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Holliday Intermediates and Reaction By-Products in FLP Protein-Promoted Site-Specific Recombination

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Holliday structures are formed and resolved by FLP protein during site-specific recombination. These structures have been isolated and are visualized in both native and partially denatured states by electron microscopy. No single-strand breaks are found within the junction, indicating that the structure results from a reciprocal exchange of strands. These structures have properties consistent with being reaction intermediates. Double-strand cleavage products and "Y structures" are also detected and appear to be by-products of the reaction. The Y structures are three-armed branched molecules with a covalently closed junction located at the FLP recombination target site. Models are discussed, suggesting that both of these novel structures are made by aberrant cleavages during formation and resolution of the Holliday intermediate.

Site-specific recombination systems appear to fall into at least two classes. One of these is referred to as the integrase family, which includes the FLP protein of the *Saccharomyces cerevisiae* 2μm plasmid, the lambda Int protein, and the Cre protein of bacteriophage P1 (2). The proteins, the reaction mechanisms, and the respective recombination target sites exhibit a number of important similarities. Each recombination site consists of a DNA spacer (also called a crossover or overlap sequence) flanked by two inverted repeats that serve as binding sites for the recombinase. The spacers are either 7 base pairs (bp) for Int (27) or 8 bp for Cre and FLP (16, 20). In each case, homology is required between the spacers of recombining partners, suggesting that DNA:DNA pairing in this region is important during at least one stage of the reaction (3, 19, 37, 38). The three recombinases make staggered cuts at or near the boundaries of the spacer and become covalently attached to the DNA via transient 3′ phosphotyrosine linkages (10, 15, 18, 36, 38). The Int protein is the only one of the three which requires accessory proteins (1, 31, 34, 42).

Two general pathways for strand exchange in these systems have been suggested. The first, called cohesive-end formation (33), requires concerted double-strand cleavage of both recombination sites followed by annealing of the resulting sticky ends and ligation of recombinant strands. The second, called the sequential model, requires single-strand cleavage, such that one strand of each recombinant partner is cleaved and exchanged to form a Holliday intermediate (21). This intermediate or chi structure would be resolved by a second cleavage, exchange, and ligation of opposing strands. Both mechanisms require homologous spacers, either for the annealing of sticky ends in the cohesive-end model or for branch migration within a Holliday intermediate. Determination of the correct mechanism has been complicated by a large body of experimental observations used to support either or both models.

Data consistent with both models comes from genetic studies. Low frequencies of recombinase-dependent gene conversion events occurring outside the Int recognition site (attP), the FLP recognition site (FRT), or the Cre recognition site (loxP) have been interpreted both as support for a Holliday mechanism (12, 41) or as evidence against such a mechanism (11, 30).

The primary evidence supporting cohesive-end formation is the appearance of double-strand cleavage products (DSCPs) in vitro (4, 25) and in vivo (24) and an apparent absence of stable Holliday structures in normal reactions, i.e., reactions involving wild-type recombinases and their respective wild-type recombination sites.

However, these observations contrast with a growing body of evidence in support of a Holliday mechanism. Chi structures, associated with 2μm circle yeast cells during meiosis, were mapped approximately to the two 599-bp repeats which contain the FRT sites (5). These chi structures could have been generated either by FLP protein or by the meiotic recombination system of the cell. For the Cre system, several mutant Cre proteins produce chi structures that are resolved to products by wild-type Cre protein (17). For the Int system, for which the greatest body of information has been obtained, synthetic att-site Holliday structures are cleaved efficiently by Int protein (22). It has now been demonstrated that recombination proceeds via two sequential and ordered sets of cleavage and religation events, forming a chi structure as an intermediate. This has been shown (i) by the use of suicide substrates to block the resolution of Holliday intermediates (35); (ii) by placing a thiophosphate at alternative sites of cleavage, thereby preventing either the formation or the resolution of the Holliday intermediate (26); and (iii) by comparing the effects of mismatches at different positions within the spacer (26, 35).

The FLP protein has been expressed in *E. coli* cells and purified to near homogeneity (9, 14, 31, 45). For the in vitro recombination reaction, FLP protein requires only a suitable buffer and ionic strength (32). In vivo, the 2μm plasmid is supercoiled, with FRT sites in inverted orientation. However, FLP protein will promote DNA inversion, insertion, or deletion reactions in vitro, depending on the location and orientation of FRT sites on the substrates used. Both linear and supercoiled DNA substrates are recombined (32, 45). High-energy cofactors and divalent cations are not required (32, 45). The stability of FLP protein is improved by the addition of bovine serum albumin (BSA) and glycerol to

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reaction mixtures. Intermolecular recombination is enhanced by the addition of polyethylene glycol (PEG). FLP protein catalyzes recombination with a relatively low turnover number (13a).

As stated above, the recombination site used by the FLP system, designated FRT for FLP recombination target (46), consists of an 8-bp asymmetric spacer separating 13-bp inverted repeats. FLP protein binds to the repeats but does not directly contact the 6 internal bp of the spacer (7). The spacer sequence can be altered significantly and yet still recombine. However, recombination efficiency is reduced when the spacers of two reacting FRT sites contain mismatches (38, 39). FRT sites containing an asymmetric, wild-type spacer can react to form products only when aligned in parallel orientation, so that the entire spacer of the FRT site is in homologous alignment. Two sites with symmetric spacer sequences can be aligned in either parallel or antiparallel orientation. This indicates that directionality in the wild-type reaction is mediated by spacer asymmetry (39). A site with a symmetric spacer (pJFS39) is approximately as reactive as the wild-type FRT. The flexibility of the spacer sequence is limited, however. A set of spacer mutations has recently been defined which reduces reaction efficiency even when spacer homology is maintained. Two features of the wild-type FRT sequence, which are required for full reaction efficiency, are polypyrimidine tracts surrounding each cleavage point and a high A+T content in the spacer (43).

This report focuses on the mechanism of strand exchange in the FLP site-specific recombination system, showing the existence of chi structures and their resolution to products by FLP protein. They are generated in reactions with the wild-type substrate and are seen in relatively high concentrations from substrates containing spacer alterations. This finding has permitted the characterization of these structures by electron microscopy, defining them as true Holliday structures (44). Some novel "Y," or three-armed, structures are identified, as well as products of double-strand cleavage events. These by-products probably arise from aberrant pathways for Holliday junction formation and resolution.

MATERIALS AND METHODS

Materials. FLP protein was prepared by ion-exchange and affinity chromatography according to a published procedure (31). Protein concentrations were measured by the method of Bradford (6). The FLP protein in the preparation used in this study is 85% of total protein, according to densitometric scanning of a polyacrylamide gel. The degree of purity was multiplied by the total protein concentration (0.148 mg/ml) to obtain the molarity of FLP protein (1.62 μM). Plasmid DNA was purified by banding twice in ethidium bromide-cesium chloride gradients. Restriction enzymes were purchased from New England BioLabs, Inc. All reagents were of analytical grade and were purchased from common vendors.

Substrates. A diagram of the recombination reaction is found in Fig. 1 along with a sequence comparison among the FRT sites used in this study. Plasmid constructions containing FRT sites have been described previously (38, 39, 43). Each FRT site was inserted into the plasmid pXF3, which is in turn a derivative of pBR322 (29), between the *Eco*RI and *Bam*HI sites. All plasmids are 2,821 bp, except for pJFS35r, which has 2,820 bp. Both pJFS36 and pJFS39 contain an *Xba*I restriction site within the spacer. Each plasmid substrate was linearized at a unique site with either *Pst*I or *Sal*I under conditions recommended by New England BioLabs. Linearization with these restriction enzymes positions the FRT site at least 276 bp away from a DNA end. The notation mutant × wild type refers to a recombination reaction between one substrate bearing a mutation and the other possessing wild-type sequences. In the present study, reactions are always intermolecular and the reacting substrates always are homologous at sequences flanking the FRT site. We therefore define parallel as that orientation in which homologous flanking sequences are aligned in these reactions and antiparallel as the opposite alignment in which corresponding flanking sequences are at opposite sides of the FRT site.

Reaction conditions. Analytical reactions (20 μl final volume) were performed in 25 mM TAPS {3-[*N*-tris(hydroxymethyl)methylamino]-propane sulfonic acid}–1 mM EDTA–0.2 M NaCl–10% (wt/vol) PEG (M_r 8,000)–20% glycerol–2.5 mg of BSA per ml–30 mM Tris and the indicated amounts of substrate DNA and FLP protein. The final pH of the reaction was 9 to 9.5, which was found empirically to slightly enhance formation of intermediates relative to experiments carried out at the standard pH 8.0. All reaction constituents except FLP protein were mixed and preincubated at 30°C for 5 min before starting the reaction with FLP protein. The reactions shown in Fig. 7B were performed under the same conditions, except that the PEG, glycerol, and BSA were omitted (31). For time courses, larger reaction volumes were employed and 20-μl aliquots were removed at each time point. Samples for zero time points were removed just before starting the reaction. Reactions were terminated by the addition of sodium dodecyl sulfate to 0.9% (wt/vol), followed by 1.5 μl of a standard solution of xylene cyanole and bromophenol blue in H_2O; electrophoresis was done in 1.5% agarose in TAE buffer (29). In cases in which supercoiled substrates were reacted with FLP protein before restriction digestion, the 20-μl reactions were terminated at 65°C for 5 min, and 10 μl of 30 mM $MgCl_2$ was added. The DNA was allowed to reanneal at room temperature and was digested with 1 μl (20 U) of *Pst*I at 37°C for 1 h before adding 3 μl of 10% sodium dodecyl sulfate.

Elution of DNA bands from agarose gels. FLP recombination intermediates were generated in 20-μl reaction mixtures containing 1.6 μg of substrate DNA (43 nM FRT site) and 162 nM FLP protein for 30 min under standard reaction conditions. The DNA species were separated by electrophoresis in 1.0% agarose gels. The gels were stained with ethidium bromide, and the DNA bands were excised with a razor blade and placed in a unidirectional electroeluter (International Biotechnologies, Inc., *Life on the Edge*, vol. 2, p. 41–43, New Haven, Conn., 1986/1987). The DNA was electrophoresed from the gel into 10 mM ammonium acetate at 100 V for 2 h, with a running buffer of 10 mM Tris hydrochloride (pH 8.0)–0.2 mM EDTA–5 mM NaCl. After precipitation with 2 volumes of ethanol, the DNA was suspended in TE (10 mM Tris [pH 7.5], 1 mM EDTA). Unfractionated samples of DNA were prepared by electrophoresing the reaction components 1 cm into the agarose to remove PEG and BSA, cutting the block of agarose from gel well to dye front, and eluting as described above.

Electron microscopy. Samples were prepared for electron microscopy by a modification of the cytochrome *c* spreading technique in which DNA is spread onto a small water drop (23). In the present experiments, no attempt was made to preserve DNA-protein structures, consequently any protein present became denatured and was incorporated into the cytochrome *c*-supporting monolayer. The DNA samples were either dialyzed against 20 mM NaCl–5 mM EDTA or used directly in TE.

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For visualization of molecules in their native state, the spreading solution contained 0.4 mg of DNA per ml, 0.01% cytochrome *c*, 7.3% formaldehyde, 14.3% formamide, 16 mM Na_2CO_3, 27 mM NaCl, and 2.5 mM EDTA. The solution was adjusted to pH 8.0 ± 0.1 (25°C) with HCl before the addition of DNA and cytochrome *c*.

For visualization of partially denatured molecules, the DNA was first diluted to 2 μg/ml in 9.0% formaldehyde–20 mM Na_2CO_3–140 mM NaCl–38 mM disodium EDTA and the pH was adjusted to 10.3 with 5.0 M NaOH before the addition of DNA. This solution was then incubated for 10 min at a temperature between 24 and 30°C. After being cooled, the solution was diluted with an equal volume of formamide and cytochrome *c* was added to 0.01%. This solution was then spread on a water drop (23). Under these conditions X and Y structures (see text) are frequently denatured at the junction position. In addition, two A+T-rich sequences are also denatured and visualized as single-stranded bubbles. These sequences were identified in plots of average A+T content (13) for the known sequence of this DNA. They are centered at coordinates 3240 (AT2) and 4200 (AT1) with respect to the map of pBR322 (New England BioLabs, Inc., 1988–1989 Catalog, p. 106, Beverly, Mass.). The *Pst*I site, centered at coordinate 3611, is 589 bp from AT1 and 371 bp from AT2. AT1 is sufficiently close to the position of the chi junction that frequently the denatured A+T-rich area and the junction area merge into a single large denatured site.

Computer-assisted length measurements were made from electron monographs by tracing and recording on a semiautomatic digitizer, as described previously (28). All measured lengths were calculated in base pairs relative to a substrate length of 2,821 bp.

Analysis of DSCPs. DSCPs (see Fig. 2) were produced during the course of FLP reactions from substrate plasmids cut with *Pst*I either before or (in the case of supercoiled substrates) after reaction with FLP protein. They were then isolated from agarose gels and suspended in 10 μl of TE, as described above. The 2,049-bp DSCP was cut with *Bam*HI and the 772-bp DSCP was cut with *Eco*RI by adding 2 μl of 10× buffer C (International Biotechnologies, *Life on the Edge*, 1986/1987) and 1 μl (20 to 25 U) of the appropriate restriction enzyme. After cleavage, each DSCP is composed of a 13-bp inverted repeat with a 4-base 5′ overhang from the restriction cut at one end and an 8-base 5′ overhang from cleavage by FLP protein at the opposite end. The *Bam*HI and *Eco*RI sites were labeled at the 3′-end with [α-^{32}P]dATP (Amersham Corp.), dGTP, and reverse transcriptase (Life Sciences, Inc.). Samples were boiled for 3 min, placed immediately in ice, and then analyzed by electrophoresis. After electrophoresis the gel, which contained 12% polyacrylamide-bis acrylamide (30:1), 8 M urea, and TBE buffer (29), was vacuum dried and visualized by autoradiography.

RESULTS

Figure 1 illustrates the general assay used for all experiments in this study. The assay is designed to generate product molecules which differ in size from the substrates. Figure 1A outlines a reaction between FRT sites containing asymmetric spacers. Only a parallel, homologous alignment of DNA substrates is permitted due to asymmetry within the spacer sequence. For all substrates, the parallel orientation is defined as that in which sequences flanking the FRT site are in homologous alignment (see Materials and Methods). If two substrate plasmids which have been linearized with the same restriction enzyme undergo recombination, the products are the same size as the substrates and comigrate on an agarose gel (not shown in diagram). However, in Fig. 1A, the restriction enzymes *Sal*I or *Pst*I are used to generate two DNA substrates with different lengths of DNA flanking the FRT site. During recombination, products are formed which are larger (4,574 bp) and smaller (1,068 bp) than those of the parental substrates. In Fig. 1B, a reaction is diagrammed in which the spacer sequences are symmetrical. In this case, site alignment may occur in either parallel or antiparallel orientation. However, only the products of the antiparallel reaction (1,544 and 4,098 bp) can be observed in these experiments. In both panels A and B, we assume that 50% of the reactions occur between identically cut substrates aligned in parallel orientation so that a maximum reaction is indicated by a 50:50 distribution between product- and substrate-sized bands. Only one FRT site is present per molecule, so all recombination events are intermolecular. The spacer sequences of the four substrates used in this study are shown in Fig. 1C.

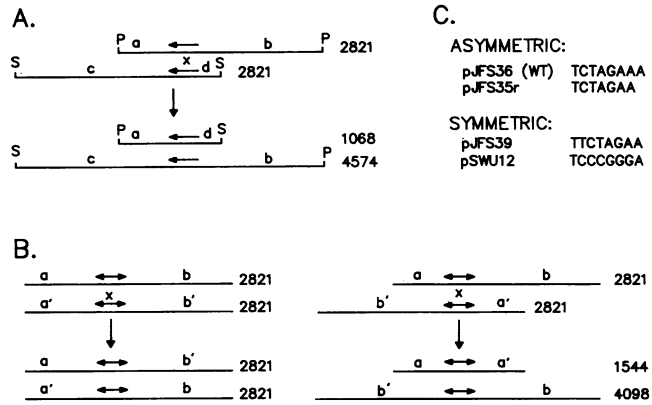


FIG. 1. Diagram of FLP protein-promoted reactions and the substrates employed. (A) Reaction between substrates with asymmetric spacers. FRT sites are in homologous, parallel alignment. When a substrate cut with *Pst*I (P) undergoes a reaction with a substrate cut with *Sal*I (S), products of different size than the substrate are formed. a and b, DNA arms surrounding the FRT site on a *Pst*I-cut substrate; c and d, DNA arms surrounding the FRT site on a *Sal*I-cut substrate. Products are 1,068 and 4,574 bp long. (B) Reaction between *Pst*I-cut substrates with symmetric spacers. The FRT site has no directionality, so substrates align in parallel or antiparallel orientation. Products from parallel, homologous alignment are the same size as the substrate. Products from antiparallel alignment are 1,544 and 4,098 bp long. All substrates are 2,821 bp long. (C) Spacer sequences of the FRT sites used in this study.

FLP-mediated recombination reactions using these four different substrates are shown in Fig. 2. Most of the DNA species evident in this gel will be described below. Lanes 4 to 6 show reactions of substrates with symmetrical spacers. In lane 4 is a reaction of pJFS39 linearized with *Pst*I. Several bands can be seen besides the normal substrate and products, including one labeled X on the left-hand side of the figure. Two bands which migrate faster than the substrate are labeled DSCP. In lanes 5 and 6 the substrate pSWU12 was employed. The 8-bp spacer in this site is symmetrical, and products of the reaction are identical in size to those of pJFS39. However, this mutant FRT site reacts six times more slowly than does pJFS39 (44). Lane 5 shows a recombination reaction of pSWU12 linearized with *Pst*I. Only a trace amount of DNA is seen in band X. In lane 6, supercoiled pSWU12 was reacted with FLP protein. The reaction

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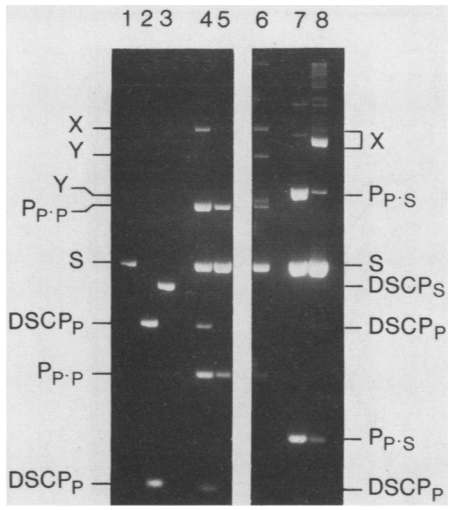


FIG. 2. Electrophoretic migration patterns of recombination reactions, using four different substrates. Lanes: 1, *Pst*I-cut pJFS36 substrate marker, 2,821 bp; 2, pJFS36 cut with *Pst*I and *Xba*I; 3, pJFS36 cut with *Sal*I and *Xba*I. (Lanes 2 and 3 contain marker DNAs which comigrate with DSCPs.) Lanes 4 to 8 show recombination reactions with the following substrate(s): 4, *Pst*I-cut pJFS39; 5, *Pst*I-cut pSWU12; 6, supercoiled pSWU12 reacted with FLP and then cut with *Pst*I; 7, *Sal*I-cut pJFS36 × *Pst*I-cut pJFS36; 8, *Sal*I-cut pJFS36 × *Pst*I-cut pJFS35r. Labels to the left refer to bands generated from substrates with symmetric spacers (lanes 4 to 6). Labels to the right refer to bands generated from substrates with asymmetric spacers (lanes 7 and 8). X and Y are explained in the text. Abbreviations: S, substrate; $P_{p\cdot p}$; product of two *Pst*I-cut substrates; $P_{p\cdot s}$, product of a *Pst*I-cut substrate and a *Sal*I-cut substrate; $DSCP_p$, $DSCP_s$, DSCPs from *Pst*I- or *Sal*I-cut substrates, respectively.

was terminated with heat before digestion with *Pst*I, as described in Materials and Methods. Two additional bands are seen in lane 6 between X and the product band. These are labeled Y.

Lanes 7 and 8 show reactions of substrates containing asymmetrical spacers, with one substrate cut with *Sal*I and the other cut with *Pst*I. Lane 7 shows the migration pattern of bands generated from pJFS36 in a wild-type × wild-type reaction. Along with the expected products, additional species are again evident. One is again labeled X. Lane 8 shows a mutant × wild-type reaction of pJFS35r × pJFS36. The spacer of pJFS35r contains only 7 bp but is otherwise the same as wild type. The X band formed during this reaction is much more abundant than the corresponding band of the wild-type pJFS36 reaction, despite the fact that product formation is much slower (data not shown). Unlabeled bands of slower mobility than X will not be dealt with in this report.

The X bands are Holliday intermediates. The DNA labeled X in Fig. 2, lanes 4 and 6, was removed from the preparative gels and prepared for electron microscopy. Electron micrographs of this DNA showed numerous chi structures. DNA isolated from band X of the *Pst*I-cut pJFS39 reaction was spread in its native state (Fig. 3a). In two separate isolations from excised gel bands, chi structures were 89% of 157 molecules counted and 55% of 143 molecules counted. Similarly, after band isolation chi structures from supercoiled pSWU12 represented 68% of the 235 molecules counted (Fig. 3b). These samples also contained a low proportion of Y structures (≤7%, see below). Chi structures were also observed in the unfractionated reaction mixtures, with 5% of 1,619 molecules for linear pJFS39 and 4% of 820 molecules for supercoiled pSWU12. In all cases, linear DNA molecules accounted for the majority of other species on the grid. The linear molecules are assumed to result from both the instability of the chi structures and the background DNA in the gel lane. In the case of unfractionated reaction samples, the major species were product and substrate molecules.

Measurements of the arm lengths generated from 85 pSWU12 chi molecules showed that the junction occurs at a unique position. Each arm was measured, and the length distribution was plotted as shown in the histogram (Fig. 4). The short and long arms are portrayed by the left (755 ± 40 bp) and the right (2,066 ± 79 bp) peaks, respectively. The length distributions are sharp and correspond well with the predicted arm lengths of 772 and 2,049 bp. From this we conclude that formation of the chi junction is site specific and occurs at the FRT recombination site.

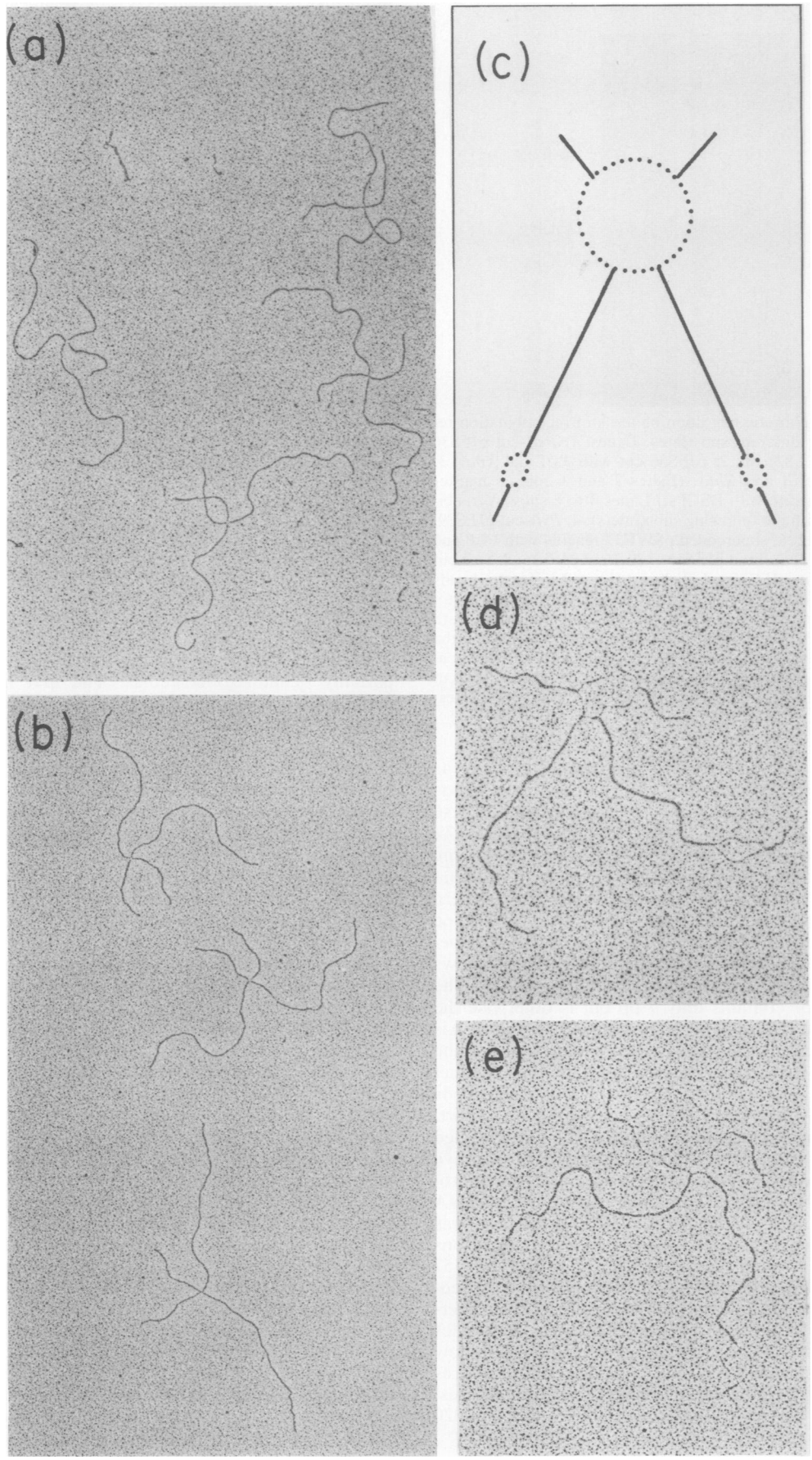
If these molecules are subjected to the appropriate partial denaturing conditions before electron microscopy (see Materials and Methods), the junction becomes preferentially denatured and reveals the state of strand continuity within the junction (Fig. 3d and e). Under these conditions, two A+T-rich regions within each substrate also denature to form bubbles at positions 183 (AT1) and 1,678 (AT2) bp on either side of the junction. The A+T-rich region of the short arm is close enough to the FRT site that the denaturation bubble frequently merges with the denatured chi junction. The conserved distance between the A+T-rich sites and the junction is further evidence that the junction occurs specifically at the FRT site.

Two important facts emerge from observations of these partially denatured molecules. The first is that there is complete strand continuity within the junction. Of 85 denatured chi structures characterized, 94% were covalently closed, with the remaining 6% broken in one strand at the junction. This is consistent with a Holliday mechanism, in which one strand of each substrate is cleaved and must be subsequently ligated to the homologous strand of the opposing substrate. With this method, we can distinguish between intact junctions and junctions possessing single nicks. Junctions with two nicks would not survive sample preparation, because spontaneous branch migration of the junction toward the two nicks would yield two linear, dissociated molecules.

The second fact is that, due to this continuity, the orientation in which the substrates were aligned at the time the initial strand exchange event occurred can be observed. This is revealed by the order in which the four double-strand arms are connected around the single-strand junction. In Fig. 5, the crossed chi structures are isomerized by rotating the bottom arms with respect to the top arms, resulting in open junctions. Parallel site juxtaposition would result in a denatured chi junction similar to that shown in Fig. 5A, showing alternating short and long arms. The alternative antiparallel alignment would result in that shown in Fig. 5B, in which two adjacent short arms are opposite two adjacent long arms. Supercoiled pSWU12, which has an FRT site with a symmetric spacer, was reacted with FLP and linearized with *Pst*I. The resulting chi structures were then band isolated and partially denatured. It was found that of 93 denatured chi junctions examined, 100% resulted from antiparallel alignment. The above identification is possible because each arm has a unique identifiable length. In addition, the identity of the arms and their polarity can be confirmed by the denatured sites AT1 and AT2 in these chi structures.

To distinguish between the possibilities that (i) parallel chi

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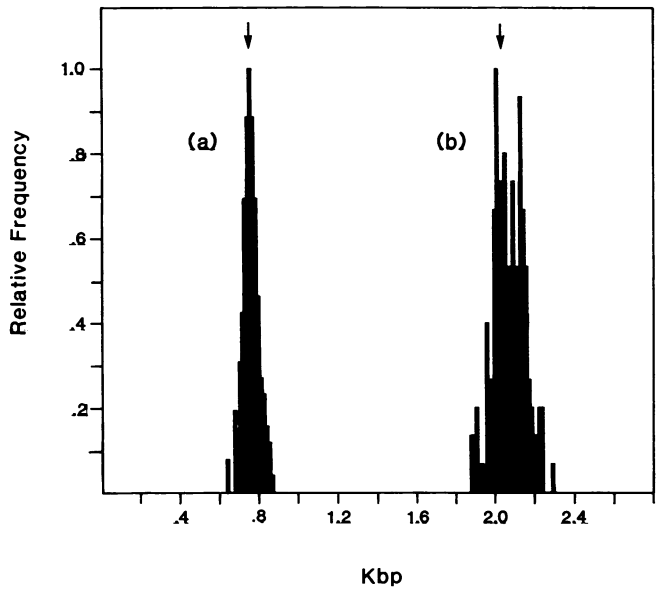


FIG. 4. Histogram showing the relative frequency of the measured arm lengths (in kilobase pairs [kbp]) of pSWU12 chi structures. Arrows mark the predicted positions (772 and 2,049 bp) for each peak. Each peak represents the distribution of measured lengths for short (a) or long (b) arms from 85 chi structures.

structures did not exist or (ii) we had missed them when we isolated DNA from a single gel band, unfractionated reaction samples were examined for chi structures. Due to the low frequency of chi structures, only 21 chi structures were examined, but all of them were derived from antiparallel reactions. In contrast, products from the parallel and antiparallel reactions have been seen to form at similar rates, when an assay was employed in which both products could be readily observed (39, 40). This suggests that chi structures formed in the parallel reaction may be less stable. Instability could be readily explained by branch migration. In the case of parallel alignment, the flanking DNA is homologous. Spontaneous branch migration to the ends of molecules which are in parallel alignment would result in linear DNA molecules equivalent in size to the original substrates. The arms of chi structures after antiparallel alignment, however, have heterologous flanking DNA surrounding the FRT site, which would prevent branch migration outside the FRT site and stabilize the Holliday intermediate during isolation. Holliday structures formed after antiparallel alignment are the ones which have been observed and characterized in this report.

Of the 79 partially denatured chi junctions characterized, 97% had an open configuration rather than the crossed configuration usually drawn for a Holliday structure. By open we mean that two arms of the Holliday structure have been rotated with respect to the other two, as shown in Fig. 5. From the present experiments we cannot determine if the rotated open configuration is actually present in solution or is a result of denaturation and the manipulations involved in the preparative procedure for electron microscopy. Regardless of this ambiguity, these structures still portray the strand continuity within the junction. Similar open configurations were also observed in lambda recombination intermediates (44).

The Holliday structures characterized thus far were isolated from reactions of substrates containing mutant spacers. To determine whether a Holliday intermediate is also operative in the wild-type reaction, the band labeled X from a pJFS36 reaction (Fig. 2, lane 7) was excised and observed by electron microscopy. Only 8% of the 317 molecules counted were actually chi structures of the type described above. The majority of the species (71%) were three-armed molecules in which all three arms were of different lengths. We reasoned that if a Holliday junction were unstable when the flanking DNA was homologous, the junction could move to the end of one substrate by branch migration. It would remain at an interior position with respect to the partner substrate (Fig. 5C). If one arm is the length of a full substrate molecule, and the sum of the lengths of the two remaining arms are equal to the length of the first, arm lengths of 1,068, 1,753, and 2,821 bp would be predicted. The arm lengths of 32 molecules were measured and found to be 1,080 ± 50, 1,740 ± 80, and 2,820 ± 96 bp, well within the range of predicted values. These structures were partially denatured in order to examine the junction for strand continuity. All partially denatured junctions contained a single strand break. This lack of continuity is consistent with a Holliday junction migrating to the end of one linear duplex DNA molecule. We believe most migrations outside the spacer occur after sodium dodecyl sulfate denaturation of FLP protein. The characteristics of these structures indicate they are vestiges of Holliday intermediates which are unstable during isolation due to extended flanking regions of homology. Their presence provides evidence that Holliday structures are generated in reactions with wild-type substrates. These three-armed structures are not the same as the Y structures described below (see Fig. 8).

When band X was excised and characterized from a reaction of mutant (pJFS35r) × wild-type (pJFS36) substrates (Fig. 2, lane 8), only 15% of 261 molecules counted were the three-armed structures identified in the wild-type reaction. Chi structures represented 64% of the molecules in the sample. This is much higher than expected since the spacers are asymmetric and flanking DNA is in homologous alignment. After partial denaturation, 100% of 44 molecules examined were derived from reactions between substrates in parallel alignment. This finding suggests that the single spacer mismatch in this cross (a deletion of 1 bp in pJFS35r) stabilizes the chi structure.

Evidence that chi structures are reaction intermediates. The appearance of Holliday structures is always observed previous to or concurrent with the appearance of recombination

FIG. 3. Chi structures as viewed by electron microscopy. (a) Chi structures produced from linear pJFS39 and found in band X. (b) Chi structures produced from supercoiled pSWU12 and found in band X. (d and e) Same as in panel b but after partial denaturation. The degree of denaturation was chosen to cause denaturation at the junction. However, two A+T-rich regions per substrate also frequently denatured (see experimental methods). The A+T-rich regions closest to the FRT site often merged with the junction to cause a large denatured area, as shown in the schematic line drawing (c). In panel d, the junction and the A+T-rich regions of the long arms have denatured, but the A+T-rich region on one short arm has not denatured and that of the other short arm has merged with the junction. In panel e, both denatured A+T-rich regions closest to the FLP site have merged with the junction, as shown in the line drawing. The measured distance from the center of the junction to the end of each arm segment is 755 ± 40 bp for the short arms and 2,066 ± 79 bp for the long arms (see text and Fig. 4).

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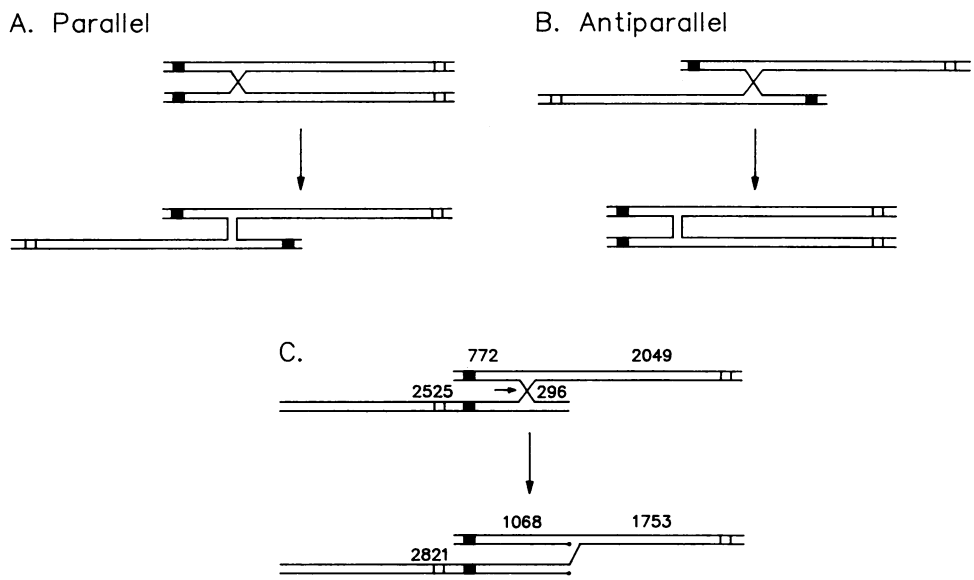


FIG. 5. Predicted Holliday intermediates as viewed by electron microscopy. (A) Parallel reaction between substrates with symmetric spacers. Rotation of arms causes an open configuration, with alternating long and short arms. (B) Rotation of arms after an antiparallel reaction results in an open junction, with two short arms opposing two long arms. (C) Parallel reaction between substrates containing asymmetric spacers. Branch migration to the end of one arm results in a three-arm structure, as shown. Although arrow depicts direction of migration in this diagram, migration in the opposite direction would yield Y structures with the same dimensions. Numbers refer to the expected distance in base pairs from junction to end of arm. ■, AT1; □, AT2.

products (data not shown). This result is consistent with the idea that Holliday structures are authentic reaction intermediates. If the chi structure is an active intermediate in recombination, its abundance might also be proportional to the rate of the recombination reaction. The rate of FLP-mediated recombination exhibits a linear dependence on the enzyme concentration (13a). To test whether the abundance of chi structure depends on enzyme concentration, time courses were performed at two concentrations of FLP protein (Fig. 6). The first contains 8 nM FLP protein and 10 nM *Pst*I-digested pJFS39, a ratio of 0.8 FLP protein monomers per FRT site. There is a lag of several minutes before products appear, and chi structures are seen at very low levels. At a 10-fold higher FLP protein concentration, there is a considerable amount of product formed at the 30-s time

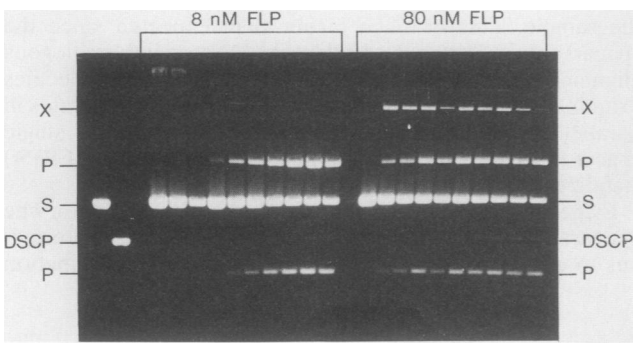


FIG. 6. Time course showing abundance of chi structure at two concentrations of FLP protein. Lane 1, Substrate marker, 2,821 bp; lane 2, pJFS36 × *Pst*I and *Xba*I marker, 2,049 bp. Time course I, 10 nM *Pst*I-cut pJFS39 reacted with 8 nM FLP protein; time course II, 10 nM *Pst*I-cut pJFS39 reacted with 80 nM FLP protein. For each time course, samples were removed at zero time and after 0.5, 1, 2, 5, 10, 30, 60, 120, and 240 min. Abbreviations are described in the legend to Fig. 2.

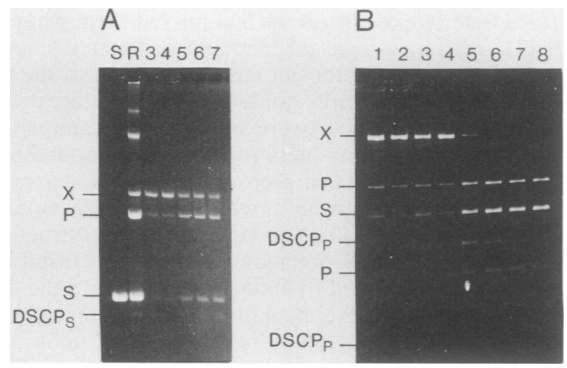


FIG. 7. Chemical competence of chi structures. (A) Resolution of chi structures to recombination products under conditions which support enzymatic turnover. Reaction mixtures included PEG, glycerol, and BSA (see Materials and Methods). Chi structures were derived from *Sal*I-cut substrates. Lanes: S, substrate marker, 2,821 bp; R, sample from original recombination reaction of *Sal*I-cut pJFS39 and FLP protein, rerun as markers. The chi band generated by this reaction was excised from the gel, treated as described in Materials and Methods, and used as the DNA source for the reactions depicted in lanes 3 to 7. Lanes 3 and 4, reaction mixture of chi structure with no added FLP protien. Samples were removed at zero time and after 60 min of incubation at 30°C, respectively. Lanes 5 to 7, reaction mixture of chi structure with 360 nM FLP protein. Samples were removed at 2, 5, and 60 min, respectively. All reactions contained approximately 4 nM FRT sites. The ratio of FLP protein to FRT site is 90:1. (B) Resolution of chi structures to recombination products under conditions which do not support enzymatic turnover. Reaction mixtures contained no PEG, glycerol, or BSA. Chi structures were generated and isolated as described in panel A, except that the substrate DNA used in the FLP reaction was precut with *Pst*I. The isolated chi structure was reacted at 3.4 nM final concentration with the indicated concentration of FLP protein. Lanes 1 to 8, 0, 4 nM, 8 nM, 15 nM, 30 nM, 60 nM, 120 nM, and 220 nM FLP protein, respectively. All reactions were terminated after 60 min at 30°C. Abbreviations are described in the legend to Fig. 2.

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point and the steady-state concentration of chi structure is much higher. This shows that a correlation exists between the rate of recombination and the observed steady-state level of chi structures.

Isolated chi structures were tested for FLP protein-dependent resolution to product (Fig. 7). After reaction of *Sal*I-cut pJFS39 with FLP protein followed by termination of the reaction with sodium dodecyl sulfate, the chi structure was separated from FLP protein and other DNA species by agarose gel electrophoresis. It was isolated from the excised gel band and introduced to reaction mixtures without (Fig. 7A, lanes 3 and 4) or with (lanes 5 to 7) newly added FLP protein. Figure 7A, lane 2, shows the reaction from which the intermediate was isolated. The chi structure generated by *Sal*I-digested substrate migrates slightly faster than one with *Pst*I-cut arms. Lanes 3 and 4 show the degrees to which the chi structures break down into product- and substrate-sized molecules in the absence of FLP protein at zero time and after 60 min of 30°C incubation, respectively. No DSCPs, which are shown below to accumulate in this reaction, are detected in these lanes. Aliquots from the reaction mixture to which FLP protein was added were taken at 2, 5, and 60 min and are shown in lanes 5 to 7. Increased prominence of product- and substrate-sized bands can be seen, along with a decrease in chi structure relative to samples in which no FLP protein was added. However, some isolated chi structure persists in the presence of FLP protein. This is probably due to repeated turnover of the products formed, resulting from the very high FLP protein concentration used in this experiment. DSCPs can be seen to accumulate, suggesting that the reaction is actively turning over (see Fig. 9). If the reaction is at steady state, Holliday intermediates should represent a constant fraction of the DNA molecules, because they would be continually formed and resolved. However, we cannot determine the proportion of isolated chi structures that have taken on nonreactive conformations under these reaction conditions.

To determine the proportion of remaining chi structures in Fig. 7A that were nonreactive, chi structures were resolved to products under conditions which would minimize reaction turnover (Fig. 7B). Chi structures generated with *Pst*I-cut pJFS39 were isolated as described above and reacted without (lane 1) or with (lanes 2 to 8) newly added FLP protein. PEG, glycerol, and BSA were omitted from the resolution reaction mixtures. At low FLP concentrations (lanes 2 to 4), little or no significant resolution of chi structure is detected over background. As the FLP protein concentration is increased (lanes 5 to 8), complete resolution of the chi structures to recombination product- and substrate-sized molecules is seen. All chi structures are therefore reactive. This experiment was performed in parallel with reaction mixtures that included PEG, glycerol, and BSA, and steady-state levels of chi structure were again present at all FLP protein concentrations (data not shown). This result supports the idea that the residual chi structures seen in Fig. 7A, lanes 5 to 7, are the result of steady-state turnover of the reaction. A high proportion of isolated Holliday intermediates are reactive and chemically competent for conversion to recombination products.

Additional structures formed during recombination. Reactions of supercoiled pSWU12 produce two DNA species that are labeled Y (Fig. 2, lane 6). The DNA from each band was isolated and prepared for electron microscopy. Electron micrographs showed many jointed molecules with three arms, thus designated Y structures. DNA in the lower Y band contained 52% Y structures of the 363 DNA molecules counted (Fig. 8A). All had one long arm and two short arms (SSL). When DNA in the upper Y band was examined, 70% of 189 molecules counted were Y structures, 100% of which had two long arms and one short arm (LLS).

The lengths of the arms of 99 SSL and 91 LLS Y structures were measured. The lengths of arms in both structures were 2,065 ± 56 bp and 756 ± 28 bp for long and short arms, respectively. Data peaks were as sharp as those of the histogram shown in Fig. 4 (data not shown). The measured arm lengths are in agreement with the predicted arm lengths of 2,049 and 772 bp pairs from *Pst*I-digested substrates. The arm lengths are also in agreement with those of a chi structure, but with one arm missing. This shows that the junction of the Y structure is located at the FRT recombination site. The junction location and the missing DNA arm place these Y structures in a different category from the three-armed molecules identified in the wild-type (pJFS36) reaction.

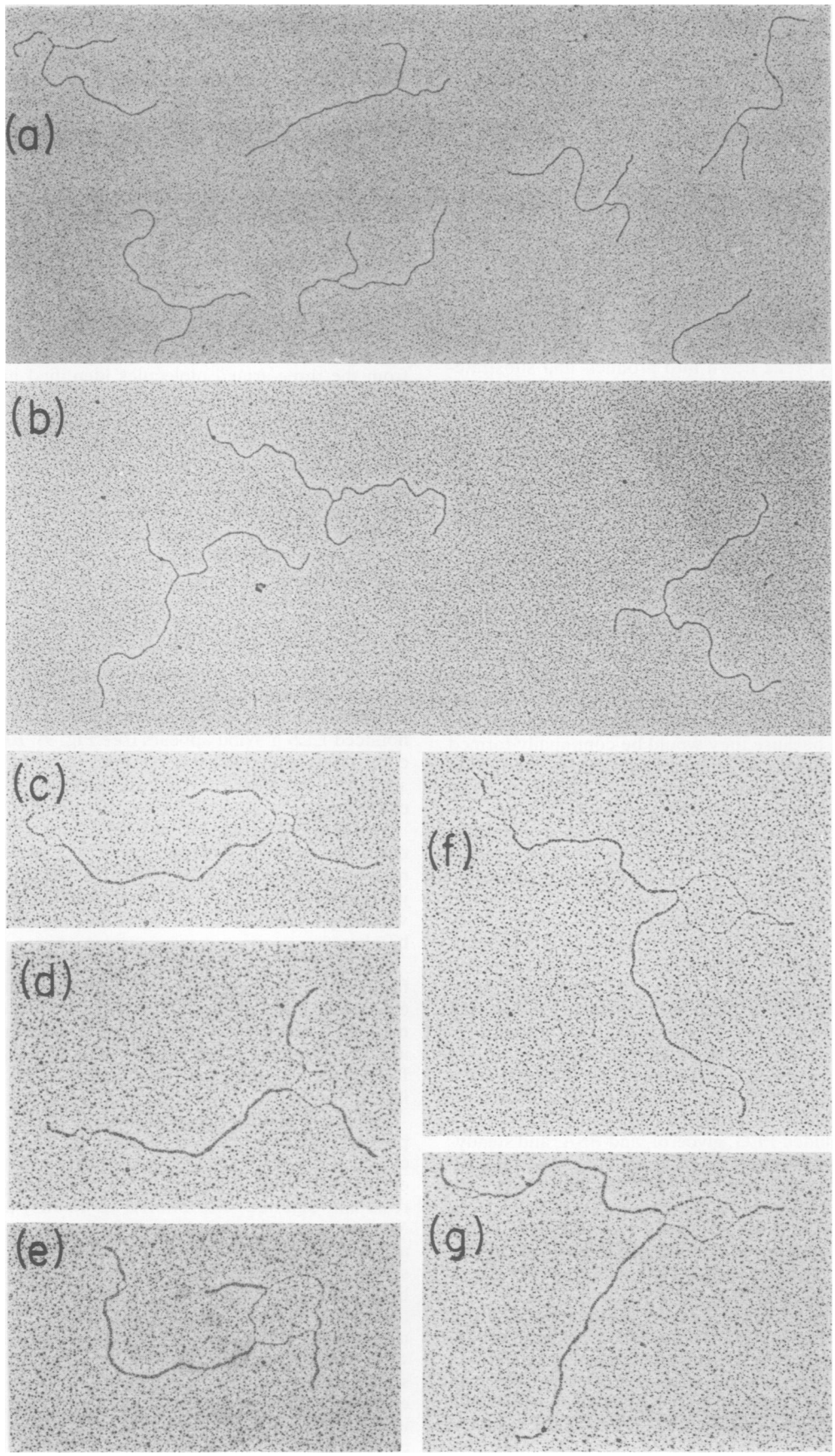
The Y structures in an unfractionated reaction of supercoiled pSWU12 are 14% of 820 molecules counted. In a separate count of 479 Y structures, 45% were LLS and the remaining 55% were SSL. This reflects an approximate 50:50 distribution between the two species, suggesting that choice of which arm to delete is random. With supercoiled pSWU12 DNA, this relative distribution is not altered over a 24-h reaction period. The LLS and SSL Y structures appear slightly later than the chi structure band in time courses (data not shown). Y structures are also found in reactions of *Pst*I-cut pJFS39 DNA, but only in small amounts, and the abundance changes with time. Reactions of pJFS39 terminated at 30 min contain less than 1% LLS and SSL Y structures. However after several hours of reaction, a pronounced LLS Y band appears (see Fig. 9). Electron microscopy after band isolation confirms this to be an LLS Y structure. An SSL Y structure was not resolved in this experiment.

Partial denaturation of both LLS and SSL Y structures opens the junction of the three arms as well as the A+T-rich regions, as shown (Fig. 8c to g). The majority of denatured junctions (90% of 91 molecules) show covalent continuity, with only a few junctions (10%) showing evidence of strand breakage.

Two DNA species, which electrophoretically migrate faster than the substrate, are products of double-strand cleavage events (labeled DSCP, Fig. 2). They comigrate with marker substrate DNA cut within the spacer region by *Xba*I and are formed by cleavage at both sides of the spacer. DSCPs derived from *Pst*I-cut substrates are 2,049 and 772 bp in length.

Analysis of the DSCPs generated from supercoiled pSWU12 revealed that approximately 50% are covalently closed at the end, forming hairpinlike structures. DNA in each DSCP band was purified after agarose gel electrophoresis and digested with *Bam*HI or *Eco*RI, as described in Materials and Methods. If the DSCP were covalently closed at the end of the FRT site to form a hairpin, the denatured single strand would be nearly twice as long as it would be if both ends were open. The fragments were analyzed by denaturing polyacrylamide gel electrophoresis. The fragments expected for both open and covalently closed ends were present in approximately equal amounts (data not shown), suggesting that roughly half the DSCPs have closed ends. When the same experiment was performed on DSCPs isolated from a reaction of *Pst*I-cut pJFS39, no covalently closed ends were detected (data not shown). Models for the

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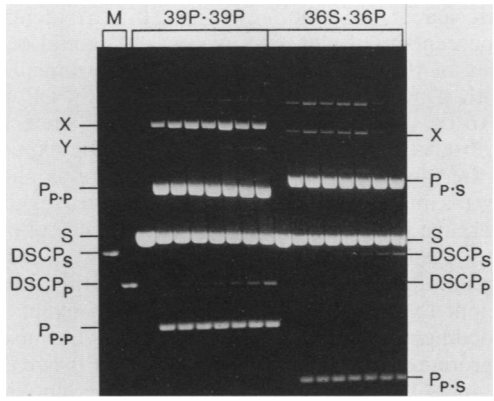


FIG. 9. Time courses showing the accumulation of DSCPs from substrates with symmetric or asymmetric spacers. Samples were removed at the beginning and after 2, 5, 10, 30, 60, 120, and 280 min. M, Markers for DSCPs generated from *Sal*I-cut (pJFS36 cut with *Sal*I and *Xba*I) and *Pst*I-cut (pJFS36 cut with *Pst*I and *Xba*I) substrates. Time course I, 44 nM *Pst*I-cut pJFS39 × 160 nM FLP protein (39P · 39P). Time course II, 22 nM *Sal*I-cut pJFS36 × 22 nM *Pst*I-cut pJFS36 × 160 nM FLP protein (36S · 36P). Labels on the left refer to the pJFS39 reaction. Labels to the right refer to the pJFS36 reaction. Abbreviations are described in the legend to Fig. 2.

formation of the Y-structures and DSCPs are discussed below (see Discussion).

DSCPs accumulate during FLP protein-mediated recombination. Figure 9 shows two time courses of FLP protein-mediated recombination reactions, with substrates containing either a symmetrical or asymmetrical spacer. The ratio of FLP protein to FRT site is approximately 4:1 in both reactions. The first time course is a reaction of *Pst*I-cut pJFS39, which contains a symmetrical spacer, with aliquots removed from 2 to 280 min. DSCPs are present at the earliest time point but continue to accumulate during the reaction period. The second time course shows samples removed from a reaction of the wild-type substrate pJFS36, also from 2 to 280 min. Since DSCPs are derived from both *Sal*I-cut and *Pst*I-cut substrates at constant total DNA concentration, each DSCP is expected to be half as abundant as the DSCPs generated by pJFS39. However, in the reaction with wild-type substrate, the DSCPs are found at much lower amounts than in the pJFS39 reaction, the first appearance occurring at 30 min. This demonstrates that DSCPs are produced from wild-type sites but are more abundant if the reactant contains a symmetrical spacer. With respect to the chi structure, the reaction appears to be at steady state at the 2-min time point with either substrate under the conditions of this experiment.

DISCUSSION

Chi structures are formed during recombination reactions catalyzed by FLP protein and have properties consistent with being intermediates in the reaction. Arm length measurements show the chi structure junction to be specifically located at the FRT site. Partial denaturation shows all four single strands of the recombinant Holliday junction to be covalently continuous, as is consistent with two sequential, reciprocal-strand exchange events during recombination. These structures appear to be intermediates in the in vitro reaction because (i) they are always observed early in the reaction, at or before products appear; (ii) after isolation they are resolved by FLP protein; and (iii) high FLP protein concentrations produce proportionally higher steady-state levels of chi structure. Their steady-state levels correlate with the rate of the reaction.

We have also identified species which appear to have had one arm of the chi structure deleted, giving them the appearance of Y structures. Supercoiled pSWU12 generates Y structures with two long arms and Y structures with two short arms in an approximate 50:50 ratio. No Y structures have been observed from reactions of linear pSWU12, but they are formed at low levels from pJFS39. Partial denaturation shows the DNA strands to be continuous within the Y structure junction. Additional products, formed by double-strand cleavage, accumulate over time. DSCPs are observed in the wild-type reaction but accumulate more rapidly in reactions employing substrates with symmetrical spacers.

We have demonstrated that Holliday structures are formed when using a substrate with the wild-type asymmetric spacer (pJFS36). Since this Holliday intermediate is unstable to isolation because of spontaneous branch migration in the homologous flanking DNA, we have done so by characterizing the stable, vestigial structure resulting from this branch migration. These are three-armed structures with predictable arm lengths and a characteristic nick at the junction. Jayaram and colleagues have also recently observed the formation of chi structures in FLP reactions with wild-type FRT sites (M. Jayaram et al., Proc. Natl. Acad. Sci. USA, in press). Substrates possessing mutations in the spacer which make the spacer symmetrical (pJFS39 and pSWU12) permit the reaction to occur with substrates aligned in either of the two possible orientations. The Holliday structures characterized here are those involving antiparallel alignment of substrates. Unlike those from the parallel reaction, these are stable after removal of FLP protein, because branch migration is restricted by nonhomologous sequences flanking the FRT sites. From currently available data, we are not able to assign quantitative or mechanistic significance to the differences in steady-state chi structure levels among substrates bearing different spacer sequences.

In reaction experiments with the substrate pSWU12, the appearance of reaction intermediates and by-products is also affected by substrate topology. This FRT site has four base-pair changes in the spacer (relative to pJFS39) that reduce recombination efficiency. Linear pSWU12 substrates produce chi forms at much reduced levels relative to pJFS39. However, the appearance of chi structures is greatly enhanced when pSWU12 is supercoiled. In contrast, supercoiled pJFS39 does not show an increase in chi structures over linear pJFS39 in FLP reactions (data not shown). The effects of DNA topology in this system have not been

FIG. 8. Y structures viewed by electron microscopy. (a) Y structures found in lower Y band (Fig. 2) from a reaction of supercoiled pSWU12. These structures contain two equal-length short arms and one long arm (SSL type). (b) Y structures found in upper Y band (Fig. 2) from a reaction of supercoiled pSWU12. These structures contain two long arms and one short arm (LLS type). The sum of S+L is equal to the length of an unreacted substrate (2,821 bp). (c, d, and e) SSL types after partial denaturation, showing strand continuity at the junction. (f and g) Partially denatured LLS type Y structures. See the legend to Fig. 3 for an explanation of the A+T-rich region that often denatures under these conditions.

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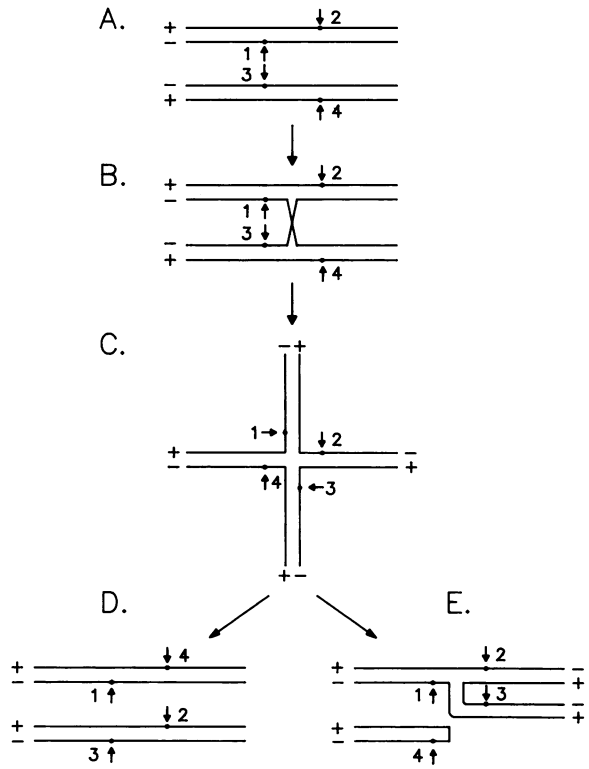


FIG. 10. Diagram showing a model for the formation of normal products versus Y structures from substrates with symmetrical spacers. (A) Site juxtaposition showing four cleavage sites. (B) Cleavage and ligation between sites 1 and 3 form a chi structure. (C) For illustrative purposes, molecule is isomerized by rotating two arms with respect to the remaining two arms. Cleavage plus ligation between sites 2 and 4 yields normal products (D), or cleavage plus ligation between sites 1 and 4 yields a Y structure and a covalently closed DSCP (E).

characterized in detail. In vivo, the reaction occurs between intramolecular FRT sites on a supercoiled plasmid.

An effort was made to increase the abundance of chi structures by reacting wild-type substrates with substrates containing single-base-pair spacer alterations. We have screened mutations at every base in the spacer for effects on the level of chi intermediate. A 1-bp deletion (pJFS35r) is the only mutation which has a large effect (Fig. 2; data not shown).

The appearance of chi structures is sensitive to reaction conditions that affect enzyme stability and that therefore affect enzyme turnover. Reactions without PEG, BSA, and glycerol do not show significant turnover (13a) and produce only a barely detectable level of chi structures (Fig. 7B; data not shown). One reason chi structures were previously unidentified in this system may be that the minimal reaction conditions were insufficient for abundant intermediate formation.

More DSCPs are produced from substrates containing a symmetric spacer than from those containing an asymmetric spacer. There appear to be at least two side pathways by which DSCPs could be formed. The first requires concerted cleavage of double strands instead of two sequential, single-strand cleavages. With an asymmetric, wild-type spacer, FLP protein may exhibit some directional preference for a primary cleavage site, as does Int protein (26, 35). The choice of a primary cleavage site would be random with a symmetric spacer, since both cleavage sites are identical. In a small percentage of cleavage events, cuts could occur on both sides of the spacer simultaneously, forming DSCPs. Even with a wild-type asymmetric spacer, FLP protein appears to be somewhat imprecise however, since DSCPs are still observed. Such products would not be expected to react further and would therefore accumulate as shown in Fig. 9. Experiments by Jayaram and colleagues suggest that any preference FLP protein may show for choice of primary cleavage site is limited (Jayaram et al., in press). The level of DSCPs generated with wild-type substrates in this study may be sufficient to account for gene conversion events previously documented in vivo (24). However, because FLP protein-promoted site-specific recombination is *rad52* independent and FLP-induced gene conversion is abolished in *rad52* mutant cells, gene conversion is not evidence that double-strand break products are true intermediates in vivo (24).

DSCPs might also be generated as part of the formation of Y structures. This second pathway involves improper resolution of the chi structure. A model for their coupled formation is shown in Fig. 10. Substrates undergo primary cleavage at sites 1 and 3 (panel A), with subsequent strand exchange and ligation, to generate a chi structure (panel B). The two bottom arms are rotated with respect to the two top arms to obtain the open configuration seen in electron micrographs (panel C). To resolve the Holliday junction, cleavages must occur at positions 2 and 4 to form normal products (panel D). Formation of Y structures would result from improper cleavage of adjacent, rather than opposite, cleavage positions. For example, cleavage at sites 1 and 4 (or at sites 2 and 3) cuts both strands of the intervening arm, releasing a DSCP. Subsequent reciprocal ligation between these adjacent positions would form two structures, a Y structure with a covalently closed junction and a DSCP with a covalently closed end (panel E). Both structures have been experimentally observed in this study. It is not clear whether the DSCPs with closed ends are mechanistically related to the similar structures recently referred to in the Int system (37). Possibly a Y structure could be further resolved by FLP protein to a single linear product molecule plus an additional DSCP, but this has not been demonstrated.

Both mechanisms for generating DSCPs could occur concurrently. Two observations suggest this is the case. The first is that no DSCPs with closed ends have been detected in reactions of pJFS39 substrates. The second is that only approximately half the DSCPs produced from pSWU12 substrates have closed ends. We suspect that if these models are valid, their relative importance differs in reactions of the two substrates. In addition, FLP protein may also generate DSCPs via some other mechanism which is not dependent on spacer symmetry, since some DSCPs are formed from wild-type FRT sites. DSCPs are also formed by Int protein from substrates with asymmetric spacers (33, 37).

The reason for the increased appearance of Y structures and DSCPs on substrates containing symmetrical spacers remains unexplained. One clue may be the structure of the Holliday junction which, as has been recently suggested, would be expected to influence resolving enzymes (8). Complete base asymmetry around the junction causes a sequence-dependent structure which is nonplanar, has non-equivalent bending angles between the arms, and cannot freely interchange strands (8). Although it is difficult to accurately predict the structure of a symmetrical junction, three possible conformations for a sequence-independent chi junction would include tetrahedral, planar-tetragonal, or

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completely flexible structures (8). With any of these conformations it would be difficult for a recombinase to accurately choose a cleavage site on the basis of DNA structure. The differences between pJFS36, pJFS39, and pSWU12 in the generation of Y structures and DSCPs may indicate that FLP protein is influenced by sequence-dependent structures at the chi junction.

The case for Holliday structures as reaction intermediates for the integrase family of recombinases is growing (17, 26, 35). However, no explanation for the observed generation of DSCPs, which had been cited as evidence for the cohesive-end formation model, has been offered. Evidence put forth in this report suggests a side pathway which allows DSCPs to be formed without contradicting the evidence that Holliday structures are mechanistic intermediates. The presence of chi structures as detailed in this study is strongly indicative of a Holliday mechanism for site-specific recombination by FLP protein.

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LITERATURE CITED

1. **Abremski, K., and R. Hoess.** 1984. Bacteriophage P1 site-specific recombination: purification and properties of the Cre recombinase protein. J. Biol. Chem. **259**:1509–1514.
2. **Argos, P., A. Landy, K. Abremski, J. B. Egan, E. Haggard-Ljungquist, R. H. Hoess, M. L. Kahn, B. Kalionis, S. V. L. Narayana, L. S. Pierson III, N. Sternberg, and J. M. Leong.** 1986. The integrase family of site-specific recombinases: regional similarities and global diversity. EMBO J. **5**:433–440.
3. **Bauer, C. E., J. F. Gardner, and R. I. Gumport.** 1985. Extent of homology required for bacteriophage lambda site-specific recombination. J. Mol. Biol. **181**:187–197.
4. **Bauer, C. E., S. D. Hesse, J. F. Gardner, and R. I. Gumport.** 1984. DNA interactions during bacteriophage lambda site-specific recombination. Cold Spring Harbor Symp. Quant. Biol. **49**: 699–705.
5. **Bell, L., and B. Byers.** 1979. Occurrence of cross strand-exchange forms in yeast DNA during meiosis. Proc. Natl. Acad. Sci. USA **76**:3445–3449.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Bruckner, R. C., and M. M. Cox.** 1986. Specific contacts between the FLP protein of the yeast 2-micron plasmid and its recombination site. J. Biol. Chem. **261**:11798–11807.
8. **Cooper, J. P., and P. J. Hagerman.** 1987. Gel electrophoretic analysis of the geometry of a DNA four-way junction. J. Mol. Biol. **198**:711–719.
9. **Cox, M. M.** 1983. FLP protein of the yeast 2-micron plasmid: expression of a eukaryotic genetic recombination system in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **80**:4223–4227.
10. **Craig, N. L., and Nash, H. A.** 1983. The mechanism of bacteriophage lambda site-specific recombination: site-specific breakage of DNA by Int topoisomerase. Cell **35**:795–803.
11. **Echols, H., and L. Green.** 1979. Some properties of site-specific and general recombination inferred from Int-initiated exchanges by bacteriophage lambda. Genetics **93**:297–307.
12. **Enquist, L. W., H. Nash, and R. A. Weisberg.** 1979. Strand exchange in site-specific recombination. Proc. Natl. Acad. Sci. USA **76**:1363–1367.
13. **Funnell, B. E., and R. B. Inman.** 1979. Comparison of partial denaturation maps with the known sequence of simian virus 40 and φX174 replicative form DNA. J. Mol. Biol. **131**:331–340.

13a. **Gates, C. A., and M. M. Cox.** 1988. FLP recombinase is an enzyme. Proc. Natl. Acad. Sci. USA **85**:4628–4632.

14. **Gates, C. A., L. Meyer-Leon, J. M. Attwood, E. A. Wood, and M. M. Cox.** 1987. Purification of FLP recombinase using sequence-specific DNA affinity chromatography. UCLA Symp. Mol. Cell. Biol. **68**:197–206.
15. **Gronostajski, R. M., and P. D. Sadowski.** 1985. The FLP recombinase of the *Saccharomyces cerevisiae* 2μm plasmid attaches covalently to DNA via a phosphotryrosyl linkage. Mol. Cell. Biol. **5**:3274–3279.
16. **Hartley, J. L., and J. E. Donelson.** 1980. Nucleotide sequence of the yeast plasmid. Nature (London) **286**:860–865.
17. **Hoess, R., A. Wierzbicki, and K. Abremski.** 1987. Isolation and characterization of intermediates in site-specific recombination. Proc. Natl. Acad. Sci. USA **84**:6840–6844.
18. **Hoess, R. H., and K. Abremski.** 1985. Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system. J. Mol. Biol. **181**:351–362.
19. **Hoess, R. H., A. Wierzbicki, and K. Abremski.** 1986. The role of the loxP spacer region in P1 site-specific recombination. Nucleic Acids Res. **14**:2287–2300.
20. **Hoess, R. H., M. Ziese, and N. Sternberg.** 1982. P1 site-specific recombination: nucleotide sequences of the recombining sites. Proc. Natl. Acad. Sci. USA **79**:3398–3402.
21. **Holliday, R.** 1964. A mechanism for gene conversion in fungi. Genet. Res. **5**:282–304.
22. **Hsu, P. L., and A. Landy.** 1984. Resolution of synthetic att-site Holliday structures by the Integrase protein of bacteriophage lambda. Nature (London) **311**:721–726.
23. **Inman, R. B., and M. Schnos.** 1970. Partial denaturation of thymine- and 5-bromouracil-containing lambda DNA in alkali. J. Mol. Biol. **49**:93–98.
24. **Jayaram, M.** 1986. Mating type-like conversion promoted by the 2μm circle site-specific recombinase: implications for the double-strand-gap repair model. Mol. Cell. Biol. **6**:3831–3837.
25. **Kitts, P., E. Richet, and H. A. Nash.** 1984. Lambda integrative recombination: supercoiling, synapsis, and strand exchange. Cold Spring Harbor Symp. Quant. Biol. **49**:735–744.
26. **Kitts, P. A., and H. A. Nash.** 1987. Homology-dependent interactions in phage lambda site-specific recombination. Nature (London) **329**:346–348.
27. **Landy, A., and W. Ross.** 1977. Viral integration and excision: structure of the lambda att sites. Science **197**:1147–1160.
28. **Littlewood, R. K., and R. B. Inman.** 1982. Computer-assisted DNA length measurements from electron micrographs with special reference of partial denaturation mapping. Nucleic Acids Res. **10**:1691–1706.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **McLeod, M., S. Craft, and J. R. Broach.** 1986. Identification of the crossover site during *FLP*-mediated recombination in the *Saccharomyces cerevisiae* plasmid 2μm circle. Mol. Cell. Biol. **6**:3357–3367.
31. **Meyer-Leon, L., C. A. Gates, J. M. Attwood, E. A. Wood, and M. M. Cox.** 1987. Purification of the FLP site-specific recombinase by affinity chromatography and re-examination of basic properties of the system. Nucleic Acids Res. **15**:6469–6488.
32. **Meyer-Leon, L., J. F. Senecoff, R. C. Bruckner, and M. M. Cox.** 1984. Cold Spring Harbor Symp. Quant. Biol. **49**:797–804.
33. **Nash, H. A., C. E. Bauer, and J. F. Gardner.** 1987. Role of homology in site-specific recombination of bacteriophage lambda: evidence against joining of cohesive ends. Proc. Natl. Acad. Sci. USA **84**:4049–4053.
34. **Nash, H. A., and C. A. Robertson.** 1981. Purification and properties of the *E. coli* protein factor required for lambda integrative recombination. J. Biol. Chem. **256**:9246–9253.
35. **Nunes-Duby, S. E., L. Matsumoto, and A. Landy.** 1987. Site-specific recombination intermediates trapped with suicide substrates. Cell **50**:779–788.
36. **Pargellis, C. A., S. E. Nunes-Duby, L. M. de Vargas, and A. Landy.** 1988. Suicide recombination substrates yield covalent

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lambda integrase-DNA complexes and lead to identification of the active site tyrosine. J. Biol. Chem. **263**:7678–7685.

37. **Richet, E., P. Abcarian, and H. A. Nash.** 1988. Synapsis of attachment sites during lambda integrative recombination involves capture of a naked DNA by a protein-DNA complex. Cell **52**:9–17.
38. **Senecoff, J. F., R. C. Bruckner, and M. M. Cox.** 1985. The FLP recombinase of the yeast 2-micron plasmid: characterization of its recombination site. Proc. Natl. Acad. Sci. USA **82**:7270–7274.
39. **Senecoff, J. F., and M. M. Cox.** 1986. Directionality in FLP protein-promoted site-specific recombination is mediated by DNA-DNA pairing. J. Biol. Chem. **261**:7380–7386.
40. **Senecoff, J. F., P. J. Rossmeissl, and M. M. Cox.** 1988. DNA recognition by the FLP recombinase of the yeast 2μ plasmid. A mutational analysis of the FLP binding site. J. Mol. Biol. **201**: 405–421.
41. **Sternberg, N.** 1981. Bacteriophage P1 site-specific recombination. III. Strand exchange during recombination at *lox* sites. J. Mol. Biol. **150**:603–608.
42. **Thompson, J. F., L. M. de Vargas, C. Koch, R. Kahmann, and A. Landy.** 1987. Cellular factors couple recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway. Cell **50**:901–908.
43. **Umlauf, S. W., and M. M. Cox.** 1988. A functional role for DNA sequence structure in a site-specific genetic recombination reaction. EMBO J. **7**:1845–1852.
44. **Valenzuela, M. S., and R. B. Inman.** 1975. Visualization of a novel junction in bacteriophage lambda DNA. Proc. Natl. Acad. Sci. USA **72**:3024–3028.
45. **Vetter, D., B. J. Andrews, L. Roberts-Beatty, and P. D. Sadowski.** 1983. Site-specific recombination of yeast 2-micron DNA in vitro. Proc. Natl. Acad. Sci. USA **80**:7284–7288.
46. **Volkert, F. C., and J. R. Broach.** 1986. Site-specific recombination promotes plasmid amplification in yeast. Cell **46**:541–550.

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