Synapsis, Strand Scission, and Strand Exchange Induced by the FLP Recombinase: Analysis with Half-FRT Sites

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We have used a previously described cross-linking assay and half-FRT site substrates to examine the requirements for synapsis, strand exchange, and strand scission. The cross-linking assay showed that the minimum functional FRT site needed for synapsis contains two inverted FLP-binding elements surrounding an 8-bp core. This indicates that four FLP molecules interact with four binding elements in a synaptic complex. The analysis using half-sites showed that the enzyme can catalyze efficient strand exchange between a half-site and the intact FRT site. The reaction occurred only if the half-site had at least 2 bp but no more than 4 bp of the adjoining core sequence. The exchange occurred exclusively at the regions of limited core homology between the respective half-site and the FRT site. The absence of strand exchange between an intact site and a half-site bearing regions of core nonhomology indicates that 1 bp of homology is not sufficient for the formation of stable recombinant structures. Qian et al. (X.-H. Qian, R. B. Inman, and M. M. Cox, *J. Biol. Chem.* 265:21779-21788, 1990) have recently shown that the FLP protein can catalyze the formation of dimeric, trimeric, and tetrameric complexes with half-FRT sites. We show that only half-sites that contained at least 2 bp of adjacent core could form stable dimer products and be cleaved by the enzyme. Stable dimers were formed between a noncleavable half-site and a cleavable half-site, suggesting that only a single cleavage event is needed for the formation of the dimer.

Site-specific recombinases promote reciprocal exchanges between short, defined, homologous DNA sequences. The use of *in vitro* systems for several different site-specific recombinases has made it possible to examine the protein-protein and protein-DNA interactions involved in the many transition steps of the reactions (9, 18, 26). This has led to the formulation of models that describe the mechanisms of the various reactions (10, 11).

The FLP protein encoded by the 2 μ m plasmid of yeast cells is one such site-specific recombinase whose *in vitro* reaction is amenable to molecular dissection (6, 8, 29). The target sequence of the FLP protein, the FRT site, consists of three 13-bp symmetry elements designated *a*, *b*, and *c* (Fig. 1A). Two of these elements are in inverted orientation and separated by an 8-bp core sequence. The recombinase catalyzes cleavage of the phosphodiester bond at the junction of the symmetry elements and the core region (4, 7). This cleavage is accompanied by the formation of a 3' phosphotyrosyl linkage (12). These two junctions are the points of strand exchange between two synapsed FRT sites (15, 21). When the protein is bound to the inverted symmetry elements, a sharp bend occurs within the core sequence (27, 28).

We recently developed a method of isolating synaptic and postsynaptic intermediates of the FLP recombination reaction (1). An analysis of these intermediates indicated that synapsis occurred by protein-protein interactions between FLP molecules bound to symmetry elements *a* and *b* of each of the paired wild-type FRT sites. In addition, we showed that synapsis occurred in the absence of core homology, and we argued that core homology was important only after synapsis to allow the progression of the reaction via a Holliday intermediate.

We have now further investigated the requirement for

synapsis by using the assay mentioned above. In addition, we have used half-FRT sites to determine the requirements for strand cleavage and strand exchange.

MATERIALS AND METHODS

Construction of plasmids. Plasmids were used as vectors as well as sources of partial FRT sites. The nomenclature of plasmids and the respective FRT sites they contained is as follows: pGP25, ($\rightarrow$][$\leftarrow$) (24); pBA116, ($\rightarrow$][$\rightarrow$] (4); and pBA114, ([$\leftarrow$) (5). (The arrows represent the 13-bp symmetry elements and their relative orientations. The square brackets represent the margin[s] of the 8-bp core region.) The full FRT site was obtained on a 123-bp *EcoRI-BamHI* fragment from plasmid pGP25. The ends of the fragment were repaired by avian myeloblastosis virus reverse transcriptase and the four deoxynucleoside triphosphates (4) prior to ligation into the respective recipient vector. Each vector was prepared for ligation by linearization with *PstI*. The *PstI* ends were made blunt by the 3' exonuclease of T4 DNA polymerase. The *EcoRI-BamHI* fragment containing the FRT site was ligated to the appropriate vector, and constructs with the core sequences in direct orientation were isolated. A 263-bp *HincII-SspI* fragment derived from plasmid pBR322 was ligated into the blunt-ended *HincII* site between the two FRT sites of each of these new plasmids. Figure 1B summarizes the various constructs and the pairwise combinations of FRT sites they contain. The reverse-core FRT site contained in pCS31 was constructed by using synthetic oligonucleotides and was identical to that contained in pLB112 (5) except that the 8-bp core was inverted with respect to its orientation in the wild-type FRT site. This plasmid was the gift of Carol Schwartz.

Purification of ³²P-end-labeled DNA substrates. Synaptic substrates were prepared by digestion of 15 μ g of the appropriate plasmid with *EcoRI* and *HindIII*. The half-site *a* and *b* substrates were generated by digestion of plasmid

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FIG. 1. (A) The FRT site. The site comprises three 13-bp symmetry elements designated *a*, *b*, and *c*. Elements *a* and *b* are in inverted orientation separated by an 8-bp core region. Element *c* is in tandem with element *b*. The sequence of the *XbaI* site is underlined, and small vertical arrows indicate the bonds cleaved by the restriction enzyme. Large vertical arrows show the sites of cleavage by the FLP protein. Horizontal bars beneath the sites indicate the sequence of the FRT site in plasmids pGP25, pBA116, and pBA114. (B) Construction of plasmids used to isolate synapsis substrates. (1) A 123-bp *BamHI-EcoRI* fragment from pGP25 was ligated into the blunt-ended *PstI* site of the parent plasmid pGP25 (i), pBA116 (ii), or pBA114 (iii). Each of these plasmids contains a partial FRT site on a *PstI*-to-*EcoRI* fragment. The lengths of these fragments and the disposition of the FRT sites are shown at the right. (2) The resulting plasmids contained two partial FRT sites and were called pLT9 (i), pLT1 (ii), and pLT8 (iii). A 263-bp *HincII-SspI* fragment from pBR322 was then ligated into the *HincII* site of pLT9, pLT1, or pLT8. (3) This gave plasmids pPS799 (i), pPS792 (ii), and pPS798 (iii). The synaptic substrates liberated from each of these plasmids with *HindIII* and *EcoRI* are shown in part 3. Lengths are approximately to scale. Bar = ~50 bp. Restriction sites: E, *EcoRI*; B, *BamHI*; H, *HincII*; P, *PstI*; I, *HindIII*. The x in brackets indicates that the site has been destroyed by the cloning procedure. The numbers are lengths of fragments (in base pairs).

pGP25 with *EcoRI* and *XbaI* or *HindIII* and *XbaI*, respectively (Fig. 1 and 2). Fragments were labeled at their 3' recessed ends by using avian myeloblastosis virus reverse transcriptase and [α - 32 P]dATP or at their dephosphorylated 5' ends by using polynucleotide kinase and [γ - 32 P]ATP. If the fragment was to be uniquely labeled, the plasmid was

digested with the first enzyme and the ends were labeled by the appropriate method. The reaction was terminated by heating at 65°C for 15 min followed by digestion with the second enzyme. The fragments were purified by polyacrylamide gel electrophoresis, electroelution, and ethanol precipitation.

FIG. 2. Synapsis assay. ^{32}P -end-labeled substrate was incubated with FLP protein, where indicated, at 22°C for 10 min. Where indicated, glutaraldehyde (Glut) was added to a final concentration of 0.02%, and the samples were incubated for an additional 3 min. The cross-linking reactions were quenched with lysine, and the samples were subjected to electrophoresis on a 3% polyacrylamide gel. Lanes: 1, untreated substrate $\rightarrow[\leftarrow\rightarrow]\leftarrow$; 2, substrate incubated with 60 U of FLP; 3, substrate incubated with 30 U of FLP followed by the addition of glutaraldehyde; 4, substrate incubated with 60 U of FLP followed by the addition of glutaraldehyde; 5 to 8, substrate $\rightarrow[\leftarrow\rightarrow]\leftarrow$ processed as described for lanes 1 through 4; 9 to 12, substrate $\rightarrow[\leftarrow\rightarrow]\leftarrow$ processed as described for lanes 1 through 4. S, substrate; RP, recombination product. SC I and SC II are synapsis complexes.

FLP preparation. The FLP used was a Sephacryl S300 fraction (50 to 85% pure) that contained 0.3 mg/ml and 30 U/ μl . This protein was the gift of Hua Pan (23a).

In vitro synapsis assays. The synapsis assay was done essentially as previously described with minor modifications (1). About 0.02 pmol of ^{32}P -end-labeled substrate was incubated with FLP protein on ice for 4 min, after which glutaraldehyde (final concentration of 0.02%) was added for 3 min. The reaction was quenched with 1 mM lysine. Samples were then immediately subjected to electrophoresis on a 3% polyacrylamide gel at 8°C . The gels were dried and exposed to X-ray film.

In vitro recombination assays. For analytical reactions, 0.02 pmol of ^{32}P -end-labeled fragment containing the half-site was combined with 0.04 pmol of the *PvuII-PvuII* fragment of plasmid pGP25 in 25 μl of solution containing 50 mM TAPS buffer [*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; pH 8.0], 0.2 M NaCl, 20% polyethylene glycol 4000, and 1.5 μg of denatured calf thymus DNA. Various concentrations of FLP protein were added, and the samples were incubated at 22°C for 20 min. The reactions were terminated by the addition of sodium dodecyl sulfate (SDS) (0.004%) and proteinase K (10 μg) and were incubated

at 37°C for 20 min, after which the samples were analyzed on a 4% native polyacrylamide gel (2).

Preparation of recombinant structures. Preparative reactions were carried out essentially as described above except that 0.2 pmol of the appropriate ^{32}P -labeled half-site was combined with 0.4 pmol of the *PvuII-PvuII* fragment of pGP25 in 75 μl of buffer containing 3 μg of denatured calf thymus DNA. In some experiments, the 5' termini of this *PvuII* fragment were also labeled with ^{32}P . Purified FLP (45 to 150 U) was added, and the samples were processed as described above. The recombinant structures were electroeluted from the gel slice and recovered by ethanol precipitation. A portion of the DNA was analyzed on an 8% polyacrylamide-urea gel as described elsewhere (1).

In vitro reactions for half-site dimer formation. Half-FRT site dimers were formed under the same conditions as were described for the recombination assays. The samples were subjected to electrophoresis on a 4% polyacrylamide gel without the addition of SDS and proteinase K.

Assay for covalent attachment of FLP to half-FRT sites. The *HindIII-XbaI* fragment of half-site *b* was ^{32}P labeled at the 5' end of the *HindIII* terminus, and the *EcoRI-XbaI* fragment of half-site *a* was ^{32}P labeled at the 5' end of the *EcoRI* terminus. About 0.16 pmol of half-site *b* with a 1-bp core, half-site *b* with a 2-bp core, or half-site *a* with a 3-bp core was incubated in 200 μl of 50 mM TAPS buffer (pH 8.0)–0.2 M NaCl–20% polyethylene glycol 4000–2% glycerol–50 μg of denatured calf thymus DNA per ml. Before addition of FLP, 20 μl of each sample was added to 10 μl of 3 $\times$ denaturing buffer (10% glycerol, 3% SDS, 62.5 mM Tris HCl [pH 6.8], 0.7 M β -mercaptoethanol) (17). Four microliters of FLP (120 U) was added to each of the remaining reactions at room temperature, and 20 μl of each reaction was added to 10 μl of 3 $\times$ denaturing buffer after 2, 4, 6, 8, 10, and 20 min of incubation. ^{32}P -labeled *Sau3A* fragments of pBR322 were used as size markers. The samples were heated at 37°C for 20 min and then electrophoresed on a 7.5% SDS-polyacrylamide gel (17). Following electrophoresis, the gel was shaken gently in water for 20 min to remove SDS. The gel was dried and exposed to X-ray film.

RESULTS

Efficient synapsis requires a pair of FRT sites, each containing two inverted symmetry elements. We previously developed a method to isolate synaptic and postsynaptic intermediates in the FLP recombination reaction (1). We also used DNase I footprinting experiments to demonstrate that the synaptic intermediates contained FLP protein bound only to elements *a* and *b* of the paired wild-type FRT sites. However, these studies did not address the minimum structural requirements of the FRT sites involved in synapsis. The wild-type FRT site consists of three 13-bp symmetry elements (*a*, *b*, and *c*; Fig. 1). Since symmetry element *c* is dispensable for recombination, the FLP protein must effect efficient synapsis and strand exchange with a minimal functional site consisting of elements *a* and *b* and the core.

To determine the minimum structural requirements for synapsis, we asked whether the enzyme could promote synapsis between the minimum FRT site and various partial FRT sites. Accordingly, plasmids that contained various pairwise combinations of the minimal wild-type and partial FRT sites were constructed (see Materials and Methods and Fig. 1). Figure 1B illustrates the disposition of the various sites on the respective substrates derived from these plasmids. The substrates were designated according to the

combination of sites they contained, i.e., $\rightarrow[\leftarrow : \rightarrow] \leftarrow$ from pPS799, $\rightarrow[: \rightarrow] \leftarrow$ from pPS792, and $] \leftarrow : \rightarrow [\leftarrow$ from pPS798.

The results of this experiment are shown in Fig. 2. Two synaptic complexes, designated SC I and SC II, were detected only in reactions containing substrate $\rightarrow[\leftarrow : \rightarrow] \leftarrow$ (lanes 3 and 4). As expected, these intermediates were absent in the reaction not treated with glutaraldehyde (lane 2). The SC I complex likely contains the FRT sites aligned in the proper configuration (as defined in reference 1), since the strand exchange products derived from this complex are of the size predicted for this alignment (data not shown). We postulate that the SC II complex contains the sites aligned in the aberrant configuration, since no strand exchange products were observed (1). The amounts of cross-linked complexes obtained from substrate $] \leftarrow : \rightarrow [\leftarrow$ (lanes 7 and 8) or substrate $\rightarrow[: \rightarrow] \leftarrow$ (lanes 11 and 12) were significantly reduced. These results suggest that the FLP protein occupies elements *a* and *b* of the FRT site ($\rightarrow[\leftarrow$) to form a complex which can then synapse in a specific way with a similar complex.

FLP enzyme requires a half-site with at least 2 bp of adjoining core to catalyze strand exchange with intact FRT site. The results given above indicate that four symmetry elements are involved in the formation of a synaptic complex. However, we observed extremely low amounts of cross-linked species in reactions involving substrates $\rightarrow[: \rightarrow] \leftarrow$ and $] \leftarrow : \rightarrow [\leftarrow$ (barely visible in Fig. 2, lanes 7, 8, 11, and 12, but discernible in longer exposures). Therefore, we wished to use a more sensitive technique to determine whether the FLP protein could catalyze strand exchange (and therefore synapsis) between a half-FRT site and the minimum functional FRT site from plasmid pGP25 (24). We therefore adapted the half-site assay described by Landy and coworkers for the lambda Int recombination system (22, 23). The basis of this assay is that if FLP catalyzes strand scission of a half-FRT site, the oligonucleotide that is generated is lost by diffusion, leaving the FLP protein covalently bound to the half-site via a phosphotyrosine bond (12) (Fig. 3, part ii).

The FRT site of pGP25 is composed of the inverted symmetry elements *a* and *b* separated by an 8-bp core region that also contains an *Xba*I restriction site. *Xba*I digestion will therefore generate two fragments: one contains element *a* with 3 bp of the intact core sequence (half-site *a*), and the other contains element *b* and 1 bp of the adjacent core (half-site *b*). Each fragment also contains a 4-nucleotide single-stranded tail. Restriction fragments containing half-sites with either element *a* or element *b* were prepared by digestion of plasmid pGP25 with either *Eco*RI-*Xba*I or *Xba*I-*Hind*III, respectively, and the ends were labeled with 32 P (Fig. 3, parts i and ii). The nonradioactive *Pvu*II-*Pvu*II fragment containing the FRT site of pGP25 (Fig. 3, part iii) was combined with each half-site and incubated with various concentrations of FLP protein. A strand transfer reaction between a half-site and the intact FRT site would result in the formation of a 32 P-labeled three-armed Y structure with a single-stranded branch point. The recombinant structure should migrate more slowly than the progenitor half-site in the polyacrylamide gel. The results of this experiment (Fig. 4A) show the presence of a major slowly migrating species (XP1). This structure was detected only in the reactions containing half-site *a* (lanes 2, 3, and 4) and not in reactions with half-site *b* (lanes 5 to 8). Two other minor bands that migrate faster than XP1 but more slowly than the substrate are also detectable (XP2 and XP3). The possible origin of

FIG. 3. Substrates used in recombination assays. (i) The *Hind*III-*Eco*RI fragment of plasmid pGP25 contains an FRT site with elements *a* and *b* and the core between them. The *Xba*I site in the core sequence is underlined, and bonds cleaved by *Xba*I are shown by small vertical arrows. The cleavage sites of the FLP protein are indicated by large arrows. (ii) Half-site *b* generated by cleavage with *Hind*III (H) and *Xba*I (X) and half-site *a* generated by cleavage with *Eco*RI (E) and *Xba*I (X). Starred nucleotides will be lost after FLP cleavage at the sites indicated by the vertical arrows. (iii) *Pvu*II-*Pvu*II (P) fragment, which contains the FRT site asymmetrically positioned with respect to the ends of the fragment. The lengths of pertinent fragments are indicated (in base pairs) by the numbers between adjacent restriction sites.

these species is discussed below. The apparent inability of the FLP protein to catalyze strand exchange between half-site *b* and the FRT site was further investigated and shown to be due to the presence of only a single base pair of the adjacent core.

One obvious difference between the *a* and *b* half-sites was that the former contains 3 bp of the core region whereas the latter contains only 1 bp (Fig. 3). We therefore wished to determine whether lengthening the duplex core region adjacent to half-site *b* rendered the site recombination competent. The appropriate nonradioactive nucleotides were incorporated into the 3' recessed end of the *Xba*I terminus of the restriction fragment containing half-site *b* to produce half-sites with 2, 3, 4, and 5 bp of the adjoining core sequence. The recombination reactions were repeated with these modified half-sites and the intact FRT site as described above. The results, shown in Fig. 4B, show the presence of recombination product (XP1) in the reactions with the modified substrates. The yield of the recombinant structure was highest when the half-site contained 2 bp of the core sequence (lanes 6 to 8). Moderate yields were observed for the half-sites with core sequences of 3 bp (lanes 10 to 12) and 4 bp (lanes 14 to 16), and a low but detectable yield was observed with the half-site containing 5 bp of the core (lanes 18, 19, and 20). One of the putative minor recombination products (XP3) was also visible. The product (XP2) corresponding to that formed with the *a* half-site might have comigrated with XP1. Thus, there is a strict requirement for 2 to 4 bp of the adjoining core region in order for FLP to catalyze an efficient strand exchange reaction between half-site *b* and the intact FRT site.

Characterization of recombination products. If the half-sites are forming stable products by recombining with a full site, we should be able to deduce the origin of recombinant structures by analyzing the size of the products by denaturing gel electrophoresis. Accordingly, recombination reactions between appropriately 5'-labeled *a* or *b* half-sites and a

FIG. 4. (A) In vitro recombination assays with half-sites *a* and *b*. The *PvuII-PvuII* fragment was incubated with half-site *a* or *b* and FLP protein at room temperature for 20 min. Reactions were terminated with SDS and proteinase K, and the mixtures were subjected to electrophoresis on a polyacrylamide gel. S1 and S2, *a* and *b* half-site substrates, respectively; XP1, XP2, and XP3, recombination products of S1; M, 32 P-labeled *Sau3A*-cut pBR322 fragments (numbers indicate lengths in base pairs). Lanes: 1 and 5, no FLP; 2 and 6, 7.5 U of FLP; 3 and 7, 15 U of FLP; 4 and 8, 30 U of FLP; 1 to 4, half-site *a*; 5 to 8, half-site *b* with 1 bp of core. (B) Formation of recombinant structures with *b* half-sites containing 1 to 5 bp of the adjacent core region. The *XbaI* end of the *b* half-site substrate was repaired in the presence of no triphosphate [b(1)]; dCTP [b(2)]; dCTP and TTP [b(3)]; dCTP, dTTP, and dATP [b(4)]; or dCTP, dTTP, dATP, and dGTP [b(5)] and reverse transcriptase to give cores with 1 to 5 bp, respectively. These substrates were incubated with the *PvuII-PvuII* fragment, and the reactions were processed and analyzed as described for panel A. Lanes: 1, 5, 9, 13, and 17, no FLP; 2, 6, 10, 14, and 18, 7.5 U of FLP; 3, 7, 11, 15, and 19, 15 U of FLP; 4, 8, 12, 16, and 20, 30 U of FLP. S, substrate. Recombinant products XP1 and XP3 are indicated.

411-bp unlabeled fragment containing a complete FRT site were carried out, and the major recombination product (XP1) was isolated. The labeled strands were analyzed on a denaturing acrylamide gel. Schematic diagrams of the simplest possible three-armed Y structures that would be formed between *a* or *b* half-sites and the full FRT site are shown in Fig. 5A.

If FLP catalyzed strand exchange between half-site *a* and the *a* symmetry element-core junction of the FRT site, the recombinant product would contain a single strand that was 323 nucleotides (nt) in length (Fig. 5A, part i) and would be detected when the 5' end-labeled *EcoRI* terminus of the half-site was used. If the exchange occurred between half-site *a* and the *b* symmetry element-core junction of the FRT site, the product would contain a 286-nt single strand (Fig. 5A, part ii). Since only the 323-nt product was observed (Fig. 5B, lane 4), the recombinant structure was formed only by an exchange between half-site *a* and the *a* element-core junction of the intact site. The product that could have resulted from an exchange between half-site *a* and the *b* element-core junction of the FRT site was not detected (lane 4). The strand labeled at the 5' end of the *XbaI* terminus had not undergone strand exchange in the formation of the recombinant structure as expected (lane 2).

Likewise, if the enzyme catalyzed a strand exchange between half-site *b* and the *b* symmetry element-core junction of the intact FRT site, as shown in Fig. 5A, part iii, the product would contain a 232-nt single strand and would be detected only when the 5' end of the *HindIII* terminus of the half-site was labeled. On the other hand, if the exchange occurred between half-site *b* and the *a* element-core junction of the FRT site, as indicated in Fig. 5A, part iv, the single strand would be 269 nt long. The results indicate that the only single strand present in the exchange product was 232 nt (see Fig. 5B, lane 8), revealing that only the exchange

between half-site *b* and the *b* symmetry element-core junction of the intact site occurred. The single strand resulting from an exchange between half-site *b* and the *a* element-core junction of the FRT site was not detected (lane 8). In addition, the recombinant strand labeled at the 5' terminus of the *XbaI* end had not undergone strand exchange (lane 6). These results indicate that the stable recombinant structures are formed in a strand exchange reaction between the respective half-sites and the intact FRT site only at the region of core homology.

As diagrammed in Fig. 5A, part i, the putative Y structure would contain a nick on the bottom strand, with FLP attached to the 3' end at the nick. Since the reactions were treated with proteinase K, this strand (183 nt) would have been detectable if the 5' ends of the 411-bp *PvuII* fragment had been 32 P labeled. Nicking of the top strand of the *PvuII* fragment would give a 220-nt strand. Therefore, the experiment was repeated using both a 5'-labeled *a* half-site and a 5'-labeled *PvuII* fragment (Fig. 6). When the entire reaction was run directly on a denaturing gel (Fig. 6A, lane 1), fragments indicative of nicking of both the bottom and top strands of the *PvuII* fragment (183 and 220 nt, respectively) were detected. However, a fragment indicative of only one of the two possible strand exchange products (323 nt) was seen. When the isolated recombinant structure XP1 was analyzed (Fig. 6B, lane 1), only the 323-nt strand exchange product and the product of nicking of the bottom strand (183 nt) were seen. Thus, the recombinant structure XP1 is consistent with that diagrammed in Fig. 5A, part i.

When the same experiment was done with the *b* half-site, similar results were obtained. In the entire reaction, both nicked strands of the *PvuII* fragment (183 and 220 nt) but only one recombinant strand (232 nt) were seen (Fig. 6B, lane 1). XP1 contained only the 232- and 220-nt strands. The

FIG. 5. (A) Possible structures formed by an FLP-catalyzed strand exchange between the *Pvu*II-*Pvu*II fragment (411 bp) and either the *a* or the *b* half-site. (i) Exchange between half-site *a* and *a* element-core junction. (ii) Exchange between half-site *a* and *b* element-core junction. (iii) Exchange between half-site *b* and *b* element-core junction. (iv) Exchange between half-site *b* and *a* element-core junction. P, *Pvu*II; H, *Hind*III; E, *Eco*RI; X, *Xba*I. The polarity (5' and 3') of strands is indicated. Sizes (in nucleotides) of unreacted, nicked, and recombinant strands are indicated by the numbers. Filled circle indicates FLP covalently bound to the FRT site. (B) Strand-denaturing gel of recombinant structures. Recombinant structures were formed in an FLP-mediated reaction containing the *Pvu*II-*Pvu*II fragment of the FRT site and either half-site *a* or half-site *b* with 2 bp of core (Materials and Methods). The respective XP1 structures were gel purified and subjected to electrophoresis on an 8% denaturing polyacrylamide-urea gel. Lanes: 1, half-site *a* substrate 5' labeled at *Xba*I end; 2, XP1 obtained from same half-site; 3, half-site *a* 5' labeled at *Eco*RI end; 4, XP1 obtained from same half-site; 5, half-site *b* 5' labeled at *Xba*I end; 6, XP1 derived from same substrate; 7, half-site *b* 5' labeled at *Hind*III end; 8, XP1 from same substrate. S1, half-site *a* substrate; P1, strand exchange product from the reaction with half-site *a*; S2, half-site *b* substrate; P2, strand exchange product from the reaction with half-site *b* with 2 bp of the core; M, markers from *Sau*3A digest of pBR322 plasmid 3' labeled with 32 P. Numbers indicate lengths of strands (in nucleotides).

structure of XP1 is thus consistent with that shown in Fig. 5A, part iii.

The nature of minor products XP2 and XP3. In order to gain some insight into the structures of products XP2 and XP3, small amounts of these products were recovered from a preparative gel used to analyze the products of reaction of a 5'- 32 P-labeled half-site (*a* or *b*) with the nonradioactive *Pvu*II fragment. Both XP2 and XP3 contained the same 323- or 232-nt labeled single strand found in XP1 (Fig. 7). We were unable to isolate preparative amounts of XP2 and XP3 when the *Pvu*II fragment was 32 P labeled. This may be because they comigrated either with XP1 (for XP2) or with the *Pvu*II substrate (for XP3). The possible origin of the recombination products XP1, XP2, and XP3 will be addressed in the discussion.

Homology of core regions is required for formation of recombinant structure XP1. Although core homology between recombining FRT sites is required for the progression of FLP-induced recombination (3), it should be noted that the nucleotide sequence of the *a* element differs from that of

the *b* element at a position 2 bp from the element-core junction of the FRT site (Fig. 1). Therefore, it was important to ascertain whether the formation of stable recombinant structures was dependent on core homology between the half-site and the intact FRT site or on homology between the symmetry elements of the half-site and the intact FRT site. To address this issue, we used an FRT site that had an inverted core region surrounded by the normal symmetry elements *a* and *b* (called reverse-core substrate). This reverse-core FRT site was obtained on an 80-bp *Eco*RI-*Bam*HI fragment. If core homology is important for formation of stable recombinant structures, half-site *a* should recombine with the *b* symmetry element-core junction of the reverse-core FRT site, whereas half-site *b* should recombine only with the *a* symmetry element-core junction of the site. Accordingly, nonradioactive reverse-core substrate was incubated with a 32 P-labeled fragment containing either half-site *a* or half-site *b* with 2 bp of the core in the presence of FLP. DNA from the resulting recombinant products was then subjected to electrophoresis on a polyacrylamide-urea

FIG. 6. Analysis of products of FLP reaction with 5'- 32 P-labeled half-sites and 5'- 32 P-labeled *PvuII* fragment. Reaction conditions were identical to those used for Fig. 5B except that the *PvuII* fragment was also 5' labeled with 32 P. (A) Entire reaction mixtures were treated with proteinase K and SDS, ethanol precipitated, and analyzed on a denaturing 8% urea-acrylamide gel. Lanes: 1, labeled *a* half-site and *PvuII* fragment with FLP (15 U); 2, same as lane 1 but no FLP; 3, labeled *a* half-site and unlabeled *PvuII* fragment with FLP; 4, same as lane 3 but no FLP; 5, labeled *b* half-site and *PvuII* fragment with FLP; 6, same as lane 5 but no FLP; 7, labeled *b* half-site and unlabeled *PvuII* fragment with FLP; 8, same as lane 7 but no FLP. Numbers in parentheses at the left indicate the lengths (in nucleotides) of the pertinent substrate strand exchange products or nicked strands. The numbers at the right are the lengths (in nucleotides) of marker fragments. (B) Bands were excised from a preparative 4% native acrylamide gel that contained the reaction mixtures described above. After electroelution and ethanol precipitation, DNA was analyzed as for panel A. Lanes: 1, XP1-*a* half-site; 2, *PvuII* substrate; 3, half-site *a*; 4, XP1-*b* half-site; 5, *PvuII* substrate; 6, *b* half-site. Numbers indicate the same fragments indicated in panel A.

gel beside denatured 32 P-labeled marker fragments. The possible results are shown in Fig. 8A.

When 32 P-labeled half-site *a* was used, the exchange product of the recombinant structure was 122 nt (P1, lane 2, Fig. 8B; Fig. 8A, part i). However, when 32 P-labeled half-site *b* was used, the exchange product was 98 nt (P2, lane 6, Fig. 8B; Fig. 8A, diagram iii). The products of exchange between the *a* half-site and the element *a*-core junction (152 nt) and between the *b* half-site and the element *b*-core junction (62 nt) were not seen. These results indicate that strand exchange in the formation of recombinant structures requires the presence of core homology and strengthen the conclusion that homology between the two recombining cores of a half-site and a full FRT site is necessary to stabilize the recombinant structure.

Only recombination-proficient half-sites form stable dimer complexes with the FLP protein. Qian et al. (25) have shown

FIG. 7. Single strands contained in recombinant products XP1, XP2, and XP3. The recombinant products were isolated from a preparative reaction mixture containing 5'- 32 P-labeled *a* half-site (lanes 1 to 4) or *b* half-site (lanes 5 to 7) and run on an 8% urea-sequence gel. Lanes: 1, *a* substrate; 2, XP1; 3, XP2; 4, XP3; 5, *b* substrate; 6, XP1; 7, XP3. Numbers indicate the same fragments as in Fig. 6A.

that the FLP protein can bind to half sites and promote the formation of stable dimers of the half-sites through strong protein-protein interactions across the core region. The ability of the protein to promote dimer formation is dependent on the length of the core sequence of the half-sites and the strand cleavage activity of the FLP protein. We were interested in learning if there might be a correlation between the ability of FLP to form dimers and to catalyze the formation of recombinant structures with half-sites. The 32 P-labeled half-sites were each incubated with FLP protein, and the reactions were analyzed on a native polyacrylamide gel (Fig. 9). FLP could bind to the single symmetry element of all the half-sites to form complex I. The slowly migrating band designated D is the putative dimer and was detected in substantial amounts in reactions containing the half-site *b* substrates with core sequences of 2 bp (Fig. 9A, lanes 6, 7, and 8), 3 bp (lanes 10, 11, and 12), and 4 bp (lanes 14, 15, and 16) but not with the half-site containing a core sequence of only 1 bp (lanes 2, 3, and 4). The highest yield of dimer was detected for the site having 3 to 4 bp of the core, and low yields were obtained with the site containing 5 bp of the core (lanes 18, 19, and 20). Smaller amounts of apparent trimeric and tetrameric species were seen along with the dimers. We identify these species as the same as the multimeric species

As shown in Fig. 9B, the dimer product was also observed in reactions with half-site *a* which had 3 bp of the adjoining core (lanes 2, 3, and 4). Half-site *a* with a 4-bp core showed a drastic reduction in dimer formation with the FLP protein (lanes 6, 7, and 8). These and the previous results suggest that dimer formation requires at least 2 bp of the core and that FLP-mediated formation of stable dimers between two half-sites is important for the strand exchange with an intact FRT site. We observed a drastic reduction in the yield of dimers and recombinant structures with a half-site with a 5-bp core, suggesting that stable dimer formation is mediated by protein-protein interactions across the core. This result is consistent with that of Ojan et al. (25).

FLP protein is unable to cleave *b* half-site having 1 bp of core. Qian et al. (25) have also observed a correlation between strand cleavage and dimer formation in reactions with half-FRT sites. The inability of the FLP protein to form stable dimers with half-site *b* having 1 bp of core could have been due to the inability of the protein to catalyze strand cleavage. To detect strand cleavage, we looked for intermediates in which FLP was covalently attached to the half-site. Unmodified half-site *b* (1 bp of core), half-site *b* with 2 or 3 bp of core, and half-site *a* were each incubated with a fixed concentration of enzyme; the reaction was terminated at various times with SDS and mercaptoethanol; and the samples were analyzed on an SDS-polyacrylamide gel (17). The results, shown in Fig. 10, indicate a time-dependent increase of an intermediate in which FLP was covalently bound to half-site *b* having core sequences with 2 bp (lanes 9 to 14) and 3 bp (lanes 16 to 21) as well as to half-site *a* (lanes 23 to 28). No covalent intermediate was observed with the unmodified *b* half-site containing only 1 bp of the core (lanes 2 to 7). These results suggest that the enzyme requires a half-site with at least 2 bp of core to catalyze strand cleavage. These results are compatible with the possibility that strand cleavage is a prerequisite for the formation of stable dimers, but it is equally likely that protein-protein interactions mediate the dimerization and that cleavage and covalent attachment follow. In fact, studies with a mutant FLP protein that is

FIG. 9. (A) Dimer formation with half-site *b* substrates. 32 P-end-labeled substrates of half-site *b* were incubated with FLP protein at room temperature for 20 min. Reaction mixtures were then subjected to electrophoresis on a polyacrylamide gel. Lanes: 1 to 4, half-site *b* with 1 bp of core incubated with 0, 7.5, 15, and 30 U of FLP, respectively; 5 to 8, 9 to 12, 13 to 16, and 17 to 20, substrates of half-site *b* with 2, 3, 4, and 5 bp of core, respectively, processed as indicated above. S, substrate; CI, complex I; D, dimer; T, trimer. (B) Dimer formation with half-site *a* substrates. Substrates of half-site *a* containing a core of 3 bp (lanes 1 to 4), 4 bp (lanes 5 to 8), 5 bp (lanes 9 to 12), 6 bp (lanes 13 to 16), or 7 bp (lanes 17 to 20) were incubated with protein and subjected to electrophoresis as indicated above. Lanes: 1, 5, 9, 13, and 17, no FLP; 2, 6, 10, 14, and 18, 7.5 U of FLP; 3, 7, 11, 15, and 19, 15 U of FLP; 4, 8, 12, 16, and 20, 30 U of FLP. S, substrate; CI, complex I; D, dimer; TR, trimer; TT, tetramer.

capable of cleavage but deficient in its ability to form stable dimers lead us to favor the second hypothesis (11a).

FLP can promote stable dimer formation between a cleavage-incompetent and a cleavage-competent half-site. We showed above that the FLP protein could not promote stable dimer formation with the *b* half-site with the 1 bp of core and could not promote strand cleavage of this site. We therefore wished to determine whether the FLP protein could mediate stable dimer formation between a noncleavable *b* half-site and a cleavable *a* half-site. Since the fragment containing half-site *a* is 56 bp longer than that containing half-site *b*, the heterodimer product of these two fragments will migrate more quickly than the homodimer product formed with half-site *a*. The complexes were resolved on a native polyacrylamide gel. As shown in Fig. 11A, a substantial amount of the heterodimer product was formed (lanes 11 and 12).

FIG. 10. Covalent attachment of FLP to half-sites. The 32 P-end-labeled half-site substrates were incubated with FLP, and aliquots were removed at various times and added to denaturing buffer (see Materials and Methods). The samples were incubated at 37°C for 20 min and then subjected to electrophoresis on a 7.5% polyacrylamide-SDS gel. Lanes: 1 to 7, half-site *b* with 1 bp of core; 8 to 14, half-site *b* with 2 bp of core; 15 to 21, half-site *b* with 3 bp of core; 22 to 28, half-site *a* with 3 bp of core. Time points were 0 min (lanes 1, 8, 15, and 22), 2 min (lanes 2, 9, 16, and 23), 4 min (lanes 3, 10, 17, and 24), 6 min (lanes 4, 11, 18, and 25), 8 min (lanes 5, 12, 19, and 26), 10 min (lanes 6, 13, 20, and 27), and 20 min (lanes 7, 14, 21, and 28) after FLP was added. S, substrate; C1a, intermediate of FLP covalently bound to half-site *a*; C1b, intermediate of FLP covalently bound to half-site *b*; M, 32 P-*Sau*3A-cut pBR322 markers. Numbers indicate lengths of fragments (in base pairs).

The heterodimer and small amounts of the presumed heterotrimer species are also seen. The heterodimer migrated faster than the homodimer product of half-site *a* (lanes 2 and 3) and more slowly than the homodimer product of half-site *b* with the 3 bp of core (lanes 8 and 9). The heterodimer product and the homodimer products were also detected in reactions containing both half-site *b* with 3 bp of core and half-site *a* (lanes 14 and 15). (No homodimer product of half-site *b* with 1 bp of core is formed [Fig. 11A, lanes 5 and 6].) This experiment suggests that only one of the two half-sites must be cleaved by the protein to result in the formation of a stable dimer.

It was possible that the *b* half-site held in the heterodimer in stable association with the *a* half-site might undergo FLP-induced cleavage. To examine this possibility, we analyzed the products of a reaction such as that whose results are shown in Fig. 11A by SDS-polyacrylamide gel electrophoresis to detect FLP covalently attached to either the *a* or the *b* half-site. Incubation of the *a* half-site with FLP resulted in detection of the expected covalent attachment of FLP to the half-site (Fig. 11B, lane 4), whereas no such attachment was detected with the *b* half-site containing 1 bp

FIG. 11. (A) Mixed dimer formation between half-sites *a* and *b*. Half-site substrates were incubated with FLP protein at room temperature for 20 min and subjected to electrophoresis on a polyacrylamide gel. Lanes: 1 to 3, half-site *a* (3 bp of core); 4 to 6, half-site *b* (1 bp of core); 7 to 9, half-site *b* (3 bp of core); 10 to 12, half-sites *a* and *b* (1 bp of core); 13 to 15, half-sites *a* and *b* (3 bp of core); 16 to 18, half-sites *b* (1 and 3 bp of cores); 1, 4, 7, 10, 13, and 16, no FLP; 2, 5, 8, 11, 14, and 17, 15 U of FLP; 3, 6, 9, 12, 15, and 18, 30 U of FLP. Sa, half-site *a* substrate; Sb, half-site *b* substrate; Cla, complex I of half-site *a*; Clb, complex I of half-site *b*; HMDa, homodimer of half-site *a*; HMDb, homodimer of half-site *b* with 3 bp of core; HTDab, heterodimer of half-sites *a* and *b*; HTTab, heterotrimers of half-sites *a* and *b*. The legend above the figure indicates the presence (+) or absence (-) of half-site *a* with 3 bp of core [a(3)] and half-site *b* with either 1 bp [b(1)] or 3 bp [b(3)] of core. (B) Cleavage and covalent attachment during mixed dimer formation. Reactions were carried out as for panel A, and reaction mixtures were analyzed for covalent attachment of FLP to the labeled half-site as described in the legend to Fig. 10. Lanes: 1, *a* half-site; 2, *b* half-site (1 bp of core) alone; 3, *b* half-site (2 bp of core); 4, *a* half-site with FLP; 5, *b* half-site (1 bp of core) with FLP; 6, *b* half-site (2 bp of core) with FLP; 7, *a* half-site and *b* half-site (1 bp of core) with FLP; 8, *a* half-site and *b* half-site (2 bp of core) with FLP. Where indicated, 0.5 U of FLP was used. *a*, *a* half-site; *b*, *b* half-site; *a*-FLP, FLP covalently attached to *a* half-site; *b*-FLP, FLP covalently attached to *b* half-site.

of core (Fig. 11B, lane 5). When both the *a* and *b* half sites were incubated with FLP, we detected FLP covalently attached to the *a* half-site but no covalent intermediate between FLP and the *b* half-site (lane 7). Control experiments using the *b* half-site with the 2-bp core showed the expected FLP-*b* half-site covalent intermediate (lanes 6 and 8). Thus, one half-site can exist in a stable dimer without being cleaved by FLP.

DISCUSSION

Requirements for synapsis. Our results suggest that the minimum functional FRT site that allows efficient formation of a synaptic complex contains elements *a* and *b* and the core. These results are consistent with our previous observation that only elements *a* and *b* of each of the two wild-type FRT sites in a synaptic complex were occupied by FLP (1). We propose that elements *a* and *b* are first occupied by FLP protein. Protein-protein interactions with another such complex result in synapsis of the two FRT sites. Thus, there is no definable role for element *c* in the synaptic reaction in vitro (24), although we cannot rule out a possible function in vivo (6, 14).

Role of core homology in recombination by half-FRT sites. Stable recombinant structures form only when there are at least 2 bp of core homology between the half-site and the full FRT site. The *b* half-sites recombine only at the homologous symmetry element-core junction of the FRT site. No recom-

binant structures were observed that resulted from strand exchange between half-site *a* and the element *b*-core junction or between half-site *b* and the element *a*-core junction. Therefore, it appears that 1 bp of homology is not sufficient to stabilize the recombinant structure, perhaps because the reaction is readily reversible. In exchanges between half-site *a* and the *a*-core junction or between half-site *b* and the *b*-core junction, there is a short stretch of homology between the single-stranded tail of the half-site (the *Xba*I end) and the core of the intact FRT site. Three-stranded-branch migration through this homology may prevent reversal of strand exchange, thus allowing the accumulation of recombinant structures. The effect of core homology was corroborated in a recombination reaction using the reverse-core FRT site.

We suggest the following pathway for synapsis and strand exchange. FLP molecules bind to elements *a* and *b* of the FRT site to form a bent DNA complex which forms a synaptic intermediate through protein-protein interactions with a similar complex. FLP catalyzes strand cleavage at one of the two cleavage junctions in each of the two FRT sites. This is facilitated by protein-protein interaction across the core. Strand cleavage then triggers an asymmetric conformational change in the intermediate that allows formation of a Holliday junction via a pair of reciprocal strand exchanges (1, 15, 21). Subunit rotation drives branch migration through the core (28) to mobilize the junction to the opposite side of the core, where cleavage and another pair of strand exchanges occur. Unlike the λ Int protein or the P1 Cre

FIG. 12. Possible structures of recombinant intermediates formed by reaction of half-site (hatched lines) with whole FRT site (solid lines). (A) Three-armed Y structure (Fig. 5A, map i) undergoes a second round of recombination with the full site via exchange of bottom strands. The result is a five-armed structure. Further rounds of recombination are possible. Such structures could form XP1. (B) Y structure undergoes FLP-induced nicking and covalent attachment of its top (solid) strand resulting in a recombinant molecule with a short gap in the top strand with FLP covalently attached at the 3' side of the gap. A 5'-OH-terminated half molecule with covalently attached FLP is also liberated. This is the likely structure of XP3. Ovals represent covalently bound FLP molecules.

protein, FLP apparently shows little preference for which strand exchange occurs first (13, 15, 16, 21, 22). Likewise, there is nothing to prevent misalignment of the site during synapsis (1). Hence, should an aberrant strand exchange be attempted (e.g., between a nick next to element *a* on one partner but next to element *b* on the other), then branch migration through the core will be impeded by heterology (3) and the exchange will be quickly reversed. We assume that this will lead to disassembly of the nonproductive synaptic complex. Thus, core homology plays a key role in preventing aberrant recombination events. Occasional failure of this homology filter or mutations within the homologous cores will lead to rare anomalous rearrangements (19) or to the evolution of altered FRT sites and altered FLP proteins that can interact with them (20).

Structure of recombinant intermediates. We have characterized the single-strand-exchange products of the recombinant intermediates XP1, XP2, and XP3 and found that each product contains either the 323-nt or the 232-nt strand, depending on whether the *a* or *b* half-site was used. The major product, XP1, also contains a nick in the recipient bottom (with *a* site) or top (with *b* site) strand. Although we have diagrammed a simple Y structure (Fig. 5A), in fact this structure may be capable of undergoing further rounds of recombination with an intact FRT site contained in the *Pvu*II fragment. An example of such a five-armed product is shown in Fig. 12A. Such a structure would be indistinguishable from the simple Y structures shown in Fig. 5A when analyzed by urea denaturing acrylamide gels. In fact, when the XP1 products are isolated by electroelution and rerun on

a higher-percentage gel than was used to isolate them, they can be resolved into three bands (data not shown), suggesting that XP1 (and perhaps XP2) may contain heterogeneous recombinant products that arise via multiple rounds of FLP-induced recombination. In fact, it is possible that the minor product XP2 is a simple Y structure and that XP1 is a mixture of more complex structures.

The XP3 products could arise from the action of FLP on a simple Y structure (Fig. 12B). A second FLP molecule might cut and covalently attach to the strand opposite the nicked strand, allowing the right bottom arm of the Y structure to be liberated. The result would be a linear structure with a short gap. The recombinant strand would be identical to that found in the Y structure. However, the fact that the products called XP3 migrated near the expected position for a linear molecule of either 320 or 226 bp is consistent with this idea.

Requirements for multimer formation with half-FRT sites. At least 2 bp of the adjoining core sequence are required for strand scission and strand exchange by a half-site. A *b* half-site with only 1 bp of the core cannot be cleaved by the FLP protein, and the enzyme is thus unable to catalyze a strand transfer reaction between this half-site and the intact FRT site. We postulate that the FLP protein interacts with these 2 bp in such a way as to position the scissile phosphodiester bond near catalytic residues of the active site. Consistent with our proposal is the observation by Bruckner and Cox that the protein establishes a phosphate contact at the T residues 3' to the bonds cleaved by the enzyme (7). Although no contacts with the second base distal to the cleavage site were observed, it is possible that the protein can contact this residue (C or T) in ways not detectable by current chemical footprinting techniques.

It is also interesting that the adjoining core of the half-site has an optimal size of 3 to 4 bp for efficient strand cleavage and strand exchange. This led us to examine the relationship between FLP-mediated cleavage and FLP-mediated dimer formation with the half-sites. Qian et al. (25) have provided strong evidence that FLP molecules bind to half-sites and promote stable dimer formation between the sites, provided the core spacing between sites does not exceed 10 bp. They suggested that the dimer is maintained by protein-protein interactions across the core region of the two half-sites. We demonstrate here that the FLP protein is unable to catalyze strand cleavage of the *b* half-site with 1 bp of core and is also unable to promote stable dimer formation with this same site. However, the enzyme will promote stable dimer formation between cleavable half-sites containing 2 bp of the adjoining core. Thus, at least 2 bp of core sequence are required for strand cleavage and dimer formation. This effect is clearly different from the core spacing effect described above. However, we found that the noncleavable half-sites with 5 bp of core sequence showed a drastic reduction in dimer formation.

FLP will promote heterodimer formation between a noncleavable half-site and a cleavage-competent half-site, suggesting that only one of the two strands needs to be cleaved in the formation of a dimer. Our result is thus consistent with that of Qian et al. (25), who demonstrated that 50% of the scissile bonds in a dimer were cleaved by the protein and that the strand cleavage activity of the protein was important for the formation of dimers. Together, these results suggest that protein-protein interactions across the core accompany a single cleavage event during which FLP attaches covalently to the FRT site. This causes a conformational change of the protein which stabilizes the dimer through stronger cross-core interactions to form the asymmetric structure

described by Qian et al. (25). We believe that it is this asymmetric complex that synapses with the intact FRT site. This leads to a single-strand exchange to form the recombinant structures.

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