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Mechanism of Cleavage and Ligation by FLP Recombinase: Classification of Mutations in FLP Protein by In Vitro Complementation Analysis

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The FLP recombinase of the 2μm plasmid of *Saccharomyces cerevisiae* is a member of the integrase family of site-specific recombinases. Recombination catalyzed by members of this family proceeds via the ordered cleavage and religation of four strands of DNA. Although the amino acid sequences of integrase family members are quite different, each recombinase maintains an absolutely conserved tetrad of amino acids (R-191, H-305, R-308, Y-343; numbers are those of the FLP protein). This tetrad is presumed to reflect a common chemical mechanism for cleavage and ligation that has evolved among all family members. The tyrosine is the nucleophile that causes phosphodiester bond cleavage and covalently attaches to the 3′-PO_4 terminus, whereas the other three residues have been implicated in ligation of strands. It has recently been shown that cleavage by FLP takes place in *trans*; that is, a FLP molecule binds adjacent to the site of cleavage but receives the nucleophilic tyrosine from a molecule of FLP that is bound to another FLP-binding element (J.-W. Chen, J. Lee, and M. Jayaram, Cell 69:647–658, 1992). These studies led us to examine whether the ligation step of the FLP reaction is performed by the FLP molecule bound adjacent to the cleavage site (ligation in *cis*). We have found that FLP promotes ligation in *cis*. Furthermore, using in vitro complementation analysis, we have classified several mutant FLP proteins into one of two groups: those proteins that are cleavage competent but ligation deficient (group I) and those that are ligation competent but cleavage defective (group II). This observation suggests that the active site of FLP is composed of several amino acid residues from each of two FLP molecules.

The FLP gene of the 2μm circle plasmid of *Saccharomyces cerevisiae* encodes a conservative site-specific recombinase (FLP) that is involved in amplification of the copy number of the plasmid (8). The FLP protein is a member of the integrase family of recombinases, whose members share a tetrad of conserved residues (arginine 191, histidine 305, arginine 308, tyrosine 343; numbers represent amino acid numbers of FLP [1, 5]). These conserved amino acids are thought to underlie a common mechanism of strand cleavage and ligation catalyzed by all integrase members.

In vitro studies have identified tyrosine 343 as the nucleophile that catalyzes cleavage of the DNA strands (9). The tyrosine covalently attaches to the 3′-PO_4 at the site of breakage and acts as the leaving group for the rejoining of the DNA strands (ligation), a process that involves the arginines at positions 191 and 308 and histidine 305 (10, 15, 16). The ligation step has recently been shown to occur independently of strand cleavage (14).

The FLP recognition target sequence contains three 13-bp symmetry elements that surround an 8-bp core region (Fig. 1). FLP binds to each of the symmetry elements and cleaves and covalently attaches to the top or bottom strands adjacent to the core region (3, 4, 6). We have recently developed assays to detect strand ligation independently of cleavage (13, 14). We therefore examined whether the FLP bound adjacent to the site of strand cleavage was the active moiety in ligation (ligation in *cis*). In contrast to the recent results of Chen et al. (7), who showed that cleavage takes place in *trans*, we found that FLP catalyzed ligation in *cis*. We also analyzed several mutant FLP proteins by an in vitro complementation test to determine which altered proteins were capable of strand cleavage and which were ligation proficient. These tests suggest that the ligation and cleavage functions of FLP lie in separate regions of the protein and identify amino acid residues that comprise the active site.

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MATERIALS AND METHODS

FLP preparations. Wild-type and mutant FLP proteins were partially purified as described previously (12). The purity of the FLP protein was approximately 15 to 50%, and the concentration was estimated by comparison with highly purified FLP protein on Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. FLP genes bearing the mutations H305L, R308K, and Y343F were obtained from M. Jayaram. (The altered FLP proteins are named as follows: the amino acid present in the wild-type protein [expressed in single-letter notation] is followed by the amino acid number, followed by the amino acid present in the mutationally altered protein. Thus, FLP H305L means that the histidine at position 305 was changed to a leucine.) The other mutations in the FLP gene were isolated in our laboratory (10, 12, 19).

Substrates. The *b* half-FRT (FRT = FLP recognition target) site was assembled by annealing two partially complementary synthetic oligodeoxyribonucleotides of the following sequences:

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1, 5′ GAG GAG TCT GAA GTT CCT ATT CTC T   3′
2, 3′             A CTT CAA GGA TAA GAG A TCT TT 5′
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The *a* half-site was assembled in a similar manner from two oligonucleotides of the following sequences:

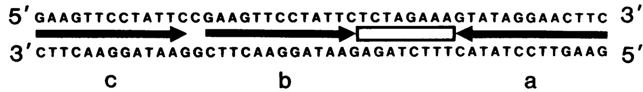
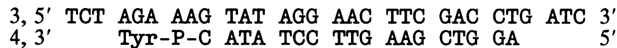


FIG. 1. Diagram of the FRT site. The FRT site is characterized by three 13-bp symmetry elements that are indicated by heavy arrows (labeled a, b, and c). Elements *a* and *b* are in inverted orientation and separated by an 8-bp core region (open box). Elements *b* and *c* are in direct orientation.


3, 5′ TCT AGA AAG TAT AGG AAC TTC GAC CTG ATC 3′
4, 3′ Tyr-P-C ATA TCC TTG AAG CTG GA 5′

Oligonucleotide 4 containing a 3′-phosphoryl tyrosine was synthesized by R. Brousseau and C. Juby at the Biotechnology Research Institute, Montreal, Quebec, Canada, as described previously (13). The other oligonucleotides were synthesized by the Carbohydrate Research Centre, Faculty of Medicine, University of Toronto. The 5′ termini of oligonucleotides 1 and 4 were labeled with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs) and annealed to the unlabeled complementary oligonucleotide as described previously (13).

Complementation assays to demonstrate ligation in *cis*. Each half-site (0.05 pmol of molecules) was preincubated with the appropriate mutant FLP protein for 10 min at 23°C in a 25-μl reaction mixture containing 50 mM Tris-Cl (pH 7.4), 33 mM NaCl, 1 mM EDTA, and 40 μg of sonicated and denatured calf thymus DNA per ml. The two complementing reaction mixtures were then mixed, and incubation was continued for a further 0 to 30 min. Reactions were terminated by addition of 3.5 μg of proteinase K (Boehringer) and 0.004% (wt/vol) SDS and incubated at 23°C for 10 min. The reactions were concentrated by evaporation and analyzed on an 8% denaturing polyacrylamide gel. Where indicated (Fig. 2), a fivefold excess of unlabeled half-site was added prior to mixing of the two reactions. In control experiments, one or both half-sites were incubated with a single protein for 25 min.

In vitro complementation test of mutant FLP proteins. The assay was done in two different ways. In one protocol, both half-sites were incubated concurrently with the FLP protein(s). The reaction mixture (25 μl) contained 50 mM Tris-Cl (pH 7.4), 33 mM NaCl, 1 mM EDTA, 40 μg of sonicated and denatured calf thymus DNA per ml, 0.015 pmol (of molecules) of each half-site substrate, and 0.03 to 1.8 μM FLP proteins, as specified in the figure legends. The reaction mixture was incubated at 23°C for 15 min and then at 30°C for 40 min. Reactions were terminated by addition of 0.004% SDS and 5 μg of proteinase K. After 30 min at 37°C, the reactions were concentrated by evaporation and analyzed on an 8% denaturing polyacrylamide gel. Alternately, each half-site was preincubated with one FLP protein for 15 min at 23°C in a 25-μl reaction mixture identical in composition to that described above. The two reaction mixtures were then combined and incubated at 30°C for 40 min. Reactions were terminated and analyzed as described above.

Ligation assay. The method described by Kulpa et al. (12) was used.

RESULTS

FLP promotes ligation in *cis*. The FLP protein binds to each of the symmetry elements of the FRT site. After synapsis of two FRT sites, cleavage and exchange of homologous strands occurs. The cleavage step involves formation of a covalent FLP-DNA intermediate. We have recently developed assays that permit the measurement of FLP-induced ligation that occurs independently of DNA strand cleavage (13, 14). We therefore wished to examine whether the FLP that was bound immediately adjacent to the cleavage site was the active moiety in ligation (ligation in *cis*). We therefore used an in vitro complementation test similar to that used by Chen et al. (7), who used this assay to show that FLP induced cleavage in *trans*.

The rationale of the experiment is shown in Fig. 2. In Fig. 2A, the *b* half-site is preincubated with a mutant FLP protein that is incapable of cleavage but is competent for ligation (FLP Y343F) and mixed with the *a* half-site that has been preincubated with a cleavage-competent but ligation-defective mutant FLP protein (FLP H305L). After the two half-sites are brought together by protein-protein interactions (cross-core dimerization) (2, 18), *trans* cleavage of the top strand of the *b* half-site occurs, liberating the trinucleotide TCT and leaving FLP covalently attached to the 3′-phosphate group via tyrosine 343. The tyrosine group involved in this activation event has been contributed by the FLP H305L that is bound to the *a* half-site. The bottom strand of the *a* half-site contains a 3′-phosphotyrosine so that it can also participate in ligation (Fig. 2A, middle [13]). Since only FLP Y343F which is bound to the *b* half-site is capable of ligation, it is possible to determine whether ligation occurs in *cis* (i.e., next to symmetry element *b*) or in *trans* (next to symmetry element *a*). If ligation occurs in *cis*, FLP Y343F will promote ligation of the top strand of the *b* half-site to either the top strand of the *a* half-site (52 nucleotides [nt]) or to the bottom strand of the *b* half-site (44 nt, bottom left). On the other hand, if ligation occurs in *trans*, then ligation products will result from ligation of the two bottom strands (40 nt) or intramolecular ligation of the strands of half-site *a* (48 nt; Fig. 2A, bottom right). Conditions are chosen such that the substrates are saturated with the respective FLP protein.

The reciprocal experiment is shown in Fig. 2B. Here the binding of the proteins to the half-sites is reversed. FLP H305L is bound to the *b* half-site, and FLP Y343F is bound to the *a* half-site. Although dimerization of half-sites can occur, no strand cleavage of the top strand of the *b* half-site can occur, since cleavage occurs in *trans* and the FLP Y343F protein lacks the active-site tyrosine. *cis* ligation by Y343F results in the joining of the bottom strands only. The formation of the intramolecular ligation product is prevented by 5′-phosphorylation of the top strand of the *a* half-site (Fig. 2B, bottom left). Since the top strand of the *b* half-site cannot participate in ligation, *trans* ligation by FLP Y343F would give no products.

As controls, we first monitored the activity of each mutant protein separately. The expected ligation products were seen when single half-sites were incubated separately with each mutant protein (Fig. 3). Incubation of half-site *a* with the FLP Y343F protein gave an intramolecular ligation product of 48 nt (lane 23), whereas the FLP H305L protein was clearly ligation defective (lane 25). When both half-sites were incubated simultaneously with the FLP Y343F protein, both intramolecular and intermolecular ligation products involving the bottom strand of the *a* half-site were formed (lane 27).

We then used the strategy described above to test for *cis* or *trans* ligation. When the FLP Y343F protein was preincubated with the *b* half-site and the FLP H305L protein was preincubated with the *a* half-site, the resultant mixture yielded significant amounts of ligation products derived from the top strand of the *b* half-site (Fig. 3, lanes 1 to 5, strands

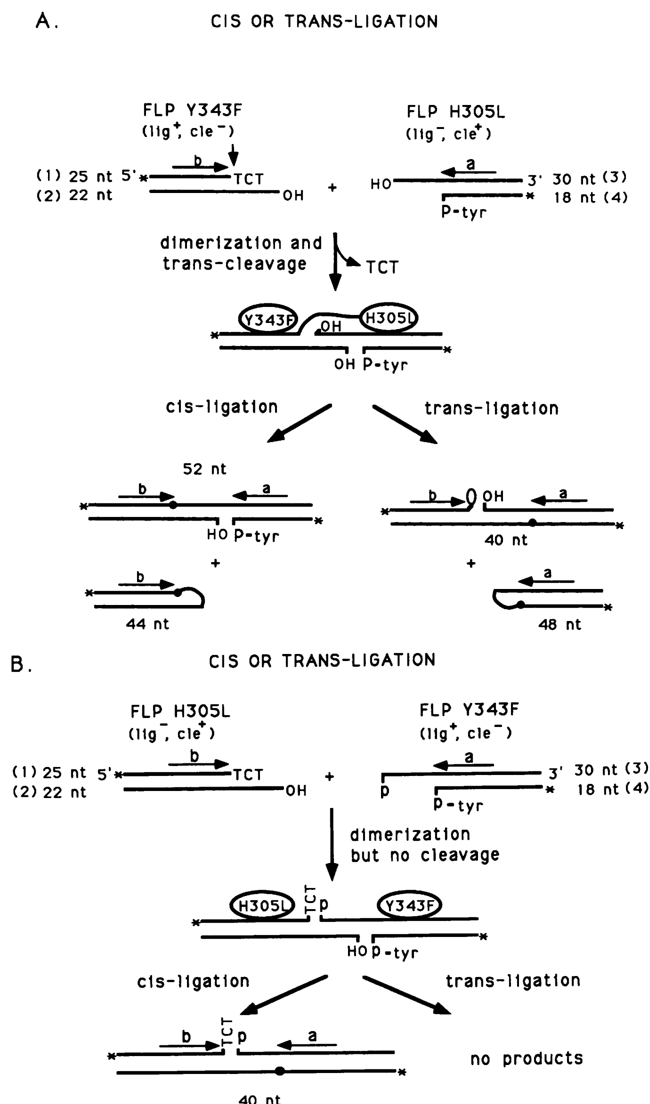


FIG. 2. Rationale for determining whether FLP ligation occurs in *cis* or in *trans*. (A) FLP Y343F is bound to the *b* half-site, and FLP-H305L is bound to the *a* half-site (top). The cleavage and ligation properties of these mutant proteins are indicated in parentheses. The *b* half-site contains a cleavage site (small vertical arrow) on the top strand at the junction between the *b* symmetry element and 3 nt of the core sequence. The bottom strand contains the entire 8 nt of the core. The *a* half-site contains the *a* symmetry element, an 8-nt single-stranded core, and a 3'-phosphotyrosine group on the bottom strand. Upon mixing of the bound substrates, dimerization occurs and FLP H305L mediates *trans* cleavage and covalently attaches at the cleavage site of the *b* half-site (middle). If the ligation mediated by FLP Y343F occurs in *cis* (bottom left), the lengths of the ligation products are 52 and 44 nt. The 52-nt product results from ligation of the two top strands of each of the two half-sites. The 44-nt product is a hairpin that results from the ligation of the cleaved *b* half-site. If ligation occurs in *trans* (bottom right), the ligation products are 40 and 48 nt. The 40-nt product results from ligation of the two bottom strands of each of the two half-sites. The 48-nt product is a hairpin that results from the ligation of the *a* half-site. The solid dots indicate phosphodiester bonds ligated, and the small open oval indicates covalently attached FLP protein. The asterisks indicate ^{32}P-labeled 5' ends. The strands are numbered 1 to 4 in parentheses. The *trans* cleavage by the H305L bound to half-site *a* (middle) is shown in the *trans*-horizontal configuration as defined by Chen et al. (7). However, it could equally well be in the *trans*-vertical or *trans*-diagonal mode as defined by those authors. The same applies to Fig. 4A and C. (B) FLP H305L is bound to the *b* half-site, and FLP Y343F is bound to the *a* half-site (top). The cleavage and ligation properties of the mutant proteins are indicated as in panel A. The *b* half-site is as described in panel A. The *a* half-site is as in panel A except that the top strand has a 5'-phosphate group instead of a hydroxyl. Upon mixing of the bound substrates, dimerization occurs. However, no cleavage occurs because FLP Y343F that is positioned *trans* to the cleavage site is cleavage incompetent (middle). If ligation mediated by FLP Y343F occurs in *cis* (bottom left), the ligation product is 40 nt in length. If ligation occurs in *trans* (bottom right), no products result because no cleavage of the *b* half-site has occurred.

1 + 3 [52 nt] and strands 1 + 2 [44 nt]). These results confirm that cleavage of strand 1 occurred in *trans* and demonstrated that ligation occurred in *cis*. However, there were also appreciable amounts of the ligation products derived from strand 4 (bottom strand of half site *a*, strands 2 + 4 and 3 + 4). To determine whether the products resulted from replacement of the FLP H305L protein bound to the *a* half-site by the Y343F protein, an excess of unlabeled half-sites was added to compete for any Y343F protein that may have dissociated from the half-site or that was free in solution. In this case (Fig. 3, lanes 11 to 15), the products derived from strand 4 (strands 3 + 4 and 2 + 4) were substantially reduced, whereas those derived from strand 1 (strands 1 + 3 and 1 + 2) were unchanged. Two unexpected products appeared upon addition of the unlabeled competitor half-sites: the 52-nt (1 + 3) product (Fig. 3, lanes 16 to 20) and the 40-nt (2 + 4) product (Fig. 3, lanes 11 to 15). Since these products cannot be competed for, they may have arisen via an exchange of the FLP molecules between one half-site and the other. The 2 + 4 product could also have arisen via a small amount of *trans* ligation by the Y343F protein bound to the *b* half-site. Because a similar mechanism could not account for the appearance of the 1 + 3 product, we tend to favor the former explanation. Experiments are in progress to determine whether exchange of subunits plays a mechanistic role in the FLP recombination reaction. We conclude that the majority of the products derived from strand 4 (lanes 1 to 5) arose as a result of binding of free Y343F to the *a* half-site.

When the mutant proteins were bound to the reciprocal half-sites (i.e., FLP H305L bound to half-site *b* and FLP Y343F bound to half-site *a*; Fig. 2B), most of the product was derived from ligation of the two bottom strands (2 + 4; lanes 6 to 10), consistent with FLP Y343F promoting ligation in *cis*. The small amount of intramolecular ligation product of strand 1 is indicative of cleavage of strand 1 in *trans* and is likely due to exchange of the FLP that was bound to the half-sites or that was free in solution. Competition using an excess of cold half-site reduced the amount of the 44-nt product (strands 1 + 2). Detectable amounts of the 52-nt product (strands 1 + 3) arose because the top strand of the cold *a* half-site was not phosphorylated and could participate in the reaction (Fig. 3, lanes 16 to 20). These results are compatible with a mechanism of ligation in *cis*; i.e., the molecule of FLP that is bound adjacent to the site of cleavage is active in ligation (Fig. 2A).

Classification of mutant FLP proteins by complementation in vitro. The foregoing experiments clearly demonstrate that cross-core dimerization of half-sites bound by different mutant FLP proteins allows a molecule of phenotype Cle$^-$ (cleavage defective) Lig$^+$ (ligation competent) and a Cle$^+$ Lig$^-$ molecule to complement each other in carrying out a cleavage-ligation reaction. We have taken advantage of this

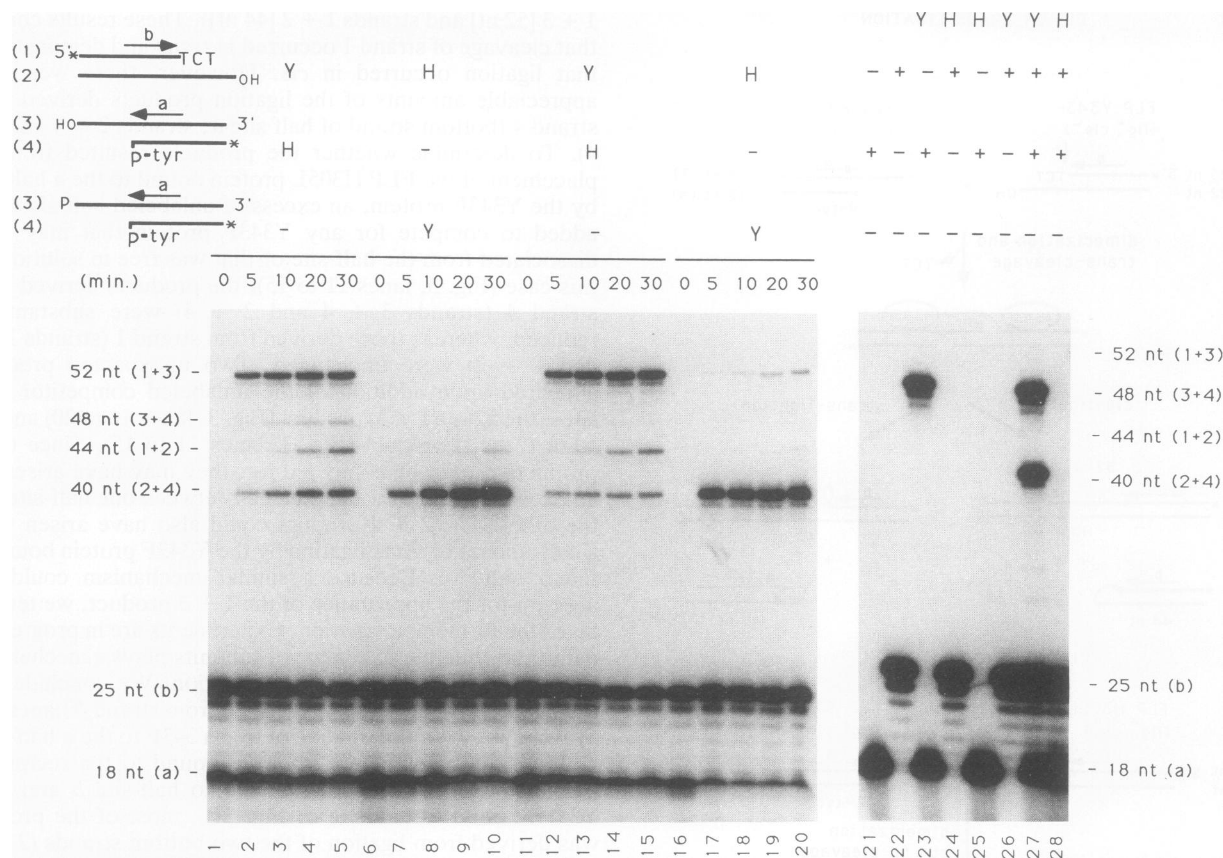


FIG. 3. FLP-induced ligation in *cis*. The reaction conditions are described in Materials and Methods. The half-sites used are diagrammed at the top left. The asterisk indicates the ^{32}P-labeled 5' end. The FLP proteins are indicated as Y (FLP Y343F, 0.08 μM) or H (FLP H305L, 0.21 μM) and are adjacent to the half-site with which they were preincubated (10 min at room temperature). After mixing of the two half-sites, samples were removed at 0 to 30 min as indicated. The reactions were terminated as described in Materials and Methods. The samples were analyzed on an 8% denaturing polyacrylamide gel. A fivefold excess of unlabeled competitor half-site was added to the reactions in lanes 11 to 20 after the preincubation period. The lengths of the substrates and products are indicated at the sides, together with the numbers of the strands from which they were derived (i.e., 1 + 3 was derived from ligation of strands 1 and 3, etc.). In lanes 21 to 28, one or both half-sites were incubated together with the FLP protein indicated, without any preincubation step.

complementation assay to monitor the behavior of various FLP mutants (10, 12, 15, 16, 17, 19) to determine whether they resembled either the FLP H305L protein or the Y343F protein in the cleavage and ligation phenotypes. In this way, we hoped to identify portions of the FLP protein required in *trans* for cleavage and in *cis* for ligation.

We had previously showed the usefulness of such an approach to uncover the ability of the R191K FLP to engage in formation of cross-core dimers with the Y343F protein (10). Thus, it was possible that these assays might uncover latent activities of mutant FLP proteins that were not apparent with previous assays. The rationale of this complementation test is shown in Fig. 4. In Fig. 4A, a mutant protein (X) that resembles H305L in being a cleavage-competent but ligation-defective protein is bound to half-site *a* and promotes *trans* cleavage. The expected products of *cis* ligation by Y343F are 52 and 44 nt. When the same protein is bound to the *b* half-site (Fig. 4B) and incubated with Y343F bound to the *a* half-site, no *trans* cleavage of the *b* half-site by the Y343F protein bound at the *a* half-site can occur, so that only *cis* ligation involving the bottom strand of the *a* half site is seen (strands 2 + 4 and 3 + 4). A mutant protein with properties similar to those of the H305L protein will produce no ligation products when complemented with FLP H305L (Fig. 4C). A mutant protein with properties similar to the FLP Y343F (i.e., ligation-competent but cleavage-defective) protein will not promote cleavage of the top strand of symmetry element *b*, and hence the only ligation products will be derived from the bottom strand of symmetry element *a* (Fig. 4D).

The altered proteins studied contained single amino acid changes in residues that are absolutely conserved among FLP-like proteins from six species of yeasts (12, 22). They include proteins bearing changes in each of the four conserved residues of the integrase family members (1, 5).

A typical complementation experiment is shown in Fig. 5, in which the FLP R308K protein was complemented with both FLP H305L and Y343F proteins. Of the three proteins, only the Y343F protein had ligation activity when assayed with the *a* half-site (lanes 2, 4, and 6). None of the three proteins had any activity when incubated with the *b* half-site alone (lanes 1, 3, and 5). Control experiments showed that wild-type FLP and a mixture of R308K and Y343F proteins produced all four expected products when incubated together with both of the half-sites (lanes 13 and 14), that the R308K and H305L proteins produced no products (lanes 15, 17, and 18), and that Y343F protein gave ligation products of strand 4 (3 + 4 and 2 + 4; lane 16). When the respective

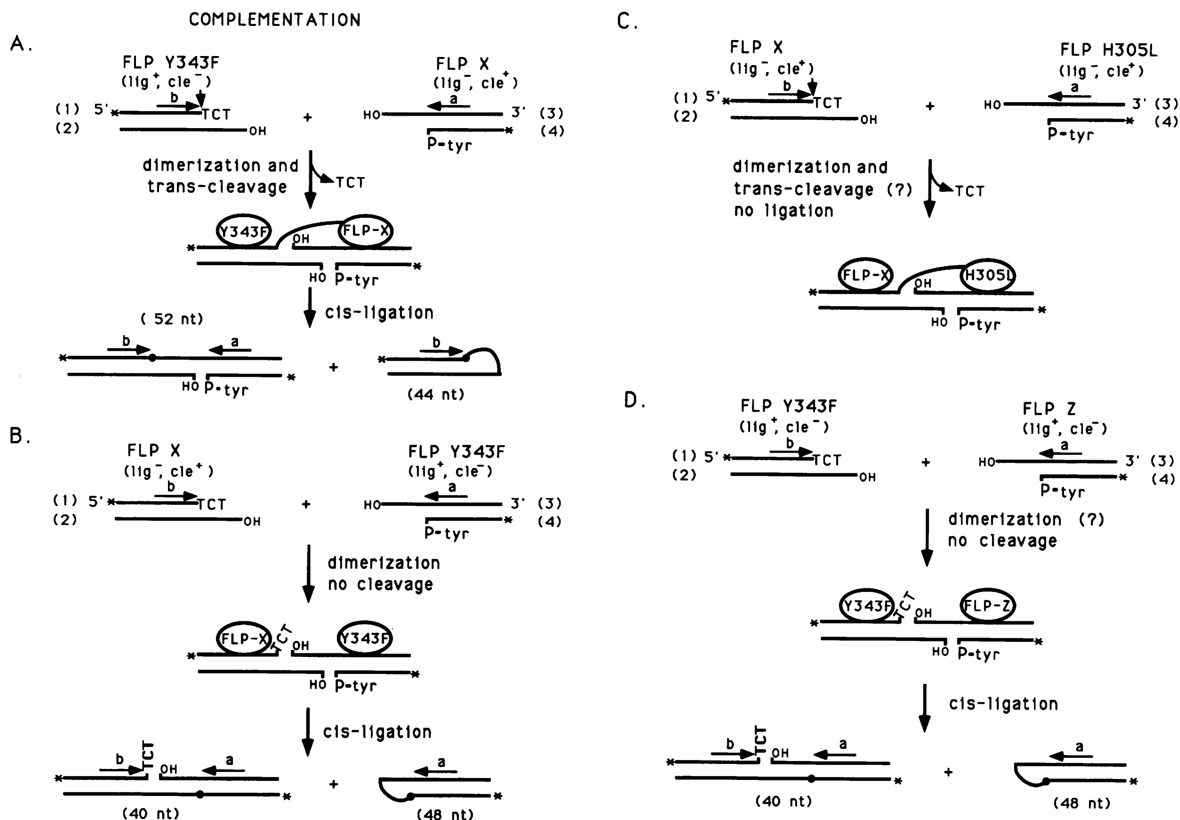


FIG. 4. Rationale for the in vitro complementation test of mutant FLP proteins. (A) FLP Y343F is preincubated with the *b* half-site, and a mutant FLP protein (FLP X) is preincubated with the *a* half-site. The cleavage and ligation properties of these mutant proteins are indicated in parentheses (top). The *b* and *a* half-sites are as described for Fig. 2A. Upon mixing of the bound substrates, dimerization occurs and FLP X mediates *trans* cleavage and covalently attaches to the cleavage site of the *b* half-site (middle). FLP Y343F mediates ligation in *cis*, giving ligation products with lengths of 52 and 44 nt (bottom) as described for Fig. 2A. The solid dots indicate ligated phosphodiester bonds. (B) FLP X is preincubated with the *b* half-site, and FLP Y343F is preincubated with the *a* half-site. Upon mixing of the bound substrates, no cleavage occurs because FLP Y343F that is positioned *trans* to the cleavage site is cleavage incompetent. Ligation mediated by FLP Y343F still occurs in *cis*, giving ligation products of 40 and 48 nt (bottom). (C) A mutant protein (FLP X) with a Lig$^-$ Cle$^+$ phenotype is preincubated with the *b* half-site, and FLP H305L (with the same phenotype) is preincubated with the *a* half-site. Upon mixing of the bound substrates as in panel A, dimerization may occur and FLP H305L may mediate *trans* cleavage and covalently attach to the *b* half-site. However, this was not assayed directly here. No ligation products result since both mutant proteins are ligation incompetent. (D) FLP Y343F (Lig$^+$ Cle$^-$) is preincubated with the *b* half-site, and a mutant FLP protein (FLP Z, also Lig$^+$ Cle$^-$) is preincubated with the *a* half-site. Upon mixing of the bound substrates, no cleavage occurs because FLP Z that is positioned *trans* to the cleavage site is cleavage incompetent. Ligation mediated by FLP Y343F occurs in *cis*, giving a ligation product with a length of 40 nt. A 48-nt ligation product results from ligation in *cis* mediated by FLP Z.

proteins were preincubated with the half-sites, the R308K protein clearly resembled the Y343F-H305L pair in the formation of ligation products predominantly derived from strands 1 + 3 and 1 + 2 (lanes 7 to 9). The R308K protein had no activity when bound to the *b* half-site and complemented by the Y343F protein bound to the *a* half-site (lane 10), confirming that cleavage occurs in *trans* and ligation occurs in *cis*. Likewise, the R308K protein did not complement the H305L protein irrespective of which protein was bound to which half-site (lanes 11 and 12). These results illustrate the feasibility of using the in vitro assay for *trans* cleavage and *cis* ligation to classify various mutant FLP proteins as to whether they resemble FLP H305L (group I, Cle$^+$ Lig$^-$) or FLP Y343F (group II, Cle$^-$ Lig$^+$).

Representative complementation experiments are shown for members of group I (FLP R191K and FLP G328R; Fig. 6A and B) and for members of group II (FLP A339D and FLP S336F; Fig. 6C and D). The results of all of the complementation experiments are summarized in Table 1, which lists the members of group I (R191K, H305L, R308K, H309L, G328R, G328E, and N329D) and group II (Y343F, S336Y, S336F, A339D, and H345L). Also listed are the properties of each of these mutant proteins as measured by various other assays.

The classification of the various mutant FLP proteins into group I or group II was confirmed by testing for complementation of (i) mutant FLP proteins in group I with one another, (ii) group II proteins with one another, and (iii) mutants in group I (other than FLP H305L) with mutants in group II (other than FLP Y343F) (data not shown). The following pairs of group I mutant proteins were unable to complement one another: R191K and H309L, R308K and H309L, L315P and R191K, L315P and G328R, L315P and N329D, R191K and N329D, N329D and H309L, and N329D and G328R. The various mutant pairs in group II that did not complement each other were S336F and A339D, S336F and H345L, and A339D and H345L. We also used mutant proteins other than H305L and Y343F to demonstrate complementation be-

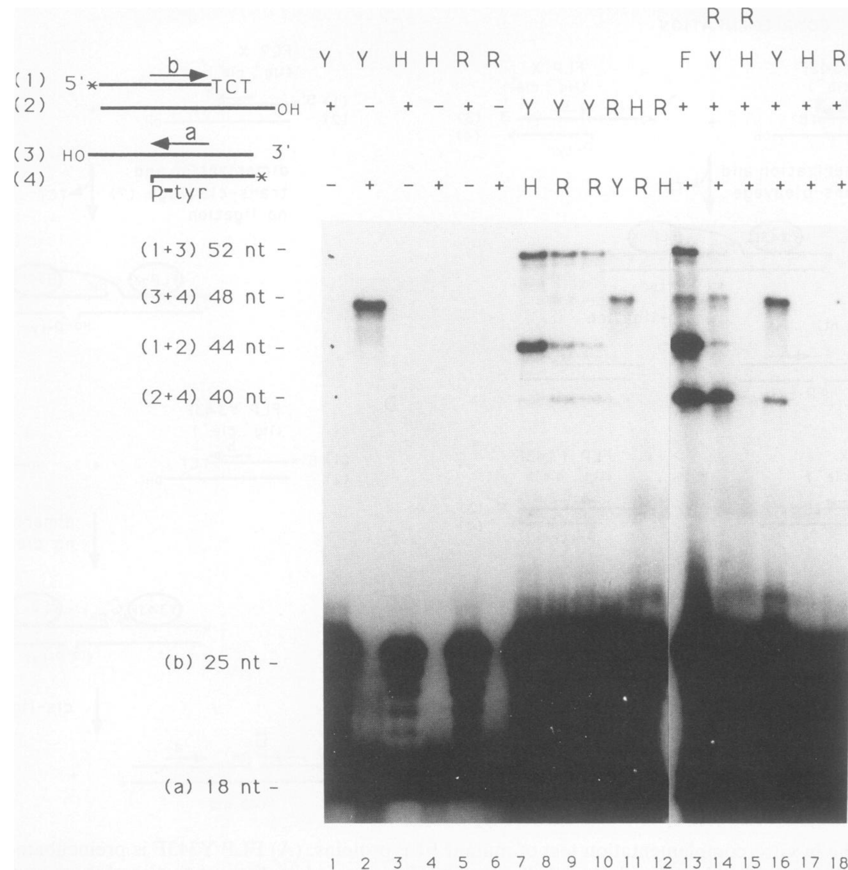


FIG. 5. In vitro complementation analysis of FLP Y343F, H305L, and R308K proteins. The reaction conditions are described in Materials and Methods. The half-site substrates used are diagrammed at the top left. The asterisk indicates the ^{32}P-labeled 5′ ends. The substrate strands are numbered 1 to 4 in parentheses. The FLP proteins are indicated as Y (FLP Y343F, 0.08 μM), H (FLP H305L, 0.21 μM), R (FLP R308K, 0.22 μM), and F (wild-type FLP, 0.03 μM). In lanes 1 to 6 and 13 to 18, the protein(s) and half-sites were mixed concurrently as indicated. In lanes 7 to 12, each substrate was separately incubated for 15 min at room temperature with the protein as indicated. The two substrate-protein mixtures were then combined and allowed to incubate for 40 min at 30°C. The reactions were subsequently terminated as described in Materials and Methods, and the samples were analyzed on an 8% denaturing polyacrylamide gel. The substrate and product lengths are indicated at the left; the numbers in parentheses indicate the sources of the ligation products.

tween groups I and II. The mutant pairs tested included G328R and H345L, R191K and H345L, and R191K and S336F.

All members of group I can perform cleavage of a half-site when complemented by a group II protein bound to another half-site, despite the fact that none of the proteins tested forms half-site dimers as measured by the assay of Qian et al. (18) and only some of the group I proteins (R191K, H305L, and R308K) can cleave a linear full FRT site. Other proteins that show no ability to cleave a linear substrate (G328R and G328E) exhibit cleavage in this complementation assay. All of the mutant proteins of group I are defective in the ligation reaction when assayed by the method of Pan et al. (13) or by the complementation assay described here.

The members of group II are all proficient in the ligation reaction and defective in cleavage activity, as assayed by half-site complementation with a member of group I. With the exception of FLP Y343F, the members of group II exhibit some cleavage activity with linear substrates or with Holliday intermediates.

Finally, the altered amino acids of members of group I and group II are spatially separated in the protein. Residues involved in ligation (group I) extend from amino acids 191 to 329, whereas those involved in cleavage in *trans* (group II) extend from amino acids 336 to 345.

DISCUSSION

We have used half-site complementation assays to show that the ligation step of the FLP recombination reaction occurs in *cis*, i.e., that the molecule of FLP that is bound adjacent to the site of cleavage catalyzes the re-formation of the phosphodiester bond. This result was implied by our previous finding that FLP can ligate a single-stranded oligonucleotide to an activated half-FRT site provided that the single-stranded oligonucleotide has some homology to the protruding single-stranded region of the half-site (14).

The design of the present experiments was guided by the recent finding that the active nucleophile in the FLP cleavage reaction is tyrosine 343 that is donated in *trans* to a FLP molecule that is bound adjacent to the site of cleavage (7). It is probable that FLP binds to a symmetry element and activates the adjacent phosphodiester bond for cleavage. After covalent attachment of the *trans* tyrosine 343 to the 3′-phosphate terminus, the 5′-hydroxyl group of an incoming strand is brought into the ligation pocket of the active site

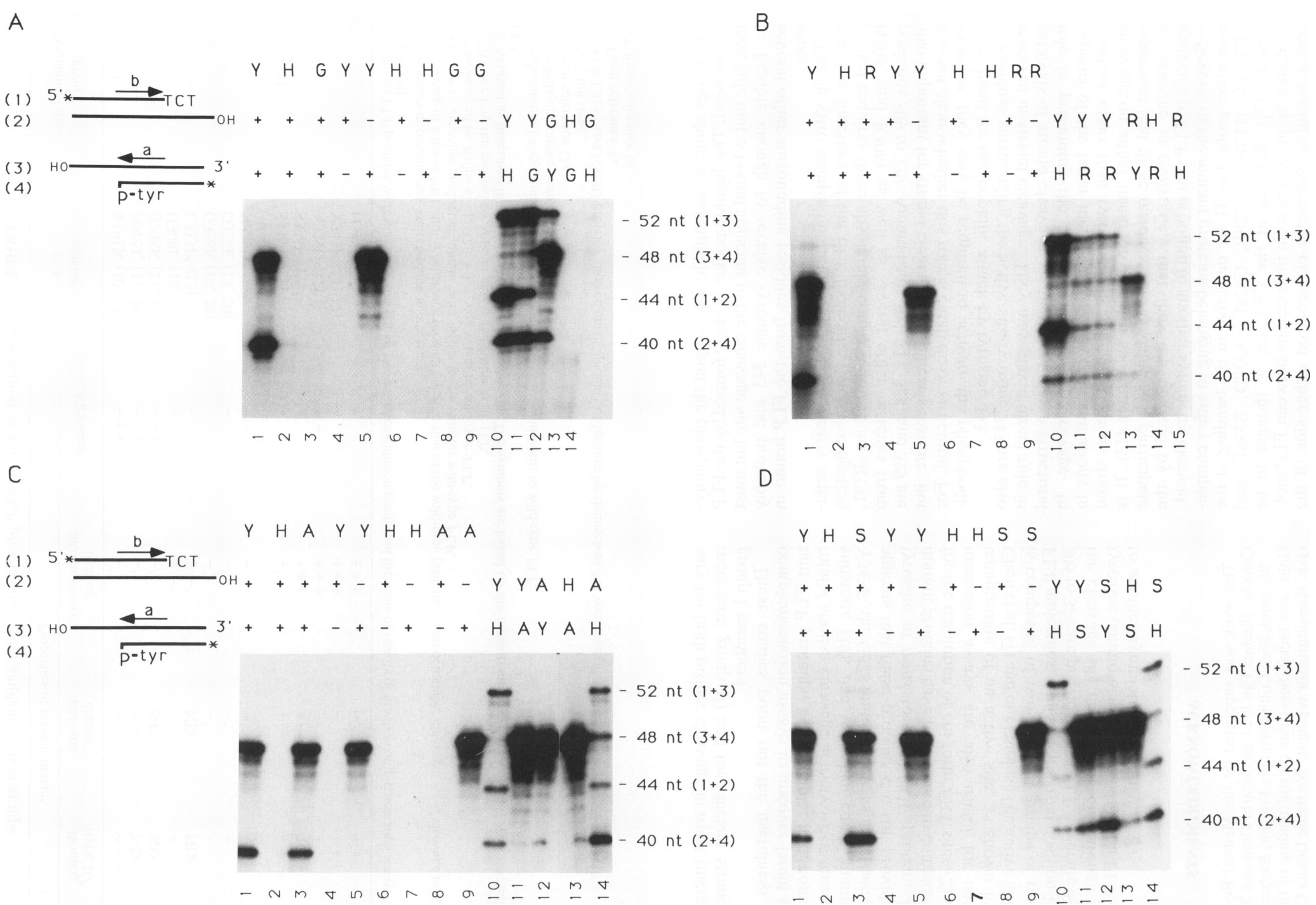


FIG. 6. In vitro complementation analysis of FLP Y343F and FLP H305L with each of FLP G328R (A), FLP R191K (B), FLP A339D (C), and FLP S336F (D). The reaction conditions are described in Materials and Methods. The product lengths and the strand numbers from which they were derived are given to the right of each autoradiogram. The half-site substrates used are diagrammed at the left. The FLP proteins are indicated as Y (FLP Y343F, 0.08 μM), H (FLP H305L, 0.21 μM), G (FLP G328R, 0.79 μM), R (FLP R191K, 0.88 μM [lanes 11, 13, 14, and 15 in panel B] or 1.8 μM [lanes 3, 8, 9, and 12 in panel B]), A (FLP A339D, 0.80 μM), and S (FLP S336F, 0.80 μM). In lanes 1 to 9, the respective protein and half-sites were mixed concurrently as indicated. In lanes 10 to 15, each substrate was separately incubated with a protein as indicated for 15 min at room temperature; the two substrate-protein mixtures were then combined and allowed to incubate for 40 min at 30°C. The reactions were subsequently terminated and analyzed on an 8% denaturing gel. Complementation results in the appearance of ligation products of strands 1 + 3 (52 nt) and 1 + 2 (44 nt).

TABLE 1. Classification of mutant FLP proteins into two complementation groups

Complementation group[a]	FLP mutant[b]	Properties of protein mutant				
		Cleavage[c]	Intramolecular ligation[d]	Half-site dimers[e]	Bending (type II)[f]	Resolution[g]
I	R191K (10, 11)	+++	−	−	+++	+/−[h]
	H305L (11, 16)	+++	−[i]	ND[j]	ND	+/−
	R308K (11, 15)	+++	−[i]	ND	ND	ND
	H309L (12)	−	+/−	−	+	+
	L315P[k] (12)	−	+/−[i]	ND	ND	ND
	G328R (19, 20)	−	−	−[l]	−	−[m]
	G328E (19, 20)	−	−	−[i]	−	−[m]
	N329D[k] (12)	+/−	−	−	−	++
II	S336Y (12)	+	+[i]	+/−	++/−	ND
	S336F (12)	+	+++	+/−	++/−	+++
	A339D (12)	+/−	+++	−	+	+++
	Y343F (14, 17)	−	+++	−	+++	−[m]
	H345L (12)	−	+++	−	++	+

[a] Mutant FLP proteins were classified into one of two complementation groups on the basis of results of in vitro complementation assays with FLP Y343F and FLP H305L.
[b] Numbers in parentheses are reference numbers.
[c] Cleavage of a linear FRT site. +, cleavage product equal to that of the wild-type protein; −, no detectable cleavage; +/−, cleavage is <10% of the wild-type level; +++, cleavage product accumulates to higher levels than for wild-type FLP.
[d] As assayed by Kulpa et al. (12). +++, equivalent to wild-type FLP.
[e] Assayed by the method of Amin et al. (2) and Kulpa et al. (12).
[f] Assayed as described by Schwartz and Sadowski (19, 20). The symbols are based on the measurements of Kulpa et al. (12). +++, equivalent to wild-type FLP.
[g] Resolution of a synthetic Holliday junction (12). +++, equivalent to wild-type FLP.
[h] Holliday junctions were cleaved but not ligated.
[i] From reference 12a.
[j] ND, not done.
[k] FLP L315P and N329D proteins showed small amounts of ligation by themselves but were complemented strongly by FLP Y343F.
[l] From reference 9a.
[m] From reference 8a.

and brings about a nucleophilic attack upon the phosphotyrosine bond. This ligation pocket is likely formed by the FLP molecule that is bound adjacent to the cleavage site but must be flexible enough to receive tyrosine 343 and probably several other surrounding residues from the FLP bound in *trans*.

The in vitro complementation experiments clearly separate two functions of the FLP molecule. An active ligation pocket requires three absolutely conserved residues (R191, H305, and R308) as well as several other residues from positions 309 to 329. Amino acids 309, 315, 328, and 329 are also important for strand cleavage on a linear substrate, but it is not clear whether this role is catalytic or structural. For example, they may be required to activate the phosphodiester bond for nucleophilic attack by tyrosine 343.

Residues surrounding the active-site tyrosine 343 are also involved in strand cleavage. They may play a direct role in catalysis of strand cleavage, or they may play a structural role in presenting the tyrosine residue for cleavage. In support of the latter idea is the finding that mutations in residues other than tyrosine 343 still allow some strand cleavage on either linear or chi-form substrates (12). It is suggested that these residues, like tyrosine 343, participate in cleavage in *trans*, i.e., that they are donated by the molecule of FLP that is bound to another symmetry element.

Several of the mutant proteins belonging to either complementation group I or II are defective in the bending of the FRT site (H309L, G328R, G328E, A339D, S336F, and H345L [12]). Bending of the FRT site is thought to be mediated by protein-protein interactions between FLP molecules bound to symmetry elements on either side of the core (19, 20). Such interactions would appear to be necessary for both proper cleavage in *trans* (members of complementation group II) and for efficient strand ligation (some group I members).

These studies point out the usefulness of the half-site complementation assays to uncover latent activities of mutant FLP proteins. For example, mutations at position 328 totally abolish cleavage activity when measured on a linear substrate (19), and yet when complemented by the FLP Y343F, the G328 mutant proteins are cleavage competent. It appears that these proteins can still provide the active-site tyrosine in *trans* to the Y343F protein. Likewise, these mutant proteins have been found to complement FLP Y343F for the resolution of chi-form FRT sites (21). These complementation assays are also useful for understanding the composition of an active site, as shown by the results of Chen et al. (7) and our studies. The active site of FLP protein is composed of two different groups of residues from two FLP molecules. Amino acids from position 191 and positions 305 to 329 are provided by a FLP molecule bound adjacent to the cleavage site and amino acids around the nucleophilic tyrosine (amino acids 336 to 345) by a FLP molecule bound to another symmetry element.

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