Functional Roles of Individual Recombinase Monomers in Strand Breakage and Strand Union during Site-specific DNA Recombination*

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The site-specific recombinase Flp from Saccharomyces cerevisiae accomplishes recombination between two target DNA sites by executing a pair of strand exchanges at either end of the strand exchange region. One round of recombination requires the cooperative action of four recombinase monomers. We demonstrate here that, in the presence of the appropriate nucleophiles, a single Flp monomer associated with its binding element can mediate strand cleavage and strand joining at the exchange site phosphate adjacent to it. Our results support a model of recombination in which pairs of Flp monomers reverse catalytic roles to mediate the first and second sets of strand breakage/union reactions. They disfavor a model that involves a relay of recombinase monomers between binding elements to assemble separate active sites for strand cleavage and strand joining. Our data are consistent with the breakage and joining reactions being carried out by a single composite active site in which some residues contribute to both reactions while others contribute to one of the two reactions.

Conservative site-specific recombination between two DNA substrates by the integrase family recombinases (Argos et al., 1986; Abremski and Hoess, 1992) is accomplished in two stepwise exchanges of two strands each, a Holliday junction being an obligatory intermediate (Craig, 1988; Stark et al., 1992; Sadowski, 1993; Jayaram, 1994). The reaction is mediated by a tetrameric assembly of the recombinase monomer. Each pair of exchanges requires two strand breakages and two strand joinings. Chemically, the strand breakage and union events are transesterification reactions. During the breakage step, the scissile phosphodiester is subjected to nucleophilic attack by the active site tyrosine of the recombinase (Evans et al., 1990). The reaction produces a 3′-hydroxyl group formed at the nick end of a deoxyoligonucleotide, and protein and exposes a free 5′-hydroxyl group. During the strand union step, the 5′-hydroxyl group formed at the nick end of a deoxyoligonucleotide is phosphorylated by T4 polynucleotide kinase in presence of [γ-32P]ATP. After 1 h at 37°C, the reaction was terminated by the addition of SDS (0.1% final concentration) and treated with proteinase K (100 μg/ml). After chloroform/phenol extraction and ethanol precipitation, the DNA was fractionated by electrophoresis in a 10% polyacrylamide gel. After staining Flp aliquots (fractionated in SDS-polyacrylamide gels) and autoradiography, the DNA was visualized by autoradiography.

Materials and Methods

Purification of Flp and Flp Variants—All reactions were done with Flp or Flp variants affinity-purified over a column containing digoxigenin units of a Flp site digoxigeninylated in TE (10 mM Tris-HCl, pH 7.8, at 23°C) using the intasamely site-specific recombinase chemistry (Evans et al., 1981). Approximately 2–4 pmol each of the appropriate digoxigenylated units were 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. Normally the radioactively labeled strand was held at one-half to one-third the concentration of the unlabeled partner strands during hybridization. To label the 5′-end of a deoxyoligonucleotide, it was phosphorylated by T4 polynucleotide kinase in presence of [γ-32P]ATP. In some cases, the 5′-hydroxyl group was phosphorylated with nonradioactive ATP. The unreacted ATP was removed by spin dialysis on a Sephadex G-25 column. Hybridization to the partner oligodeoxynucleotide(s) was done in TE.

Strand Transfer Reactions in Half-sites—The conditions for half-site strand transfer were essentially the same as those described previously (Chen et al., 1992a). Normally the reactions contained approximately 0.02–0.05 pmol of the half-site and an excess of Flp (8–10 pmol of Flp/pmol of Flp binding element) in a reaction volume of 30 μl. Reactions were terminated by the addition of SDS (0.1% final concentration) and treated with proteinase K (100 μg/ml) reaction for 1 h at 37°C. After chloroform/phenol extraction and ethanol precipitation, the DNA was fractionated by electrophoresis in 10% denaturing polyacrylamide gels (acrylamide:bis-acrylamide, 19:1). After autoradiography.

As we see, the recombinase use two separate active sites or a single active site to perform these two steps? During a round of recombination, does a recombinase monomer retain its association with the target DNA element to which it is initially bound? Or is there dissociation and redistribution of the protein monomers from their original configuration? The nondissociative mode would be consistent with a unitary active site mechanism for strand cleavage and strand joining, whereas the dissociative mode would be more consistent with a binary active site mechanism. We address here these fundamental mechanistic issues of recombination chemistry using the integrase family site-specific recombinase Flp from Saccharomyces cerevisiae.

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Catalytic Contributions of Flp Monomers in Recombination

In Situ Assay for DNA Cleavage and Strand Transfer in Immobilized Flp-DNA Complexes—Complexes formed by association of Flp or a Flp variant with a labeled DNA target were separated by electrophoresis on native polyacrylamide gels. Complexes of interest were then excised from the gel after their position was determined by autoradiography. The gel slices were immersed in standard recombination medium (Chen et al., 1992a) containing 100 mM tyramine-HCl, and gently shaken for 15 min at 30°C. They were then rinsed briefly with 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). DNA was extracted from these slices by standard procedures and fractionated by electrophoresis in 10% denaturing polyacrylamide gels (acrylamide to bisacrylamide, 19:1). The bands corresponding to unretracted substrate, cleavage product, and recombinant product were identified by autoradiography.

General Methods—Restriction enzyme digestions, isolation of plasmid DNA, and other miscellaneous procedures were done as described by Maniatis et al. (1982).

RESULTS

Since recombination is the sum of two sets of strand cleavage and joining events, each involving a pair of DNA strands, it is convenient to conceptually divide the recombinase tetramer functionally into a dimer of dimers (or two pairs of monomers). Two models for recombination may then be considered and are outlined in Fig. 1. These conform to the discovery that a Flp monomer does not cleave the scissile phosphodiester adjacent to it but cleaves that distal to it across the spacer (or strand exchange region) (Chen et al., 1992a, 1993; Lee et al., 1994).

In the "equal tetramer model" shown in Fig. 1A, recombination is carried out by two functionally equivalent pairs of monomers. We may imagine that the pair of monomers bound to one side of the spacer provide the pockets for strand cleavage and strand joining at that end. They receive the nucleophiles for cleavage (Tyr-343) in trans from the pair of Flp monomers bound across from them. Similarly, they receive the nucleophiles for strand joining (5'-hydroxyl groups) from the Flp-nicked DNA strands. Following formation of the Holliday intermediate, a conformational change within the recombination complex reverses the role of the monomer pairs. The pair of Flp monomers that were catalytically quiescent during the formation of the Holliday intermediate (except for donation of the active site tyrosine) now provide the active site pockets for the cleavage/joining chemistry that resolves the Holliday junction into mature recombinants. The pair of monomers that were initially active are now quiescent, and their role is limited to supplying the catalytic tyrosine residues.

In the "unequal tetramer model" (Fig. 1B), the functional contributions of each of the two pairs of protein monomers to recombination are nonequivalent. For example, one pair is specified to make nearly exclusive contributions toward assembling the strand cleavage pocket; this pair accepts the appropriately oriented Tyr-343 residues from the second pair. The latter pair contributes almost exclusively to building the strand joining pocket, except for donating the catalytic tyrosine residues for strand cleavage. This pair accepts the 5'-hydroxyl groups from Flp-nicked DNA as the active nucleophiles for the strand joining reaction. Since recombination calls for phosphoryl transfers performed at one end of the spacer to be repeated at the other, limiting the functions of each pair of Flp monomers to either cleavage or joining necessitates the translocation of protein monomers between two Flp binding elements (Fig. 1B). Variations of this model in which recombinase replacement occurs from solution rather than across binding elements may also be conceived.

Although the physical separation of the two binding elements of a recombination target site by two helical turns (21 base pairs from midpoint to midpoint) on the linear DNA would seem to preclude protein translocation, this is deceptive. In the Flp-occupied state, the binding elements are arranged virtually in tandem to each other by virtue of the large protein-induced DNA bend of 140° or more centered within the spacer (Schwartz and Sadowski, 1990; Chen et al., 1992b). Trans DNA cleavage by a Flp monomer and consequent covalent attachment of Flp to the phosphate located across the spacer from it may indeed trigger protein displacement. Thus the relative spatial disposition of the Flp monomers during recombination does not run counter to the possibility of switching protein partners between two binding elements. The idea of the swap cannot be discounted by energetic considerations either. Protein DNA contacts broken at one binding element would be

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FIG. 1. The "equal tetramer" (A) and "unequal tetramer" (B) models assigning functional roles to Flp monomers during one round of recombination. A, two full site substrates occupied by four Flp monomers are assembled in a synaptic complex. The Flp monomers at the left (stippled ovals) provide the catalytic centers for strand cleavage and for strand joining at the left to generate a Holliday junction. The active nucleophiles (Tyr-343 for cleavage and 5'-hydroxyl for joining) are derived from the Flp pair at the right (unfilled ovals) and Flp-nicked DNA, respectively. A reconfiguration of the recombination complex (isomerization?) reverses the functional roles of the left and right protein pairs, and thus reverses their catalytic contributions. The chemistry of strand cleavage and transfer is now repeated at the right end to resolve the Holliday junction. B, the two Flp monomers at the left (tyrosine acceptors) can only provide the strand joining pockets. Following strand cutting, the left and right protein pairs switch positions to effect strand joining. Two such swaps would be required for every round of recombination.
remade at the other, and the system would be essentially isoenergetic prior to and following the swap. Furthermore, recent experiments reveal that the loss of a single G-C contact is sufficient to destabilize a Flp monomer associated with a binding element and markedly increase its dissociation rate (Lee et al., 1994).

Although the scheme shown in Fig. 1 is simpler and hence more attractive than that in Fig. 1B, there is no strict experimental evidence that a single Flp monomer can mediate both the cleavage and the joining events required for one strand exchange. We describe below experimental strategies that attempt to distinguish between models 1A and 1B.

**A Half-site Substrate Containing one Flp Binding Element Associates with a Single Flp Monomer**—Our rationale was to design an experimental system in which the role of a given Flp monomer in a single cleavage/joining reaction could be addressed. A half-site substrate, initially designed for analysis of the integrase reaction (Nunes-Duby et al., 1989) and subsequently adapted for the Flp system (Qian et al., 1990; Amin et al., 1991; Serre et al., 1992), contains one recombinase binding element, one cleavage site, and one 5'-hydroxyl group that can act as a phosphoryl acceptor during strand joining. Furthermore, the chemistry involved in the cleavage/joining reaction within a half-site (see Fig. 4A) is equivalent to that required to generate one of the four recombinant strands resulting from a normal recombination event. When a half-site was bound by a Flp mutant lacking the active site tyrosine, Flp(Y343F), a single half-site-protein complex (Qian et al., 1990; Serre et al., 1992) could be identified by electrophoresis on a 10% native polyacrylamide gel (HS I; Fig. 2, lane 2). Higher order complexes (predominantly HS II; presumably two equivalents of HS I associated noncovalently) in addition to HS I were produced by wild type Flp (Fig. 2, lane 1). We inferred that HS I contained one Flp monomer associated with the unitary Flp binding element within the half-site from the experiment shown in Fig. 2B. Here a full site containing a pair of Flp binding elements was bound by Flp(Y343F) (Fig. 2B, lane 1) or by a hybrid protein obtained by fusion of Flp(Y343F) with glutathione S-transferase (Fig. 2B, lane 2) or by a mixture of Flp(Y343F) and GST-Flp(Y343F) in roughly equimolar amounts (lane 3, Fig. 2B). The two complexes formed by Flp(Y343F) are designated C IF and C IIF; the corresponding complexes formed by GST-Flp(Y343F) are labeled C IG and CIIG. In the reaction with the mixture of proteins, the complex II was split into three complexes in a roughly 1:2:1 molar ratio. In contrast, the complex I did not show the band split. The simplest explanation of these data is that complex I was formed by binding of a single protein monomer to the labeled substrate, and complex II was formed by binding of two protein monomers, one to each of the two binding elements. Thus complex II in the mixed reaction included the additional species, C IIF-G, representing the heterodimeric protein association on the DNA. The small amount of the band comigrating with the CIIF-G band in lane 2 can be accounted for by some free Flp(Y343F) produced by breakdown of the hybrid protein during purification. The one to one association between a Flp monomer and a Flp binding element...
The results are in agreement with those reported by Amin (1991) for binding reactions containing mixed half-sites. A half-site results in a unitary protein-DNA complex containing one half-site and one protein monomer. Furthermore, binding of Flp(Y343F) to a half-site results in a unitary protein-DNA complex containing one half-site and one protein monomer.

Half-site Recombination within a Gel-immobilized Protein-DNA Complex—Trapping of a half-site in association with a single recombinase monomer provides an enzyme-substrate complex in which the protein-DNA dynamics during one round of strand breakage/union steps can be analyzed. Recent experiments have shown that a half-site bound by the catalytically inactive mutant Flp(Y343F) can be induced to undergo cleavage by the tyrosine mimic, tyramine, added exogenously (Lee et al., 1993; see Fig. 4A). The resulting product in which the 3′-phosphate at the cleavage point is covalently linked to tyramine is a good substrate for the strand joining reaction. Can the same bound monomer of Flp(Y343F) facilitate both cleavage and joining in presence of tyramine? Following electrophoretic fractionation, the gel slice corresponding to HS I species formed by each of the two proteins and gave no indication of a hybrid complex (data not shown).

We also ensured that there was only one molecule of half-site per molecule of HS I by incubating two half-sites of different lengths (HSa and HSb in Fig. 3A), either separately or as an equimolar mixture with Flp(Y343F). Each half-site yielded a single HS I complex with Flp(Y343F) individually (HS Ia and HS Ib; Fig. 3, B and C, lanes 3 and 4), and in combination, they failed to yield a hybrid complex (Fig. 3, B and C, lane 5). Panel C represents a set of binding reactions similar to those in panel B, except that the time of electrophoresis was increased to obtain greater resolution of the HS Ia and HS Ib complexes. Even under these conditions, no trace of a hybrid complex was detected. With wild type Flp, The HS I pattern was similar to that obtained with Flp(Y343F) (Fig. 3, B and C, lanes 6–8). However, the Flp half-site dimer (HS II complex) was split into three components, corresponding to the predicted two homodimeric species (HS I-a-a and HS I-b-b; Fig. 3B, lanes 6 and 7) and one heterodimeric species (HS I-a-b; Fig. 3B, lane 8). These results are in agreement with those reported by Amin et al. (1991) for binding reactions containing mixed half-sites.

In summary, the half-site and full site binding results certify that each binding element within a Flp target site associates with one Flp monomer. Furthermore, binding of Flp(Y343F) to a half-site results in a unitary protein-DNA complex containing one half-site and one protein monomer.

FIG. 3. Binding reactions containing a mixture of two half-sites. Two half-sites, HSa and HSb, of different lengths were labeled at the 5′-end on the strands that are not cleaved by Flp by the kinase reaction. Phosphorylation ensures that recombination between the two half-sites or within a half-site (between the two strands) is not possible in the binding mixtures. The binding reactions were fractionated by electrophoresis in a 10% non-denaturing polyacrylamide gel. The duration of the run was adjusted such that the protein-DNA complexes of interest were well resolved. Under these conditions, the shorter of the two half-sites, HSa, migrated to the bottom of the gel. The lower portion of the gel showing the relative mobilities of the free substrates is displayed in panel A. The upper region of the gel where the protein-DNA complexes are located is shown in panel B. HS Ia and HS Ib refer to the monomeric half-site-Flp complexes. Homodimeric and heterodimeric configurations derived from the monomeric species are denoted by HS I-a-a, HS I-b-b and HS I-a-b. Panel C represents the HS I region of a gel in which a set of reactions was fractioned until the free substrates had run off the gel, thus increasing the resolution between HS Ia and HS Ib. Lanes 1 and 2 are incubation mixtures containing either HSa or HSb but no added protein. Lanes 3–5 and 6–8 represent binding reactions with Flp(Y343F) and wild type Flp, respectively. For each of the two proteins, lanes from left to right indicate reactions containing HSa alone (lanes 3 and 6), HSb alone (lanes 4 and 7), or a roughly equimolar mixture of HSa plus HSb (lanes 5 and 8). The bands seen above HS Ib in the wild type lanes are higher order Flp-half-site complexes.

implies that the smallest DNA-protein complex obtained with the half-site must contain one recombinase monomer bound to one half-site molecule. Consistent with this inferred molar stoichiometry, a binding reaction of the labeled half-site in Fig. 2A with an equimolar mixture of Flp(Y343F) and GST-Flp(Y343F) revealed only two discrete complexes corresponding to the HS I species formed by each of the two proteins and gave no indication of a hybrid complex (data not shown).

Strand Cleavage and Strand Joining within a Flp(Y343F)-Half-site Complex in the Presence of an Exogenous Nucleophile—The inference that a single Flp monomer can activate the target dieter during the two transesterification reactions of recombination was confirmed by the tyramine reaction carried out in solution. The rationale is based on the finding that a mutant of Flp altered at Tyr-343 and His-305, Flp(H305L,Y343F) can support neither strand cleavage (C) nor
strand joining (P) in a half-site in the presence of added tyramine (Fig. 5A, lane 2). The labeled half-site was prebound with Flp(Y343F) on ice for 10 min. After the addition of 30°C, Flp(Y343F,H305L) was added in molar ratios of 1, 2, 4, and 8 (with respect to Flp(Y343F)) to the reaction together with tyramine (Fig. 5B). The cleavage and strand transfer products were assayed after a 15-min incubation period at 30°C. The amounts of strand transfer product obtained was approximately the same irrespective of the absence of the double mutant (Fig. 5A, lane 3) or its presence in up to an 8-fold molar excess relative to Flp(Y343F) (Fig. 5B, lane 4). The slight decrease in the product band in lane 4 of Fig. 4B is best explained by partial replacement of Flp(Y343F) by the Flp double variant prior to strand cleavage. Densitometric quantitation of bands P and C normalized to HS (from appropriately exposed autoradiograms) revealed the same ratio of strand transfer product to cleavage product in all of the lanes. Preferential protein replacement following strand cleavage should have skewed this ratio in favor of the cleavage product. Similar results were obtained when the prebound complex was mixed with up to nearly a 250–500-fold molar excess of the cold substrate and concomitantly treated with tyramine (Fig. 5C, lanes 1–5). By contrast, in reactions where the two proteins were mixed in the same molar ratios as in Fig. 5B and reacted with the labeled substrate in the presence of tyramine, there was a progressive decrease in the amounts of the cleavage and strand transfer product (Fig. 5D, lanes 1–4). These results corroborate the notion that, following strand cleavage by tyramine, there is no dissociation of Flp(Y343F) from the labeled half-site prior to strand joining. If there had been protein dissociation, the excess double mutant should have occupied the emptied sites (Fig. 5B), or the free protein should have been titrated out by the excess cold half-site (Fig. 5C), inhibiting strand transfer within the labeled half-site in either case.

The above experiment does not exclude the possibility that between the cleavage and joining steps there might still have been protein transplacement at a half-site by a monomer bound to a second half-site molecule rather than by one from solution. For example, in the unequal tetramer model shown in Fig. 1B, protein exchanges are depicted as occurring from within the DNA-occupied state. To test the validity of this idea, the competition experiment shown in Fig. 5 was modified as follows (see Fig. 6). The labeled half-site bound by Flp(Y343F) was challenged with an excess of the unlabeled half-site prebound by Flp(Y343F,H305L) at the same time that tyramine was added to the reaction. The cleavage-joining reaction was not significantly inhibited over a 160-fold excess of the competitor protein-DNA complex (compare the reactions shown in Fig. 6, lanes 4–8, to that in Fig. 6, lane 3).

Thus the tyramine-aided half-site strand cleavage and exchange mediated by a monomer of Flp(Y343F) is insensitive to challenge by free DNA, free protein, or DNA-associated protein. We conclude that the bound Flp(Y343F) monomer stays
attached to half-site through the two chemical steps of the reaction and does not dissociate from it in between.

**DISCUSSION**

In the results reported here, we have used a half-site substrate associated with a single monomer of a Flp variant lacking the active site tyrosine to explore the mechanism of recombination. We show that a single monomer can provide the active site for the strand cleavage and strand joining steps that yield one recombinant DNA strand. The cleavage step is not followed by dissociation of the recombinase monomer and the assembly of strand joining pocket from a second recombinase monomer. Thus a Flp monomer can orient the scissile phosphodiester in DNA for attack by the phenylade nucleophile derived in trans (either from Tyr-343 of a second Flp monomer during the normal reaction or from tyramine in the simulated reaction). The same monomer can then orient the phosphodiester formed between DNA and tyrosine (or tyramine) during cleavage for attack in trans by the nucleophile derived from the 5'-hydroxyl of the Flp-cleaved DNA. Our current data therefore argue against the scheme proposed in Fig. 1B. They strongly support the "cis activation/trans nucleophilic attack" paradigm for recombination (Lee and J ayaram, 1993), in which the target phosphodiester(s) are oriented in cis by Flp bound next to them, and reactive nucleophiles are then supplied in trans for executing the chemical steps. Results from the present study are consistent with and extend previous analyses of half-site reactions using step arrest Flp variants (Chen et al., 1992a; Pan et al., 1993). However, the earlier studies did not rigorously establish that the two chemical steps of a single recombination event can be carried out by one and the same Flp monomer.

The half-site strand transfer reaction is mechanistically faithful to the normal full site reaction. The rules for assembling the Flp active site by sharing of catalytic residues between protein monomers are the same in half-sites and in full sites (Whang et al., 1994). The trans mode of DNA cleavage initially demonstrated in half-sites (Chen et al., 1992a; Yang and J ayaram, 1994) holds true for full sites as well (Lee et al., 1994). Similarly, the sensitivity of the strand joining step to the presence of adjacent base complementarity within the spacer segment is identical in half-sites and in full sites (Lee and J ayaram, 1995). We feel justified, therefore, in extrapolating the half-site results into a simple mechanistic scheme for the apparently complex phosphoryl transfers required to complete recombination between two double-stranded DNA partners. The reaction follows the path presented in Fig. 1A. In this model, the two Flp monomers to the left make the principal active site contributions in cis for the cleavage/joining reactions that generate the Holliday intermediate, and they utilize the chemical reactivity of appropriate nucleophiles derived in trans. In repeating the phosphoryl transfer steps at the other end of the spacer to resolve the Holliday junction, the two Flp monomers to the right take over the catalytic functions, effectively switching roles with their left partners. We propose that this role reversal is associated with an isomerization step, perhaps involving some branch migration of the Holliday junction. We suggest that the molecular dynamics associated with this event operate on the recombination complex as a whole with no need for dissociation of recombinase monomers from their sites of occupancy. The figure is not meant to imply that the reaction takes place with a defined order of strand exchanges, initiated at the left and terminated at the right. In principle, the reaction could well be initiated at the right and terminated at the left. Thus, although there is functional symmetry of the four Flp monomers in the context of a complete recombination event, there is clear asymmetry between pairs of Flp monomers during each of the two cleavage/exchange reactions. How this asymmetry is imparted to the system remains an interesting and open question.

If a single Flp protomer has the potential for providing the active sites for strand cleavage and strand joining, how are they accommodated within the protein structure? Given the structural constraints of DNA, the two transphosphorylation steps that complete one cleavage/joining event must take place in close proximity. Hence, if the reactions are accomplished by two active sites that are physically removed from each other, there must be significant protein movement relative to DNA to disengage the cleavage pocket and engage the joining pocket. On the other hand, given the mechanistic similarity of strand cleavage and strand joining, a single, composite active site may carry out the breakage and union reactions. We favor the single active site hypothesis. It is conceptually simple and is easily accommodated by the cis activation/trans nucleophilic attack model. It is also consistent with the step arrest phenotypes of Flp variants altered at the invariant integrase family residues (Arg-191, His-305, Arg-308, and Tyr-343). Two of the tetrad residues, Arg-191 and Arg-308, contribute to strand cleavage as well as strand joining; both reactions are abolished in the absence of either of the two residues (Parsons et al., 1988, 1990; Lee et al., 1992; Yang and J ayaram, 1994). On the other hand, Tyr-343 is required only to provide the nucleophile for cleavage (Evans et al., 1990); it is dispensable in the joining reaction (Pan and Sadowski, 1992; Lee and J ayaram, 1993). Similarly, His-305 is not essential for cleavage in full sites (Parsons et al., 1988), but it is essential for joining in full sites and half-sites (Parsons et al., 1988; Lee and J ayaram, 1993; Pan et al., 1993; Yang and J ayaram, 1994). However, contrary to the expecta-
tion from full site assays, little or no half-site cleavage occurs in the absence of His-305. A satisfactory explanation for this apparent contradiction may have to wait till more is known about the structures of Flp-half-site and Flp-full site complexes. It is possible that the requirement of His-305 at the cleavage step may be fulfilled by another residue in the full site complex, but not in the half-site complex. Nevertheless, the identification of some catalytic residues that are common to the cleavage and strand transfer steps (Arg-191 and Arg-308), and of others that are unique to each of the two steps (Tyr-343 for cleavage and perhaps His-305 for joining) blends nicely with the idea of one composite active site. Within this active site, the required conformational flexure to switch from the cleavage mode to the joining mode may be minimal and may occur concomitant to the cleavage reaction itself. It is likely that the chemical nature and relative dispositions of the target diester (the scissile DNA phosphodiester or the 3'-phosphotyrosyl bond) and of the active nucleophile (Tyr-343 or 5'-hydroxyl group) may stabilize the functional configuration of the strand cleavage and joining pockets via induced fit. The observation that a Flp monomer can bind to a precleaved substrate and mediate strand transfer within it (Pan and Sadowski, 1992; Lee and Jayaram, 1993) would be consistent with this notion.

In summary, by using a combination of the half-site substrate, step-arrest Flp mutants, and an active site nucleophile mimic, we have provided a model that assigns functional roles to individual recombinase monomers during the chemical steps involved in one round of recombination.

APPENDIX

Direct Estimation of DNA-Protein Stoichiometry in Flp-DNA Complexes

The binding reactions reported here using two DNA substrates of different lengths and Flp(Y343F) or, conversely, a single DNA substrate and two Flp(Y343F) variants of different molecular masses are consistent with the interpretation that the DNA-protein complexes identified by gel electrophoresis have a molar stoichiometry of no more than one Flp monomer per Flp binding element on the DNA. We have confirmed this inference by a direct estimation of the protein and DNA contents of these complexes by using a method described by Mack et al. (1992) with some modifications. In our assays we estimated protein amounts by digital analysis of bands stained with Coomassie Brilliant Blue rather than by the Western blot method employed by Mack et al. (1992). Binding reactions were done with a full site or a half-site DNA substrate (Fig. 7) in which one strand was end-labeled with 32P. Each substrate was prepared by mixing 0.5 pmol of labeled plus 199.5 pmol unlabeled top strand (assuming one A260 unit = 33 μg/ml single-stranded DNA) with a 3-fold excess (600 pmol) of the bottom strand in the hybridization medium. Under these conditions, virtually all of the single strand was converted to the double-stranded form. All DNA estimates were based on the specific radioactivity of the labeled strand of the input substrate. Concentration of Flp(Y343F) in the preparation used in the binding assays was estimated using a molar extinction coefficient, E280 = 6.0 × 10^4 M^-1 cm^-1, derived by adding the tyrosine, phenylalanine, and tryptophan extinctions from the predicted amino acid composition of Flp. The intensities of the Coomassie Brilliant Blue-stained bands obtained after SDS-polyacrylamide electrophoresis of a set of Flp(Y343F) standards produced a linear curve in the concentration range relevant to the test samples (see Figs. 7 and 8). The reliability of this method of protein estimation was verified by applying it to a >95% pure preparation of the transposase protein of phage Mu (gift from J.-Y. Yang and R. M. Harshey), whose theoretical molar extinction coefficient E280 was calculated as = 1.2 × 10^5 M^-1 cm^-1 (data not shown).

After incubating the DNA substrate and Flp(Y343F) under conditions that convert over 50% of the half-site and full site

![Fig. 7. Electrophoretic refractionation of Flp(Y343F) and DNA from gel-isolated half-site complex HS I and full site complex C IIF.](http://www.jbc.org/)

A, the gel-isolated HS I complexes obtained with the half-site substrate (one Flp binding element) from six separate binding reactions were re-fractionated in a 10% SDS-polyacrylamide gel. Standard mixtures of Flp(Y343F) and the half-site DNA fractionated under the same conditions are displayed below. Protein bands were visualized by Coomassie Brilliant Blue staining; DNA bands were revealed by autoradiography. B, the gel-isolated complexes C IIF formed by the full site substrate (two Flp binding elements) from six independent binding reactions plus the corresponding standards were treated as described in A.
into the complexes HS I and C IIF (see Fig. 2), respectively, the reactions were fractionated in native polyacrylamide gels. The complexes HS I and C IIF were identified by autoradiography. The gel slices corresponding to them were carefully excised, placed in the wells of 10% SDS-polyacrylamide gels, and refractionated by electrophoresis at approximately 8 V/cm to separate the protein and DNA components (Laemmli, 1970). Mixtures of standard aliquots of Flp(Y343F) and the DNA substrate (the half-site or the full site) were fractionated simultaneously on identical gels to provide the reference for DNA and protein quantitation. After the run, the gels were cut into DNA and protein sections (the DNA migrates well ahead of the protein during electrophoresis). The gel sections containing protein were stained with Coomassie Brilliant Blue and photographed, whereas those containing DNA were dried and subjected to autoradiography. The protein and DNA bands were quantitated from the negatives and x-ray films, respectively, using Image 1.41 software (NIH).

The protein and DNA profiles obtained upon fractionation of the isolated HS I and C IIF complexes formed by the half-site and full site substrates, respectively, are shown in Fig. 7, A and B (top). The corresponding patterns for the standard DNA/protein mixtures are also displayed (Fig. 7, A and B, bottom). The data from Fig. 7 are quantitatively represented in Fig. 8. The average molar ratio of Flp(Y343F) to the half-site (one Flp binding element) in the HS I complex derived from six independent binding reactions was 0.73 ± 0.09. The equivalent ratio for the full site (two Flp binding elements) in the C IIF complex was 1.95 ± 0.16. These values are most consistent with the interpretation that, in both HS I and C IIF, one monomer of Flp(Y343F) is present per one Flp binding element.

In summary, we have shown by direct protein and DNA measurements that there is a one to one association between Flp(Y343F) and the DNA element to which it binds. Combined with our demonstration that the HS I complex formed by Flp(Y343F) and trapped within polyacrylamide can be induced to undergo the chemistry of recombination by providing tyramine (the nucleophile mimic of the missing tyrosine; see Fig. 4), this result strongly supports the hypothesis that the protein unit that orients the phosphodiester for nucleophilic attack during strand cleavage is indeed a monomer of Flp. Thus one round of recombination between two DNA partners, requiring four strand breakage/joining events, is likely mediated by a tetramer of DNA-bound Flp (as depicted in the model of Fig. 1A). It is unnecessary to invoke the possibility that additional interactions between substrate-associated Flp and Flp from solution may contribute to the reaction.

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Fig. 8. Molar stoichiometry of Flp(Y343F) to Flp binding element in HS I and C IIF. The standard graphs for DNA and protein were prepared from digital image analysis of the data shown in Fig. 7A. The protein and DNA contents within HS I and C IIF were derived by using these graphs. The Flp(Y343F)-DNA stoichiometry calculated for each binding reaction is tabulated. The mean value from the six estimates together with the standard deviation is shown.
Catalytic Contributions of Flp Monomers in Recombination

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