Alcoholysis and Strand Joining by the Flp Site-specific Recombinase

MECHANISTICALLY EQUIVALENT REACTIONS MEDIATED BY DISTINCT CATALYTIC CONFIGURATIONS*

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The strand joining step of recombination mediated by the Flp site-specific recombinase involves the attack of a 3′-phosphotyrosyl bond by a 5′-hydroxyl group from DNA. The nucleophile in this reaction, the 5′-OH, can be substituted by glycerol or other polyhydric alcohols. The strand joining and glycerolysis reactions are mechanistically equivalent and are competitive to each other. The target diester in strand joining can be a 3′-phosphate covalently linked either to a short tyrosyl peptide or to the whole Flp protein via Tyr-343. By contrast, only the latter type of 3′-phosphotyrosyl linkage is a substrate for glycerolysis. As a result, in activated DNA substrates (containing the scissile phosphate linked to a short Flp peptide), Flp(Y343F) can mediate the joining reaction utilizing the 5′-hydroxyl attack but fails to promote glycerolysis. Wild type Flp promotes both reactions in these substrates. The strand joining and glycerolysis reactions are absolutely dependent on the catalytic histidine at position 305 of Flp. Our results fit into a model in which a Flp dimer, with one monomer covalently attached to the 3′-phosphate, is essential for orienting the target diester or the nucleophile (or both) during glycerolysis. The requirement for this dimeric complex is relaxed in the strand joining reaction because of the ability of DNA to orient the nucleophile (5′-OH) by complementary base pairing. The experimental outcomes described here have parallels to the “cleavage-dependent ligation” carried out by a catalytic variant of Flp, Flp(R308K) (Zhu, X.-D., and Sadowski, P. D. (1995) J. Biol. Chem. 270, 23044–23054).

The Flp site-specific recombinase of Saccharomyces cerevisiae is a member of the integrase family of recombinase proteins (2, 3). These proteins carry out recombination “conservatively,” i.e. without DNA degradation or synthesis, and in the absence of exogenous energy input. This “break-join” mechanism follows from the ability of integrase-type recombinases to carry out two types of transesterification reactions, first to break the parental DNA strand and then to form the recombinant strand. The cleavage and joining steps of recombination follow a basic topoisomerase I strategy. Strand cleavage is mediated by an active site tyrosine residue, Tyr-343 in Flp. As a result, a 3′-phosphotyrosine bond and a 5′-hydroxyl group are formed at either side of the nick. Strand joining in the recombinant mode is chemically a reversal of the cleavage step, except that the reaction occurs between the broken strands of two DNA partners.

Several interesting features of the cleavage and joining steps of the Flp reaction have been revealed (4–7). The active Flp entity for strand cutting is a dimer (8, 9). Within the dimer, one Flp monomer, bound adjacent to a scissile phosphodiester bond, orients it for attack by Tyr-343 of the second Flp monomer bound across the strand exchange region (or spacer). Thus, the target phosphate is oriented in cis, while the cleavage nucleophile is donated in trans. The strand exchange reaction also follows the “cis-orientation/trans-nucleophilic attack” paradigm. In this case, the target phosphate belongs to the 3′-phosphotyrosine bridge, and the nucleophile is the 5′-hydroxyl group formed on the partner DNA. Once the target has been activated by Flp, a variety of exogenous nucleophiles can mimic the action of the native ones. Thus, strand breakage can be effected by tyramine, phenol, or hydrogen peroxide (6). Similarly, the 3′-phosphotyrosyl bond formed by Flp cleavage can be attacked by glycerol or polyhydric alcohols, by water (or the hydroxide ion), or even by a vicinal 2′-hydroxyl group in a DNA-RNA hybrid substrate (10, 11).

A simplified form of the recombination reaction (that involves the breakage and reformation of only one phosphodiester bond) can be performed using suitably designed “half-site” substrates (12, 13). These substrates were fashioned after the half-sites employed for the analysis of λ integrase-mediated recombination (14). Although reactions with half-sites may lack some of the geometric constraints and conformational attributes of the normal reaction, they have been remarkably useful in revealing the chemical and mechanistic aspects of Flp recombination (8, 15, 16).

The first step during the “half-site reaction,” as during normal recombination, is the covalent linkage between the cleaved strand and Flp via a 3′-phosphotyrosyl bond. The other product of cleavage is a short oligonucleotide (with a 5′-hydroxyl end) that cannot remain stably hydrogen-bonded to the uncleaved complementary strand. As a result, the 5′-hydroxyl group of the uncleaved strand can attack the phosphotyrosyl bond to form a hairpin product, thus releasing Tyr-343 from attachment to DNA. The reaction can be effectively stopped at the cleavage step if the 5′-hydroxyl is blocked by phosphorylation. Furthermore, an artificial (activated) half-site that mimics the cleaved intermediate by harboring a 3′-phosphate linked to tyrosine, tyramine, or a short tyrosyl peptide can undergo
The sequences of the oligonucleotides used to construct the substrates used in this study are listed. The Flp binding elements are shown in capital letters. The scissile phosphodiester position is indicated by “p.” The half-site was assembled from HT (top strand) and HB (bottom strand). The three full sites were constructed from TL and B (top left and bottom strands) as the common oligonucleotides and TR (right top strand) as the variable oligonucleotide.

### Table I

<table>
<thead>
<tr>
<th>Oligonucleotides for constructing the half-site</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>HT</td>
<td>5′-aagcttgtcGAAAGTCTCTATACpctttt-3′</td>
</tr>
<tr>
<td>HB</td>
<td>5′-tctagaaaaGATATAGGAACCTCGca-3′</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Oligonucleotides for constructing the full sites</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>5′-ggtttgctctGAAAGTCTCTATACpctttt-3′</td>
</tr>
<tr>
<td>B</td>
<td>5′-gagctctgtctagatgacGAAAGTCTCTATCTCpcttcgagaaaGATATAGGAACCTCGca-3′</td>
</tr>
<tr>
<td>TR1</td>
<td>5′-ttctagagaATAAGAAGAATCTCCgcttctcagcgtcgtc-3′</td>
</tr>
<tr>
<td>TR2</td>
<td>5′-aatctagaaATAAGAAGAATCTCCgcttctcagcgtcgtc-3′</td>
</tr>
<tr>
<td>TR3</td>
<td>5′-ctagagaATAAGAAGAATCTCCgcttctcagcgtcgtc-3′</td>
</tr>
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Hairpin formation when provided with a Flp mutant lacking Tyr-343, namely Flp(Y343F) or Flp(Y343S) (6, 7).

In a previous analysis using half-sites, we established that the alcohololysis and the strand joining reactions of Flp share overall mechanistic similarity. The target diester is the same diolyl phosphodiester of DNA as alternative nucleophiles. The outcomes of the “activated” half-sites (with the normally reactive 3′-phosphate linked to a short tyrosyl peptide and not to full-length Flp) bound by Flp or Flp(Y343F). The reason for this difference is not clear. A plausible explanation is that a dimeric configuration of Flp, with perhaps one monomer covalently bound to DNA, is a prerequisite for positioning the polyhydric alcohol in its reactive orientation. During strand joining, on the other hand, the potential to orient the 5′-hydroxyl group by the base pairing ability of DNA may overcome the requirement for the Flp dimer. However, based on previous experiments, we cannot rule out the possibility that the observed differences between strand ligation and alcohololysis might merely be an artifact of the special configuration of a half-site substrate.

### EXPERIMENTAL PROCEDURES

#### Purification of Flp—Wild type Flp and the Flp variants were purified as described previously (17). The concentration of Flp or a Flp variant in a given preparation was estimated according to the procedure of Lee and Jayaram (16).

#### Synthetic DNA Substrates—Oligonucleotides for the construction of the substrates were synthesized in an Applied Biosystems model 393A DNA synthesizer using phosphoramidite chemistry (18) and purified as described by Christiansen et al. (19). The sequences of the individual DNA strands are listed in Table I. The half-site was obtained by annealing the two strands HT and HB (Table I). In all of the full sites, two of the component oligonucleotides (TL and B) were common. The spacer differences were introduced via the third oligonucleotide (TR1-TR3). The relevant features of the full sites are described under Table I and diagrammed in Fig. 1A. In addition, for clarity, the substrates used in each experimental set are schematically represented in the appropriate figure. The preparation of the “activated” forms of the half- and full-sites is described below.

For assembly of the half-site or the full sites, 10–20 pmol of each of the appropriate oligonucleotides were mixed in TE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, pH 8.0), heated to 80 °C for 10 min, and cooled slowly to room temperature. The radiolabeled oligonucleotide was hybridized with the complementary strand(s) as that of the input labeled strand. The resulting DNA was annealed to its complementary partner(s). Thus, the substrate concentration in a given reaction was practically the same as that of the input labeled strand.

#### Assembly of Activated Substrates—The activated substrates were obtained following the same general hybridization strategy described for the nonactivated substrates, except that the 5′-phosphate end of the labeled strand was linked to a short Flp peptide via Tyr-343. For this purpose, the labeled strand was prepared as follows. First, a half-site containing the 5′-end label in the cleavable strand and a 5′-phosphate block in the second strand was treated with Flp in repeated cycles to obtain ~50% conversion into the cleaved product. After proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation, the DNA was fractionated in a 12% denaturing polyacrylamide gel (5% bisacrylamide) to separate the cleaved (peptide-linked) strand from the uncleaved strand. The desired band was excised from the gel, purified by standard protocols (10), and used for substrate assembly by hybridization.

#### Phosphorylating DNA Substrates at the 5′-End—The phosphorylation reaction was used either to label a given DNA strand with 32P or to prevent it from acting as a polynucleotidyl acceptor. The 5′-end of an oligonucleotide was labeled by incubation with T4 polynucleotide kinase in the presence of γ-32P ATP as the phosphoryl donor. For blocking the 5′-end, the oligonucleotide was phosphorylated by the kinase reaction in the presence of a molar excess of unlabeled ATP. The unreacted ATP was removed by spin dialysis on a G-50 column.

#### Assays for Flp-mediated Strand Transfer or Alcohololysis—Flp-mediated DNA joining or alcohololysis was investigated by reacting 0.025 to 0.05 pmol of the 5′-end labeled full sites with approximately 0.5 pmol of wild type Flp or Flp variants in a 200-μl reaction volume for 30 min at 30 °C. All reactions were performed in Flp reaction buffer containing 100 mM Tris (pH 7.5), 10 mM KCl, 10 mM MgCl2, 1 mM DTT, and 0.1% NP-40. After incubation, the reaction was terminated by the addition of SDS (0.1%) final concentration and either ethanol-precipitated directly or treated with proteinase K (10 mg/ml) for 1 h at 37 °C prior to ethanol precipitation, as stated. The DNA was fractionated by electrophoresis in 12% denaturing (5% bis-acrylamide) polacrylamide gels, and the reaction products were visualized by autoradiography or by phosphor imaging.

#### Assays for the Formation of the Cleaved Protein-DNA Complex from Half-Site or Full Site Substrates—The activated half- or full sites, appropriately labeled at one 5′-end, were treated with wild type Flp or Flp(H305L) under the conditions described for the strand transfer assays (see above). The reactions were terminated by the addition of SDS, but treatment with proteinase K was omitted. Samples were run in 12% SDS-polyacrylamide (3% bisacrylamide) gels, and the protein-DNA complexes were identified by autoradiography or by phosphor imaging.
Assays for the Formation of Complexes between a Half-site and Flp or Flp Variants—Approximately 0.05 pmol of the labeled half-site with or without a preactivated 3'-DNA-peptide end was used per reaction. Incubations with Flp or Flp variants was carried out for 15 min at room temperature in 20 μl of Flp reaction mixture (see the strand transfer protocol described above). The samples were subjected to electrophoresis in 6% nondenaturing polyacrylamide gels (3% bisacrylamide), and the DNA-protein complexes were visualized by autoradiography (or phosphor imaging).

RESULTS

The substrates used in this study mimic a normal Flp full site by containing two Flp binding elements placed in opposite orientations adjacent to the scissile phosphodiester bonds spaced 8 nucleotides apart on the top and bottom strands. However, they differ from the normal full site in that one of the two strands is discontinuous. This discontinuity was intentionally built into these substrates, so that they are comparable to those in which strand discontinuity is introduced as a result of cleavage by Flp (see below).

Three full sites, FS1, FS2, and FS3, containing, respectively, a nick, two nucleotide mismatches, and a small gap (3 nucleotides) adjacent to the scissile phosphate (Fig. 1A) were tested in assays for Flp-mediated strand transfer or alcoholysis. Two different types of the full site substrates were employed. The first type (the nonactivated full sites) contained three nucleotides 3'-9 to the scissile phosphodiester bond adjacent to the "left" Flp binding element. They were capable of undergoing Flp-mediated Alcoholysis and Strand Joining.

FIG. 1. Assays for Flp catalysis. A, the various full sites employed for studying Flp strand transfer or glycerolysis by Flp and Flp variants are schematically represented. The left section shows the nonactivated full sites (FS1, FS2, FS3) labeled at the left end on the top strand (asterisk). Cleavage positions on the top strand adjacent to the left Flp binding element (a) and on the bottom strand adjacent to the right binding element (b) are denoted by the short vertical arrows. The large arrowheads represent the relative head to head orientation between a and b. Although the top strand in these substrates is discontinuous, the phosphodiester position (p) preceding the trinucleotide (TTT) is cleavable by Flp. The activated full sites, in which the scissile phosphate is linked to a short Flp peptide (YX) via a 3'-phosphotyrosyl bond, are illustrated in the right section. B, the assays employed to study the cleavage, strand joining, and glycerolysis activities of Flp are represented using the full site FS1 as an example. The left flow chart shows reactions mediated on a nonactivated full site. Upon strand cleavage via Tyr-343-mediated transesterification, Flp gets covalently linked to the 3'-phosphate end of the left binding site (a), and the trinucleotide (TTT) diffuses away. Following cleavage, a second transesterification reaction using either the 5'-OH end of the spacer or glycerol will yield ligated products with concomitant release of Flp from covalent linkage to DNA. The right flow chart shows two possible reaction pathways that can occur in activated full sites. In pathway I, Flp first cleaves the 3'-phosphotyrosyl bond by Flp, and the scissile phosphate forms covalent attachment to the whole protein. Strand joining or glycerolysis can then occur as described for the nonactivated full site. In pathway II, the cleavage step by Flp is skipped. In this reaction, a Flp monomer noncovalently bound adjacent to the activated phosphate promotes transesterification between the 5'-OH of the spacer and the phosphotyrosyl bond.
mediated strand cleavage, with the concomitant release of a trinucleotide segment (Fig. 1A, left panel). In the second type, the full sites were already in their activated state by the attachment of a short Flp-derived tyrosyl peptide (indicated as XY) to the phosphate at the exchange point (Fig. 1A, right panel). The ability of Flp or Flp variants to mediate phosphoryl transfer from these substrates to the 5'-OH end of an acceptor strand or to glycerol was assayed as schematically illustrated in the right part of Fig. 1B for the full site containing a nick (FS1). For reference, the equivalent “nonactivated” substrates were assayed for strand transfer to DNA or to glycerol following cleavage by the full-length Flp as depicted in the left part of Fig. 1B for FS1.

Flp-assisted Strand Joining within Activated Full Sites—Reactions with half-site and full site Flp substrates have shown that the strand joining step of recombination is sensitive to the homology of 2-3 base pairs at each end of the strand exchange region (or spacer) between recombination partners (20–22). In accordance with the cis-activation/trans-nucleophilic attack model for Flp-mediated recombination (6), these results have led to the proposal that base pairing serves to orient the DNA-nucleophile (5'-hydroxyl) for attack on its target diester (3'-phosphotyrosyl-Flp). Later analysis (10) with half-sites has suggested that the Flp active site can also orient polyhydric alcohols to attack the 3'-phosphotyrosine bond in a reaction that is competitive with the 5'-hydroxyl attack.

To further examine the mechanistic similarities between “strand joining” and alcoholysis, we first tested the role of complementary base pairing during strand joining in a set of activated full sites (FS1–FS3; Fig. 2). In the 5'-end-labeled strand of these substrates, the 3'-phosphate (corresponding to the normal cleavage point) was linked via Tyr-343 to a short proteolytic peptide derived from Flp. Strand joining was assayed by incubating the activated substrates with wild type Flp under standard conditions as described under “Experimental Procedures.” Following termination of the reactions, samples were split into two and analyzed with (odd numbered lanes) or without (even numbered lanes) proteinase K digestion. The control in lane 1 was a reaction in which the substrate FS1 (shown over lanes 2 and 3) was incubated in the absence of Flp and was treated with proteinase K prior to electrophoresis. Incubation of FS1 (containing a fully matched spacer) with Flp led to its efficient conversion into L (lanes 2 and 3), the expected 62-nucleotide recombinant strand resulting from transfer of the activated phosphate to the 5'-OH of the nicked spacer. The formation of L was prevented by phosphorylation of the 5'-spacer end of FS1 (lanes 4 and 5). Instead, a trace of a faster migrating product band (not labeled in Fig. 2) was observed. This minor product can be explained by phosphoryl transfer to the 5'-OH end exposed on the unlabeled bottom strand by Flp cleavage at the right end of the spacer (see Fig. 1). The adjacent mismatch in FS2 significantly impaired strand transfer (compare L in lanes 6 and 7 to that in lanes 2 and 3), whereas the adjacent gap in FS3 virtually eliminated strand transfer (lanes 8 and 9). These results are consistent with the critical role of complementary base pairing in facilitating the orientation and proximity of the 5'-hydroxyl for strand joining.

The additional bands above and below the substrate band (S) in the proteolysed samples (odd numbered lanes) deserve comment. The faster moving S′ is the result of further protease digestion of the short Flp-derived oligopeptide covalently attached to the activated substrates. Note that S′ was formed by proteinase K treatment of FS1 even in the absence of prior incubation with Flp (lane 1). The collection of bands (CI) above S (and therefore larger than S) indicate peptides linked to the labeled strand that could have arisen only from proteinase K action on the full-length Flp monomer. These results are consistent with previous observations that the phosphotyrosyl phosphodiester is a target for nucleophilic attack by Tyr-343 of Flp (1, 23). Thus, formation of L in reactions with FS1 (lanes 2 and 3) can be accounted for by two modes of strand transfer: (i) by a single step reaction via the direct 5'-hydroxyl attack of the activated phosphate in FS1 (see also Fig. 3B) or (ii) by a two-step reaction via the initial formation of the Flp-DNA covalent intermediate. The presence of proteolytic products larger than S in reactions with full sites blocked or compromised in strand joining is supportive of the second mechanism. However, the first mechanism is also operational, as indicated by reactions with the cleavage minus variant, Flp(Y343F) (see below; Fig. 3). The lower yield of cleavage in the phosphorylated form of FS1 (lane 5) compared with FS2 (lane 7) or FS3 (lane 9) might result either from the presence of the adjacent phosphate group in FS1 or from the differences in the conformational flexibilities of these substrates.

Flp-mediated Alcoholysis within Activated Full Sites—Our previous experiments on alcoholysis in half-sites (10) poses the
The ability of activated full sites to support alcoholysis mediated by wild type Flp or Flp(Y343F). A, reactions mediated by wild type Flp. Lane 1, activated DNA substrate FS1 incubated without Flp. Lanes 2–13, reactions with spacer-phosphorylated (P) or unphosphorylated (OH) versions of FS1, FS2, and FS3. Substrates containing the unphosphorylated spacer were reacted with Flp in the absence of glycerol; those containing the phosphorylated spacer were reacted with Flp in the absence of glycerol. A longer exposure of the relevant portion of the autoradiogram comprising lanes 3–5 is shown at the right. The alcoholysis product is marked A. Other abbreviations and labeling of reaction products are as described under Fig. 2.

The contrasting results with Flp and Flp(Y343F), in conjunction with the previous findings with half-sites (10), suggest that a dimer configuration of Flp is necessary, but not sufficient, for alcoholysis. They are consistent with the hypothesis that cleavage and concomitant attachment of the 3’-phosphate to Flp is a prerequisite for the alcoholysis reaction. It should be noted that, consistent with this interpretation, there was a good correlation between the observed alcoholysis within a full site (Fig. 3A) and its apparent the unphosphorylated spacer with that expected for the replacement of the tyrosyl peptide from the 3’-end of the labeled strand by glycerol (10). The extent of strand transfer to the 5’-hydroxyl from these substrates (namely, the formation of L) agreed well with the results shown in Fig. 2. FS1 was superior to FS2, and FS3 was inactive.

In contrast to Flp, Flp(Y343F) failed to mediate alcoholysis within any of the activated full sites (Fig. 3B, lanes 4, 5, 8, 9, 12, and 13) and its apparent following dilemma. If the alcoholysis and strand joining reactions are mechanistically equivalent and competitive, why does alcoholysis, in contrast to strand joining, not occur in an activated half-site (3’-phosphate covalently linked to a short Flp-derived tyrosyl peptide) treated with Flp or Flp(Y343F)? From earlier binding studies, it is known that a normal half-site (in its nonactivated state) gives only a monomeric complex with Flp(Y343F) (one half-site to one protein monomer) but produces a dimeric complex with Flp (two half-sites to two protein monomers) (13, 16). The following questions are therefore relevant. Is a dimeric configuration of Flp essential when the nucleophile in the ligation step of recombination is glycerol or a polyhydric alcohol rather than the normal 5’-hydroxyl group? Within the dimeric configuration, is it also necessary for a whole Flp monomer to be covalently linked to DNA for alcoholysis to occur? And finally, does a normal half-site differ from its activated counterpart in its capacity to dimerize in the presence of wild type Flp?

To address the above issues, we first assayed the ability of wild type Flp or Flp(Y343F) to mediate alcoholysis within activated full sites (Fig. 3, A and B). Since a monomer of Flp or Flp(Y343F) can bind to each of the two binding elements in these substrates, both proteins should be able to assemble dimers on them. However, only wild type Flp, and not Flp(Y343F), can form covalent attachment to DNA by cleavage of the phosphotyrosyl bond. In order to avoid competition with strand transfer to the 5’-hydroxyl of DNA, alcoholysis was assayed using substrates harboring 5’-phosphoryl ends adjacent to the activated phosphate (lanes 3–5, 7–9, and 11–13 in Fig. 3, A and B). The phosphoryl acceptor in the alcoholysis assays was glycerol. As a control, strand ligation to 5’-hydroxyl spacer ends in the absence of added glycerol was also assayed (lanes 2, 6, and 10; Fig. 3, A and B). The reactions were analyzed by electrophoresis without protease treatment. This was done intentionally to avoid obfuscation of the alcoholysis product by those resulting from proteolysis. With increasing amounts of exogenously added glycerol (2.5 and 5 M), wild type Flp yielded corresponding amounts of the product band marked A for all three substrates, FS1–FS3 (Fig. 3A; lanes 4, 5, 8, 9, 12, and 13; lanes 4’ and 5’ are longer exposures of lanes 4 and 5). The migration of A was consistent with that expected for the replacement of the tyrosyl peptide from the 3’-end of the labeled strand by glycerol (10). The extent of strand transfer to the 5’-hydroxyl from these substrates (namely, the formation of L) agreed well with the results shown in Fig. 2. FS1 was superior to FS2, and FS3 was inactive.

In contrast to Flp, Flp(Y343F) failed to mediate alcoholysis within any of the activated full sites (Fig. 3B, lanes 4, 5, 8, 9, 12, and 13). However, Flp(Y343F) was as efficient as Flp in mediating strand ligation in FS1 and FS2 (Fig. 3B, lanes 2 and 6; compare with Fig. 3A, lanes 2 and 6). The contrasting results with Flp and Flp(Y343F), in conjunction with the previous findings with half-sites (10), suggest that a dimer configuration of Flp is necessary, but not sufficient, for alcoholysis. They are consistent with the hypothesis that cleavage and concomitant attachment of the 3’-phosphate to Flp is a prerequisite for the alcoholysis reaction. It should be noted that, consistent with this interpretation, there was a good correlation between the observed alcoholysis within a full site (Fig. 3A) and its apparent
cleavage by Flp (see Fig. 2). For example, FS1 appeared to be poorly cleaved by Flp (note the levels of CI in Fig. 2, lane 5) compared with FS2 (lane 7) and FS3 (lane 9) and yielded the least amount of the alcoholysis product (Fig. 3A, lanes 4 and 5). It required twice as long an exposure of the autoradiogram shown in Fig. 3A (left) for detection of the A bands in lanes 4' and 5' (right).

**Binding of Flp and Flp Variants to Activated Half-sites—Can the ability of Flp to mediate alcoholysis in a half-site but not in a nonactivated half-site (10) be explained by differences in the nature of the protein-DNA complexes formed by the two types of substrates? It is known that a dimer of a Flp-half-site complex (F-HS II) (Fig. 4; see below) is capable of strand cleavage, whereas the monomeric complex (F-HS I) (Fig. 4) is not (13, 16). Covalent linkage of Flp to DNA following cleavage is probably responsible for the stabilization of the F-HS II complex. The inability of several Flp variants to execute strand cleavage in half-sites and their failure to produce the F-HS II complex (12) are well correlated. If so, can the failure to detect alcoholysis in an activated half-site in the presence of Flp (10) be correlated to the absence or a reduction in F-HS II formation?

In Fig. 4A, the complexes formed by a normal half-site (HS) with Flp, Flp(Y343F), and Flp(H305L) are displayed. As has been seen previously, only Flp was able to produce F-HS II along with smaller amounts of higher order complexes (C). In fact, at our assay conditions, most of the complexes formed between wild-type Flp and the half-site exist in the dimeric form (FSII). On the other hand, the two Flp variants yielded only F-HS I. With the activated half-site (Fig. 4B), all three proteins yielded the monomeric complex equivalent to F-HS I. No F-HS II equivalent was observed with Flp(Y343F) or with Flp(H305L). Even with wild-type Flp, the yield of this dimeric complex was approximately 5% of the F-HS II formed with the normal half-site (compare lanes 2 of Fig. 4, A and B). Note that the total amount of the complexes (F-HS I plus F-HS II) was roughly the same for Flp-half-site binding or Flp-activated half-site binding. In Fig. 4C, the ability of Flp and Flp(H305L) to cleave the normal or activated form of the half-site was assayed directly. In concordance with previous results, the formation of cleavage complexes correlated well with the formation of the dimeric complex F-HS II (Fig. 4, A and B). Thus, the normal half-site was cleaved efficiently by Flp (lane 3), whereas the cleavage reaction was strongly impaired on the activated half-site (lane 4). Neither one of the half-sites was cleaved by Flp(H305L) (lanes 5 and 6).

The lack of alcoholysis in activated half-sites can, therefore, be explained by the poor cleavage of these sites by Flp and the consequent failure to assemble a dimeric complex competent in accommodating the active orientation of glycerol or polyhydric alcohols. Glycerolysis from the trace amount of the dimeric complex formed by wild-type Flp would most likely not have been detected in our assays.

**Formation of Covalent Flp-DNA Complexes in Activated and Nonactivated Full Sites—**The preceding experiments (Figs. 2–4) suggest the following scenarios for the strand ligation and the alcoholysis reactions. Both require a 3'-phosphotyrosyl bond as the target for nucleophilic attack. However, the ligation step can take place with a single Flp monomer bound adjacent to the 3'-phosphate linked to tyrosine (or a tyrosine mimic) (6, 7) or to short tyrosyl peptide (Refs. 10 and 23; this study). For the alcoholysis step, in addition to the bound adjacent Flp monomer, the target phosphotyrosyl bond must be part of a whole Flp monomer.

In order to test this hypothesis more critically, the formation of Flp-DNA covalent adducts was directly assayed in the normal and activated forms of FS1, FS2, and FS3 (Fig. 5, A and B). These full sites contained either a 5'-OH or a 5'-phosphorylated spacer end adjacent to the reactive phosphate position. The reaction products were fractionated in 10% SDS-polyacrylamide gels and visualized by autoradiography to detect the covalent DNA-protein complex (labeled FD). All of the nonactivated full sites (indicated by S) were cleaved by wild-type Flp to yield FD (Fig. 5A, lanes 7–12). The apparent meager level of FD in lane 7 is illusory. In FS1 (containing a base paired spacer end with a 5'-hydroxyl), the complex was rapidly converted into the ligated product (L; see Fig. 2, for example) that comigrated with the substrate band (S). The joining reaction (and proportional reduction in FD) occurred to a lesser extent in FS2 (containing an unpaired spacer end with a 5'-hydroxyl) as well (lane 9). Formation of FD was observed in reactions of Flp with the activated substrates also (Fig. 5B, lanes 7–12). The extent of cleavage, however, was uniformly lower relative to the nonactivated substrates. Here again, the strong reduction in FD in lane 7 (barely detectable even after longer exposures) and in lane 9 was due to the joining reaction mediated by the 5'-hydroxyl group. However, the strikingly low yield of FD in lane 8 cannot fit this explanation, since the 5'-phosphate end would be blocked in the joining reaction. It is possible that the presence of this neighboring phosphate might inhibit the cleavage of the phosphotyrosyl bond by Flp. One may imagine that this phosphate directly competes with the scissile phosphate for critical catalytic residues involved in transition state stabiliza-

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1 The abbreviations used are: F-HS I and F-HS II, monomeric (one Flp monomer plus one half-site molecule) and dimeric (two Flp monomers plus two half-site molecules) complex, respectively; FD, Flp-DNA cleavage complex.
Flp or Flp(H305L) was incubated with the nonactivated (A) or with the activated (B) full sites and analyzed for the formation of the cleaved, covalent protein-DNA complex (see “Experimental Procedures” for details). The uncomplexed substrate DNA is labeled S, and the covalent Flp-DNA complex is labeled FD.

The cleavage of the phosphotyrosyl bond and formation of FD was suggested by the experiments in Fig. 2 as well (CI in lane 5 of Fig. 2).

The strong correlation observed between the reactivity of a full site in the formation of the covalent Flp-DNA adduct (Fig. 5) and its potency in the alcoholysis reaction (compare lanes 8, 10, and 12 of Fig. 4B with lanes 5, 9, and 13 of Fig. 3A) lends further support for Flp-mediated cleavage being a prerequisite for alcoholysis.

The cleavage of the phosphotyrosyl bond and formation of FD were also assayed using the Flp variant Flp(H305L). The full sites in their nonactivated state were cleaved by (H305L) (Fig. 5A, lanes 13–18). Unlike the case with wild type Flp, little difference between the phosphorylated and unphosphorylated forms of the substrates was seen. This result is consistent with the extremely slow kinetics of the strand joining reaction mediated by Flp(H305L) (6, 24). The His-305 variant of Flp has previously been found to cleave full sites (with no strand discontinuity) efficiently and to accumulate the cleavage product (25). However, it fails to cleave a half-site or cleaves it extremely inefficiently (26). The weaker cleavage by Flp(H305L) than Flp observed here may be due to the strand discontinuity in the substrates tested. Thus, mutations of His-305 appear to render cleavage by Flp particularly sensitive to substrate configurations. None of the activated full sites were cleaved by Flp(H305L) (Fig. 5B, lanes 13–18). Even trace amounts of FD were not detected after prolonged exposures of the autoradiogram. The lack of reaction probably reflects the inability of Flp(H305L) to orient the phosphotyrosyl bond for nucleophilic attack by Tyr-343.

**Test for the Ability of Flp(H305L) to Mediate Alcoholysis—** Since Flp(H305L) could mediate formation of the DNA-protein complex in nonactivated full sites (FS1–FS3, Fig. 5A), we tested the ability of this protein to mediate alcoholysis in two of these substrates (FS1 and FS3; Fig. 6). Alcoholysis was readily observed in reactions with wild type Flp and phosphorylated forms of FS1 and FS3 (bands marked A in lanes 5 and 7, respectively). Alcoholysis was weak in the unphosphorylated FS1 due to the competing strand joining reaction (band L in lanes 2 and 3). However, it was detectable upon longer exposure of the autoradiogram (compare lane 3’ to lane 2’ on the right). No alcoholysis was observed with Flp(H305L) in any of the substrates (lanes 8–13). Consistent with its impaired strand joining activity, Flp(H305L) produced much smaller amounts of L from unphosphorylated FS1, compared with Flp (compare lanes 8 and 9 to lanes 2 and 3).

Thus, although Flp(H305L) can form the target 3’-phosphotyrosyl linkage required for alcoholysis, it cannot carry out the reaction to completion. Presumably, His-305 is a critical catalytic residue for directing the nucleophilic attack by glycerol (as it is for directing the 5’-hydroxyl attack during strand joining).

**Discussion**

The analysis of the chemical mechanism of Flp-mediated site-specific recombination has been facilitated by a number of relatively small molecular weight reagents that mimic substrates of the normal reaction (6, 10, 27). In the strand cleavage reaction, Tyr-343 of Flp can be functionally substituted by a number of exogenously supplied small nucleophiles such as phenol, tyramine or hydrogen peroxide (6, 27). Similarly, in the strand joining reaction, the 5’-hydroxyl group can be replaced by polyhydric alcohols (in particular glycerol), by water (or the hydroxide ion), and by the adjacent 2’-hydroxyl group from a ribonucleotide (10, 11). The 5’-hydroxyl attack can be promoted by a Flp monomer lacking Tyr-343, but properly positioned with respect to a 3’-phosphate covalently linked to tyrosine or a short tyrosyl peptide; however, the same substrate-protein configuration fails to support glycerolysis. Experiments described here provide a reasonable explanation for this apparent discrepancy and offer a plausible mechanism for expanding the catalytic capacity of a given active site via protein subunit cooperativity.

**Nucleophile Orientation by Flp during Strand Joining and Glycerolysis—** The primary conclusion from this study is that Flp can utilize more than one mechanism for functionally aligning the attacking nucleophile in reactions that are equivalent to the strand joining reaction. Consistent with earlier results, the activated substrates used here reinforce the role of base complementarity in bringing the 5’-hydroxyl in line with the phosphotyrosine bond (20). They also reveal that the phosphotyrosyl bond can be cleaved by Tyr-343 from a second Flp monomer lacking Tyr-343, but properly positioned with respect to a 3’-phosphate covalently linked to tyrosine or a short tyrosyl peptide; however, the same substrate-protein configuration fails to support glycerolysis. Experiments described here provide a reasonable explanation for this apparent discrepancy and offer a plausible mechanism for expanding the catalytic capacity of a given active site via protein subunit cooperativity.
more rigidly fixed when it is part of the cleaved Flp complex than when it is part of a short Flp peptide. This additional orientation effect, provided by the protein being bound to its cognate sequence, is likely to be more significant for glycerolysis than for strand joining. The latter reaction has the advantage that the 5'-hydroxyl group can be precisely oriented by complementary base pairing. Alternatively, the “restricted” Flp dimer formed by cleavage of the activated substrates might provide additional contacts for glycerol within the reaction pocket. Thus, the cleavage reaction can, in principle, stimulate glycerolysis by a dual mechanism, by orienting the target diester as well as the nucleophile.

The rather puzzling observation that wild type Flp can mediate glycerolysis in “normal” half-sites but cannot do so in activated half-sites (10) can also be explained by the orientation advantage conferred by the cleavage reaction. As shown in this study, the activated half-sites are poor substrates for cleavage by Flp and cannot give rise to stable dimeric Flp-half-site complexes. Furthermore, we noticed that Flp(H305L), a Flp variant that can cleave the full site substrates but is strongly depressed in strand joining, cannot yield alcoholysis. This result would be consistent with His-305 being an essential catalytic residue for this reaction and further supports the proposed common chemical mechanism for strand joining and glycerolysis.

A situation analogous to the lack of glycerolysis in the absence of Flp-DNA attachment has also been described for the strand joining reaction. Zhu and Sadowski (1) found that Flp(R308K) cannot mediate normal strand joining in an activated half-site or full site substrate. They noticed that this defect results from the inability of the mutant protein to cleave the phosphotyrosyl bond and form DNA linkage with the whole protein. In nonactivated half-sites or full sites, though, where cleavage by the mutant protein is not impeded, strand joining occurs efficiently. Zhu and Sadowski (1) have called this phenomenon “cleavage-dependent ligation.” In the framework of our model, the poor strand joining reaction can be accounted for by the inability of the mutant protein to orient the scissile phosphate contained within a short peptide-DNA linkage. Hydrogen bonding between Arg-308 and the nonbridging oxygen atoms of the phosphate group is likely to be critical for this step, as suggested by the crystal structure of the Cre protein (like Flp, a member of the integrase family) complexed with DNA (28). However, strand joining can be restored if the phosphotyrosyl bond is restrained by covalent attachment to a DNA-bound protein monomer.

**Catalytic Versatility of Complex Active Sites: Expression of Novel Activities**—The recombination active site is a complex active site. In addition to carrying out the chemistry for strand breakage and strand joining, the active site must also deal with the conformational logistics of coordinating these phosphoryl transfers in time and space and directing them to occur between DNA partners. The normal recombination reaction is carried out by four Flp monomers acting cooperatively. Consistent with the logic of evolving and optimizing a complex catalytic function by combining (or expanding on) simpler ones, the Flp active site has been shown to harbor cryptic nuclease and topoisomerase activities (11). Since the catalytic residues for carrying out these elementary chemical transactions have

**Fig. 6.** Test for the ability of Flp(H305L) to mediate alcoholysis on nonactivated or activated full sites. The nonactivated or activated full sites were treated with wild type Flp (lanes 2–7) or Flp(H305L) (lanes 8–13) in the absence or the presence of added glycerol. Abbreviations and labeling of reaction products are as described for Fig. 3. An overexposure of lanes 2 and 3 (A) and lanes 2–5 (B) is depicted at the right. The alcoholysis product A is detectable in 3' (A) and in 3' and 5' (B) locations.
already been configured within the recombinase active site, it is not surprising that it can carry out (given the appropriate substrates) related yet novel reactions with varying efficiencies. The utilization of glycerol and other polyhydric alcohols by Flp in place of the normal 5’-hydroxyl group from DNA is therefore not particularly surprising. However, the optimal substrate-enzyme configurations for the standard reaction and an artificial reaction are likely to be different from each other. It is quite plausible, however, that only modest amino acid substitutions are required in order to unmask a normally cryptic (or weak) activity resident within a given active site. Since the active form of Flp in recombination utilizes a composite of four active sites, it offers a relatively large template for accommodating conformational flexibilities that permit “induced substrate fit,” and prime the emergence of catalytic variations. As demonstrated by our observations, the active site contained entirely within a single Flp monomer expresses the strand joining function but is inactive in glycerolysis. However, when provided with the catalytic cooperativity of a second Flp monomer, the composite active site can carry out both strand joining and glycerolysis.

During the progression of recombination along its normal kinetic path, there would be little opportunity for the expression of the alcoholysis or the hydrolysis reaction (10) by Flp. However, under special circumstances that impede or block the normal reaction path, these activities could have important implications. For example, if recombination is blocked following strand cleavage, removal of the protein-DNA linkage would be a potential first step in a repair pathway that recognizes the DNA damage and restores the integrity of the broken strand (29).

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REFERENCES