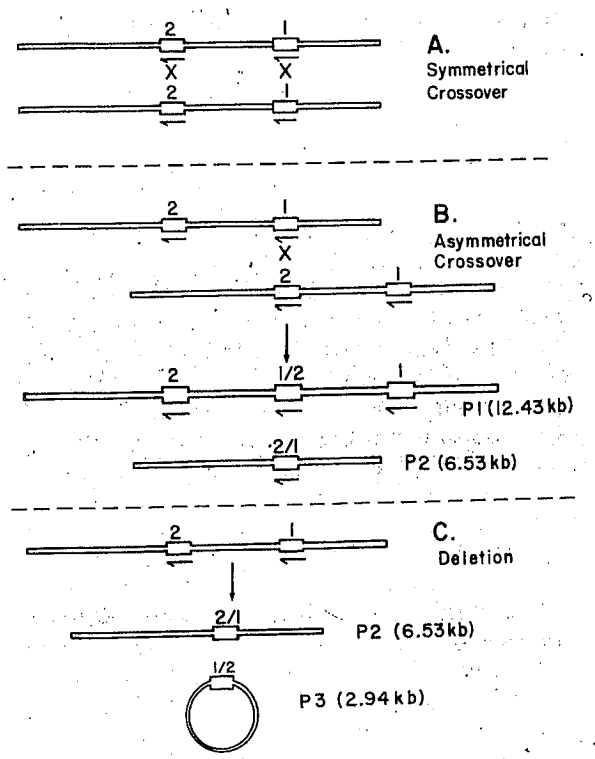


Figure 3. FLP-mediated recombination. Reactions employing a linear substrate with two recombination sites in direct repeat.

trated in Figure 3, the products are a circular DNA molecule (P3, 2.94 kb), derived from the DNA between the recombination sites, and a linear DNA molecule (P2, 6.54 kb). P2 contains the replication origin and is equivalent to the plasmid pMMC10.

Intermolecular recombination has two different consequences. The recombination sites in Figure 3 are distinguished arbitrarily as sites 1 and 2. A symmetric recombination event (1×1 or 2×2) will generate products that are identical to the substrates. This reaction cannot be observed in our assay. An asymmetric recombination event (1×2 or 2×1), however, will result in one product (P1) that is larger than the substrate and another (P2) that is smaller. The smaller recombination product is equivalent to the P2 product of intramolecular recombination. P1 has been enlarged by 2.94 kb (one linear P3 equivalent) relative to the substrate. A second asymmetric recombination event involving P1 and pMMC3 will result in the generation of another molecule of P2 and an additional product, which is now 2.94 kb larger than P1. Continued intermolecular recombination will therefore tend to produce a number of products larger than the substrate, which differ by the addition of 2.94-kb segments; only one smaller product, P2, accumulates. Since P2 is generated by both intramolecular and intermolecular recombination, it is of limited utility in distinguishing these reactions. P1 is uniquely diagnostic of intermolecular recombination, whereas P3 is diagnostic of intramolecular recombination.

A typical experiment is illustrated in Figure 4. A 200-μl reaction volume was employed in this experiment, and 20-μl aliquots were removed at the indicated times and treated as described in Materials and Methods. Several features of FLP-protein-promoted genetic recombination are evident in this experiment. First, this reaction proceeds efficiently with a linear substrate. This was reported previously by Vetter et al. (1983), who employed an in vitro system derived from yeast extracts. Second, both intramolecular and intermolecular recombination events are evident in reaction A. Intermolecular recombination results in the ladder of bands (P1) corresponding to product molecules larger than the substrate. We have observed up to seven separate recombination products in this ladder. Intramolecular deletion results in the formation of the circular product, P3. This product is also found to be a series of bands in these experiments. It will be demonstrated below that these bands are separate topoisomers of P3 and that adjacent bands are related by a change in linking number of 1.

One other feature of this in vitro system is evident in Figure 4. The time courses designated A and B were carried out under identical conditions, except that FLP protein from two different glycerol gradient fractions was employed. Deletions are observed in reaction A but not in reaction B. If the two fractions are mixed, the resulting mixture does not promote deletion reac-

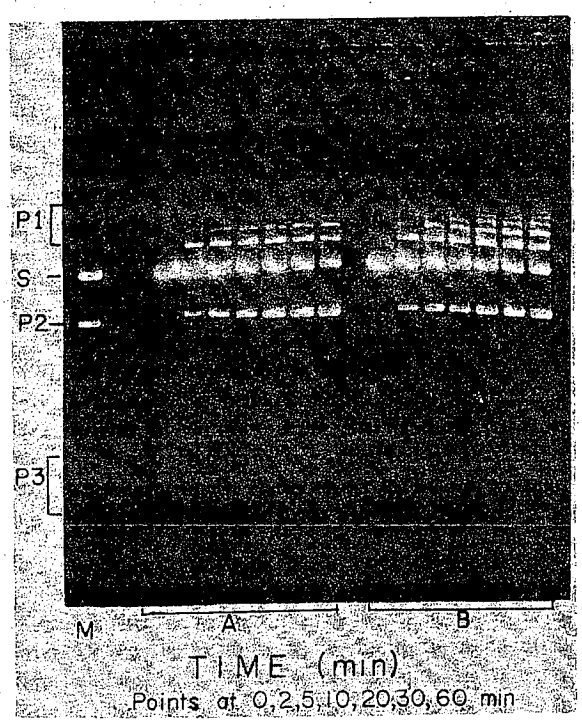


Figure 4. Intramolecular and intermolecular recombination promoted by FLP protein in vitro. (M) Marker DNA including pMMC3 (S) and pMMC10 (P2) DNAs, each linearized with *Bam*HI. (P1, P3) Reaction products described in the legend to Fig. 3 and in the text. The two reaction time courses (A, B) were carried out with different FLP protein fractions as described in the text.

tions under these conditions. We do not understand the basis for this difference in capability to carry out intramolecular recombination. It is clearly influenced by other factors in the protein fractions. It is also influenced by reaction conditions, since fractions that do not promote intramolecular recombination under these conditions have been observed to do so when the ionic strength has been increased (L. Meyer-Leon, unpubl.).

The requirements for this reaction are very simple. No high energy cofactors are required, and although Mg^{++} is included in the standard reaction conditions, a divalent cation is not absolutely required. If 10 mM $MgCl_2$ is included, an optimal reaction is observed at NaCl concentrations of 100–200 mM, whereas if the Mg^{++} is deleted, the concentration of NaCl required for an optimal reaction is increased to 250–350 mM. Thus, the only important factors appear to be the presence of a substrate with an intact recombination site and appropriate ionic strength.

Two types of site-specific recombination, deletions and insertions, are presented in Figure 4. The FLP protein, however, is normally involved in an inversion reaction in vivo, and an example of an FLP-protein-promoted inversion reaction in vitro is illustrated in Figure 5. This reaction employs as a substrate the plasmid pMMC1 (Fig. 2), which contains two recombination sites in inverted orientation. Recombination can be demonstrated by cleavage of this plasmid with the restriction endonucleases *Bam*HI and *Nco*I. Supercoiled pMMC1 is presented in Figure 5, lane 1. Cleavage with *Bam*HI and *Nco*I results in the production of two products shown in lane 2. An inversion of the sequences between the two recombination sites should alter the restriction digest pattern. Inversion will result in two new restriction products that are intermediate in size between the large and small substrate fragments. These are designated P in Figure 5. If supercoiled pMMC1 is reacted with FLP protein for 90 min and subjected to no further treatment, a DNA pattern results, which is illustrated in Figure 5, lane 3. An aliquot of this DNA, after the FLP-protein-promoted reaction, was subjected to digestion by *Bam*HI and *Nco*I, yielding the pattern illustrated in Figure 5, lane 4. This result indicates that FLP-protein-promoted inversion has produced an approximately 50/50 mixture of the two forms of this plasmid. This result would be expected if the inversion reaction proceeded to equilibrium.

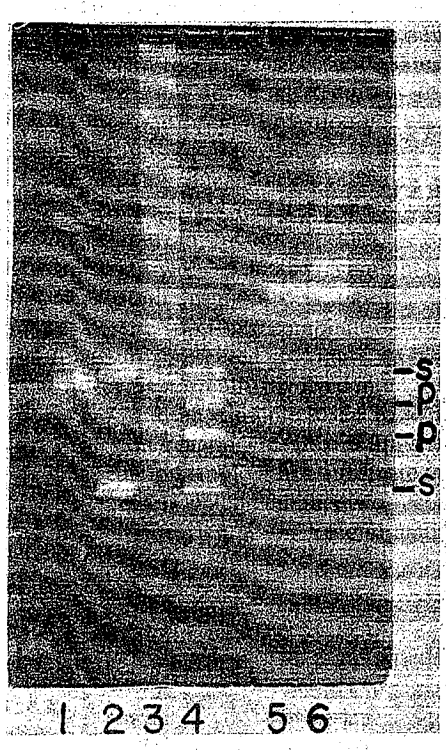


Figure 5. FLP-protein-promoted inversion in vitro. Lane identification: (*1*) Supercoiled pMMC1 DNA; (*2*) pMMC1 DNA after cleavage with *Bam*HI and *Nco*I; (*3*) supercoiled pMMC1 DNA after reaction with FLP protein for 90 min; (*4*) DNA of lane *3* after cleavage with *Bam*HI and *Nco*I; (*5*) pMMC1 DNA after cleavage with *Bam*HI; (*6*) DNA of lane *3* after cleavage with *Bam*HI. Agarose gel electrophoresis was carried out as described in Materials and Methods.

Results presented in Figure 5 demonstrate that intermolecular recombination occurs with this substrate as it does with pMMC3. Cleavage of pMMC1 with *Bam*HI should result in production of a single DNA band whether or not an inversion reaction has occurred. The band resulting from *Bam*HI cleavage of unreacted pMMC1 is shown in Figure 5, lane 5. After reaction with FLP protein, cleavage of pMMC1 DNA with *Bam*HI results in two additional bands (lane 6). These are derived from an asymmetric intermolecular recombination reaction involving pMMC1. If the recombination sites of pMMC1 are arbitrarily designated site 1 and site 2, an asymmetric recombination reaction is defined as a reaction of site 1 on one DNA molecule with site 2 on the other.

An alternative assay for FLP-protein-mediated recombination, which measures only intermolecular recombination, is illustrated in Figure 6. A plasmid containing only one FLP recombination site is employed. Two linear substrates that differ in their endpoints are generated by cleavage with one of two restriction enzymes, x and y. Both substrates are identical in size. If two linear molecules cleaved by enzyme x are recombined, the products will be identical to the substrates and the reaction will not be observed. If a DNA molecule cleaved by enzyme x is recombined with the same DNA molecule cleaved by enzyme y, however, two new products will be generated. One of these will be larger than the substrates and one will be smaller.

Several examples of this type of assay are illustrated in Figure 7. For each set of reactions, the four lanes correspond to four different levels of FLP protein (1–4 μl of an FLP protein fraction equivalent to fraction V; the amount of protein fraction increases in 1-μl increments from left to right) employed in the reaction. In the first set of reactions (599 × 599), the plasmid pMMC10 (6.53 kb) linearized with either *Cla*I or *Pvu*II is employed as substrate. This plasmid contains one complete copy of the 599-bp repeat. Recombination

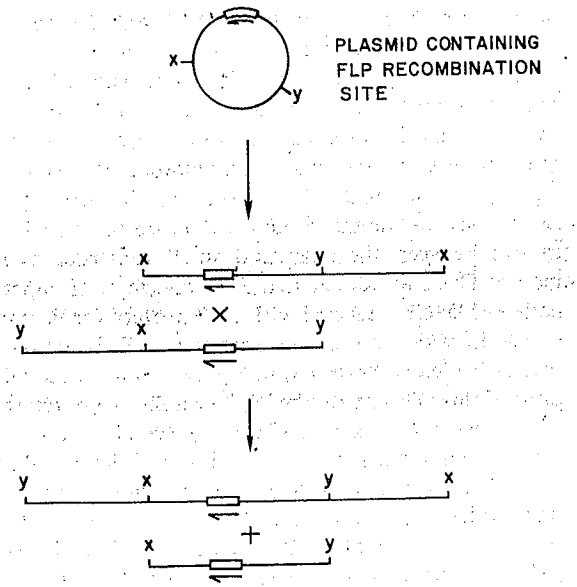


Figure 6. Assay for FLP-protein-promoted intermolecular recombination. (x,y) Arbitrary restriction endonuclease cleavage sites.

between *Cla*I-cleaved pMMC10 and *Pvu*II-cleaved pMMC10 should generate two products, which are 9.43 kb and 3.63 kb in length. Products that correspond closely in size to the expected products are evident in Figure 7. The second set of reactions employs the plasmid pJFS2 (3.33 kb) linearized with either *Eco*RI or *Pst*I. The expected products (4.08 kb and 2.57 kb) are again observed. The plasmid pJFS2 contains 166 bp from the 599-bp, 2-micron plasmid repeat. This segment includes all sequences necessary for FLP-protein-promoted recombination. In the third set of experiments, pMMC10 linearized with *Cla*I is recombined with pJFS2 linearized with *Bam*HI. The expected recombination products (5.09 kb and 4.77 kb) are again observed. In these and a series of similar experiments, we have found no evidence indicating that sequences within the 599-bp repeat, but outside of the 56-bp fragment spanning the *Xba*I site, affect this recombination reaction. The function of this long repeated sequence thus remains unclear. The fourth set of reactions employs the plasmid pMMC8, which contains a single recombination site that has been altered by the addition of 4 bp at the *Xba*I site. Alteration of sequences within this *Xba*I site abolishes FLP-promoted recombination in vivo in both yeast and *E. coli*. This altered site is also nonfunctional in our in vitro system. The reaction illustrated involves pMMC8 DNA linearized with either *Cla*I or *Pst*I. The products, if they were produced, would be 5.29 kb and 6.85 kb. This provides another piece of evidence, that the FLP protein produced from *E. coli* promotes a recombination reaction in vitro, which is consistent with all of the known properties of FLP-protein-promoted recombination in yeast.

Possible Mechanisms for FLP-promoted Recombination

The properties of FLP-protein-mediated recombination in vitro, as described above, closely resemble the properties of the *cre/lox* recombination system derived from bacteriophage P1 (Abremski et al. 1983). The two reactions are nearly indentical in that they proceed efficiently with linear substrates, promote all three types of site-specific recombination, and exhibit no requirement for additional proteins or factors. As pointed out above, the recombination sites also exhibit significant homology. We decided to examine this identity further by carrying out experiments designed to determine the mechanism by which recombination sites were aligned in the FLP system. A deletion reaction was carried out with supercoiled plasmid containing two recombination sites in direct repeat. In the case

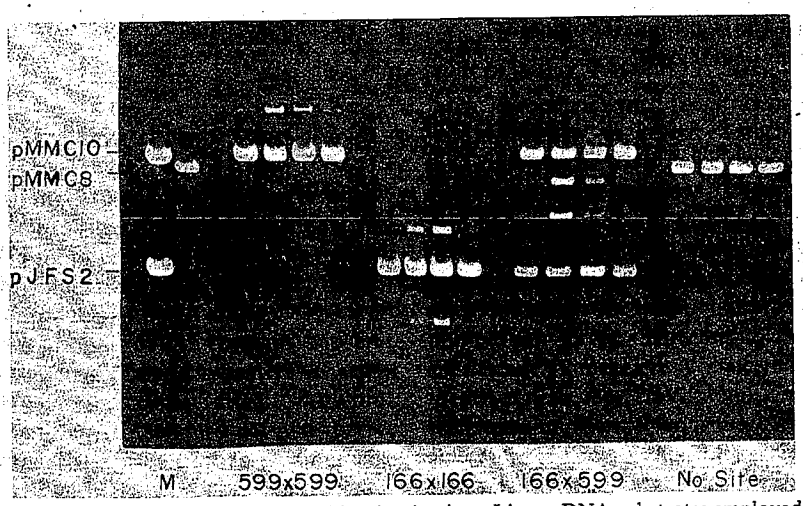


Figure 7. FLP-protein-promoted intermolecular recombination in vitro. Linear DNA substrates employed in these experiments are identified in the first two lanes (M). Reactions are described in detail in the text. Agarose gel electrophoresis was carried out as described in Materials and Methods.

of recombination mediated by Cre protein, about 50% of the deletion products are free and the remainder are present as catenanes interlinked one time. As described by Abremski et al. (1983), this provides evidence that Cre-protein-mediated recombination proceeds by a tracking mechanism in which the location of one recombination site is followed by a processive search on the same DNA molecule for a second recombination site. For a detailed description of this mechanism, see Krasnow and Cozzarelli (1983). An alternative mechanism involves "random collisions" in which a recombinase bound at one recombination site locates another site by randomly binding and releasing other DNA segments. Our results indicate that FLP protein utilizes a random collision mechanism for site-specific recombination. A series of FLP-protein-mediated deletion reactions involving both linear and supercoiled pMMC3 DNA are illustrated in Figure 8. These experiments employ a glycerol gradient FLP protein fraction that was found to promote intramolecular deletion efficiently. Supercoiled pMMC3 DNA before and after reaction with FLP protein is shown in Figure 8, lanes 1 and 2, respectively. Cleavage of the DNA in lane 2 with *Bam*HI (lane 3) yields the pattern of reaction products illustrated in Figure 4. The series of bands corresponding to the circular recombination product diagnostic of a deletion reaction all migrate ahead of the DNA smear evident in lane 2. FLP-protein-promoted deletion, unlike the Cre-protein-promoted reaction, does not produce free deletion products. The products, therefore, must all be catenated. The smear in lane 2 has not yet been analyzed but may represent a complex assortment of catenated and knotted products.

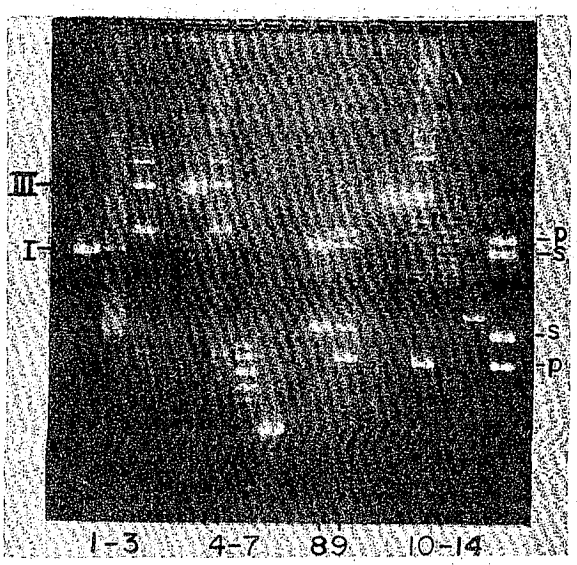


Figure 8. Evidence for a random collision mechanism in FLP-protein-promoted recombination. Lane identification: (*1*) Supercoiled pMMC3 DNA; (*2*) supercoiled pMMC3 DNA after reaction with FLP protein; (*3*) DNA from lane *2* after digestion with *Bam*HI; (*4*) pMMC3 DNA linearized with *Bam*HI; (*5*) DNA from lane *4* after reaction with FLP protein; (*6*) pJFS8 DNA relaxed with calf thymus type-1 topoisomerase; (*7*) supercoiled pJFS8 DNA; (*8*) pMMC3 DNA after cleavage with *Bam*HI and *Nco*I; (*9*) DNA from lane *5* after cleavage with *Nco*I; (*10*) pMMC3 DNA linearized with *Nco*I; (*11*) DNA from lane *10* after reaction with FLP protein; (*12*) pMMC10 DNA relaxed with calf thymus type-1 topoisomerase; (*13*) supercoiled pMMC10 DNA; (*14*) DNA from lane *11* after cleavage with *Bam*HI. All reactions were carried out for 90 min. Agarose gel electrophoresis was carried out as described in Materials and Methods. See text for details.

An FLP-protein-promoted deletion reaction employing pMMC3 DNA linearized with *Bam*HI is presented in Figure 8, lanes 4–7. This substrate is shown before and after reaction in lanes 4 and 5, respectively. The results may be compared with those obtained in Figure 4. A supercoiled plasmid of approximately 3 kb, pJFS8 (the structure of this plasmid will be described elsewhere), is shown in lane 7 to provide a marker that is approximately the same size as the 2944-bp circular deletion product. This 3-kb plasmid was relaxed with calf thymus type-1 topoisomerase, as described in Materials and Methods, to provide a series of bands differing in their topological linking number in steps of 1 (lane 6). Although these bands are somewhat larger than those produced by the pMMC3 deletion reaction, the patterns are similar and provide evidence that this series of bands represents a series of topoisomers of the small circular deletion product. The small deletion product can be uniquely cleaved with the restriction enzyme *Nco*I. The larger deletion product is not cleaved by this enzyme. If pMMC3 DNA linearized with *Bam*HI is further treated with *Nco*I both before and after reaction with FLP protein, the results in lanes 8 and 9, respectively, are obtained (Fig. 8). The new bands evident in lane 9 (designated P) correspond to the linearized 6.54-kb and 2.94-kb deletion products. This experiment demonstrates that the series of putative topoisomers of the small circular deletion product are reduced to a single band by cleavage with *Nco*I as expected.

A different linear substrate is generated if pMMC3 is cleaved only with *Nco*I. In this case, the circular product generated by FLP-protein-mediated deletion is 6.54 kb, and the linear product is 2.94 kb. The circular product is equivalent to the plasmid pMMC10. This substrate is presented in Figure 8 before and after reaction with FLP protein in lanes 10 and 11, respectively. The plasmid pMMC10 (supercoiled) is presented in lanes 12 and 13, after and before relaxation with calf thymus type-1 topoisomerase, respectively. The recombination products evident in lane 11 include the linear 2.94-kb deletion product and a series of bands that correspond exactly with the pMMC10 topoisomers. The circular product of this recombination reaction is again present as a series of topoisomers that differ in their topological linking number in steps of 1. In this case, the distance between the recombination sites on the linear substrate is greater, and the range of topoisomers of the circular deletion product observed is correspondingly increased. The pattern of topoisomers observed is nearly identical to that generated by complete relaxation of pMMC10 supercoiled DNA by

a type-1 topoisomerase under similar conditions. The topoisomer most likely reflects the expected thermal fluctuations in the DNA helix at the time of the recombination event (Depew and Wang 1975; Pulleyblank et al. 1975). If the DNA in lane 11 is cleaved with *Bam*HI, it is reduced to the pattern presented in lane 14. This pattern is identical to that observed in lane 9, as expected, and demonstrates that cleavage of the circular product with an enzyme that should cleave it once reduces the topoisomer pattern to a single band (top band in lane 14). This experiment is consistent with a random collision mechanism for FLP-protein-promoted recombination. All of the products of a deletion reaction involving a supercoiled substrate are catenated. This piece of evidence alone does not eliminate a tracking mechanism. FLP protein, however, promotes intermolecular recombination efficiently. Intermolecular recombination must involve random collision to some extent.

DISCUSSION

The properties of the in vitro system for FLP-protein-promoted, site-specific recombination described above are consistent with the properties of the same reaction as observed in vivo in yeast and in yeast extracts. FLP protein produced from *E. coli* promotes a site-specific recombination reaction that has simple requirements, proceeds with equal efficiency on supercoiled and linear substrates, and requires an intact FLP recombination site. All three types of site-specific recombination are observed. These properties are very similar to those reported for the *cre/lox* recombination system of bacteriophage P1.

We note at least one apparent difference between the two systems, however. Cre-protein-mediated deletion reactions involving a supercoiled DNA substrate result in the production of free deletion products, whereas no free deletion products are observed in a similar experiment carried out with FLP protein. Our results suggest that the FLP protein promotes site-specific recombination via a random collision mechanism.

The experiments described above were carried out with partially purified fractions of FLP protein. It is unclear at present to what extent the conclusions outlined above are affected by contaminants in these fractions. Confirmation of these results, therefore, awaits the total purification of the FLP protein.

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