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Active-Site Assembly and Mode of DNA Cleavage by Flp Recombinase during Full-Site Recombination

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A combination of half-site substrates and step arrest mutants of Flp, a site-specific recombinase of the integrase family, had earlier revealed the following features of the half-site recombination reaction. (i) The Flp active site is assembled by sharing of catalytic residues from at least two monomers of the protein. (ii) A Flp monomer does not cleave the half site to which it is bound (DNA cleavage in *cis*); rather, it cleaves a half site bound by a second Flp monomer (DNA cleavage in *trans*). For the λ integrase (Int protein), the prototype member of the Int family, catalytic complementation between two active-site mutants has been observed in reactions with a suicide *attL* substrate. By analogy with Flp, this observation is strongly suggestive of a shared active site and of *trans* DNA cleavage. However, reactions with linear suicide *attB* substrates and synthetic Holliday junctions are more compatible with *cis* than with *trans* DNA cleavage. These Int results either argue against a common mode of active-site assembly within the Int family or challenge the validity of Flp half sites as mimics of the normal full-site substrates. We devised a strategy to assay catalytic complementation between Flp monomers in full sites. We found that the full-site reaction follows the shared active-site paradigm and the *trans* mode of DNA cleavage. These results suggest that within the Int family, a unitary chemical mechanism of recombination is achieved by more than one mode of physical interaction among the recombinase monomers.

The Flp protein of *Saccharomyces cerevisiae* is a conservative, site-specific DNA recombinase that belongs to the Int (λ integrase) family of recombinases (1, 3). Members of this family execute recombination in two sequential steps. The first pair of strand cleavage-joining reactions produces a Holliday intermediate which, following branch migration, is resolved into recombinants by the second pair of cleavage-joining reactions. The Int family recombinases use an active-site tyrosine as the nucleophile to attack the scissile phosphodiester during the strand breakage step. In Flp, this tyrosine residue is Tyr-343 (9). The active-site tyrosine is one of the invariant tetrad residues of the Int family (1, 3). The other three invariant residues are two arginines and a histidine (the RHR triad; Arg-191, His-305, and Arg-308 in Flp). The strand cleavage reaction results in covalent attachment of the recombinase to the 3′ phosphate of DNA and exposure of a 5′-hydroxyl group at the nick. Strand joining in the recombinant mode is then effected via nucleophilic attack, by the 5′-hydroxyl group from the nicked strand of one DNA substrate, on the 3′-phosphotyrosyl bond formed within the partner substrate.

The Int family recombinases exist in solution as monomers and bind to DNA as monomers. Four recombinase monomers must act cooperatively to accomplish one round of recombination. Two concerted break exchanges must be made at one end of the strand exchange region (spacer) to form the Holliday junction. The process then needs to be repeated at the other end of the spacer to resolve the junction into mature recombinants. How does an Int family recombinase coordinate the breakage-joining events within the two DNA substrates taking part in recombination? Does the recombinase have a built-in mechanism by which it avoids abortive partial reactions within an incompletely assembled reaction complex?

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The active-site configuration of two Int family members, Flp and the *Zygosaccharomyces rouxii* recombinase R, inferred from recombination reactions containing half-site substrates and step arrest mutants of the recombinases suggests potential solutions to the problems addressed above (6, 7, 13, 17, 24). A monomer of Flp or R harbors a partial active site; a complete active site is assembled by contribution of residues from more than one recombinase monomer. In the shared active site, three of the invariant Int family residues, the RHR triad (Arg-191, His-305, and Arg-308 in Flp; Arg-207, His-317, and Arg-320 in R), are derived from one monomer; the active-site tyrosine (Tyr-343 in Flp and Tyr-358 in R) is provided by a second monomer (6, 17). Assembly of a functional active site from partial active sites neatly accommodates the observation that in reactions with half sites, an Flp or an R monomer does not cleave the substrate to which it is bound but rather cleaves the substrate bound by a second recombinase monomer (*trans* DNA cleavage; 6). The partial active site, together with the *trans* mode of DNA cleavage, suggests possible mechanisms for postponing the chemistry of recombination until the complex is fully organized, for simultaneously assembling two active sites for coordinated strand cleavages, and for coupling the cleavage reaction with the conformational switch required for strand joining between partner substrates.

The catalytic strategies displayed by Flp and their mechanistic implications in recombination suggest that they may be universal to the Int family. One set of experiments with λ Int using suicide *attL* substrates supports this notion. Catalytic complementation in pairwise combinations of the RHR triad mutants of Int with the active-site tyrosine mutant has been demonstrated (10). This result is strongly suggestive of *trans* DNA cleavage by Int. However, other results obtained by using suicide *attB* substrates and synthetic Holliday junctions are more parsimoniously explained in terms of *cis* DNA cleavage by Int (16).

The mechanistic dilemma posed by the Int results raises fundamental issues regarding the mechanism of Int family

site-specific recombination. First, is the apparent *cis-trans* duality a basic feature of the reaction? Second, are there multiple modes of active-site assembly within this family? Finally, is the half-site reaction mechanistically distinct from the full-site reaction? We have devised an experimental design in which full sites carrying mismatches in the spacer region serve as substrates in complementation tests with step arrest Flp mutants. Our results fully support the shared active-site paradigm during full-site recombination. Furthermore, the mode of DNA cleavage is *trans*. We have found no evidence of *cis-trans* duality in Flp recombination.

MATERIALS AND METHODS

Purification of Flp. Wild-type Flp and Flp variants were partially purified essentially as described by Prasad et al. (20). Strand cleavage and strand transfer assays were carried out with these preparations. Some reactions were done with 90 to 95% pure proteins obtained by an affinity purification protocol (18). Assays with affinity-pure proteins yielded the same results as those done with the less pure proteins. Fusion proteins composed of Flp (or a Flp variant) and glutathione *S*-transferase (GST) were purified in accordance with the procedure detailed by Yang and Jayaram (24). Protein concentrations were estimated by comparing densitometric scans of gel-fractionated aliquots stained with Coomassie brilliant blue to similar scans done with bovine serum albumin as the standard. These estimates were relatively crude and were only accurate within a factor of 2 or so.

Synthetic recombination sites. Oligodeoxynucleotides for construction of full sites were synthesized in an Applied Biosystems 380A DNA synthesizer by using phosphoramidite chemistry (4). Normally, 10 to 20 pmol of each of the two appropriate oligodeoxynucleotide pairs was mixed in TE (10 mM Tris-HCl [pH 7.8] at 23°C, 1 mM EDTA [pH 8.0]), heated to 65°C for 10 min, and cooled slowly to room temperature. The relevant features of these sites are described in Results and displayed in the figures. The complete sequences of the substrates are available upon request.

The 3′ end of a deoxyoligonucleotide was labeled with α-^{32}P-labeled cordycepin phosphate. Labeling at the 5′ end was done by the T4 polynucleotide kinase reaction by using [γ-^{32}P]ATP as the phosphoryl donor. For some experiments, the 5′ ends were phosphorylated with unlabeled ATP. The unreacted cordycepin phosphate or ATP was removed by spin dialysis on a G-25 column. Hybridization to the partner oligodeoxynucleotide was done in TE.

Strand cleavage assays. Strand cleavage reactions were done under standard recombination conditions (6). Normally, 0.05 pmol of the 3′ end-labeled substrate was reacted with approximately 0.5 pmol of Flp or a Flp variant (roughly 5 pmol of Flp per pmol of the binding element) in 30 μl of the reaction mixture. Incubations were done at 30°C for 30 min. Reactions were stopped by immersing samples in a boiling water bath for 5 min. After addition of sodium dodecyl sulfate (final concentration, 0.1%) and proteinase K treatment (100 μg per sample for 1 h at 37°C), samples were phenol-chloroform extracted and DNA was precipitated with ethanol. The DNA pellet was recovered by centrifugation, washed twice with 80% ethanol, and dried in vacuo. Strands were denatured in 95% formamide at 95°C, and samples were fractionated by electrophoresis in 10% denaturing polyacrylamide gels (acrylamide-bisacrylamide ratio, 19:1). Cleavage products were identified following autoradiography.

Strand transfer assays. The synthetic full sites (approximately 45 to 50 bp long, carrying *Eco*RI and *Hin*dIII overhangs at the ends) were poor substrates in strand transfer. To increase the efficiency of the reaction, assays were done with the monomeric form of a radioactively labeled full site and the concatemeric form of the unlabeled full-site partner. The concatemer was prepared as follows. The full site phosphorylated at the 5′ end on both strands was ligated at room temperature for 3 h under conditions that gave concatemers containing an average of 8 to 10 U of the monomer. The strand transfer reactions were done with the normal protocols described previously (6). The ratio of the labeled substrate to the monomeric equivalent of the unlabeled substrate was approximately 1:5. In these assays, approximately 6 to 8 pmol of Flp per pmol of the Flp-binding element was present in a reaction volume of 30 μl. The samples were processed and fractionated as described for the strand cleavage assay.

Assay for formation of DNA-protein covalent adducts. Reactions were carried out under strand transfer conditions with a substrate labeled at the 5′ ends on both strands. Reactions were quenched by addition of an equal volume of a stop mixture containing 250 mM Tris-HCl (pH 7.8), 4% sodium dodecyl sulfate, 40% glycerol, and 300 mM β-mercaptoethanol. Suitable aliquots were heated at 95°C for 4 min and fractionated by electrophoresis in 8% polyacrylamide gels (12). The gels were rinsed in distilled water with gentle shaking, dried, and subjected to autoradiography.

General methods. Restriction enzyme digestions, isolation of plasmid DNA, and other miscellaneous procedures were done as described by Maniatis et al. (14).

RESULTS

The normal Flp reaction uses two double-stranded DNA substrates, each containing a copy of the Flp recombination target sequence (Fig. 1A). These are referred to as full-site substrates. A recombination event between two full sites requires the cooperative action of four Flp monomers and involves the breakage and reformation of four phosphodiester bonds within DNA (two breakage-union steps within each substrate partner). The disposition of a target-bound Flp monomer with respect to the four scissile phosphodiester bonds can be described as *cis*, *trans* horizontal, *trans* vertical, or *trans* diagonal. Conceptually, a full site can be split into two half sites, a left half site and a right half site. Half-site substrates (Fig. 1B), originally designed for the λ Int reaction (15) and subsequently adapted for the Flp reaction (2, 21, 22), have simplified the mechanistic analysis of site-specific recombination. A half site contains one Flp-binding element, one scissile phosphodiester, and one 5′-hydroxyl group that can act as a phosphoryl acceptor. Hence, it is capable of undergoing one strand cleavage and one strand-joining reaction, precisely half of the chemistry that a full site undergoes during a normal recombination event. However, while interactions between two Flp monomers within a full site are constrained by the continuous DNA segment between them (the strand exchange region or the spacer), half sites are not subject to such constraints. Hence, two half sites could potentially interact with each other in a variety of modes that may not be accessible to two full sites (Fig. 1B). A legitimate concern, then, is that direct extrapolations from half-site to full-site reactions may not always be valid. To overcome this impediment, the analyses described here were done with appropriately modified full-site substrates (Fig. 2).

Pairwise complementation between Flp(Y343F) and the RHR triad variants of Flp during strand cleavage in full sites. It is known that a Flp variant altered at one or more of the RHR triad positions in combination with a second variant

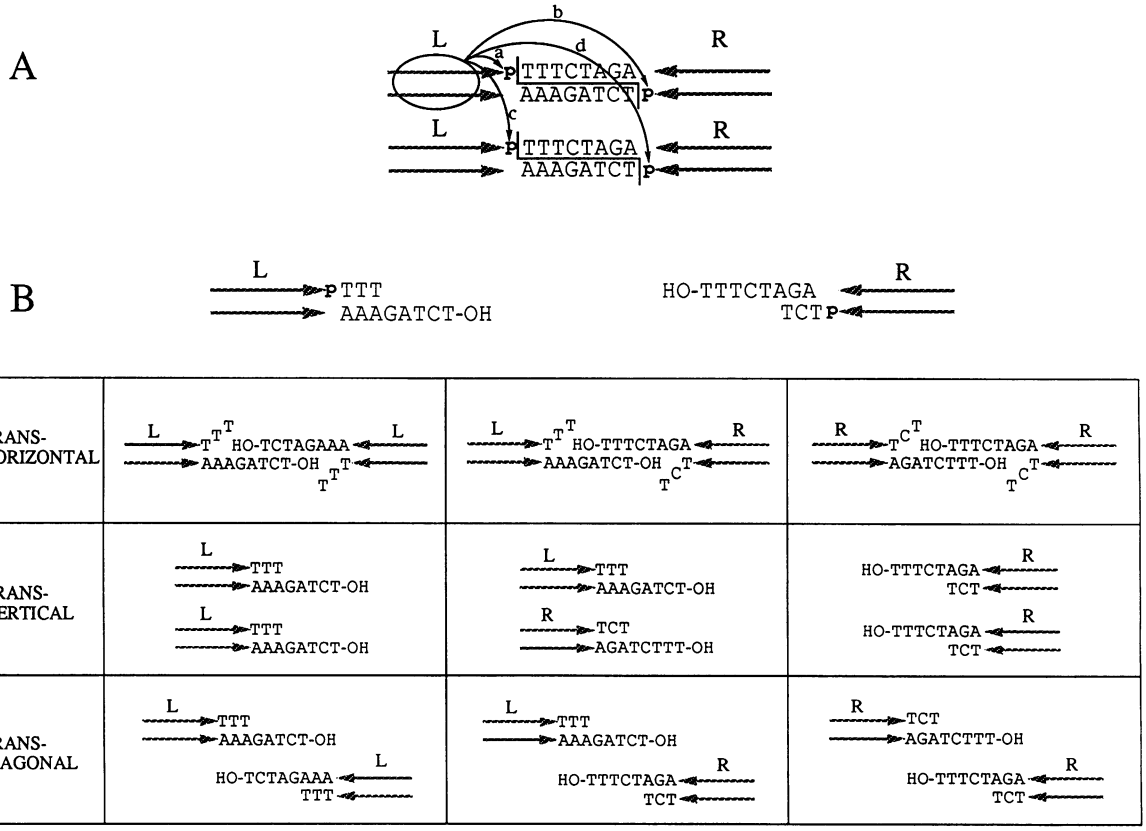

FIG. 1. Full-site and half-site substrates for Flp site-specific recombination. (A) Each full site contains two invertedly oriented Flp-binding elements (parallel arrows) bordering the strand exchange region (spacer). There is a one-to-one association between a binding element and a Flp monomer. Conceptually, a full site can be split into a left half site (L) and a right half site (R). The phosphodiesters involved in recombination between two full sites are indicated (p). The placement of a Flp monomer with respect to these phosphodiesters can be described as *cis* (a), *trans* horizontal (b), *trans* vertical (c), or *trans* diagonal (d). (B) A half site contains one Flp-binding element and one scissile phosphodiester. When the site is cleaved, the short spacer segment on the cleaved strand does not remain stably hydrogen bonded to its complementary sequence. Hence, it is effectively lost from the reaction center by diffusion. The 5′-hydroxyl group of the spacer on the noncleaved strand can then act as a phosphoryl acceptor to complete a half-site recombination event. Details of half-site reactions have been previously described (for example, see reference 22). Whereas two Flp monomers bound to a full site are restricted in their interactions by spacer connectivity, two half sites, each associated with a Flp monomer, are not subject to this constraint. They could potentially assume the configurations indicated. These correspond to variations of the *trans* interactions depicted in panel A.

lacking the active-site nucleophile, Flp(Y343F), can mediate a strand cleavage and a strand joining event within a half-site substrate (6, 17). However, attempts to obtain catalytic complementation between a triad mutant and Flp(Y343F) in full sites have not been successful. This is not surprising. In a recombination complex containing two monomers of each mutant oriented appropriately, a Holliday junction may be formed. Since the junction cannot be resolved into recombinants, the exchange reaction is reversed to restore the parental configuration. Demonstration of complementation therefore required the use of a suicide substrate in which the reaction intermediates could be readily trapped. We discovered that mismatches at certain positions within the strand exchange (spacer) region of a full site can strongly inhibit the strand-joining step of recombination, thus effectively transforming the sites harboring such mismatches into suicide substrates (unpublished data). For example, the substrate shown in Fig. 3 contains two adjacent mismatches each (bubbles) neighboring the cleavage points at the left and right ends of the spacer (Fig. 2). This substrate was cleaved efficiently by wild-type Flp. However, since strand joining was markedly slowed down (unpublished results), the cleavage product accumulated (lane 2 in Fig. 3). As expected, no cleavage was obtained with Flp(Y343F) (lane 3 in Fig. 3). Flp variants in which either of the two arginine residues from the RHR triad (Arg-191 and Arg-308) were changed failed to produce the cleavage product (lanes 4 and 8 in Fig. 3). It is known that Flp variants of the triad histidine can yield cleavage in a full site but are severely diminished in the ability to reseal strands (19). However, in a full site with the double bubble, cleavage by the histidine variants was significantly lowered relative to that obtained with wild-type Flp (compare lanes 6 and 2 in Fig. 3). It is possible that the absence of His-305, combined with the mismatched spacer configuration, perturbs the normal protein interactions that lead to catalysis. The histidine variants are also known to test as cleavage incompetent when provided with half-site substrates (23). When a triad arginine variant of Flp was mixed with Flp(Y343F), they complemented each other, as evidenced by the cleavage detected within the bubbled full site (lanes 5 and 9 in Fig. 3). Complementation was obtained when Flp (Y343F) was paired with the His-305 variant as well. Whereas cleavage with the His-305 variant alone was weak (lane 6 in Fig. 3), the complementing pair yielded much higher levels of cleavage (lane 7 in Fig. 3).

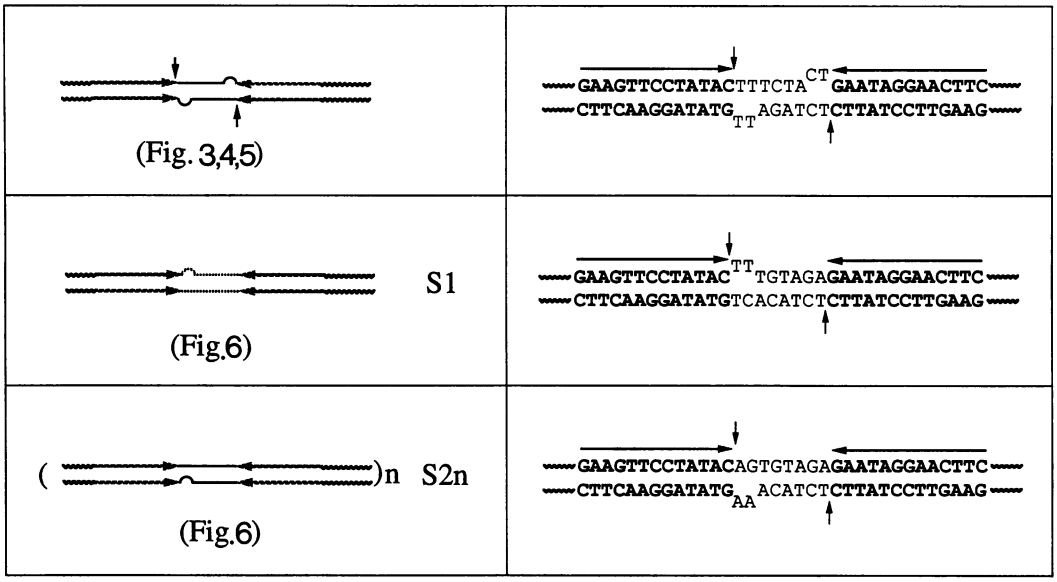

FIG. 2. Full-site substrates containing spacer mismatches. The sequences of the synthetic full sites used in the strand cleavage and strand transfer assays are shown. The Flp-binding elements are in boldface. Sequences flanking the Flp target site are represented by wavy lines. The positions of spacer mismatches (bubbles) are indicated. The experiments in which they were used are indicated by the corresponding figure numbers. S2n refers to a concatemer of S2 (8 to 10 monomeric units, on average). The spacer mismatches in S1 and S2 are such that strand swapping between the two substrates (following strand cleavage) would produce perfect complementarity.

These results are consistent with the partial active-site model arrived at from half-site reactions (6). According to this model, a mutant pair can build an active site in which the RHR triad is contributed by Flp(Y343F) and Tyr-343 is contributed by the triad mutant. Therefore, strand cleavage becomes possible.

The nucleophile in the cleavage reaction by a pair of complementing Flp variants is Tyr-343. The partial-active-site model is based on the tacit assumption that strand cleavage is executed by the lone Tyr-343 present within a pair of the complementing protein monomers. The model would break down if, in the absence of Tyr-343, a substitute nucleophile in the form of a serine, threonine, cysteine, or another tyrosine could take up its function. We have ruled out the possibility of *cis* cleavage by a nucleophile other than Tyr-343 in complementation reactions with half sites (24). In the full-site context, activation of a surrogate nucleophile within Flp(Y343F) as a result of allosteric interactions among the protein monomers is not impossible. To test the surrogate nucleophile hypothesis, the assay was done with a bubbled full site labeled at the 5′ end on both strands and a complementing pair of Flp variants made up of a normal-sized protein and a 30-kDa larger protein partner obtained as a fusion with GST. The protein-DNA adduct formed by Flp and the GST-Flp hybrid upon DNA cleavage can be distinguished by the difference in electrophoretic migration between them (lanes 2 and 3 in Fig. 4). Flp (Y343F) or GST-Flp(Y343F) did not yield the cleavage product, as expected (lanes 4 and 6 in Fig. 4). Flp(H305L) and GST-Flp(H305L) yielded low levels of the cleavage products with the expected mobilities (lanes 5 and 7 in Fig. 4). The low level of strand cleavage by these proteins was as predicted by the results shown in Fig. 3. However, in partnership with Flp (Y343F), they produced elevated levels of cleavage commensurate with catalytic complementation (lanes 8 and 9 in Fig. 4). When the complementing partners were GST-Flp(Y343F) and Flp(H305L), the size of the cleavage product matched that obtained from reactions with Flp (lane 8 in Fig. 4). When the reaction contained the reciprocal pair, Flp(Y343F) and GST-Flp(H305L), the cleavage product corresponded in size to that obtained with GST-Flp (lane 9 in Fig. 4).

The pattern of DNA-protein adducts observed in the complementation reactions demonstrates that cleavage is carried out exclusively by the protein partner that harbors the active-site tyrosine. The simplest interpretation of the results is that it is indeed Tyr-343 that performs strand cleavage. The more complex scenario in which the active species is derived from the triad variant but is a surrogate nucleophile rather than Tyr-343 is not excluded. However, this possibility is strongly discounted by the fact that no complementation was obtained between Flp(Y343F) and a double variant altered at a triad position and Tyr-343 (data not shown).

The donor of Tyr-343 during catalytic complementation can be mutated at all triad positions. The shared-active-site model for Flp predicts that a Flp variant doubly or triply mutated in the RHR triad would be as competent as the single mutant in catalytic complementation with Flp(Y343F) provided their binding affinities for the target DNA do not differ significantly. This prediction has been verified in half-site recombination (7). We tested two triad double mutants and a triad triple mutant in combination with Flp(Y343F) in the cleavage assay with the bubbled full site (Fig. 5). Individually, neither the triad mutants nor Flp(Y343F) could effect strand cleavage (lanes 3, 4, 6, and 8 in Fig. 5). In contrast, each pair formed by mixing Flp(Y343F) and a triad mutant in roughly equimolar amounts exhibited approximately the same levels of complementation (lanes 5, 7, and 9 in Fig. 5). A second strong prediction of the partial-active-site model is that a triad mutant that also lacks Tyr-343 in combination with wild-type Flp will produce a catalytically inactive protein pair. This prediction could be directly tested in half-site reactions, since one could load the double mutant on a radioactively labeled half site and the wild-type protein on an unlabeled half site and then monitor

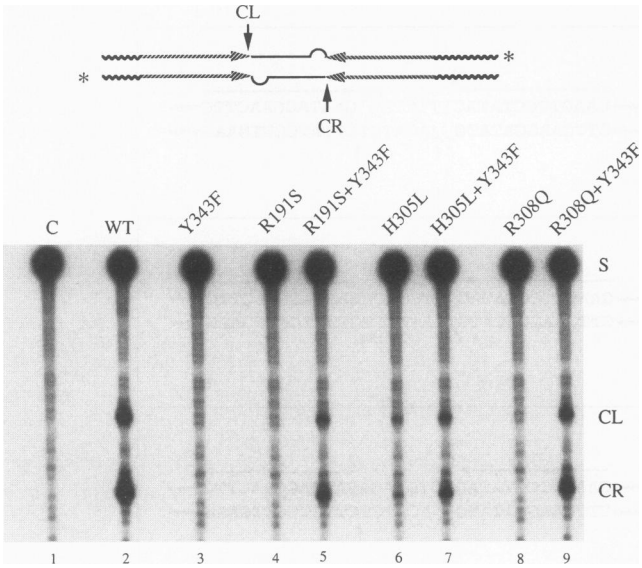

FIG. 3. Complementation between Flp(Y343F) and triad mutants of Flp in full-site substrates. The full site used in the assays is schematically represented at the top (Fig. 2). The parallel arrows represent the Flp-binding elements; the short vertical arrows indicate the points of strand cleavage by Flp. The two mismatched positions within the spacer adjacent to the cleavage sites are shown by the bubbles. DNA sequences unrelated to recombination are symbolized by the wavy lines. The asterisks stand for the ^{32}P label at the 3′ ends. Products of cleavage from the top strand (left) and the bottom strand (right) are labeled CL and CR, respectively. The substrate band is designated S. The lane marked C is a reaction in which no Flp or Flp variant was added. WT, wild type.

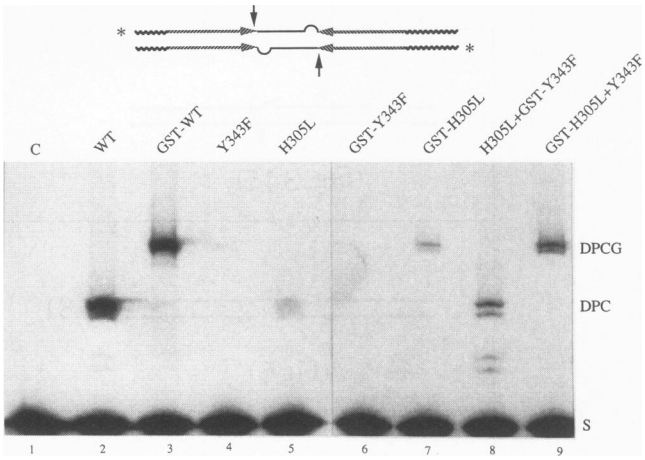

FIG. 4. Identification of the protein partner responsible for strand cleavage during catalytic complementation. Strand cleavage assays were carried out by using the substrate used in reactions shown in Fig. 3. The radioactive label was placed at the 5′ end of each strand (asterisks). The covalent DNA-protein complex resulting from strand cleavage by Flp (or a Flp variant) is called DPC; that derived from GST-Flp (or a GST-Flp variant) is called DPCG. The doublets corresponding to DPC or DPCG are most likely cleavage products derived from the top and bottom strands. The substrate DNA band is marked S. Flp and the Flp variants used in the reactions are indicated above the appropriate lanes. Lane C is a control reaction without added Flp or Flp variants. WT, wild type.

strand transfer only from the labeled substrate upon mixing of the prebound complexes (7). Thus, the background of wild-type recombination could be kept hidden. However, difficulty in selectively binding a protein monomer to one of the two normal binding elements of the full-site substrate poses an impediment to the rigorous testing of this prediction. Nevertheless, the degree of cleavage reduction obtained upon the mixing of roughly equal amounts of Flp and Flp(H305L, Y343F) was consistent with inactivation of the wild-type partner in a double-mutant–wild-type protein pair (data not shown).

Strand transfer in bubbled full sites by pairwise combination of Flp(Y343F) and triad variants of Flp. According to the partial-active-site–*trans*-cleavage model, during complementation between a Flp triad mutant and Flp(Y343F), cleavage should occur on the scissile phosphodiester adjacent to the bound Flp(Y343F) (the *cis* configuration in Fig. 1A) and away from the triad mutant (the *trans* configuration in Fig. 1A) (6, 7). Once the DNA 3′-phosphotyrosyl bond has been formed, Flp(Y343F) can facilitate the strand-joining reaction by using the 5′-hydroxyl group as the nucleophile (13, 17). In the bubbled full-site substrate, the spacer mismatch inhibits the joining reaction. However, if one used a pair of substrates (S1 and S2n in Fig. 6), each mismatched within its spacer but fully matched with the partner's spacer, strand joining within a substrate (parental mode) would be suppressed but that between partners (recombinant mode) would be encouraged. Even when the lack of a second pair of exchanges (as with a pair of complementing mutants) would tend to reverse this reaction, one might expect to trap some of the strand transfer product. With wild-type Flp, strand transfer products were formed from this pair of substrates (lane 3 in Fig. 6). The heterogeneity of strand transfer products results from the fact that one of the two DNA substrates (the nonradioactive one) was a concatemer of a single full site (S2) obtained by ligation. We resorted to this trick because, under our assay conditions, strand transfer efficiency was increased severalfold by increasing the length of at least one of the recombination partners. The size of the recombinant strand would depend on the site within S2n at which crossover occurred. In reactions containing Flp(Y343F) in combination with a triad single mutant or a triad double mutant, strand transfer was indeed detected (lanes 6 to 8, 10 to 12, 14 to 16, and 18 to 20 in Fig. 6). The low level of reaction compared with the wild type is not surprising. Since a single-strand transfer requires a matched pair of cleavage events within the two substrates (on the top strands or the bottom strands), only a reaction complex containing two appropriately positioned Flp(Y343F) and triad mutant monomers would be successful in completing an exchange event. Further, unlike, the wild-type reaction, the mutant pair reaction cannot execute the second pair of exchanges that yields the mature recombinant product. One would expect, therefore, that reversal of the first exchange (due to the absence of a second exchange) would be more pronounced in a reaction containing the complementing partners than in the wild-type reaction.

The successful execution of strand exchange within a full site by a complementing pair of Flp variants fully corroborates the inference from half-site reactions that Flp(Y343F) (harboring an intact RHR triad) can facilitate not only strand cleavage but also strand joining by using a Tyr-343 residue and a 5′-hydroxyl group, respectively, donated in *trans*.

DISCUSSION

The partial-active-site–*trans* DNA cleavage model for Flp was first proposed to account for the pattern of DNA cleavage and strand transfer in half-site substrates by complementing

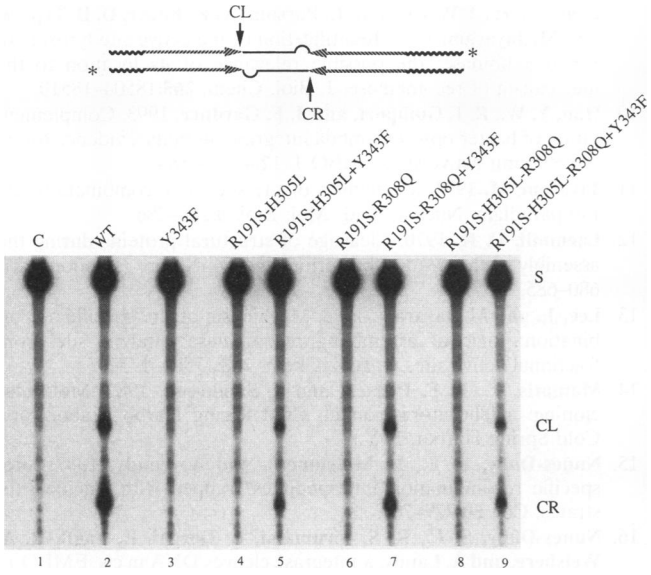

FIG. 5. Complementation between Flp(Y343F) and double or triple triad mutants of Flp in full-site cleavage. The substrate was labeled at the 3′ ends. The proteins used in the assays are indicated above the lanes. The substrate band (S) and the cleavage products (CL and CR) are labeled as in Fig. 3. A control reaction without Flp or Flp variants is shown in lane C.

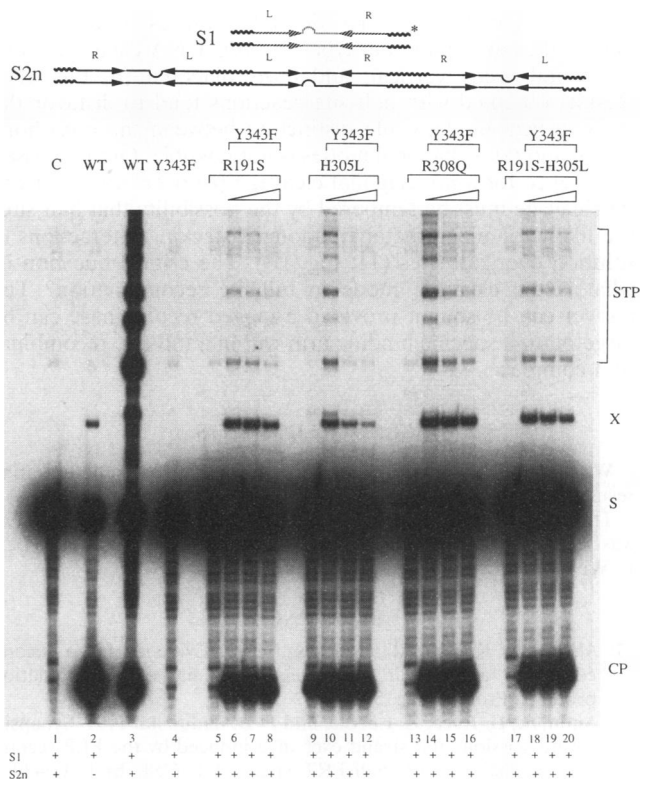

FIG. 6. Strand transfer in full sites by pairwise combinations of Flp step arrest mutants. The substrates used in the assay are schematically shown at the top. The labeled substrate S1 contained ^{32}P at the 3′ end of the top strand and two spacer mismatches (5′TT3′ and 3′TC5′) adjacent to the top-strand cleavage point. The unlabeled substrate was a concatemer of S2 obtained by self-ligation (S2n). S2 also contained two mismatched spacer positions next to the top-strand cleavage site (5′AG3′/3′AA5′). Following strand swapping between S1 and S2, the spacers would be fully matched (5′AG3′/3′TC5′ and 5′TT3′/3′AA5′). Strand cleavage and strand transfer products are designated CP and STP, respectively. S represents the substrate. The heterogeneity in strand transfer products results from the multiplicity of crossover points within S2n. For each reaction set with a complementing protein pair, the leftmost lane represents a reaction with the triad variant alone (at the same molar concentration as in the rightmost lane). The next three lanes represent reactions containing the triad variant and Flp(Y343F) in approximate molar ratios of 1:1, 1:1.5, and 1:2, respectively. Roughly 3 pmol of Flp(Y343F) was present per pmol of the Flp-binding element. Lane C represents an assay with no Flp or Flp variant added to the reaction. The Flp reaction shown in lane 2 contained S1 but not S2n. The product X likely arose by cleavage within S1 and subsequent phosphoryl transfer to the 5′-hydroxyl group of the unannealed top-strand oligodeoxynucleotide of S1 present in the reaction. The size of X, as measured against standard molecular size markers, fits this explanation. Note the presence of X in a reaction containing S1 alone (lane 2). WT, wild type.

pairs of catalytic Flp mutants (6, 7, 13). The validity of the model was then verified for the R recombinase from *Z. rouxii* in half-site strand transfer reactions (24). The simplicity and functional parsimony of the model led us to speculate that the rules of active-site assembly and of DNA cleavage are likely to extend beyond the yeast site-specific recombinases and encompass the entire Int family. Experiments with λ Int and suicide *attL*, *attB*, or Holliday junction substrates have yielded rather paradoxical results (10, 16). While one set of results supports the Flp paradigm (10), the other set casts doubt on the generality of the model (16).

Flp assembles a functional active site from partial active sites during full-site recombination. The apparent *cis* DNA cleavage by Int in full *attB* sites and Holliday junctions calls into question the pertinence of the active-site assembly by Flp during a half-site reaction to that during normal recombination. The design of full sites containing mismatched spacers has allowed us to address this issue directly. Complementation by step arrest variants of Flp to mediate strand cleavage in full sites supports the shared-active-site configuration during a normal recombination reaction. In a reaction carried out by a pair of Flp step arrest mutants, cleavage is executed by the protein partner harboring Tyr-343, thus virtually excluding the operation of an aberrant pathway during complementation.

Recently, mechanistic analyses of another Int family recombinase, Xer (responsible for stable chromosome partitioning in *Escherichia coli*), have become possible (5). Recombination in this system requires the combined action of two recombinases, XerC and XerD. The binding arms of the Xer target site encode specificities for each of the two protein monomers. The cleavage pattern observed with Xer is most easily explained by *cis* DNA cleavage, although one particular type of *trans* cleavage (*trans* vertical or partner *trans*; 6, 16) cannot be ruled out.

The λ Int and Xer examples, contrasted with Flp, imply that Int family recombinases do not conform to the same rules in building their active sites. However, one suspects that, within the fully assembled active sites of these proteins, key catalytic residues must have the same relative spatial disposition. This would account for the fact that they follow the same chemistry of recombination. Global diversity and limited homology, which are the hallmarks of this family (3), would then make a strong case for mechanistic convergence (8, 16) among proteins that execute chemically identical reactions.

Which mode of *trans* cleavage does Flp follow? While our results strongly support *trans* DNA cleavage during full-site

recombination by Flp, they do not allow us to distinguish among the three potential types of *trans* DNA cleavage, *trans* horizontal, *trans* vertical, and *trans* diagonal (6; Fig. 1A). Results obtained with half-site reactions tend to disfavor the *trans*-vertical mode, while distinction between the *trans*-horizontal and *trans*-diagonal modes is not possible. Our expressed preference for *trans*-diagonal cleavage (6) over *trans*-horizontal cleavage must be tempered by the possibility that half sites are likely to enjoy greater freedom of stacking interactions in solution over full sites (11; Fig. 1B). The critical question is: what is the cleavage mode in full-site recombination? The answer can be sought provided a tagged recombinase can be targeted to a specific binding arm within a full-site recombination complex.

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