DNA Splicing by an Active Site Mutant of Flp Recombinase

POSSIBLE CATALYTIC COOPERATIVITY BETWEEN THE INACTIVE PROTEIN AND ITS DNA SUBSTRATE*

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Each strand transfer catalyzed by the Flp recombinase is the composite of two transesterification reactions. The active nucleophilic species in the two reactions are the catalytic site tyrosine (Tyr-343) of Flp and the 5'-hydroxyl from the Flp-nicked DNA substrate, respectively. A "half recombination site" is capable of undergoing this pair of transesterifications in the presence of Flp. When the substrate is a half-site containing a chiral phosphorothioate at the exchange point, the Flp reaction yields a product in which the phosphate chirality is retained. A mutant of Flp that lacks the active site tyrosine, Flp(Y343F), is incapable of mediating strand transfer in a full-recombination site but can execute strand transfer in a half-site. The efficiency of this reaction is about 2% of that of the wild type reaction. The activity of Flp(Y343F) is critically dependent on the length of the half-site spacer. Furthermore, in this reaction, the strand cleavage and strand exchange steps cannot be uncoupled. These results strongly suggest a direct attack by the 5'-hydroxyl of the half-site spacer on the phosphodiester at the normal strand transfer point.

Phosphate esters and phosphate anhydrides play an ubiquitous role in biology. Phosphoryl transfers are, therefore, among the most common biological reactions. Such reactions include those catalyzed by self-splicing introns, spliceosomes, DNA recombinases, and topoisomerases, DNA and RNA polymerases, kinases, ligases, and nucleases. The basic chemical feature of these reactions is the attack by a nucleophile (usually a sugar hydroxyl or the hydroxyl from a protein amino acid) on a susceptible phosphate group. The RNA or protein enzymes that catalyze these reactions may exhibit commonalities in their mechanism of action.

There are two well characterized families of site-specific DNA recombinases; the Int and Hin/resolvase families (for a review, see Craig (1988)). The Int family uses the hydroxyl of a specific tyrosine of the recombinase in the transesterification reaction that breaks the DNA chain and reveals a 5'-hydroxyl in preparation for the strand union reaction. The hydroxyl group then attacks the phosphodiester formed by the strand cleavage and strand exchange steps cannot be uncoupled. These results strongly suggest a direct attack by the 5'-hydroxyl of the half-site spacer on the phosphodiester at the normal strand transfer point.

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MATERIALS AND METHODS

Reconstruction of the Flp(Y343F) Clone by the Cassette Replacement Procedure—The Flp(Y343F) clone was originally obtained by in vitro mutagenesis of the wild type Flp template (Prasad et al., 1987). To exclude the possibility of any template contamination, the
Flp(Y343F) clone was reconstructed as follows. The DNA segment coding for the carboxyl-terminal two-thirds of Flp(Y343F) was cloned into pUC19. From the resultant plasmid, a small DNA piece that includes the Phe-343 mutation was excised and replaced by a short stuffer DNA fragment containing the SnaBI restriction enzyme site (see Fig. 1). The engineered Flp(Y343F) insert was then cut out and spliced in-frame to the DNA segment encoding the amino-terminal portion of Flp in an inducible expression vector (Jayaram, 1985). After confirming the presence of the Phe-343 mutation (TTT) and the changed Thr-341 codon (ACT) in the plasmid construct, a purified Escherichia coli clone containing this plasmid was processed for the Flp(Y343F) preparation (Prasad et al., 1987; Parsons et al., 1990). All steps of purification were done with freshly prepared buffers and other solutions and with columns, tubing, and chromatography media (including the Flp affinity matrix) that had not been previously used for Flp isolation.

Purification of Flp—Wild-type Flp or a Flp variant was partially purified essentially as described by Prasad et al. (1987). Further purification was achieved by chromatography on an affinity column containing oligomeric Flp-site oligodeoxynucleotides linked to CNBr-activated Sepharose (Parsons et al., 1990). The final purity of wild type and mutant preparations was approximately 90% as judged by Coomassie Brilliant Blue staining in situ in SDS-polyacrylamide gels. Flp concentrations were derived from densitometric scans of aliquots of the protein stained with Coomassie Brilliant Blue in situ in SDS-polyacrylamide gels compared to similar scans obtained with a range of known amounts of Flp(Y343F).

Half Recombination Sites—Oligodeoxynucleotides for construction of half sites were synthesized in an Applied Biosystems DNA synthesizer (Model 380A) using phosphoramidite chemistry (Beaucage and Caruthers, 1981). Normally 12 to 24 pmol each of the appropriate oligodeoxynucleotide pairs was mixed in TE (10 mM Tris-HCl, pH 7.5 (at 25°C), 1 mM EDTA, pH 8.0), heated to 65°C for 10 min and cooled slowly to room temperature.

When required, the spacer 5'-hydroxyl was phosphorylated by treating the oligodeoxynucleotide with polynucleotide kinase in the presence of an excess of unlabeled ATP. The unreacted ATP was removed by spin-dialysis on a G-25 column. Hybridization to the partner oligodeoxynucleotide was done in TE.

Half-site Substrate Containing Phosphorothioate at the Strand Exchange Point—The top strand oligodeoxynucleotide, 5'CCCGAAGTTCCCTATTB'C, containing 12 of the 13 nucleotides that form part of the protein stained with Coomassie Brilliant Blue in situ in SDS-polyacrylamide gels was ethanol-precipitated. The aqueous phase was ethanol-precipitated and the DNA pellet was washed three times with 80% ethanol, dried in vacuo, and dissolved in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0). The DNA solution was passed over a short G-25 column equilibrated with TE prior to use in strand transfer reactions. The expected double stranded oligonucleotide following the Klenow fill-in reaction is as follows.

5'CCCGAAGTTCCCTATTB'C
3'GGGCTTCAGAATTAGCCCG
Sequences 1 and 2

The lowercase letters represent the spacer nucleotides, the underlined uppercase letters constitute the Flp binding region, and the normal uppercase letters (except the OH at the 5'-end) indicate sequences that are not relevant to the strand transfer reaction. The radioactively labeled phosphates are shown in underlined p letters marked by asterisks, and the phosphorothioate groups are indicated by p.

Strand Transfer Reactions in Half-sites—The conditions for half-site strand transfer were essentially the same as those for full-site recombinations described previously (Parsons et al., 1990). Reactions were terminated by the addition of SDS (0.1% final concentration) and treated with proteinase K (100 µg per reaction for 1 h at 37°C). After chloroform-phenol extraction and ethanol precipitation of the aqueous phase, the DNA was fractionated by electrophoresis in 10% denaturing polyacrylamide gels (19:1 cross-linked) and stained with ethidium bromide. Since the reactions contained a radioactively labeled substrate, the strand exchange products were visualized by autoradiography.

Analysis of Strand Transfer Products from the Phosphorothioate-substituted Half Site—Strand transfer reaction was carried out with Flp according to standard protocols for 30 min at 30°C. The strand transfer product was separated from the substrate by electrophoresis in 10% denaturing polyacrylamide gels (19:1 cross-linked) and revealed by autoradiography. The radioactive bands of interest were cut out from the gel, and DNA was recovered by standard procedures.

Snake venom digestion was done in 50 mM Tris-HCl, pH 9.0, for 8 h at 32°C with a total of 0.012 enzyme units (Boehringer-Mannheim; specific activity, 1.5 units/mg protein) per reaction added 4 h apart in two equal aliquots. Digestion with P1 (Boehringer-Mannheim; 300 units/mg protein) was done in 10 mM sodium acetate, pH 5.3, 0.5 mM ZnSO4, for 8 h at 32°C with a total of 1 enzyme unit per reaction added in two 0.5-unit aliquots 4 h apart. The limit digestion products were identified by electrophoresis in 20% denaturing polyacrylamide gels.

Quantification of Strand Exchange—To estimate relative extents of strand exchange, autoradiograms were scanned using a Bio-Rad densitometer (Model 1650), and the area under the peaks corresponding to product bands of interest were integrated.

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

RESULTS AND DISCUSSION

Flp(Y343F) Mediates Strand Transfer in Half Sites—The nuclease attack on the scissile phosphate during the strand cleavage step of recombination is carried out by Tyr-343 of Flp (Evans et al., 1990) (see Fig. 1). Mutants of Flp that lack this active site tyrosine are able to bind substrate normally, but are incapable of DNA cleavage (Prasad et al., 1987). Hence a Flp variant in which Tyr-343 is replaced by Phe, Flp(Y343F), would be expected to be inactive in strand transfer.

The reactions of a Flp half-site with wild type Flp and two Flp variants, Flp(Y343F) and Flp(H305L), are shown in Fig. 2A. A half site, containing one Flp binding site and one strand cleavage site, can undergo intra- or intermolecular strand

FIG. 1. Schematic representation of Flp recombination. Recombination of a pair of strands between two substrates by Flp is schematically shown. The phosphodiester that is involved in the two transesterification reactions is indicated (p). This phosphodiester joins the last nucleotide (C) of the Flp-binding element and the first nucleotide (t) of the strand exchange region. The first transesterification step links the 3'-phosphate on CA to Tyr-343 of Flp and leaves 5'-OH on the spacer. In the second transesterification step, the 5'-hydroxyl on the t attacks the phosphotyrosyl bond on the cleaved partner strand. A similar pair of cleavage and exchange steps on the bottom strand (dashed lines) will complete recombination.
Half-site Strand Transfer by Flp(Y343F)

Fig. 2. Half-site strand transfer by Flp and two-step-arrest mutants of Flp. A, the half-site reaction is schematically shown at the top. The thick arrows represent the Flp-binding sequence. The asterisk indicates the 32P label. The 5' and 3'-ends of DNA strands are shown by the circle and the split-arrowhead, respectively. Cleavage of the substrate (S) by Flp, loss of the trinucleotide TTT, and phosphoryl transfer to the 5'-hydroxyl of the bottom strand results in the hairpin product (P). The reactions were fractionated by electrophoresis in a 10% denaturing polyacrylamide gel. Lane 1, no Flp control; lanes 2 and 3, Flp; lanes 4 and 5, Flp(Y343F); lanes 6 and 7, Flp(H305L). The reactions were done at 30°C for 30 min and contained approximately 4 pmol of Flp or Flp variant/pmol of substrate. B, Flp or the indicated Flp variant was incubated under recombination conditions for 15 min with the same half site as in A, except that the 5'-spacer hydroxyl was phosphorylated. The reactions were fractionated on a 10% nondenaturing acrylamide gel (29:1 cross-linked). Complexes labeled I and II are presumably monomeric and dimeric protein-half-site assemblies, respectively. The substrate band is denoted by S. Lanes from left to right are binding reactions containing approximately 0, 3, and 6 pmol of protein/pmol of half site for Flp and Flp(Y343F), and 0, 2, 4, and 6 pmol of protein/pmol of half site for Flp(H305L).

transfer in the presence of Flp to give hairpin or pseudo-full-site products (Fig. 2A, lanes 2 and 3; Serre et al., 1992). The predominant product in a single half-site reaction is the hairpin (Serre et al., 1992). The sum of the two products was assessed by electrophoresis in 10% denaturing polyacrylamide gels. Since the hairpin and the pseudo-full site contain the same radioactive strand, they comigrate in a denaturing polyacrylamide gel (Fig. 2A; P). Flp(Y343F) gave rise to a strand transfer product that had the same mobility as the normal Flp product. The yield of this product was approximately 2-3% of that from a normal Flp reaction (Fig. 2A; lanes 4 and 5) and three to four times higher than that from a reaction by Flp(H305L) (lanes 6 and 7; Fig. 2A). Flp(H305L) is capable of strong cleavage, but incapable of strand exchange in a full-site reaction, and executes weak cleavage and weak strand transfer in a half-site reaction (Parsons et al., 1988; Serre and Jayaram, 1992). That the reaction by Flp(Y343F) was distinct from that of Flp or Flp(H305L) was suggested by the results shown in Fig. 2B. Association of Flp with a half site gave rise to two predominant complexes (HS I and HS II), a significantly smaller quantity of HS III, and a trace amount of HSIV (Fig. 2B; Serre et al., 1992). HS II likely corresponds to the dimeric Flp-half site complexes described by Qian et al. (1990), in which one half of the sites was Flp-cleaved. Such dimeric complexes could not be produced by cleavage minus Flp mutants. The low level of HS II yielded by Flp(H305L) correlated well with its weak strand transfer activity (compare levels of P formed (lanes 6 and 7, Fig. 2A) to HS II levels formed (Fig. 2B) by Flp(H305L)). In contrast, Flp(Y343F) yielded no detectable HS II complex (Fig. 2B), but produced higher levels of half-site strand transfer than Flp(H305L) (Fig. 2A; lanes 4 and 5). One interpretation of this result is that half-site strand transfer by Flp(Y343F) occurs by a mechanism that is independent of strand cleavage by the protein.

The Flp(Y343F) in the above experiments was expressed by a clone that was reconstructed by a "cassette replacement" procedure that virtually excludes contamination of the clone by the wild type gene (see "Materials and Methods" and "Appendix"). This precautionary measure is not fool-proof against wild type contamination due to possible transcriptional and translational errors in the E. coli host. However, given the significant catalytic activity seen with Flp(Y343F) and the distinctive features of the Flp(Y343F) reaction (described below), the contamination theory is quite untenable. Two other Flp variants of position 343, Flp(Y343S) and Flp(Y343G), behaved identical to Flp(Y343F) in the half-site recombinations (data not shown).

The authenticity of the strand exchange product formed by Flp(Y343F) was inferred by a set of reactions in which the spacer length of the bottom strand of the half-site was altered from the normal 8 to 6 and 4 nt. In each case, the product of the Flp reaction and that of the Flp(Y343F) reaction comigrated during electrophoresis in a denaturing gel (see Fig. 3; discussed in more detail later). In addition, when the spacer was shortened to 2 nt or the 5'-spacer hydroxyl of
The half-site was phosphorylated, no strand exchange product was detected from the Flp and Flp(Y343F) reactions (data not shown). This set of results is interpreted to mean that in the Flp(Y343F) reaction, strand cleavage and transfer points were no different from those in the normal Flp reaction. The unexpected reaction observed with Flp(Y343F) forces one to the premise that the removal of the normal site tyrosine unmasked a strand transfer mechanism that remained cryptic in the normal reaction course.

Flp(Y343F) Strand Transfer Reaction Is Distinct from the Wild Type Reaction—The distinction between strand transfer by wild type Flp and the variant protein was verified by using half-sites with altered spacer lengths (Serre et al., 1992). Reactions contained a left half-site, 5′-end labeled with 32P on the strand that undergoes cleavage by Flp (Fig. 3). This strand was the same for the half-site variants as well. The unlabeled strand contained an 8-nt spacer for the normal site and 6 and 4 nt for the variant sites. Thus, in these experiments “variable spacer lengths” refer only to the bottom strands of the half sites. When the spacer was shortened to 6 from 8 nt, the net yield of strand transfer product (P) in the wild type reaction was approximately the same (in the reactions shown in Fig. 3, the product ratio was 0.8 to 1; compare lanes 6 and 10). This shortening had virtually no effect on the Flp(Y343F) reaction as well (lanes 11 and 7 or lanes 12 and 8). More pertinent was the result with the 4-nt spacer. From this substrate, wild type Flp yielded roughly as much product as from the substrate with the 6- or 8-nt spacer (lane 2); on the other hand, the product yield with Flp(Y343F) was barely above the lower limits of detection (lane 3). The band labeled X seen in the Flp reaction from the half-site with 4-nt spacer (lane 2) is likely the result of some contamination of the substrate by a half site containing a longer spacer (perhaps 6 nt). The level of this contaminant (assuming it to be the substrate with the 6-nt spacer) was likely not high enough to elicit a product in the Flp(Y343F) reaction.

To conclusively rule out Flp contamination of Flp(Y343F), experiments shown in Fig. 4 were done. In one set of reactions (Fig. 4; left three lanes of each panel), the substrates were individual half sites containing 8, 6, and 4-nt-long bottom strand spacers. In the second set (Fig. 4; right two lanes of each panel), the substrate with the 8-nt bottom strand spacer was mixed with an equimolar amount of one of the other two substrates (containing 5- or 4-nt bottom strand spacer). Reactions were done with wild type Flp (30-min incubation), Flp(Y343F) (3 cycles of 30-min incubation), and with Flp(Y343F) deliberately contaminated with approximately 5–1% Flp (30-min incubation). The entire reactions with Flp(Y343F) and the Flp(Y343F)/Flp mixture, and 2% of each of the reactions with Flp were fractionated on 10% denaturing polyacrylamide gels (19:1 cross-linked). The substrate or substrate mixture used in a reaction is indicated at the top of the corresponding lane. The substrate and product bands are denoted by S and P, respectively.
substrate with the 8-nt spacer. The product from the substrate with the 4-nt spacer was below the levels of detection. The results were strikingly different when Flp(Y343F) was laced with approximately 0.5-1% Flp. Product formation was now evident in the reaction with the half site containing the 4-nt spacer by itself, or in a mixed substrate reaction (8- and 4-nt spacers). The wild type contribution in the mixed protein reaction must be roughly one-fifth to one-third of the total (given that Flp(Y343F) is 2% as active as Flp, and the protein is contaminated with 0.5-1% Flp). This level of Flp contribution to the Flp(Y343F)/Flp mixture can fully account for the extent of strand transfer obtained from the half site with the 4-nt spacer, either by itself or in presence of a second half site (8-nt spacer). In the individual reactions, the ratios of the product from the half site with the 4-nt spacer to those from half sites with 6- and 8-nt spacers were 1:5 and 1:6, respectively. In the mixed substrate reaction (8- and 4-nt spacers), the corresponding ratio was approximately 1:9.

Thus, the mixed substrate-mixed protein results vouch for the differences between Flp and Flp(Y343F) activities toward the different substrate types. While the Flp activity is virtually unaffected by the change in spacer length from 8 or 6 to 4 nt, the Flp(Y343F) activity drops precipitously as a result of the same change. The presence of a clear product signal with 1% Flp/99% Flp(Y343F) from the half site containing the 4-nt spacer (Fig. 4; lanes 3 and 5 of the right panel) and the virtual absence of this signal with Flp(Y343F) alone (Fig. 4; lanes 3 and 5 of the central panel) show that potential contamination of Flp(Y343F) by Flp must be much less than 1%. Yet, the Flp(Y343F) reactions with half sites containing 6- or 8-nt spacers yield nearly 2% signal relative to the corresponding Flp reactions (Fig. 4; lanes 1 and 2 of the central and left panels). This contradiction makes the “contamination hypothesis” unacceptable.

**What Is the Active Nucleophilic Group in the Flp(Y343F) Strand Transfer Reaction?**—The two steps of the normal Flp catalyzed reaction involve two nucleophilic substitutions. The first one is initiated by the hydroxyl group of Tyr-343 and results in DNA strand breakage and formation of a phosphotyrosyl linkage. The second one, carried out by the 5'-hydroxyl of the nicked strand of the partner substrate, breaks the phosphotyrosyl bond, reforms the DNA phosphodiester, and produces a recombinant strand (Parsons et al., 1988; Evans et al., 1990).

What then is the active nucleophile in the Flp(Y343F) reaction? One may envisage two possibilities that are not mutually exclusive (Fig. 5, A and B). In the absence of Tyr-343, a surrogate nucleophile from the protein (X, Fig. 5A), perhaps the hydroxyl from a serine or a threonine or a second tyrosine or the thiol group from a cysteine, could weakly substitute for it. In this case, the reaction would proceed via the normal two step mechanism; the first transesterification causing strand cleavage and the second transesterification causing strand exchange (Fig. 5A). On the other hand, the half site/Flp(Y343F) combination contains a nucleophile that is conspicuously absent in the normal full site reaction scheme; the 5’-hydroxyl of the spacer DNA. Recall that, in the full site reaction, a 5'-hydroxyl is exposed at the strand exchange point only after strand cleavage has been executed. The spacer hydroxyl of the half site can potentially mimic the hydroxyl of Tyr-343 and directly attack the scissile phosphodiester. Strand transfer would then be reduced from the normal two-step cleavage and exchange reaction to a concerted single step reaction. This is depicted in the “concerted break-join” model of Fig. 5B. The conformational freedom of the active hydroxyl demanded by this model can be provided by the virtually single stranded nature of the half site spacer.

The surrogate nucleophile model and the concerted break-join model make contrasting predictions for a half-site reaction in which the Flp cleavage site is normal, but in which strand transfer is precluded by phosphorylating the spacer hydroxyl or by making the bottom strand spacer too short (2 nt) to be reactive. In this situation, the surrogate nucleophile, if it does exist, should still be able to carry out strand cleavage. On the other hand, if strand transfer is a single step reaction mediated directly by the spacer hydroxyl, elimination of strand exchange should simultaneously eliminate cleavage.

The two models were tested by doing a reaction in which one half of the substrate molecules was labeled at the 5'-end with 32P on the cleavage strand by (γ-32P)ATP using polynucleotide kinase, and the other half was labeled at the 3'-end of the cleavage strand with 3'-[α-32P]cordycepin 5'-triphosphate using terminal deoxynucleotidyl transferase (the mixed substrate is marked S in Fig. 6; see also Fig. 5). Strand transfer was measured by transfer of the 5’ label from substrate 1 into the recombinant (P), while strand cleavage was measured by the release of the 3’ label from substrate 2 as a tetranucleotide (C). The results of reactions with two half-site substrates, one in which the bottom strand contained the normal 8-nt spacer (lanes 1, 3, 4, and 7) and the other in which the spacer was shortened to 2 nt (lanes 2, 5, 6, and 8) are shown in Fig. 6A. Note that “spacer length,” when mentioned, refers to the bottom strand of the half site. In these assays, the wild type Flp concentration was reduced to levels that gave product yields comparable to or lower than those obtained with Flp(Y343F). The recombinant and cleavage products formed from the two half sites at higher concentrations of Flp are shown in lanes 3 and 4 of Fig. 6B. With wild type Flp, the normal half site yielded recombinant (P) and cleavage (C) products in roughly equimolar amounts (lane 7, Fig. 6A; see also lane 3 of Fig. 6B). In contrast, the half-site with the 2-nt spacer yielded no recombinant, but gave the same or slightly elevated levels of the cleaved tetranucleotide relative to the normal substrate (compare lane 7 with lane 8 of Fig. 6A and lane 3 with lane 4 of Fig. 6B). From a number of experiments, this increase in cleavage was estimated to be roughly 1.5–2-fold. The difference in the relative intensities of P and C in lane 3 of Fig. 6B compared with lanes 3 and 4 of Fig. 6A reflects the difference in the specific activities of the 5’- and 3’-end-labeled substrates in the two experiments. The reaction of Flp(Y343F) with the normal half site was quite similar to that of Flp; the recombinant and cleavage products were realized in approximately equal quantities (lanes 3 and 4, Fig. 6A). However, with the half site containing the 2-nt spacer, the result was strikingly different. No recombinant was obtained, but the cleavage was sharply reduced (approximately 20–25-fold; lanes 3 and 6, Fig. 6A). Identical results were obtained when the experiment was repeated with a half site containing a 5’-phosphorylated 8-nt spacer in place of the half site with the 2-nt spacer. Phosphorylation of the spacer hydroxyl abolishes strand transfer in both wild type and Flp(Y343F) reactions (data not shown). However, the cleavage event persisted in the wild type reaction, whereas it was completely abolished in the Flp(Y343F) reaction. The large negative effect of strand transfer inhibition on substrate cleavage (20–25-fold) in the Flp(Y343F) reaction and the relatively small effect (1.5–2-fold in the opposite direction) in the Flp reaction imply that the predominant, if not the exclusive, mechanism for the Flp(Y343F) reaction is as depicted in the concerted break-join model. The weak cleavage (less than one-twentieth of that seen under strand transfer conditions; compare lanes 5 and 6 with 3 and 4) seen in the Flp(Y343F) reaction even
when strand transfer was inhibited may be accounted for by phosphodiester hydrolysis or, less likely, by phosphoryl transfer to a "surrogate nucleophile" within Flp(Y343F). This result indicates that, when the half site is bound by Flp or by Flp(Y343F), the exchange site phosphate becomes susceptible to hydrolysis. A similar situation obtains in Tetrahymena pre-ribosomal RNA (see Cech, 1990). Here, the 5'- and 3'-splice site phosphodiester bonds are slowly cleaved even in the absence of guanosine, whose 3'-hydroxyl normally carries out cleavage of the phosphodiester at the 5'-splice site.

Overall, the above results reveal two key features of the Flp(Y343F) reaction that distinguish it from the Flp reaction. First, Flp(Y343F) is active toward half sites only if the bottom strand spacer ends in a free 5'-OH; wild type Flp can cause top strand cleavage in a half site whose bottom strand spacer ends in a 5'-phosphate. Second, unlike the wild type Flp-half-site reaction, the Flp(Y343F)-half-site reaction shows much tighter coupling of DNA cleavage to strand transfer. Both results argue strongly for a direct attack by the spacer hydroxyl from the bottom strand on the phosphate at the normal Flp cleavage point within the top strand.

Assuming the active nucleophile in the Flp(Y343F) reaction to be the spacer 5'-hydroxyl, there has to be enough conformational flexibility for this species to approach the cleavage site phosphate. This steric feature of the reaction is substantiated by the large disparity in the reactivity of the half sites with 6- and 4-nt spacers in the presence of Flp(Y343F). The 4-nt spacer (AAAG) is severely constrained in its conformational mobility through hydrogen bonding to the three spacer nucleotides (TTT; see Fig. 3) of the top strand. Consequently, the full strand exchange potential of this spacer remains unexpressed. The very weak activity of this substrate can be accounted for by DNA breathing. In the wild type reaction, the attack by the spacer hydroxyl is preceded by strand cleavage and removal of the trinucleotide, TTT, from the top strand. Therefore, the 4- and 6-nt spacers have nearly equal flexibility and show roughly equal reactivity.

Stereoreactivity of Phosphoryl Transfer in Flp Recombination—Substitution of one of the nonbridging oxygen atoms of a phosphate within a DNA chain by sulfur produces a chiral center at the phosphorous atom. Chirality of the phosphorothioate substrate allows one to test whether a phosphoryl transfer reaction proceeds with retention or inversion of configuration. Phosphoryl transfer reactions can, in principle, proceed by a dissociative pathway (Sn1), an in-line associative pathway, or an adjacent associative pathway (Sn2). The stereochemistry of the final reaction product, inversion, retention, or racemization of configuration, can place constraints on the possible range of reaction mechanisms, although, in the absence of direct observation of intermediates, they cannot prove a particular mechanism. Results from a number of enzyme reactions indicate that, in general, enzymes that carry out a phosphoryl transfer in a single step (or strictly, an odd number of steps) do so with inversion of configuration and those that carry out the transfer in two steps (or strictly, an even number of steps) do so with retention of configuration (reviewed in Eckstein (1985); Floss et al. (1984); Knowles (1980)). This set of observations would be consistent with an in-line nucleophilic substitution mechanism involving a trigonal bipyramidal intermediate. It is therefore an enzymological tenet that inversion of configuration provides "acceptable" proof for a one-step phosphoryl transfer; conversely, retention supports a two-step transfer mechanism. In the context of recombination, it has been shown that RNA cleavage catalyzed by the Tetrahymena rRNA intron and transfer of the nicked end of phage Mu to target DNA by the Mu transposase are accompanied by inversion of phosphate configuration (McSwiggen and Cech, 1989; Mizuuchi and Adzuma, 1991). These results were interpreted to mean that the nucleophiles in the two reactions, the 3'-hydroxyl of guanosine in RNA cleavage and the 3'-hydroxyl of DNA in transposition, directly attack the target phosphate.

In the normal Flp reaction, which is generally accepted to be a two-step event, one would expect the phosphate undergoing transfer to retain its configuration (see Fig. 7). The stereoreactivity of the half-site reaction was tested by using a half site in which the cleavage site phosphate was replaced by phosphorothioate (the R<sub>s</sub> form) as described under "Materials and Methods." The 5'-neighbor of the phosphorothioate was a C that carried a 5'-phosphate labeled with 32P.
The stereospecificities of P1 nuclease (active on the S\(_p\) isomer but not the R\(_p\) isomer) and snake venom phosphodiesterase (active on the R\(_p\), but not S\(_p\) isomer) were used to monitor the stereochromical consequence of strand transfer in this substrate (Fig. 8). The limit digest of the R\(_p\) substrate (Fig. 8; lane 1) with P1 and snake venom produced a labeled dinucleotide (pCpsT) and a labeled mononucleotide (pC), respectively (Fig. 8; lanes 5 and 6). The digestion pattern of the gel-isolated strand transfer product (Fig. 8; lane 2) from the wild type reaction with snake venom or P1 was qualitatively similar to that of the substrate (Fig. 8; lanes 3 and 4), indicating retention of stereochromical configuration. The above result is consistent with the two-step strand transfer mechanism, with each step producing stereochemical inversion of phosphate configuration (see Fig. 7). It may be noted, however, that the stereochromy does not absolutely rule out two steps, each of which results in retention of configuration. Unambiguous stereochromical assignment to the product of the Flp(Y343F) reaction was difficult because of its low yield, the significant phosphate contamination of the commercial phosphoro-thioate, and the potential stereochromical preference for one of the two forms (S\(_p\) and R\(_p\)) of the substrate.

Thoughts on Self-catalyzed and Proein-mediated Phospho-transfer Reactions in Nucleic Acids: Evolution of Catalysis—Although we have not absolutely ruled out the possibility that the Flp(Y343F) reaction is mediated by a surrogate nucleo-phil from the protein, we prefer the mechanism involving direct attack by the DNA hydroxyl for its general implications in a variety of phosphoryl transfer reactions. We explain strand transfer by the catalytically inactive Flp(Y343F) as follows. The protein is able to bind substrate normally and make the DNA contacts relevant to the first transesterification reaction of recombination, namely DNA cleavage. In the protein-DNA complex, the phosphate at the cleavage point,
which is known to be contacted by the Flp protein (Bruckner and Cox, 1986), is rendered particularly susceptible to nucleophilic attack. We imagine that, in the case of wild type Flp, chirality as the substrate. Hence, the limit digestion pattern of the hairpin with snake venom and P1 will be the same as that of the substrate.

so with no net gain of DNA, while the transposase indirectly causes gain of DNA. The site-specific recombinase makes four breaks and joints with no loose ends left behind to be tied up later. The first step of recombinase reaction consumes a substrate and a target phosphodiester, but also generates one each of the two species required for the second step. The transposase has evolved to add phosphodiesters during DNA rearrangements: duplication of itself and of the target. To ensure this, the first half of the transposase reaction produces a reactive nucleophile but does not produce a potential target diester. This would indeed be desirable if reciprocity of strand transfer is to be avoided. Transposition, like recombination, breaks four phosphodiester bonds; but only two new ones are immediately formed. It is left to the replication/ligation machinery of the bacterial host to join the loose ends, thereby increasing the number of phosphodiester in the process.

The Flp(Y343F) reaction has implications for RNA-mediated catalysis (Cech, 1990; Pace and Smith, 1990; Altman, 1991; Wirsching et al., 1991) suggests that "once an appropriate binding cavity is achieved, reaction pathways commensurate with the intrinsic chemical potential of proteins ensue." The Flp(Y343F) reaction indicates that, in some instances, the chemical potential of the substrate may also be tapped.
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APPENDIX

![Diagram of DNA fragment coding for the carboxyl-terminal portion of Flp(Y343F) clone, which was initially obtained by site-directed mutagenesis of the wild type template. The sequences of the oligonucleotide cassettes used for sequence replacements are shown. The asterisk indicates the Phe-343 codon (TTT). Plasmid 4 is the expression vector in which the amino-terminal portion of Flp is fused to the XbaI.](image)

**FIG. 1. Reconstruction of Flp(Y343F) from the initial clone by the “cassette” replacement procedure.** Details of the reconstruction of Flp(Y343F) starting from the original Flp(Y343F) clone are described under "Materials and Methods." Plasmid 1 contains the Flp(Y343F) clone, which was initially obtained by site-directed mutagenesis of the wild type template. The sequences of the oligonucleotide cassettes used for sequence replacements are shown. The asterisk indicates the Phe-343 codon (TTT). Plasmid 4 is the expression vector in which the amino-terminal portion of Flp is fused to the lambda Pr promoter. Ba, Bsp; Be, BclI; E, EcoRI; S, SnaBI; Sp, SphI; X, XbaI.

**FIG. 2. Activity of Flp(Y343F) expressed from the reconstructed clone.** Flp(Y343F) was expressed in *E. coli* from plasmid 5 shown in Fig. 1 and purified to near homogeneity by a procedure that included a final step of affinity purification. The activity of this protein was assayed by the standard half-site assay. S is the labeled substrate, and P is the strand transfer product. Lane 1, no protein control; lane 2, two 30-min cycles of the Flp(Y343F) reaction with approximately 4 pmol of protein per pmol of half site; lanes 2–5, reactions with increasing aliquots of the extract from temperature-induced *E. coli* cells containing plasmid 4 of Fig. 4A carried through the Accell-CM chromatography step of the standard Flp purification procedure (Prasad et al., 1987).

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