Topological Analysis of the Role of Homology in Flp-mediated Recombination*

Naiyer Azam, Julie E. Dixon, and Paul D. Sadowski‡

From the Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Recombination by the Flp recombinase of Saccharomyces cerevisiae is known to be inhibited by heterology of the overlap regions of the two recombining DNA targets (FRT sites). We have used topological analysis to show that Flp can promote two rounds of intramolecular recombination between heterologous FRT sites contained within the same supercoiled plasmid. The products are in parental nonrecombinant configuration. Thus, heterology may appear to “block” recombination by rendering the heteroduplex recombinant products unstable, thus favoring a second round of recombination to homoduplex (but parental) products. Hence, homology in the core region is not a requirement for the recombination reaction by Flp but for the formation of recombinant products.

Flp is a conservative site-specific recombinase that is a member of the integrase family (1). Flp is encoded by the 2-μm circle, an autonomously replicating, multicopy plasmid of Saccharomyces cerevisiae (2), and plays a role in the amplification of the 2-μm plasmid (3–5).

The Flp recognition target (FRT)1 sites lie within two 599-bp inverted repeats of the plasmid. An FRT site contains three 13-bp Flp binding sites, symmetry elements a, b, and c (Fig. 1). Two symmetry elements (b and c) lie in the same orientation, while symmetry element a lies in reverse orientation separated from symmetry element b by an 8-bp asymmetrical core. The deletion of symmetry element c has no detectable effect on recombination either in vivo or in vitro (6–8).

Flp cleaves the FRT site at the margins of the 8-bp core and becomes covalently attached to the 3′-phosphate via Tyr-343 within the active site (9–11). Cleavage occurs by the cooperation of two Flp molecules and is said to occur in trans (12, 13). The Flp molecule that acts as the tyrosine donor is bound to a symmetry element across the core from the cleavage site (14). Two reciprocal strand exchanges generate a four-armed Holliday intermediate, which is then resolved by another pair of reciprocal strand exchanges to yield reciprocally recombinant products (15–18).

A site-specific recombinase carries out strand exchange in a unique “sense.” This means that for a given reaction, the direction of the strand exchanges (clockwise or counterclockwise) in relation to the helical axis of the paired DNA targets is always the same rather than random. The topological products resulting from recombination of supercoiled substrates produced by the λ integrase or the Tn3 resolvase were found to have an invariant stereostucture: the nodes are positive in sign for the λ integrase and negative for the Tn3 resolvase (19–21). Recombination of a relaxed excision substrate by the Flp recombinase produces only unlinked products rather than a mixture of linked and unlinked circles (22). This is consistent with recombination in a unique sense within a synapse of unique topology. It could also be due to recombination in either sense within synapses of opposite sign.

It has been observed that although the core sequence can be changed (23, 24), efficient recombination requires that the cores of the two recombining FRT sites be homologous to one another. Therefore, Flp, like other members of the integrase family (25), shows impaired recombination between two FRT sites whose core regions are not homologous.

Why is homology in the core required for Flp-mediated recombination? One step in the Flp reaction where the homology of the cores might be sensed is during the synapsis stage. However, previous studies of Flp-mediated synaptic complexes ruled out a requirement for homology at this stage (26). Another stage where homology might be sensed is in the rejoining of the cleaved strands. Recent studies do point to the importance of homology of the base pair immediately adjacent to the site of cleavage for ligation by Flp (27, 28). However, core heterologies that are further removed from the sites of cleavage also block recombination but have no effect upon ligation efficiency (Ref. 23 and this work). A third possibility is that the homology might be required to permit branch migration of the Holliday junction through the core region to the site of the resolution (29, 30). However, it has recently been shown that free mobility of the Holliday junction is not required by Flp and other integrases for the resolution of the Holliday intermediate (31–34). We were therefore led to consider other possible reasons for inhibition of recombination by heterologies between recombining FRT site cores.

One such mechanism might be that the heterology could be inhibiting the appearance of recombination products but not recombination. By this hypothesis, recombination of FRT sites bearing heterologous cores would lead to the formation of mismatched recombinant products, but these would be unstable and quickly recombine again to give homoduplex (nonrecombinant) products (35).

If recombination is assayed using linear substrates, the products of two rounds of recombination would be indistinguishable from the starting substrates. However, if the two recombining FRT sites are located on the same supercoiled molecule, topological analysis can be used to distinguish two rounds of recombination from simple reversal of the reaction. Flp recodes supercoiled substrates by a mechanism of “random collision” (22). This means that during synapsis and subsequent recombination of the two FRT sites, random numbers of the interdomainal supercoils are trapped as nodes between the

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‡ To whom correspondence should be addressed. Tel.: 416-978-6061; Fax: 416-971-2494; E-mail: p.sadowski@utoronto.ca.

1 The abbreviations used are: FRT, Flp recognition target; bp, base pairs(s); kb, kilobase pair(s).

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recombining sites (Ref. 22, Fig. 2). When the FRT sites are in inverse orientation, the initial products are knots that have odd numbers of nodes (3, 5, 7, 9, etc.). When the FRT sites are in direct orientation, the products are catenated circles bearing even numbers of nodes (2, 4, 6, 8, etc.). These products are in recombinant configuration. If the sense of the strand exchange by Flp is the same in each round (see above), when the products of the first round undergo a second round of recombination, the products are knots with odd numbers of nodes (for excision substrates) or knots with even numbers of nodes (for inversion substrates), respectively, and both are in a nonrecombinant configuration (Ref. 22, Fig. 2).

We have used topological analysis to study recombination between two FRT sites that are heterologous at two positions, 4 bp from each of the Flp cleavage sites. As expected, these mismatches inhibited recombination. In confirmation of the above hypothesis, topological analysis showed that such substrates had undergone two rounds of recombination to yield nonrecombinant molecules of the expected topology. We did similar studies using a substrate where the heterology resided immediately adjacent to one of the cleavage sites. We found that the substrate had undergone two rounds of recombination to yield nonrecombinant products. Thus heterology between two FRT sites appears to block formation of recombinant products by promoting two sequential rounds of recombination.

MATERIALS AND METHODS

The enzymes PstI, HindIII, AvaI, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. Avian myeloblastosis virus reverse transcriptase was from Life Sciences. Pancreatic DNase I was from Worthington. Shrimp alkaline phosphatase was from U.S. Biochemical Corp. Human topoisomerase I was from TopoGen Inc. E. coli tRNA was from Sigma. The Biotrans Nylon Membrane (0.2 μm) used for DNA transfer was from ICN Biomedicals Inc. The [α-32P]dATP and [γ-32P]ATP were from DuPont NEN. E. coli XL1-Blue cells [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac I’ proAB lacYIΔ15Tn10 (TetR)] were from Stratagene. The oligonucleotides were supplied by the Biotechnology Service Center, University of Toronto and are shown in Table 1.

Bacterial Strain and Plasmid Purification—XL1-Blue cells were used for construction and propagation of plasmids. Cells were made competent for transformation with calcium chloride (36). All of the plasmids were purified from 1-liter cultures using ethidium bromide/cesium chloride equilibrium density centrifugation as described previously (37).

Plasmid Construction and DNA Fragments—The plasmid pB1A04 (9.2 kb) contains two FRT sites in direct orientation (37). The 8.4-kb plasmids, pNA1 and pNA2, have two wild type FRT sites in direct and inverse orientations, respectively (Fig. 3), and were constructed by ligating three pairs of the kinased and annealed complementary oligonucleotides (numbers 1, 2, 3, 4, 5, and 6 in Table 1) containing the mutant and wild-type FRT sites, into the large fragment from HindIII-digested pB1A04. The plasmids pNA1 and pNA2 are designated as +/−/+ and +/−/−, respectively (Fig. 3). The plasmids pTF1 and pTF2 (8.4 kb) contain one wild-type FRT site and one FRT site with mutations in the core region at locations 1 and 3, respectively. The sites 1, 2, 4, 5, and 6 in Table 1 designate the Flp cleavage sites adjacent to the wild-type FRT site (Ref. 22).

FIG. 1. The FRT site and the mutated substrates. In each diagram, the double-stranded Flp binding sites, symmetry elements a, b, and c, are denoted by horizontal arrows. The Flp cleavage sites adjacent to symmetry elements a and b are shown by filled and open circles, respectively. Panel a, the sequence of the wild-type FRT site with three 13-bp Flp binding sites and the 8-bp asymmetrical core (filled rectangle). Panel b, mutated core sequences of the FRT site; the mutations are indicated with asterisks.

FIG. 2. Topology of Flp recombination of supercoiled substrates (after Beatty et al. (22)). Panel a, the FRT sites are in inverse orientation (i). Synapsis traps two interdomainal supercoils (ii, iii), and recombination (iv) introduces a third node, N3, to yield a recombinant three-noded knot (v). A second round of recombination produces a four-noded knot that is in the parental configuration (vi). The signs of the nodes in ii are arbitrary, as is the "sense" of the strand rotation. Panel b, directly oriented FRT sites (arrows). The substrate contains two FRT sites (i), which synapse in parallel with the entrapment of one node, N1 (ii). Recombination (iii) introduces a second node, N2, so that the products are two recombinant catenated circles (iv), where the number of nodes is even (here n = 2, but n could be 4, 6, 8, etc., depending upon the number of interdomainal nodes present at step ii). A second round of recombination in the same sense as iv introduces another node, N3, but the products are nonrecombinant (v). As illustrated, the product is a knot with three nodes.
second FRT site into the unique AcaI site of pNA5 by ligating the kinased and annealed pairs of complementary oligonucleotides (numbers 2, 5, 8, 9, and 10). The plasmid pNA6 was designated as “xx”. The plasmid pNA7 (8.4 kb, Fig. 3a) contains one wild-type FRT site and one having a single mutation adjacent to the a cleavage site at position +4 (Fig. 1b, ii). It was constructed by ligating the 5’-phosphorylated and annealed pairs of complementary oligonucleotides (numbers 1, 2, 5, 6, 11, and 12) into HindIII-digested pBA104. The plasmid pNA8 (8.4 kb, Fig. 3a) is similar to plasmid pNA7 except that it has a mutation adjacent to the b cleavage site at position +4 (Fig. 1b, ii). It was constructed in a similar fashion using the complementary pairs of oligonucleotides 1, 2, 5, 5 and 6 and substituting oligonucleotides 13 and 14 for 11 and 12, respectively. The plasmid pNA9 (8.4 kb) had mutations at both positions +4 and –4 and was created by substituting oligonucleotides 15 and 16 for 12 and 12 in pNA7 (Fig. 1b, iv). The plasmids pNA7, pNA8, and pNA9 are designated as “+/xx”, “-/xx”, and “+/+x”, respectively. All plasmids are shown in Fig. 3. The mutations were confirmed using the T7 sequencing kit (Pharmacia Biotech Inc.).

**Flp Preparation**—Wild-type Flp protein (>90% pure) was a Sephacryl S-300 fraction, purified as described previously (38). Protein concentrations were determined using the method of Bradford (39).

**Conditions for Flp Reactions**—A standard Flp reaction (0.07 ml) contained 50 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl2, 0.6 pmol of ethanol-precipitated, and suspended in a small volume of TE buffer as substrate plasmid DNA, and 2–10 pmol of Flp. The reaction was preincubated at 30 °C and was initiated by the addition of Flp. The reaction was terminated after 20 min, and the DNA was phenol-extracted, ethanol-precipitated, and suspended in a small volume of TE buffer as described by Beatty et al. (22). The 20-min time was chosen because it was early in the reaction before the appearance of the site-specific topoisomerase activity of Flp that occurs late in the reaction (Ref. 22; see “Discussion”). This activity would render the detection of knotted structures after DNase digestion problematical. Similar results were obtained at 14 min. The DNA was then subjected directly to electrophoresis, digested with DNase I to induce nicking, or digested with PstI to determine the extent of recombination. Nicking with pancreatic DNase I and reaction termination were carried out as described (22) except that the reaction mixture contained 15 ng of pancreatic DNase I and 0.2 mg/ml of ethidium bromide.

For intermolecular recombination reactions, the plasmids pNA1, pNA7, pNA8, and pNA9 were digested with either BstEII or BstBI restriction enzyme, respectively, and the 0.4- and 5.1-kb fragments were isolated from agarose gels and used as the substrates. The 0.41-kb fragments were 32P-labeled using reverse transcriptase and [α-32P]dATP. The substrates (0.41 and 5.1 kb) bearing identical FRT sites were incubated with 10 pmol of Flp at 30 °C for 50 min. The samples were run on a 0.8% agarose gel.

**Agarose Gel Electrophoresis**—Horizontal agarose gels (0.8%) in Tris-phosphate buffer were used to separate topoisomers, knots, or catenanes of different linking number (22). Gels were photographed on a Fotodyne model 3–5000 ultraviolet transilluminator using Polaroid type 55 film.

**Digestion of Agarose Gel Track with PstI in Situ**—An agarose gel track was excised after staining with ethidium bromide, digested with 900 units of PstI, and run in a second 1.2% (w/v) agarose gel in TPE buffer essentially as described previously (22).

**Southern Blotting**—The DNA present in an agarose gel was transferred to Nylon membranes by the procedure described by Sambrook et al. (40). Hybridization with nick-translated 32P-labeled pNA1 DNA was performed as described (22).

**RESULTS**

Flp substrates that bear heterologous core regions fail to undergo Flp-mediated recombination as assayed both in vivo and in vitro (23, 41, 42). We wished to test the hypothesis that failure to observe recombination between FRT sites with heterologous core regions might be due to the use of assays that would not detect two rounds of recombination. Previous assays for recombination between heterologous FRT sites have involved at least one linear DNA partner, where the products of two rounds of recombination would have been indistinguishable from the starting parental substrates. To circumvent this difficulty, we used supercoiled plasmids containing homologous and heterologous FRT sites so that topological analysis could be used to detect two rounds of recombination if they were occurring.

**Heterology Blocks Intramolecular Recombination When FRT Sites Are on Supercopied Molecules**—We first wished to confirm that heterology between two FRT sites inhibited the appearance of recombinant products generated via intramolecular recombination. We therefore subjected supercoiled plasmids bearing heterologous FRT sites that were in either direct or inverse orientation to treatment with Flp. Plasmids pNA1 and

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**Table I**

| Oligonucleotide | Sequence |
|-----------------|----------|
| 1               | 5'-AGCTTGATCCGAGATTTCTATCCCAGGAGT-3' |
| 2               | 5'-GATAGGACTTGGATCCGAGTACCA-3' |
| 3               | 5'-TCTATTTCCATAGAAGATATG-3' |
| 4               | 5'-TTTCTAGAGAAGTAGATAG-3' |
| 5               | 5'-GAACTTCCCTAGGTAAAGTATAG-3' |
| 6               | 5'-AGCTTTGCTCTGAGTTCCTATAC-3' |
| 7               | 5'-TCTGACTTCCCTAGGAAAGATATG-3' |
| 8               | 5'-CCGCGCTTCTGAGTTCCTATAC-3' |
| 9               | 5'-CTTCGAGTCCCTAGGAAAGATATG-3' |
| 10              | 5'-CCGCGCTTCTGAGTTCCTATAC-3' |
| 11              | 5'-TCTATTTCCCTAGGAAAGATATG-3' |
| 12              | 5'-CTTCGAGTCCCTAGGAAAGATATG-3' |
| 13              | 5'-CTTCATTCCCTAGGAAAGATATG-3' |
| 14              | 5'-CTTCATTCCCTAGGAAAGATATG-3' |
| 15              | 5'-CTTCATTCCCTAGGAAAGATATG-3' |
| 16              | 5'-CTTCGAGTCCCTAGGAAAGATATG-3' |

**FIG. 3.** The structure of the plasmids used for the study. Panel a, excision substrates (pTF1, pNA1, pNA7, pNA8, and pNA9). The FRT sequences (arrows) are in direct orientation (left). The FRT sites may be wild-type (+) or mutant (x). The sequences of the mutations are given in Fig. 1. With pNA1 (+/+), Flp produces two circular products 6.7 and 1.7 kb in length (right), each of which contains a single PstI site (P). The numbers indicate the size (kb) of the fragments produced by PstI digestion. Panel b, inversion substrates. i, pTF2 and pNA2. The FRT sequences (arrows) are in inverse orientation. The bottom FRT site is wild-type (+) in both plasmids, whereas the top FRT site is wild-type in pNA2 (+) but mutant (x) in pTF2. Inversion of a segment of DNA between the two target sequences leads to a change in the sizes of the fragments produced by digestion with PstI. As in pNA1 (left), these are 4.4 and 4.0 kb, but after recombination mediated by Flp, the product (right) has PstI fragments of 5.3 and 3.1 kb. ii, pNA6 containing two mutated FRT sites (x; see Fig. 1). Inversion changes the sizes of the fragments produced by Sty1 digestion from 3.2 and 1.2 kb to 2.4 and 2.0 kb. S, Sty1 sites.
Homology and Flp Recombination

pNA2 carry two intact (wild-type) FRT sites in direct and inverse orientation, respectively (Fig. 3). Plasmids pTF1 and pTF2 are identical to pNA1 and pNA2 except that one of the two FRT sites in each plasmid has a mutated core region. Plasmid pNA1 (+/+), gave readily detectable bands in an agarose gel that was stained with ethidium bromide. The products from the Flp reactions were nicked with DNase I. After DNase digestion, the interdomainal supercoils are trapped topologically as nodes, and the various species of nicked catenanes or knots can be resolved by agarose gel electrophoresis. Knots with greater numbers of topologically constrained nodes migrate more quickly during electrophoresis because of the increasingly compact structure.

Accordingly, the products from the Flp reactions were nicked with DNase I and run on a high resolution agarose gel. The excision substrate (pNA1; +/+ ) gave a ladder of bands (Fig. 5, lane 2) that corresponded approximately to every band of a topoisomerase I-relaxed standard (also pNA1). Therefore, the number of nodes of each form varied by one. The bands (catenanes) occupying the even positions (2, 4, 6, 8, etc.) were more intense than the bands in the odd-noded positions because the samples were taken relatively early in the reaction (22). The former correspond to the recombinant products. However, the bands occupying the odd positions (3, 5, 7, 9, etc.) arise from a second round of recombination of catenanes and correspond to nonrecombinant knots. These results are the same as found previously (22). When the inversion substrate pNA2 (+/+ ) was treated with Flp, nicked, and run on a high resolution agarose gel (Fig. 5, lane 5), a series of bands was obtained with a spacing that corresponded predominantly to the odd-numbered positions of the topoisomerase I-relaxed standard. These correspond to the recombinant products (knots) that arise from the first round of recombination. The bands occupying the even positions were very faint and result from two rounds of recombination. They therefore correspond to nonrecombinant knotted products. These results are in accordance with our previous findings (22). Furthermore, these knot and catenane ladders provide an accurate standard with which to compare the topological products obtained with the heterologous substrates, pTF1 and pTF2.

The excision substrate with one mutated FRT site (pTF1)
gave a ladder of bands (Fig. 5, lane 3) that comigrated with bands occupying the odd positions (3, 5, 7, 9, etc.) of the Flp knot ladder standard (lane 5). Comparison with the products from plasmid pNA1 (lane 2) showed that these bands correspond to the odd-numbered positions (nonrecombinant) that arose as a result of a second round of recombination of catenanes.

In contrast, the inversion substrate with one mutated FRT site and one wild-type FRT site (pTF2) gave a ladder of bands (Fig. 5, lane 4) corresponding to bands occupying the even positions (2, 4, 6, 8, etc.) of the Flp-induced ladder (lane 2). These bands correspond to nonrecombinant knots that arose as a result of a second round of recombination of the Flp-induced knots. Inspection of predominant bands in lanes 3 and 4 shows that they are clearly interleaved with one another as well as with the predominant bands in the adjacent marker control ladders (lanes 2 and 5).

To confirm the results of Fig. 4 indicating that the plasmids pTF1 and pTF2 did not yield even minute amounts of recombinant structures, we cut out the individual tracks from the gel shown in Fig. 5, digested the DNA in situ with PstI, and ran the digestion products in a second agarose gel. The results (not shown) confirmed that the odd-noded knots derived from the +/x excision substrate (pTF1) and the even-noded knots obtained from the +/x inversion substrate (pTF2) were in a parental, nonrecombinant configuration. The catenanes and knots from the two wild-type substrates gave the expected recombinant configuration. This result is consistent with the interpretation of the findings in Fig. 5, namely that the bands corresponding to knotted products arose from two rounds of recombination.

**Topology of Products of Recombination between Two Mutated, Homologous FRT Sites**—It was important to be sure that the results shown in Fig. 5 were not the result of some aberrant topology peculiar to the mutated FRT site used. We therefore constructed a plasmid containing the two identical mutated FRT sites in inverse orientation. We show in Fig. 6 that Flp-mediated recombination under identical conditions to those in Fig. 5 led to an apparent decrease in the superhelical band (Fig. 6, lane 2) and the appearance of a smear running faster than the supercoiled plasmid. The Flp-treated DNA was nicked and run on a high resolution agarose gel. As shown in Fig. 6, lane 4, the nicking resulted in the appearance of a ladder of bands corresponding approximately to every other band of a topoisomerase I-relaxed standard (also pNA7, lane 3). These correspond to recombinant products. This result is totally in accordance with what we observed with pNA2, an inversion substrate with two wild-type FRT sites, and with previous studies (22). Therefore, a substrate with two mutated FRT sites obeys the topological rules of the wild-type FRT sites.

**Effects of Heterologies at Positions Immediately Adjacent to the Flp Cleavage Sites**—Homology in the core immediately adjacent to the Flp cleavage site has been shown to be important for the ligation reaction (27, 28). It was of interest to study the effect of heterologies at these positions on recombination of supercoiled substrates. We therefore constructed plasmids in which one of the two FRT sites contained a mutation adjacent to the a cleavage site (pNA7, +/xa), to the b cleavage site (pNA8, +/xb) or to both cleavage sites (pNA9, +/xc). In each case the two FRT sites were in direct orientation.

We first determined the effect of this heterology upon intramolecular recombination, and in the case of each of the plasmids, recombination was undetectable (data not shown). Examination of the topological products of the reactions of supercoiled substrates after nicking with DNase I showed that plasmid pNA7 (+/xa) had undergone two rounds of recombination (Fig. 7). The products were odd-noded knots (Fig. 7, lane 2) as judged by their mobility relative to the catenane/knot ladder in the adjacent lane 1. This was consistent with the results obtained previously with plasmid pTF1 (see Fig. 5, lane 3).

**Fig. 5. The action of Flp on excision and inversion substrates; analysis of DNase I-nicked products.** The nicked products of the Flp reaction were analyzed by high resolution agarose gel electrophoresis. For clarity of photographic reproduction, the gels were transferred to nylon membranes, which were probed with 32P-labeled pNA1. Lane 1, topoisomerase I-relaxed pNA1 standard. Lane 2, nicked product of excision substrate, pNA1 after treatment with Flp. Note that a ladder of bands is observed in which both the even positions (4, 6, 8, etc.) and the odd positions (3, 5, 7, 9, etc.) are occupied; the even positions are more prominent than the odd positions. II, nicked circular substrate; I, position where supercoiled substrate would have run. Lane 3, nicked products of excision substrate, pTF1 after treatment with Flp. Reaction conditions were identical to those in lane 2. A ladder of bands is observed in which only the odd positions are occupied. These bands interdigitate with the predominant bands in the marker control catenane ladder in lane 2. Lane 4, nicked products of inversion substrate, pTF2 after treatment with Flp. A ladder of bands is observed in which predominantly the even positions are occupied. These correspond to even-noded knots. Note that these bands interdigitate with the bands in the odd positions in the two adjacent lanes. Lane 5, nicked products of excision substrate, pNA2, after treatment with Flp. A ladder of bands is observed in which the predominant bands are in the odd positions and faint bands are also visible in the even positions. The symbols above the lanes indicate the orientation and sequence of the two FRT sites. +, wild-type site; x, mutated site; →→, direct orientation; ←←, inverse orientation.

**Fig. 6. The action of Flp on inversion substrate (pNA6) and analysis of nicked products.** Lane 1, untreated pNA6; lane 2, Flp-treated pNA6; lane 3, topoisomerase I-relaxed pNA6 standard; lane 4, Flp-treated, DNase I-nicked pNA6. Reaction mixtures are described under "Materials and Methods." A ladder of bands is observed in which the odd positions (3, 5, 7, etc.) are occupied. The odd positions represent knots that are in a recombinant configuration. The symbols are those used in Fig. 5.
Homology and Flp Recombination

It has long been known that productive recombination by site-specific recombinases requires homology in the “overlap” regions between the two recombining sites (23, 25, 43, 44). Homology does not seem to be required for synapsis by either Flp (26) or the λ integrase (45–47). Homology of the base pairs immediately adjacent to the cleavage site is critical for the ligation reaction (27, 28).

It was originally postulated that heterologies blocked the branch migration of the Holliday intermediate that was necessary for its resolution (29, 30). However, recent studies showing that a Holliday junction can be immobilized at a site distant from the resolution point can still be efficiently resolved (31–34) led us to entertain an alternate hypothesis, namely that heterologies between recombining FRT cores appear to block recombination because they favor the occurrence of two successive rounds of recombination. After the first round, the two products would each have mismatched bases in their cores. These mismatched cores are unstable and would quickly recombine again to yield products with homoduplex cores. This recombinant product of wild-type excision substrate, pNA1 (+/+), pNA7 (+/xa), pNA8 (+/xb), and pNA9 (+/xc): analysis of nicked products. Lane 1, nicked product of wild-type excision substrate, pNA1, after treatment with Flp. A ladder of bands is observed in which both the even positions (4, 6, 8 etc.) and the odd positions (3, 5, 7, 9 etc.) are occupied. Note that the even positions are more prominent than the odd positions. II, nicked circular substrate; I, position where supercoiled substrate would have run. Lane 2, reaction conditions were identical to those in lane 1 except that plasmid pNA7 was used. A ladder of bands is observed in which only the odd positions are occupied. These bands interdigitate with the predominant bands from the marker catenane/knot ladder in lane 1. Lane 3, plasmid pNA8. No knotted products are seen. Lane 4, plasmid pNA9. No knotted products are seen. The symbols above the lanes indicate the orientation and sequence of the two FRT sites.

However, when the heterology resided adjacent to the b cleavage site or to both a and b cleavage sites, no topological products of the Flp reaction were evident (Fig. 8, lanes 3 and 4).

It was important to show that the mutation introduced into the core region adjacent to the a or b cleavage sites did not affect recombination with a similarly mutated FRT site. To assay recombination between two identical FRT sites we isolated end-labeled 0.41-kb and unlabeled 5.1-kb fragments from the plasmids pNA1, pNA7, pNA8, and pNA9 to use as substrates (see “Materials and Methods”). As shown in Fig. 8 (lane 6), the intermolecular recombination between the two substrates containing the FRT site mutated within the core adjacent to the a cleavage site resulted in accumulation of products corresponding to 0.78 and 4.7 kb, and the reaction was as efficient as that for the reaction between the substrates containing wild-type FRT sites (Fig. 8, lane 5). However, the substrates containing the FRT site with mutations within the core region adjacent to the b cleavage site (Fig. 8, lane 7) or to both the a and b cleavage sites (Fig. 8, lane 8) showed markedly impaired recombination. These mutations did not appear to impair binding or cleavage by Flp (data not shown). The reason for this inhibition of the recombination reaction is under investigation. Nevertheless, the experiments show that a heterology adjacent to one of the Flp cleavage sites has the same effect on recombination as a heterology four nucleotides from the cleavage site.

DISCUSSION

Previous assays with linear substrates may have failed to detect these products because they would appear to be identical to the starting material. However, topological analysis of intramolecular recombination on supercoiled substrates can detect the “footprint” of these recombination events as nodes that have been topologically trapped in knotted substrates. The results shown in Fig. 5 clearly show that Flp promotes two rounds of recombination of mismatched FRT sites. Flp may sense the mismatched cores of the two products and quickly recombine them once more to yield homoduplex sites in which the products are in the same nonrecombinant configuration as the starting substrates but are topologically distinct. The partially single-stranded character of the mismatched cores may speed strand exchange.

Does this second round of recombination occur by a “processive” or “distributive” mechanism; i.e. does the synaptic complex, once assembled, catalyze both rounds of recombination, or does the complex dissociate after the first round and then reassemble to promote the second round? Members of the resolvase-invertase family of site-specific recombinases are known to catalyze multiple rounds of processive recombination
as well as distributive recombination when confronted with target sites having heterologies in the overlap region (43, 44, 49). The result is that the heterologies block the appearance of recombination products.

While the resolvase-invertase family members assemble a precisely ordered synapse, an integrase and Flp initiate synopsis via the mechanism of “random collision,” whereby variable numbers of interdomainal supercoils are topologically trapped during the first stage of the reaction. The result is the accumulation of catenanes or knots of the “torus” class (20). The products of two rounds of distributive recombination of an inversion substrate would be knots of the “granny” class that contain even numbers of nodes. The knotted products of two rounds of processive recombination also have even numbers of nodes but are of the “twist” class. They are also nonrecombinant. Neither our previous studies nor the present studies distinguish the two classes of knots, and hence they do not directly address the processive or distributive nature of the recombination.

Recent results of Waite and Cox (50) suggest that the Flp synaptosome may be quite stable. These workers observed a concentration-dependent dissociation step needed for the disassembly of the Flp-FRT site complex. At high Flp concentrations such as the ones used here, this complex had the stability to endure for the entire course of the reactions studied in our experiments. Thus multiple rounds of recombination within such a complex are possible.

Is processive recombination incompatible with synopsis by random collision? The work of Stark et al. (51) argued that recombinases of the integrase family that proceed through a Holliday intermediate cannot undergo processive recombination without “resetting” of the recombinase molecules in the synaptic complex. Resetting is required to approximate the two strands that are destined to begin the next round of recombination. A second round without resetting would simply lead to reversal of the topological outcome of the first round. It is possible that initial formation of the synapse occurs randomly but that it is stabilized after one round of recombination. Resetting may occur within the synaptic complex, allowing a second round of recombination without dissociation (52).

A mutation immediately adjacent to the a cleavage site had a similar effect to mutations in the middle of the core, i.e. two rounds of recombination occurred. It was surprising that this heterology permitted two rounds of recombination, since impairment of ligation might have been expected to block strand exchange. It is possible that blockage of initiation of strand exchange at the a site directs initiation to the b site. The resulting Holliday intermediate may then be resolved despite the heterology at the a site. It is also possible that the second round of recombination occurs without sealing of the nicks. Alternatively, the supercoiling of the substrate may bypass the requirement for homology in the ligation reaction that is seen on linear substrates. These results emphasize that the homology requirement for ligation is insufficient to account for the need for homology between two recombinating FRT sites (see also Ref. 47).

Introduction of a similar mutation at the b cleavage site dramatically impaired recombination even between two homologous FRT sites. Since this mutation did not impair DNA binding or cleavage, we suspect that it interferes with strand exchange.

We have considered the possibility that the nonrecombinant products could have arisen by the action of a topoisomerase II-like activity of Flp. If, during parallel synopsis promoted by Flp, double-strand passage of one FRT through the other occurred, then nonrecombinant twist knots containing odd numbers of nodes could arise for the excision substrate. Similarly, even-noded twist knots could arise in the inversion substrate. Since Flp does exhibit a topoisomerase II activity that ultimately will remove the nodes that have been trapped in an inversion substrate after multiple rounds of recombination (22), this is not a trivial possibility.

However, this topoisomerase II activity becomes evident late in the reaction in the inversion substrate only. The present results were obtained with both excision and inversion substrates. Kinetic studies showed that the topoisomerase II activity was due to the accumulation of knots and presumably the associated strain caused by their nodes (53). The reactions we show in the present study were taken at much earlier times, when even the wild-type substrate had not yet shown topoisomerase II activity.

If the results are due to topoisomerase II activity, then how does the heterology between the cores trigger the double-strand breakage needed for the double-strand passage? The cleavage of each of the two FRT sites would occur in trans before the assembly of the synaptic complex (14). From studies with half-FRT sites (54), it is assumed that only one of the two strands of each FRT site in the synaptic complex is cleaved, since double-strand cleavage of the FRT site is rarely seen. Therefore, the heterology would be sensed and then trigger the cleavage of the other strand. Although this mechanism may seem contrived, it should be noted that Burgin and Nash (47), remarkably, did observe Int-mediated double-strand cleavage of an attB substrate that was dependent on the presence of core heterologies between this substrate and the attP-containing intasome.

Topoisomerase II activity was also considered as an explanation for the results of recombination of heterologous sites in the case of the resolvase-invertase group (43, 44) but was rejected on topological grounds. Therefore, although topoisomerase activity of Flp could give the topological products observed between heterologous FRT sites, we favor the explanation that the knots arise from a second round of recombination of the heteroduplex products of the first round.

In summary, the Flp recombinase senses heteroduplex molecules that result from recombination of FRT sites having heterologous cores. A second round of recombination leads to topologically distinct products of identical sequence to the starting substrate. In this respect, Flp resembles the members of the resolvase-invertase family of site-specific recombinases.

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