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# Reactions between Half- and Full-FLP Recombination Target Sites

## A MODEL SYSTEM FOR ANALYZING EARLY STEPS IN FLP PROTEIN-MEDIATED SITE-SPECIFIC RECOMBINATION*

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**The FLP recombination target (FRT) can be cut in half so that only one FLP protein binding site is present (a "half site"). FLP protein binds the half sites and joins them into dimeric, asymmetric head-to-head complexes held together chiefly by strong noncovalent interactions. These complexes react with full (normal) FRT sites to generate a variety of products. Analysis of these DNA species reveals that the reaction follows a well-defined reaction pathway that generally parallels the normal reaction pathway. The system is useful in analyzing early steps in recombination, since the identity of the products in a given recombination event unambiguously pinpoints the order in which the cleavage and strand exchange reactions occur. Two conclusions are derived from the present study: (i) Formation of the dimeric head-to-head complex of half sites is a prerequisite to further steps in recombination. (ii) The identity of the base pairs at positions 6 and −6 within the FRT site has a subtle effect in directing the first strand exchange event in the reaction to predominantly one of two possible cleavage sites. In addition, results are presented that suggest that a DNA-DNA pairing intermediate involving only two base pairs of the core sequence is formed prior to the first cleavage and strand exchange. DNA-DNA interactions may therefore not be limited to the isomerization step that follows the first strand exchange.**

Conservative site-specific recombination generates precise and efficient DNA rearrangements at well defined DNA sites (for reviews, see Craig, 1988; Landy, 1989). The yeast 2-$\mu$m plasmid encodes a site-specific recombination system that permits the plasmid to circumvent host cell cycle control of plasmid replication for the purpose of increasing plasmid copy number (Futcher, 1986; Volkert and Broach, 1986; Murray *et al.*, 1987; Reynolds *et al.*, 1987). The recombinase is designated FLP and is a member of the integrase family of site-specific recombinases.

The integrase family, which includes the λ integrase and the cre recombinase of bacteriophage P1 (for reviews, see Argos *et al.*, 1986; Sadowski, 1986; Craig, 1988; Landy, 1989; Cox, 1989) is defined by a number of mechanistic features of the reactions catalyzed by the respective recombinases. A second family of recombinases is called the resolvase/invertase family and includes the hin invertase (*Salmonella typhimurium*), gin invertase (bacteriophage Mu), and the resolvases of Tn3 class transposons (Craig, 1988). The general pathway for recombination reactions promoted by integrase-class recombinases is presented in Fig. 1 and based on studies carried out with a number of systems. The protein-DNA intermediate formed in the first step is a 3′-phosphotyrosine linkage for integrase-class recombinases, leaving a free 5′-hydroxyl group at the cleavage site (the intermediate in invertase/resolvase-class reactions is a 5′-phosphoserine). The initial cleavage step involves only two of the four cleavage sites, and strand exchange yields a Holliday structure intermediate. Although the first cleavage is shown as occurring to the *left* of the core in Fig. 1, there is actually no prescribed order, and the first set of cleavages occurs on the opposite side with equal probability. The Holliday intermediate has been identified during site-specific recombination only for integrase-class reactions (Nunes-Düby *et al.*, 1987; Hoess *et al.*, 1987; Kitts and Nash, 1988; Jayaram *et al.*, 1988; Meyer-Leon *et al.*, 1988, 1990). There are two Holliday structures on the pathway, related by an isomerization step that can become rate-limiting at 0 °C (Meyer-Leon *et al.*, 1990). The last two steps of the reaction parallel the first two. Many details of this pathway remain to be worked out.

The minimal FRT[1] site *in vivo* and *in vitro* consists of an eight base core region flanked by 13-base pair inverted repeats. The two FLP recombinase binding sites within the FRT include the single base pair at either end of the core sequence and the 11 contiguous base pairs in the adjoining repeats (Andrews *et al.*, 1985; Bruckner and Cox, 1986; Senecoff *et al.*, 1988). There are no identified protein-DNA interactions in the central 6 base pairs of the core sequence. The core sequence can be altered substantially without affecting FRT function, although two reacting FRT sites must have homologous cores. One FRT site with an altered core sequence that has been used in many studies is the FRT site in plasmid pJFS39 (Senecoff and Cox, 1986) (Fig. 2). The core in this FRT site is palindromic, differing from the normal FRT core at five of the six central positions.

The requirement for homology in the core sequences of two reacting recombination sites led to consideration of the possibility that an unusual four-stranded DNA structure was formed as a prerequisite to cleavage and strand exchange (Kikuchi and Nash, 1979; Nash and Pollock, 1983; Griffith and Nash, 1985). A variety of careful studies have failed to provide support for this idea, and it has gradually given way to the idea that core homology is required only in the isom-

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[1] The abbreviations used are: FRT, FLP recombination target; SDS, sodium dodecyl sulfate; TAPS, 3-([tris(hydroxymethyl)methyl] aminopropanesulfonic acid; kbp, kilobase pair(s); bp, base pair(s); PEG, polyethylene glycol.

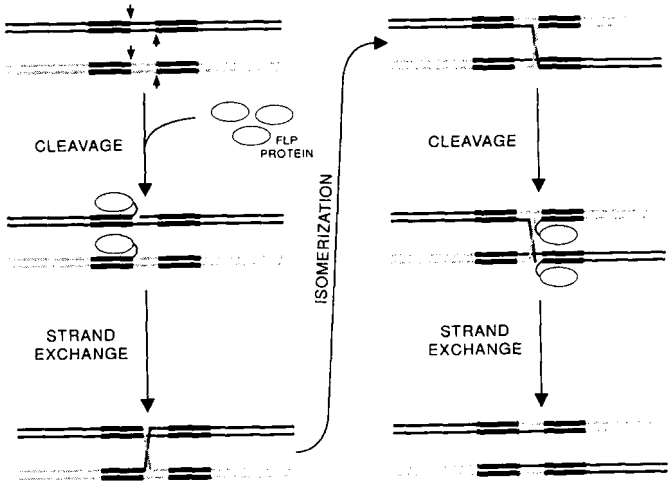



FIG. 1. **Recombination pathway for FLP protein-mediated recombination.** The major steps are illustrated for recombination of normal (unaltered) FRT sites. The *heavy bars* represent the 13-bp repeats. *Short arrows* indicate the sites of FLP-mediated DNA cleavage. FLP protein is shown only when it is covalently linked to the DNA, although four FLP monomers are probably present throughout the reaction.

erization step (Weisberg *et al.*, 1983; Nunes-Düby *et al.*, 1987; Kitts and Nash, 1987) in integrase-class reactions. In particular, several studies have shown that the first strand exchange can occur in spite of one or more core mismatches (Nunes-Duby *et al.*, 1987; Kitts and Nash, 1987). Core mismatches generally block the isomerization step, and Holliday structures formed from two sites with different cores generally revert to substrates.

One useful approach to dissecting this recombination reaction involves the use of half-recombination sites that include only one of the two recombinase binding sites. These are referred to simply as half sites. This approach was pioneered by Nunes-Düby *et al.* (1989) in a study showing that λ att half sites were competent for all steps in the reaction, up to and including the first set of cleavage and strand exchanges, when reacted with a complete att site.

Half sites have also proven to be useful in dissecting early steps in the recombination reaction promoted by the FLP recombinase. Qian *et al.* (1990) found that half-FRT sites were cleaved by FLP protein. This did not reflect binding of FLP protein to a single half site, however, but instead required the formation of a dimeric head-to-head complex of two half sites held together by strong noncovalent interactions. Within this complex, one of the two half sites was cleaved. This and other evidence showed that the complex was asymmetric, and the asymmetry was protein-based rather than DNA-based. In this work, the half sites were generated by restriction digestion so that the terminus of the second strand of the half site (the strand not cleaved by FLP protein) ended in a 5′-phosphate. Thus there was no free 5′-OH group available to react with the 3′-phosphotyrosine protein-DNA intermediate generated in the cleavage reactions.

As noted in Qian *et al.* (1990), this complex will react with full-FRT sites and we report here the results of a detailed characterization of that reaction. While this work was being completed, Amin *et al.* (1991) and Serre (1991)[2] communicated to us their results with half-FRT sites in reactions with full sites. Amin *et al.* (1991) showed that at least two base pairs of the core sequence were required in the half-FRT site to observe dimer formation and cleavage, provided additional evidence for asymmetry in the dimeric complex, and observed the formation of molecules they identified as Y structures that would be formed in a strand exchange between half-FRT sites and full-FRT sites. Serre *et al.* (1991)[2] demonstrated that if the second strand of the half-site ends in a 5′-OH, this will react with the 3′-phosphotyrosine DNA-protein linkage formed in the cleavage step to permit the formation of full-FRT sites and half sites with closed (hairpin) ends. They also showed that some recombinant products were formed in reactions between half sites and full sites that implied the existence of Y structure intermediates in the pathway. We show here that some half sites react with full sites in a well defined reaction that parallels all of the steps in a normal site-specific recombination reaction, yielding products that reveal a number of mechanistic details about the course of the first steps in the reaction. The Y structure intermediates and the recombinant products in this reaction are visualized by electron microscopy and characterized in detail.

[2] M.-C. Serre, B. R. Evans, H. Araki, Y. Oshima, and M. Jayaram, submitted for publication.

## MATERIALS AND METHODS

*Enzymes and Chemical Reagents*—FLP protein was purified by a published procedure (Meyer-Leon *et al.*, 1987; Gates *et al.*, 1987) with the exception that preparations were further purified by means of standard chromatography on orange A-agarose (Amicon Corp.). The FLP protein preparation used is >64% homogeneous as determined by SDS-polyacrylamide gel electrophoresis. FLP protein concentration was determined using the method of Bradford (1976), and the reported concentrations have been corrected for the degree of purity of the preparation. The specific activity of the preparations was $3.7 \times 10^6$ units/mg total protein (the preparation used in Fig. 11 and the *right panel* of Fig. 12 had a specific activity of $4.9 \times 10^6$ units/mg), with units defined as in Meyer-Leon *et al.* (1987). Restriction enzymes were purchased from New England Biolabs or from Promega. Agarose was from Bethesda Research Laboratories. [α-$^{32}$P]dTTP was from Amersham Corp. PEG (polyethylene glycol; molecular weight, 8,000) and glycerol were from Sigma and J. T. Baker, Inc., respectively. Proteinase K (type XX VIII) was from Sigma. Avian myeloblastosis virus reverse transcriptase was from Life Science, Inc. TAPS was from Research Organics Inc. 4′-Amino-4,5′,8-trimethylpsoralen was from Calbiochem. All chemicals were reagent grade or better.

*Plasmid and DNA Manipulation*—All plasmids used in this work were previously described: pXHQ01 (Qian *et al.*, 1990); pJFS36 and pJFS39 (Senecoff *et al.*, 1986); pSWU11, pSWU13, and pSWU19 (Umlauf and Cox, 1988). Features important to this work are summarized in Fig. 2. All plasmid DNAs were isolated by banding twice in cesium chloride gradients in the presence of ethidium bromide. The *Ase*I-cut pJFS39 was 3′ end-labeled with [α-$^{32}$P]dTTP as described in Qian *et al.* (1990), and purified by passing through a Sephadex G-50-spun column twice (Maniatis *et al.*, 1982). The labeling efficiency at both DNA ends was equivalent (data not shown). Reaction products of half- with full-site reactions were isolated from agarose gel by electroelution into a dialysis bag according to Maniatis *et al.* (1982). All DNA concentrations are reported in terms of total FRT sites or half-FRT sites.

*Reaction Conditions*—All reactions were carried out at 30 °C in a solution (20 μl) containing 25 mM TAPS (pH 8.0), 1 mM EDTA, 5% (w/v) PEG, 10% (v/v) glycerol, 200 mM NaCl, and 5.4 nM half- and 5.4 nM full-site DNA. PEG and glycerol were omitted in the reaction mix prepared for the SDS-K$^+$ precipitation experiments (described below). FLP protein concentrations are 45 nM, unless otherwise stated. If reactions were to be sampled at various times, a solution containing $n \times 16$ μl (where $n$ was the number of aliquots to be taken in a given experiment) of all components except FLP protein were prepared, and incubated at 30 °C for at least 5 min. Then 16 μl of solution was removed to serve as a zero time control. The reaction was initiated by adding $(4n - 4)$ μl of 224 nM FLP protein. Aliquots (20 μl) of reaction solution were removed at the indicated time and treated with 2 μl of 10% SDS. Alternatively, aliquots were treated with 1 μl of 10% SDS and 1 μl of 20 mg/ml proteinase K and incubated at 37 °C for 15 min.

*SDS-K$^+$ Precipitation*—The DNA with covalently linked FLP protein was separated from the free DNA by SDS-K$^+$ precipitation (Trask *et al.*, 1984). A 60-μl reaction solution (half site plus full site)

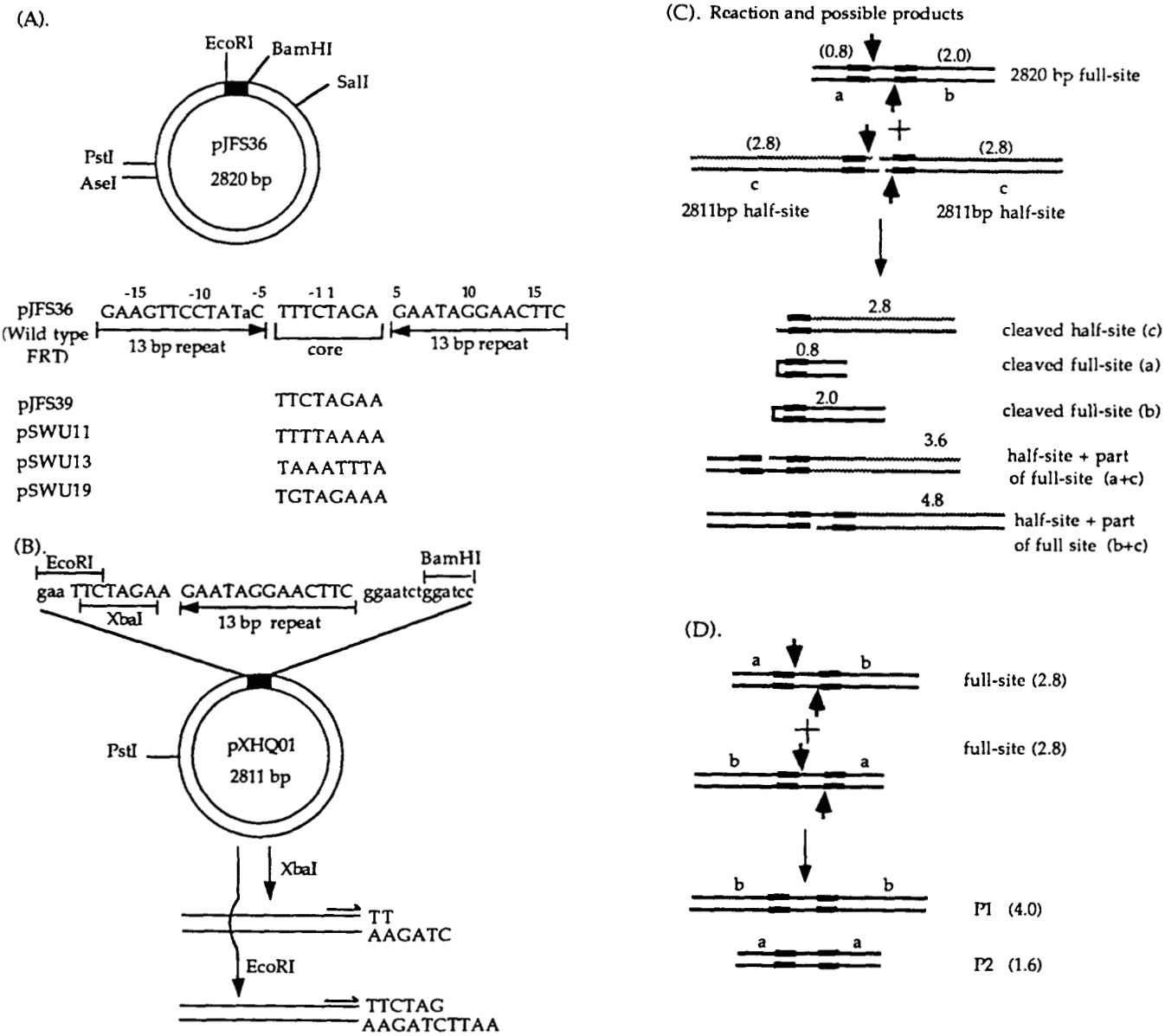



FIG. 2. **DNA substrates used in this study, and potential recombination products.** *A*, plasmids pJFS36, pJFS39, pSWU11, pSWU13, and pSWU19. These plasmids are identical except for sequence differences in the core sequence in each FRT site as indicated, and the *left* and *right* 13-bp repeats are switched in the pSWU series relative to the pJFS series. The *number* above pJFS36 FRT refers to the relative position of each base pair in the FRT site (Bruckner and Cox, 1986). The FRT sites are immediately flanked by *Eco*RI and *Bam*HI restriction sites on the plasmid. *B*, the plasmid pXHQ01, source of the half-FRT site substrates. The FRT site in this plasmid represents a cloned half site, with only one FLP protein binding site. Cleavage with *Xba*I or *Eco*RI produces substrates terminating in the core sequences shown. *C*, possible products from reactions between FRT full and half sites. The *heavy bars* represent the 13-bp repeats. *Short arrows* indicate the site of DNA cleavage. All five products that could be generated from the half-site × full-site reaction are shown. The *numbers* in *parentheses* indicate the lengths of each molecule, rounded off for convenience. These approximate numbers are used in all figures except Fig. 5 and Table I. *D*, normal FRT site reaction and products. The designations of *heavy bars* and *short arrows* are the same as in *C*. Products P1 and P2 are derived from the antiparallel juxtaposition of two substrate molecules.

as described above was incubated at 30 °C for 30 min. To this was added 6 μl of 10% SDS, followed by addition of 250 μl of buffer A (10 mM Tris-Cl, pH 8.0, 20 μg/ml acetylated bovine serum albumin, 2.8 μg/ml calf-thymus DNA, and 1% SDS). The solution was vortexed, then warmed at 37 °C for 2–4 min. This was followed by addition of 25 μl of 2.5 M KCl, then the solution was vortexed again and put on ice for 10 min. The solution was centrifuged for 2 min in an Eppendorf centrifuge. The supernatant (~280 μl) was set aside and treated as described below. The DNA pellet was washed with 1.0 ml of ice-cold buffer B (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM KCl). The DNA pellet was completely dispersed in the buffer and vortexed for 30–60 s, then centrifuged for 2 min. This wash step was repeated once. The final pellet was resuspended in 0.5 ml of buffer C (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, and 20 μg/ml tRNA). To 400 μl of the DNA/buffer C solution was added 1 ml (2.5 volume) of 100% ice-cold EtOH. The solution was mixed, and after 30 min at −20 °C it was centrifuged. The DNA pellet was dissolved in 30 μl of Tris and EDTA. An aliquot (10 μl) of this solution was treated with 0.5 μl of RNase A (10 mg/ml) for 15 min at 37 °C, then 1 μl of 10% SDS was added before loading onto an agarose gel for electrophoresis.

The supernatant from the first centrifugation was mixed with 2 μl of 5 μg/μl of tRNA, then precipitated with EtOH/NaOAc. The DNA pellet was dissolved in 30 μl of Tris and EDTA. An aliquot (8 μl) of

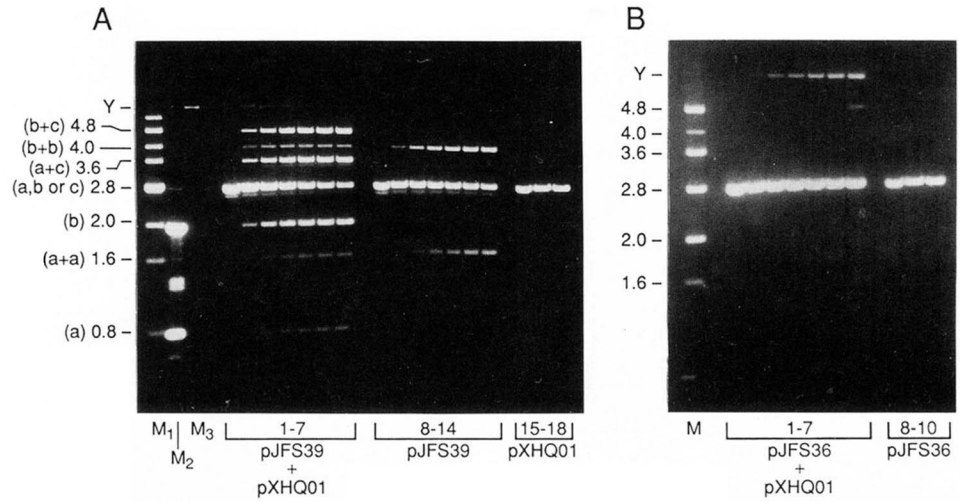



FIG. 3. **Reactions between half-FRT sites and full-FRT sites.** *A*, reaction between pXHQ01 and pJFS39. Reaction contained 45 nM FLP protein, 5.4 nM *Pst*I-cut pJFS39 and (or) 5.4 nM *Xba*I-cut pXHQ01 as indicated below the gel figure. *Lanes 1–7* and *8–14* represent timecourses with points (*left* to *right*) at 0 (no FLP protein), 2, 5, 10, 15, 30, and 60 min. *Lanes 15–17* represent a less extensive timecourse with points at 0 (no FLP protein), 2, and 30 min. Lane M1 contains the DNA size markers as indicated by the labels (in kbp). Lane M2 contains *Xba*I- and *Pst*I-cut pJFS39 DNA. Lane M3 contains Y structure DNA (isolated from the reaction in *B* and characterized in later figures). The vertical labeling shows not only the size of each DNA marker but also indicates which DNA arms should make up linear DNA molecules of each size (see Fig. 2). The small amount of DNA in the band just below the substrate is an unidentified contaminating species present in this preparation of pJFS39 (also present in Figs. 11 and 12). The experiments have been reproduced in detail with substrate that does not have such a DNA band (not shown). *B*, reaction between pXHQ01 and pJFS36. *Lane M* contains the same reaction as in *lane 7* of *A* to serve as DNA size markers. *Lanes 1–7* are the reaction between *Pst*I-cut pJFS36 and *Xba*I-cut pXHQ01. The timecourse, *lanes 1–7*, are points taken at 0 (no FLP protein), 2, 5, 10, 15, 30, and 60 min. *Lanes 8–10* are the reaction of *Pst*I-cut pJFS36 alone with timepoints at 0, 5, and 60 min, respectively.

this solution was also treated with RNase A as above and used for electrophoresis. The amounts of DNA used for electrophoresis in both cases are calculated to represent the same volume of the original reaction mixture.

*Gel Electrophoresis and DNA Quantitation*—All gel electrophoresis in this study was performed in 1.2% agarose gels run in 1 × Tris acetate/EDTA (Maniatis *et al.*, 1982) buffer at 30–40 V/35 cm for overnight. The gel was stained in 0.5 μg/ml EtBr for 0.5 h, and photographed. In the case that the labeled DNA in each band was to be quantified, the gel was put on the DE-81 paper and dried, and the DNA bands were visualized by autoradiography. The bands were localized on the gel with the aid of developed x-ray film and cut out. Quantitation of bands was carried out by liquid scintillation counting (Bio-Safe II Fluor from Research Products International Corp.) on a Beckman LS-3801 counter. The relative concentrations of reaction products were calculated as follows. In these reactions the FRT full site, labeled at both ends as described above, gives rise to six products. Two of these are derived from the reaction of two full sites and hence are also labeled at both ends. The other four are derived from reactions of FRT full sites with unlabeled FRT half sites, and are labeled at only one end. The radioactivity in each band derived from the latter four reactions was corrected (multiplied by a factor of two) so that the data reflected the relative concentration of each DNA species. The total or corrected radioactivity (as appropriate) of a given product band was then divided by the sum of the total radioactivities of all the bands in the gel lane. Each product is thereby expressed in terms of the fraction of the starting substrate that undergoes the reaction to yield this product. Note that any unlabeled half-site DNA remaining in a lane is not accounted for in this calculation.

*Electron Microscopy*—DNA species produced from the reaction between pXHQ01 (*Xba*I) half sites and pJFS39 (*Pst*I) full sites were separated by agarose gel electrophoresis and isolated by electroelution from the gel. The DNA species were then prepared for electron microscopy in several ways as follows. 1) Undenatured samples were prepared by diluting DNA to 1 μg/ml total DNA in a solution (13 μl) containing 16 mM $Na_2CO_3$, 11 mM NaCl, 5 mM $Na_2EDTA$, 10 mM HCl, 7% formaldehyde, 14.3% formamide, and 0.014% cytochrome *c*. From this solution, 7 μl was spread by the drop method (Inman and Schnös, 1970). 2) Partially denatured samples were prepared by diluting the DNA to 1 μg/ml in a solution (10 μl) with 20 mM $Na_2CO_3$, 14 mM NaCl, 7 mM $Na_2EDTA$, 16 mM NaOH, and 9.3% formaldehyde. Solutions were then incubated at either 30 °C or 40 °C (determined empirically for each sample) for 10 min. After cooling to room temperature, 10 μl of formamide and 2 μl of 0.1% cytochrome *c* were added, and 7 μl of the resulting mixture was then spread by the drop method. (3) Samples to be completely denatured were first 4′-amino-4,5′,8-trimethylpsoralen cross-linked (Umlauf *et al.*, 1990) with DNA and 4′-amino-4,5′,8-trimethylpsoralen concentrations at 4.5 nM (molecules) and 0.2 μg/ml, respectively. The sample was dialyzed against 20 mM NaCl, 5 mM $Na_2EDTA$, then adjusted to 5% formaldehyde and heated to 70 °C for 15 min. The DNA was then diluted as for the undenatured samples (but without HCl). The Y structure molecules from the pXHQ01 (*Xba*I) half site with pJFS36 (*Pst*I) full-site reaction was also isolated and prepared for electron microscope examination either under the undenatured conditions or partially denatured conditions described above, as appropriate.

## RESULTS

*Experimental Design*—The goal of this study was to characterize reactions between half-FRT and full-FRT sites. The substrates used in the study, along with the reaction itself, are illustrated in Fig. 2. Products and intermediates were analyzed by agarose gel electrophoresis and electron microscopy. The half sites were derived from the plasmid pXHQ01. In the presence of FLP protein, the half sites generated by *Xba*I cleavage of pXHQ01 form head-to-head dimeric complexes held together noncovalently by FLP protein (Qian *et al.*, 1990). The DNA in this complex is thus arranged as it is in a full site, and we refer to this as a pseudo-full site (this definition of pseudo-full site is somewhat different than that used by Serre *et al.* (1991)[2]). The pseudo-full site formed by *Xba*I-cleaved pXHQ01 has a normal length (8 bp) core sequence identical to the core sequence of the FRT in pJFS39, and it lacks the base change at position −6 that distinguishes the two FLP protein binding sites present within all of the full FRT sites. The full sites used in most of these experiments were *Pst*I-cleaved pJFS36 or pJFS39.

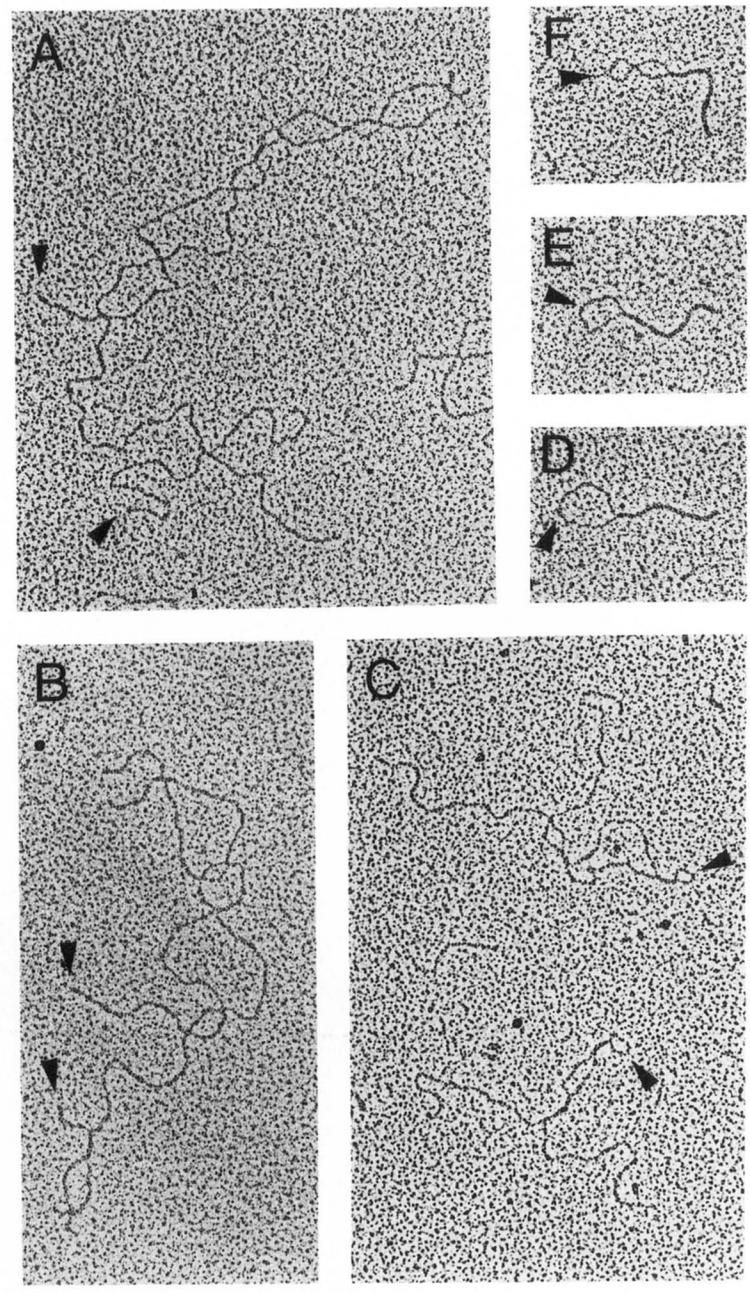



FIG. 4. **Electron microscopy of the products observed in Fig. 3.** Samples in *A*, *B*, and *C* are isolated products, corresponding to the 4.8, 3.6, and 2.0-kbp bands, respectively, produced in a reaction like that in *lanes 1–7*, *A*, Fig. 3. Samples *D*, *E*, and *F* are the isolated 0.8-kbp product from the same reaction. The samples shown in *A*, *B*, and *C* were cross-linked with 4′-amino-4,5′,8-trimethylpsoralen and denatured before spreading. The samples in *D*, *E*, and *F* were partially denatured before spreading. The *arrow* in *A* and *B* indicates the ends derived from the break in one strand, or the loops at one end of the DNAs in *C–F*.

As in Qian *et al.* (1990), half sites in the present study were generated by *Xba*I- or *Eco*RI-restriction digestion and thus all have a terminal 5′-phosphate on the strand opposite to that cleaved by FLP protein. This precludes the many inter- and intra-half-site reactions observed by Serre *et al.* (1991)[2] with synthetic half sites ending with 5′-OH that generate full FRT sites and half sites with closed ends (hairpins). In the present study, reaction of the half sites is halted at the first cleavage step unless full FRT sites are added to the reactions (Fig. 2C). We note that the full site can react with other full sites, and in some cases (*i.e.* when the core sequence is palindromic) discrete products are generated as outlined in Fig. 2*D*.

The lengths of the DNA arms attached to the FRT or half-FRT sites are also given in Fig. 2 (rounded to the nearest 0.1 kbp for convenience). The lengths of various combinations of these arms are also noted in Fig. 2*C*, as these represent potential products of any reaction between half and full sites.

*The Reaction of Half and Full Sites*—A reaction between *Xba*I-cut pXHQ01 (half site) and *Pst*I-cut pJFS39 (full site) is presented in Fig. 3*A* (*lanes 1–7*). As controls, the reactions of pJFS39 alone and pXHQ01 alone are presented in *lanes 8–14* and *15–18*, respectively. The pJFS39 reaction yields the expected 4.0 (P1) and 1.6 (P2) kbp products (see Fig. 2*D*). In the reaction between half site and full site, five new bands are observed in addition to P1 and P2. Since no reaction is observed with pXHQ01 alone, the new species must result from a half-site/full-site reaction. As noted in Fig. 3*A*, four of these bands comigrate with linear DNA markers that correspond to either cleavage of the full site (0.8- and 2.0-kbp bands) or a combination of one arm of the full site with a half site (3.6 and 4.8-kbp bands; see Fig. 2*C*). These four new bands represent the major products of the reaction, and the P1 and P2 bands are much depressed relative to the reaction of pJFS39 alone. A fifth band, labeled Y, is also seen. This band appears early in the reaction, then remains at a steady

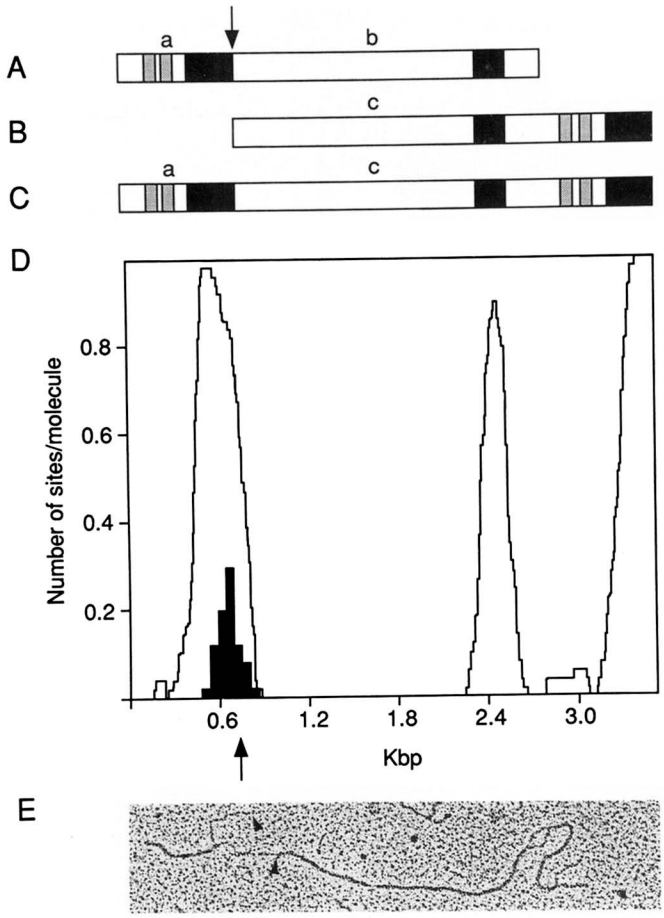



FIG. 5. **Analysis of the 3.6-kbp recombination product.** A nick is present at the FRT site in one of the products from a half- × full-site reaction. *A-C*, respective predicted denaturation maps of the full site (pJFS39, 2, 820 bp), half-site (pXHQ01, 2, 811 bp) substrates, and the putative product (*ac*) shown in Fig. 2C (3,584 bp). The maps were derived from the base sequence, as previously described (Funnell and Inman, 1979), using an averaging segment length of 200 bp and a cut-off window of 60% A + T (*solid areas*) or 50% A + T (*shaded areas*). Maps have been aligned so that the FRT sites correspond to the *arrow* shown above *A*. The segments *a–c* are the same as those shown in Fig. 2. *D*, the experimental denaturation map (shown as the *open histogram*) for the 3.6-kbp product. Both the total length (3,584 bp) and the position of the AT-rich regions correspond to the expected product (*a* + *c*), which is formed by a recombination event occurring at the FRT sites of the *left* segment (773 bp) of the full-site substrate shown in *A* and the half-site substrate (2,811 bp) shown in *B*. The *filled histogram* shows the position of a nick in one of the strands that was observed in 44 out of 49 molecules measured. The average position of the nick corresponds, within experimental error, to the FRT site (shown by the *arrow* below this *panel*). The peak position corresponds to 15 molecules. *E*, electron micrograph of a partially denatured 3,584-bp product molecule. The single-stranded ends resulting from a nick are indicated by the *arrows*.

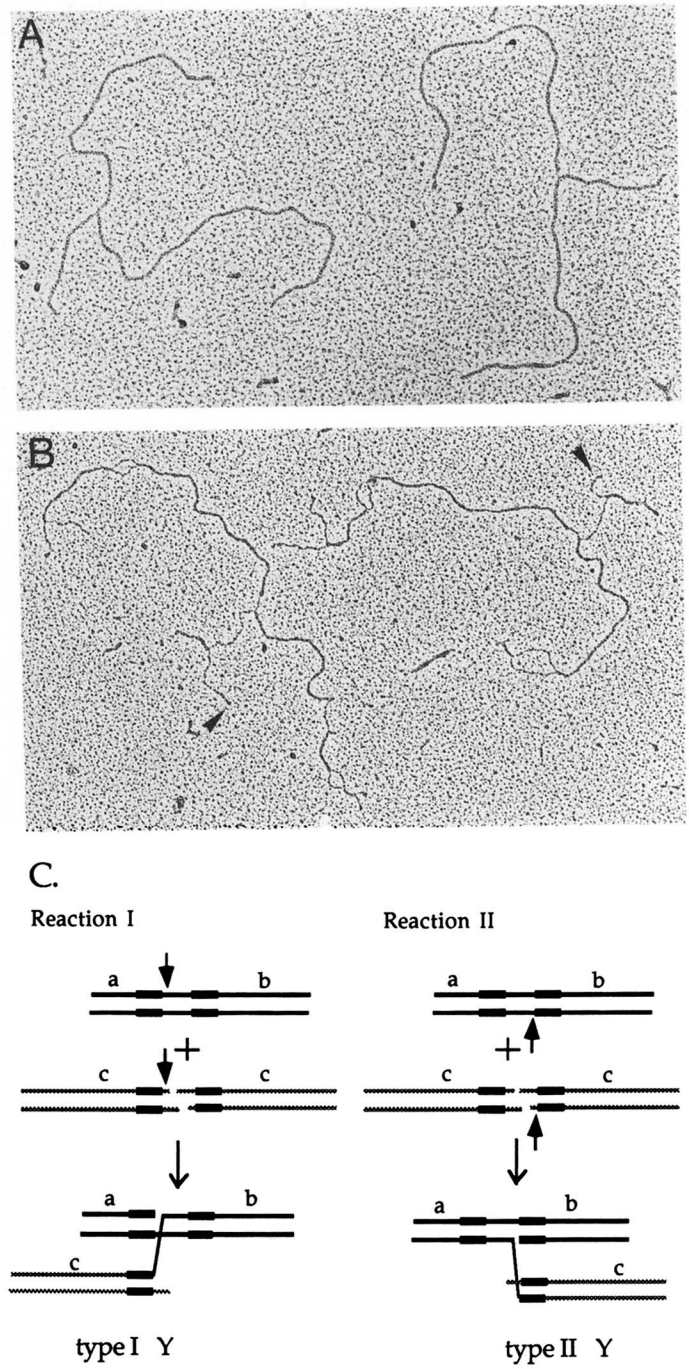



FIG. 6. **Electron microscopy of the Y band in Fig. 3*B*.** The Y band from a reaction between pJFS36 and pXHQ01 was isolated and spread (*A*), or partially denatured, then spread (*B*). *Arrows* in *B* indicate the ends that define the broken strand within the Y structures. *C* shows the two possible reactions between the half-FRT site and the full-FRT site and the structure of the Y expected from each reaction. The strand cleavage and exchange starts at the *top* strand or *bottom* strand, respectively, for reaction *1* and *2*. The *heavy bars* indicate the 13-bp repeat. The *heavy arrows* indicate the cleavage sites.

state or slowly declines as the reaction progresses, suggesting that it represents a reaction intermediate. The Y band comigrates with a three-armed DNA marker derived as described in the figure legend. All reaction aliquots were treated with SDS in this experiment, so that none of these species represent DNA fragments held together by protein-protein or other noncovalent interactions.

A reaction between the pXHQ01 half sites and pJFS36 is presented in Fig. 3*B*. The core sequence of the full FRT site in pJFS36 has the normal FRT sequence, which differs from the pJFS39 FRT core site (from which the half sites are derived) at five of the six central positions. The major product of this reaction was the Y band. At later timepoints a small amount of the 4.8-kbp product is observed. In some trials of this experiment the 3.6-kbp species also appeared, but always at levels much reduced relative to the 4.8-kbp species. The Y product accumulated in this experiment, in contrast to the result in Fig. 3*A*.

Each of these product bands was excised from agarose gels and examined by electron microscopy. The vast majority of the 4.8-, 3.6-, 2.0-, and 0.8-kbp bands consisted of linear DNA

TABLE I

*Measured lengths of reaction products and intermediates observed by electron microscopy*

| Product | Expected length of species in Fig. 2C based on sequence | Measured length |
|---|---|---|
| | *bp* | |
| 0.8 kbp | 773 | 724 ± 41 |
| 2.0 kbp | 2,047 | 1,969 ± 46 |
| 3.6 kbp | 3,584 | 3,504 ± 92 |
| 4.8 kbp | 4,858 | 4,796 ± 111 |
| Y structure arms[a] | | |
| Shortest | 773 | 773 ± 26 |
| | 2,047 | 2,035 ± 41 |
| Longest | 2,811 | 2,823 ± 42 |

[a] For Y structures, the relative lengths of the three arms were determined; *i.e.* the shortest arm in the measured molecules is 27.4% as long as the longest arm. The numbers shown represent the best fit to the expected arm lengths.

with measured lengths not significantly different from the putative products shown in Fig. 2*C*. When the 4.8- and 3.6-kbp species were completely denatured, many molecules had a strand break at a position which, by eye, corresponded to the expected FRT position for these putative products (Fig. 2*C*). Electron micrographs of such molecules are shown in Fig. 4, *A* and *B*. In these completely denatured samples there was also a significant number of random breaks (50% and 7% in the 4.8- and 3.6-kbp species, respectively), most of which were generated during sample preparation.

We next examined partially denatured molecules to more completely define the nature of the products. In addition to providing more structural information, this protocol generated fewer random strand breaks. The results for the 3.6-kbp species are summarized in Fig. 5. The predicted position of denatured sites for the full- and half-site substrates are shown in Fig. 5, *A* and *B*, and Fig. 5*C* shows the expected map for the putative 3.6-kbp product. These denaturation maps were based on A + T content of the DNA sequences as in Funnell and Inman (1979). *D* (open histogram) shows the experimental denaturation map derived from 49 molecules from the 3.6-kbp band, and we conclude, on the basis of the similarity between *C* and *D*, that the 3.6-kbp product does in fact have the structure shown in Fig. 2*C*. The position of the nick (*shaded histogram* in *D*) closely corresponds to the position of the FRT site.

The 4.8-kbp species was analyzed in the same way as shown in detail above for the 3.6-kbp species (data not shown). Analysis of 67 partially denatured 4.8-kbp molecules showed that they conformed to the putative structure shown in Fig. 2*C* and that an irregularity (consistent with a nick or small gap) occurred 2.01 ± 0.05 kbp from the left end, which again corresponds closely with the FRT site position.

The 2.0- and 0.8-kbp species proved to be quite distinct. A total of 116 molecules of the 0.8-kbp species was examined after partial denaturation (Fig. 4, *D–F*). The majority of these molecules (93%) were closed at one end so that the molecules represented an extended hairpin. The rest were either open at both ends or ambiguous. According to the denaturation patterns (not shown), the looped ends corresponded to the left ends as drawn in Fig. 2*C* for the 0.8-kbp species, and therefore occur unambiguously at the FRT site. The 2.0-kbp species has a high GC content at its left end (as drawn in Fig. 2*C*), and this end did not open up under the partial denaturation conditions used here. These molecules were therefore examined after they were completely denatured (Fig. 4*C*). From a total of 118 molecules, about 87% were closed at one end. This result, as well as the result for the 0.8-kbp species, indicates that these are molecules in which the pJFS39 FRT site has been cleaved with an interstrand closure to yield either the *a* or *b* arm with a closed end at the FRT site.

The Y band was also examined, using material from an experiment like the one in Fig. 3*B*. Undenatured samples showed that more than 95% of these molecules were branched Y structures (Fig. 6*A*). Arm lengths were measured for 69 of these molecules, and found to be 773, 2,035, and 2,823 (± 40) base pairs (Table I). This corresponds well to the 773, 2,047, and 2,811-bp lengths expected for a Y structure derived from a half-site × full-site reaction.

These Y structures were partially denatured and spread again (Fig. 6*B*). Of 52 molecules that unambiguously have a single-stranded region near the junction (95% of the total molecules had this structure), 100% had a break in one of the three strands at the junction. An A + T-rich region exists in the 0.8-kbp arm near the FRT site, and denaturation in this region allowed assignment of the broken strand. Interestingly, 51 of the molecules (98%) had a DNA strand break in the 0.8-kbp arm (*a* arm), indicating that the half sites are covalently linked to the 2.0-kbp arm (*b* arm). In 31 of these 51 molecules, the other broken end was located in the half-site (*c*) arm, providing unambiguous identification of the Y structures as molecules derived from reaction 1 in Fig. 6*C*. Only one molecule had a break in the 2.0-kbp arm (type II Y in Fig. 6*C*). These results suggest cleavage at the side of the pJFS36 core sequence corresponding to reaction I illustrated in Fig. 6*C*. Notably, the cleavage and strand exchange needed to generate the type I Y structure occur on the side of the core where the full site and half site share 2 bp of homology. Only 1 bp of homology exists on the other side.

All of the four reaction products were examined and aligned relative to the FRT site using denaturation maps as in Fig. 5. In addition, the lengths of each of the four linear products were measured (Table I). In each case the measured length corresponded closely to the lengths expected for the molecules illustrated in Fig. 2*C*. The relative lengths of the arms of undenatured Y structures also corresponded closely to those expected.

The products of the reaction between half sites and pJFS39 (Fig. 3*A*) were quantified in reactions employing end-labeled pJFS39 substrates as described under "Materials and Methods." This experiment was repeated four times, and a typical timecourse is shown in Fig. 7*A*. The data from all four trials was averaged and presented in Fig. 7*B*. Several things stand out in this experiment. First, the products did not appear in the same amounts. The 4.8- and 0.8-kbp species appeared at 1:1 ratios, as did the 3.6- and 2.0-kbp species; however, the 3.6- and 2.0-kbp species accumulated at nearly twice the rate and yield of the other two. The difference is explained below.

To determine if any of the reaction products included covalently bound protein, the SDS-potassium treatment of Trask *et al.* (1984) was used to precipitate protein-DNA complexes. The data are presented in Fig. 8. The data indicate that some of the recombinant products (*a* + *c* and *b* + *c bands*) were precipitated, suggesting covalent linkage to protein. None of the full-site cleavage product bands (the hairpins derived from the *a* or *b* arms) were precipitated. All the material from these bands remained in the supernatant. This suggests that at least some of the molecules of each recombinant species have covalently linked protein.

We also demonstrated that the formation of the dimeric half-site complexes characterized by Qian *et al.* (1990) was a prerequisite for the full-site × half-site reaction. When *Eco*RI-cut pXHQ01 was used in the reaction with pJFS39, no Y

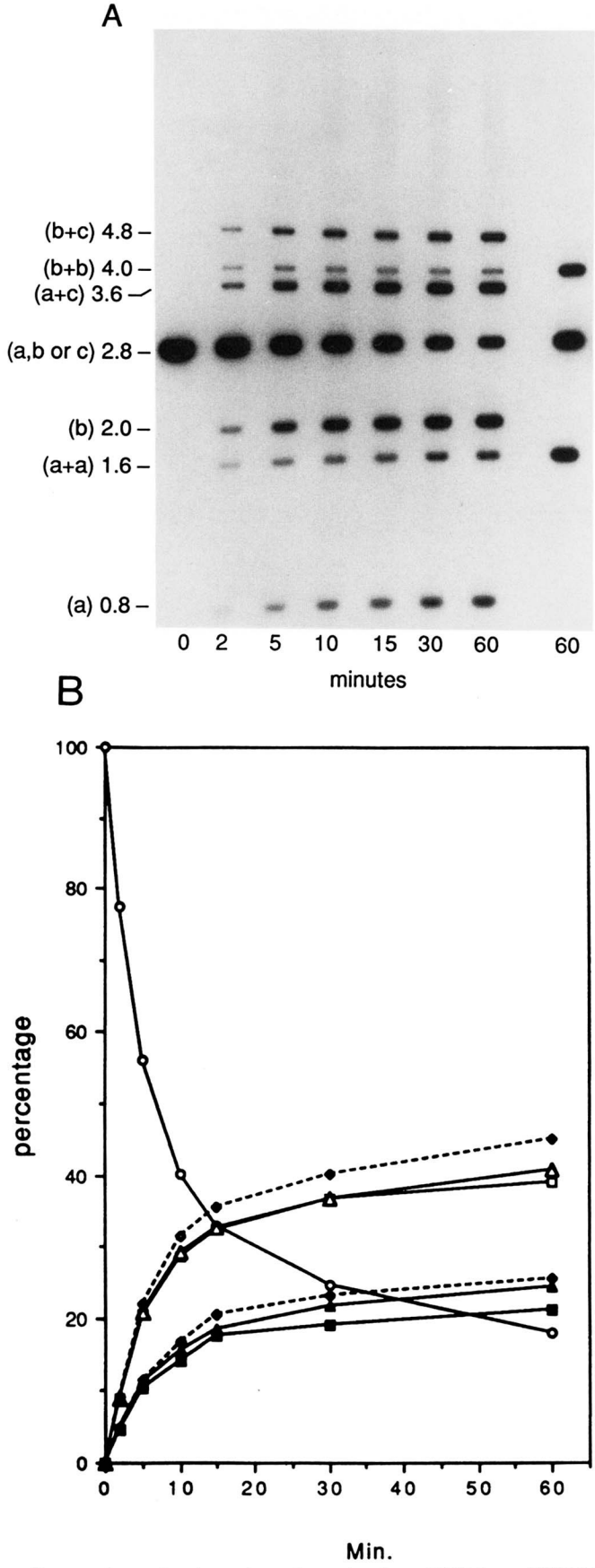



FIG. 7. **Quantitation of products in the pJFS39 × pXHQ01 (half-site) reaction.** *A*, the pJFS39 were cut with *Ase*I and labeled at the 3′ end with [$\alpha$-$^{32}$P]dTTP. The reaction contains 5.4 nM *Xba*I-cut pXHQ01 and 5.4 nM labeled pJFS39 and 45 nM FLP protein. The *numbers* below each *lane* indicate the reaction time in minutes. The last lane is a 60-min reaction with pJFS39 alone as a control. The vertical labeling is similar to that in *A*, Fig. 3. *B*, the DNA bands in *A* were cut out and quantified as described under "Materials and Methods." Curve assignment: ○, substrate (a, b); ■, (b + c); ▲, (a); □, (a + c); △, (b); ◆, uncorrected (b + c); and ◇, uncorrected (b). The correction of b + c and b curves for secondary reactions with the pJFS39 products are described under "Materials and Methods." The percentage is the molar ratio of product to starting pJFS39 substrate.

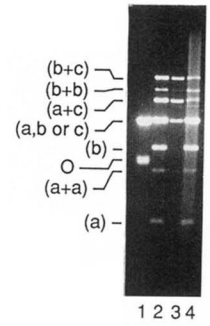



FIG. 8. **SDS-K$^+$ Precipitation of pJFS39 with pXHQ01 reaction.** Substrates pJFS39 and pXHQ01 were cut with *Pst*I and *Xba*I, respectively. *Lane 1* is the DNA substrates without FLP protein. *Lane 2* is the FLP reaction without SDS-K$^+$ precipitation. *Lanes 3* and *4* are the precipitate and supernatant from SDS-K$^+$ precipitation after a 30-min FLP reaction. The vertical labeling is similar to that in *A*, Fig. 3. The band labeled *O* was an extra DNA in this preparation of markers that is irrelevant to the experiment. The background in *lane 4* was caused by the presence of calf thymus DNA added during the procedure. The DNA amounts in *lanes 2–4* represented the same amount of the original reaction volume, for convenient comparison between lanes.

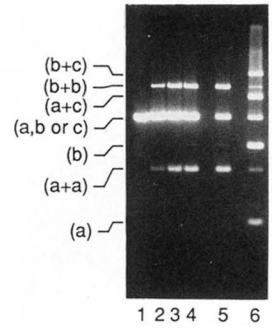



FIG. 9. **Reaction between pJFS39 and *Eco*RI-cut pXHQ01 half site (see Fig. 2).** The substrates in each set of reaction are as follows. *Lanes 1–4* are *Pst*I-cut pJFS39 plus *Eco*RI-cut pXHQ01. *Lane 5* is *Pst*I-cut pJFS39 alone. *Lane 6* is a control reaction with *Pst*I-cut pJFS39 and *Xba*I-cut pXHQ01. The timecourse in *lanes 1–4* is 0 (no FLP protein), 5, 30, and 120 min. The reaction times in *lanes 5* and *6* are both 120 min. The vertical labelings are similar to that of Fig. 3*A*.

structures or products were observed except for the P1 and P2 products generated by pJFS39 itself (Fig. 9). This half site has a large core sequence that precludes formation of the dimeric half-site complex as shown by Qian *et al.* (1990).

Together, the data indicate that the reaction follows the ordered pathway outlined in Fig. 10. This pathway parallels the normal reaction pathway presented in Fig. 1, except that one of the substrates is a dimeric complex of half sites. The initial cleavage step releases a dinucleotide from the half site, so that the ensuing strand exchange is not reciprocal and yields only one covalent bond. As shown, cleavage on the 0.8-kbp arm side of a *Pst*I-generated full site (*a*) would yield a covalent link between the *b* arm and a half site (*c*). The intermediate observed in our experiments would be a Y struc-

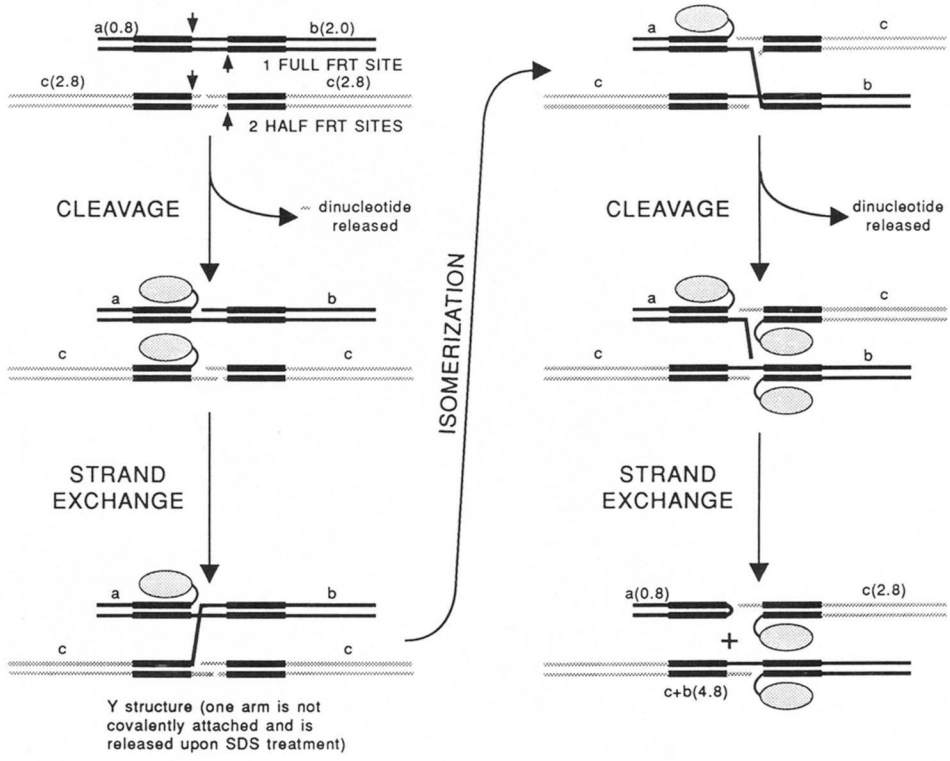



FIG. 10. **Proposed pathway for the reaction between half-FRT sites and full FRT sites examined in this study.**

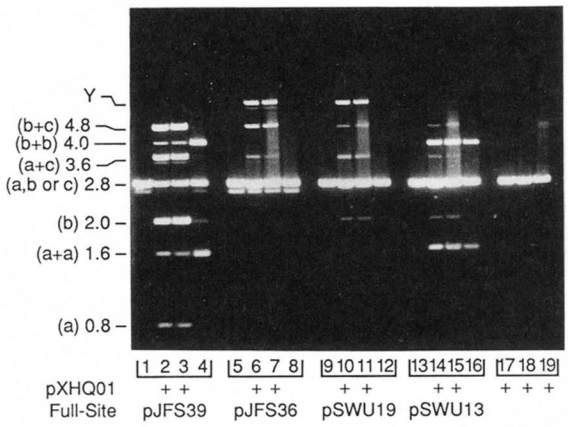



FIG. 11. **Reaction of different full-FRT sites (altered core sequences) with the pXHQ01 half site.** The DNA substrates used in each reaction are indicated below the corresponding reaction set. The pXHQ01 half site and the full sites were cut with *Xba*I and *Pst*I, respectively. Each reaction contains 84 nM FLP protein, 5.4 nM pXHQ01, and (or) 5.4 nM full site and was incubated at 30 °C for 120 min. The first lane in each set has no FLP protein. The reaction in the second lane was treated with proteinase K as described under "Materials and Methods." The reaction in the third and fourth lanes in each set were treated with SDS.

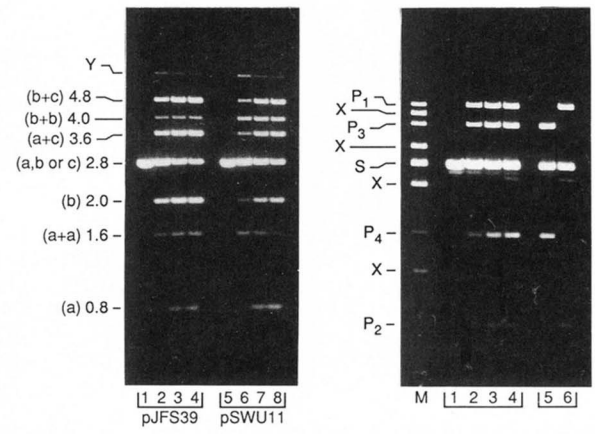



FIG. 12. **Reaction between pSWU11 (full site) and pXHQ01 (half site).** *Left panel*: *Xba*I-cut pXHQ01 was incubated with *Pst*I-cut pJFS39 or *Pst*I-cut pSWU11 as indicated. The timecourse in each case (*left* to *right*) is 0 (no FLP protein), 5, 30, and 60 min. *Right panel*: lack of reaction between pJFS39 and pSWU11 full FRT sites. *Lane M*, DNA size markers generated in a reaction between *Pst*I-cut pJFS39 and *Sal*I-cut pJFS39. The *X* bands represent the products of a cross-reaction between the two substrates. P1 and P2 are the products observed from a reaction of *Sal*I-cut pJFS39 alone (or *Sal*I-cut pSWU11, *lane 6*). P3 and P4 are derived from a reaction of *Pst*I-cut pJFS39 alone (or *lane 5*). *Lanes 1–4* are the reaction of *Pst*I-cut pJFS39 with *Sal*I-cut pSWU11. The timecourse (from *left* to *right*) is 0 (no FLP protein), 5, 30, and 120 min. *Lanes 5* and *6* were 120-min reactions of *Pst*I-cut pJFS39 and *Sal*I-cut pSWU11, respectively.

ture, since SDS treatment would remove the second noncovalently linked half site. After the isomerization step, the second set of cleavages would result in release of a dinucleotide from the second half site. Neither of the two protein-DNA linkages formed at this point are resolved, however. The 5′-OH terminus attached to the *a* arm instead undergoes an intramolecular reaction to generate a hairpin, a reaction analogous to those synthetic half sites lacking 5′-phosphate termini observed by Serre *et al.* (1991).[2] The hairpin in this case is diagnostic of this second set of cleavages, since there is no potential source of hairpin structures in the first set of cleavages (the half-site termini here have 5′-phosphate groups). Fig. 10 shows the course of the reaction when the first strand exchange occurs on the left, and this sequence produces only the 4.8- and 0.8-kbp products. If the reaction instead initiates with a strand exchange on the right side, the 3.6- and 2.0-kbp products are generated instead. Thus the products generated in a given recombination event tell us where the first strand exchange occurred.

The Y structures are the manifestation of Holliday-like intermediates in the reaction, generated via the first two steps

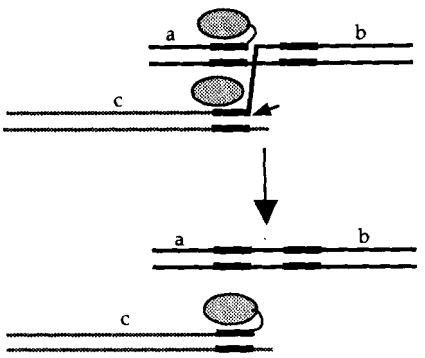



FIG. 13. **A possible pathway for regeneration of FRT-full site substrates from Y structures.** Cleavage of the Y structure at the *arrow* would generate a free 5′-OH that could act as a nucleophile to regenerate the full site. The reaction shown is not the reverse of the first two steps in Fig. 10, because the dinucleotide lost in step 1 of the Fig. 10 pathway is not present.

of Fig. 10. The kinetic behavior of the Y structures is consistent with a role as reaction intermediate. In addition, heterologous sequences in the core, which tend to inhibit the isomerization step (De-Massy *et al.*, 1989), still permit formation and accumulation of Y structures. We note that since a dinucleotide is lost from the half site in the first cleavage step and the first strand exchange is non-reciprocal, formation of Y structures is not directly reversible in this system. When the isomerization step is inhibited by heterology in the core, the Y structures accumulate (Fig. 3*B*) rather than revert to substrates as Holliday structures would.

In this pathway, every reaction initiated on the "*a*" side of the full site will generate a *b*–*c* recombinant with two nucleotides missing from one strand, plus a hairpin derived from the *a* arm. The 1:1 correspondence between the 4.8- and 0.8-kbp products (*b* + *c* and *a*) is consistent with this prediction. The third product is a half site indistinguishable in size from substrates. When recombination is initiated on the right or *b* side of the core, an *a*–*c* recombinant product (3.6 kbp) should be produced 1:1 with a "*b*" arm hairpin (2.0 kbp), as observed in Fig. 7. Both of the recombinant products (*a*–*c* and *b*–*c*) plus some of the half sites should have covalently linked protein, as suggested by the experiment in Fig. 8.

The 2-fold enhancement in production of the 3.6- and 2.0-kbp products relative to the other two is best explained by examining the sequence of the pJFS39 full site. The only base pair difference that distinguishes the two sides of the core is that at position −6 of the *a* arm relative to position 6 of the *b* arm (Fig. 2). The T at position 6 maintains the polypyrimidine tract at this position, while the A at −6 disrupts the corresponding polypyrimidine tract (in the complementary strand) in the *a* arm. Evidence has been presented previously that the *b* arm sequence is somewhat more effective in recombination (Senecoff *et al.*, 1986), and this appears here as a 2-fold bias in favor of initiating the reaction on the *b* side of the core. This bias is fortuitous in these experiments, since it renders the 1:1 relationship of the 4.8/0.8- and 3.6/2.0-kbp products more transparent than would be the case if all were generated in the same amounts.

We note that the products of the reaction of pJFS39 alone (P1 and P2) can also react with half sites. P1 has two *b* arms (which duplicates the favorable nucleotide at position 6), and this should be more reactive than P2. P2 was present in 2-fold excess relative to P1 in this experiment, indicating a selective depletion of P1 in reactions with half sites. P1 and P2 are generated in equal amounts in reactions of pJFS39 alone (Fig. 7 control; quantitation not shown). A reaction between P1 and half sites should generate the 4.8- and 2.0-kbp products. A slight excess of the 4.8-kbp product over the 0.8-kbp product is actually observed in Fig. 7 as a result of this reaction (*dashed line* in Fig. 7*B*). The data in Fig. 7*B* (*solid lines*) have been corrected for depletion of P1 relative to P2, which reflects the selective reaction of P1 with half sites.

*Effects of Core Heterology on Early Reaction Steps*—As indicated above, the products of a given recombination event unambiguously pinpoint the side of the core at which the reaction was initiated. We can also trace the origin of any Y structures generated in the reaction. The system is capable of detecting subtle effects of sequence changes that bias early steps and direct the first strand exchange to one side of the core or the other. Inasmuch as the protein components in the dimeric complex that cleaves the DNA are conformationally distinct (Qian *et al.*, 1990; Amin *et al.*, 1991), the bias in the first cleavage step must reflect effects of sequence on the assembly of the asymmetric protein dimer. We have already noted a subtle effect of sequence changes at position 6/−6, and the effects of homology in generating Y structures in the reaction between pJFS36 and half sites. Additional experiments were carried out to examine effects of heterology in the core on this reaction.

Some of these results are presented in Fig. 11. Here the reaction of the half sites and PJFS36 (as in Fig. 3*B*) is presented at longer timepoints and contrasted with a reaction with pSWU19. The bias favoring the 4.8-kbp product over the 3.6-kbp product can be seen in the pJFS36 result, presumably reflecting the 2 bp of core homology on the *a* side of the core. The pSWU19 core is the inverse of pJFS36 in that it has 1 bp of homology on the *a* side of the core and 2 bp of homology on the *b* side. The reaction of half sites with pSWU19 shows a corresponding bias that now favors the 3.6-kbp product over the 4.8-kbp product. Note that neither pJFS36 nor pSWU19 reactions produce P1 or P2, since the cores are asymmetric. A reaction between half sites and pSWU13 is also shown in Fig. 11. In this reaction there is only 1 bp of homology at either side of the core, and all products of the half-site × full-site reaction are greatly reduced. The P1 and P2 products observed in this case are derived from the reaction of pSWU13 alone, which has a symmetric core and clearly reacts well on its own (see Umlauf *et al.*, 1988). Notably, Y structures do not accumulate in this reaction.

Reactions were also carried out with pSWU11, which differs in sequence from pJFS39 at positions 2 and −2, leaving 2 bp of homology on both sides of the core. An efficient reaction of half sites with pSWU11 is observed (Fig. 12) that is almost the same as the reaction with pJFS39. Heterology at these internal positions of the core clearly is unimportant in the half-site reaction with full sites. There is no reaction between pSWU11 and pJFS39 full sites, however (Fig. 12*B*), demonstrating that the structural constraints during isomerization are not as great in the half-site × full-site reaction as they are in the normal reaction between two full sites.

## DISCUSSION

Our primary conclusion is that half-FRT sites undergo an FLP-mediated recombination reaction with full sites that largely parallels a normal recombination event. The system is especially useful in dissecting early steps in recombination. We find that (I) formation of a head-to-head dimeric complex of half-FRT sites (mediated by noncovalent interactions between FLP monomers) is a prerequisite for the reaction; (II) sequence changes at positions 6 or −6 in the FRT site have

subtle effects in directing the first strand exchange steps to one side of the core or the other; and (III) two base pairs of homology at the ends of the core sequences of the reacting sites are needed to observe efficient formation of the Y structure product of the first cleavage and strand exchange steps.

This model system has several advantages over the normal reaction for studying early steps in site-specific recombination. In the normal reaction, formation of Holliday structures is readily reversed if isomerization is blocked, and when Holliday structures are not seen it is hard to determine whether the experimental parameter in question blocked isomerization or blocked an earlier step in the reaction. In this system, the corresponding intermediate is a Y structure that cannot revert directly to substrates because of the release of the dinucleotide in the forward reaction. The full-site substrate might be regenerated by the pathway in Fig. 13, discussed in more detail below. In any case, the first cleavage and strand exchange steps are more readily detected and measured in the half-site × full-site reaction. In addition, the overall reaction follows a course that is prescribed by the location of the first strand exchange steps; *i.e.* an initial cleavage and strand exchange on one side of the core yields products that are distinct from those generated when the initial cleavage and strand exchange is on the other side of the core. This permits the measurement of subtle effects of sequence changes in FRT on early reaction steps.

One set of sequence changes that affect the early course of the reaction involves positions 6 and −6, which lie outside the core within the FLP binding sites (Fig. 2). When a T is found at position 6 and an A in position −6 (in the respective pyrimidine strands) within a full site, the first strand exchange with half sites occurs at the side of the core near position 6 twice as often as it occurs on the other side of the core. No evident bias directing the first strand exchange to one side of the core or the other has been observed previously (*e.g.* Jayaram *et al.* (1988)) although the system described here is probably more sensitive to small effects than methods previously applied.

Both Amin and Sadowski (1991) and Serre *et al.* (1991)[2] have provided evidence that half sites can react with full sites through the first cleavage and strand exchange steps to generate Y structures. Here, we have characterized these structures by electron microscopy and provided evidence that they are not end products but can be intermediates in a more elaborate reaction.

An important precursor to Y structures is the head-to-head dimeric complex of half-FRT sites characterized by Qian *et al.* (1990). Half-site substrates in which formation of this dimeric complex was blocked did not react with full sites. This result reinforces the idea that an asymmetric dimer of FLP protein bound to an FRT site represents an early intermediate in FLP-mediated recombination.

This model system does not reproduce all features of the normal reaction. In particular, some mismatches in the core sequence (for example, at positions 2 and −2) are tolerated well in the half-site × full-site reaction yet block the normal reaction completely (Fig. 12). Conversely, any sequence changes that inhibit the half-site × full-site reaction are likely to have even more dramatic effects on the corresponding steps of the normal reaction.

Particularly important are the effects of heterologous sequences at positions 3 and −3. Core mismatches at these positions greatly inhibit the half-site × full-site reaction, and completely block the normal reaction. Amin *et al.* (1991) demonstrated that two base pairs of core sequence are necessary for the cleavage of half sites and noted a need for homology at these positions that they interpreted as the previously reported homology requirement during the isomerization step. Although we didn't observe the complete lack of reaction when core sequences differed at these positions reported by Amin *et al.* (1991), we note that our reaction conditions are somewhat different and the timepoints shown in Fig. 11 (120 min) are much longer than those used by Amin *et al.* (1991). We believe our results agree well with their observations that these two base pairs must be present and the same in the two FRT sites for recombination to proceed efficiently. The base pair at position −3 has no specific contacts with FLP protein since base changes at this position do not affect the homologous full-site reaction (Umlauf and Cox, 1988).

A homology requirement implies a DNA-DNA interaction at some point in the reaction. In integrase class recombination events, this could reflect formation of a novel 4-stranded DNA intermediate prior to the first strand exchange, or DNA-DNA interactions during the isomerization step (which is in part an abbreviated branch migration process). *If* loss of the dinucleotide in the first strand exchange renders the formation of Y structures irreversible, and *if* core mismatches at positions 3 and −3 affect only the isomerization step, then Y structures should accumulate when core mismatches at these positions are present. Significantly, Y structures do not accumulate in the reaction between the half sites and a full site with heterologous base pairs at both −3 and 3 (pSWU13, Fig. 11), and homologous base pairs at only one of these positions have a strong effect in directing the first strand exchange.

The key issue then becomes the irreversibility of Y structure formation. If Y structures can be cleaved to reform full sites as in Fig. 13, then the lack of Y structures in the pSWU13 reaction of Fig. 11 could indicate that Y structures are simply unstable without homology at positions 3 and −3. In this case they might form but be rapidly cleaved to reform full sites. This would be consistent with the view that DNA-DNA interactions occur only during the isomerization step. On the other hand, if Y structures are formed irreversibly, then the lack of Y structures in the pSWU13 reaction (Fig. 11) must reflect inhibition of the first strand exchange step and possibly the first cleavage step. This would imply the existence of a DNA-DNA pairing intermediate, either prior to cleavage or just prior to the ligation of strands to form the Holliday intermediate in the first strand exchange (*i.e.* after cleavage, there may be a step in which strands are switched and a limited region of homology is sampled prior to joining the cleaved ends to new partners; see Kitts and Nash (1987)). We note that isolated and deproteinized Y structures (treated with proteinase K) are converted only to recombinant products and not reversed when more FLP protein is added (the Fig. 13 pathway is not observed).[3] It is not clear that this experiment faithfully replicates the situation existing immediately after Y structure formation, however, and more work is required to define the possible pathways of Y structure resolution.

Several other studies (Nunes-Düby *et al.*, 1987; Kitts and Nash, 1987; De-Massy *et al.*, 1989) have provided evidence that core sequence mismatches block recombination at the isomerization step, and have found no evidence for the existence of a paired DNA-DNA intermediate in the synaptic complex that is formed prior to cleavage. Both the Landy and Nash groups have provided evidence that nonhomology at the position in λ att sites equivalent to position −4 in FRT (in the sense it is also adjacent to the cleavage point) doesn't block the first strand exchange (Ricket *et al.*, 1988; Nunes-

[3] X.-H. Qian, unpublished results.

Düby *et al.*, 1989; Nash and Robertson, 1989) as it appears to do in the present study. The results reported here encourage further work to determine if a modified and limited version of a 4-stranded pairing intermediate, in which the two DNAs are homologously aligned at the two core base pairs adjacent to the first cleavage site, is a key early intermediate in the reaction.

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