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Wild-type Flp recombinase cleaves DNA *in trans*

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Site-specific recombinases of the Integrase family utilize a common chemical mechanism to break DNA strands during recombination. A conserved Arg-His-Arg triad activates the scissile phosphodiester bond, and an active-site tyrosine provides the nucleophile to effect DNA cleavage. Is the tyrosine residue for the cleavage event derived from the same recombinase monomer which provides the RHR triad (DNA cleavage *in cis*), or are the triad and tyrosine derived from two separate monomers (cleavage *in trans*)? Do all members of the family follow the same cleavage rule, *cis* or *trans*? Solution studies and available structural data have provided conflicting answers. Experimental results with the Flp recombinase which strongly support *trans* cleavage have been derived either by pairing two catalytic mutants of Flp or by pairing wild-type Flp and a catalytic mutant. The inclusion of the mutant has raised new concerns, especially because of the apparent contradictions in their cleavage modes posed by other Int family members. Here we test the cleavage mode of Flp using an experimental design which excludes the use of the mutant protein, and show that the outcome is still only *trans* DNA cleavage.

Keywords: Flp/integrase family/site-specific recombinase/*trans* cleavage

Introduction

The Integrase family, headed by the Int protein of phage lambda, is a large family of site-specific recombinases comprising over a hundred members. The hallmark of the family is a tetrad motif, R-H-R-Y, which until recently was thought to be invariant (Esposito and Scocca, 1997; Nunes-Düby *et al*., 1998, and references therein). It has now been noted that the histidine residue is not absolutely conserved within the family. Nevertheless, biochemical analyses (Gronostajski and Sadowski, 1985; Pargellis *et al*., 1988; Parsons *et al*., 1988, 1990; Evans *et al*., 1990; Chen *et al*., 1992b; Friesen and Sadowski, 1992; Lee *et al*., 1992; Arciszewska and Sherratt, 1995) as well as currently available structural information (Guo *et al*., 1997; Hickman *et al*., 1997; Kwon *et al*., 1997; Subramanya *et al*., 1997; Gopaul *et al*., 1998) point to a common catalytic role for the tetrad motif. The two arginines of the RHR triad activate the labile phosphodiester bond adjacent to the bound recombinase monomer; the tyrosine residue then provides the nucleophile to break this bond. The histidine may contribute to the cleavage reaction by either stabilizing the leaving group or directly participating in the phosphodiester activation. In the Flp protein, the site-specific recombinase encoded by the yeast 2 micron plasmid, the tetrad residues correspond to Arg191, His305, Arg308 and Tyr343. Secondary structure alignments of a large number of the Int family members (Esposito and Scocca, 1997; Nunes-Duby *et al*., 1998), as well as the structural relatedness between type I topoisomerases and Int type recombinases in their catalytic pockets (Guo *et al*., 1997; Cheng *et al*., 1998; Redinbo *et al*., 1998), have unveiled two more conserved catalytic residues. One of these is a lysine residue situated within a β-stranded region, occasionally substituted by an arginine. The other is a conserved histidine which in some recombinases is replaced by a tryptophan. The equivalent residues in Flp are Lys223 and Trp330, respectively.

The Int family members utilize a type IB topoisomerase mechanism to carry out the strand breakage and joining steps of recombination (Figure 1). Each of the two core sites taking part in the reaction contains two inverted DNA elements, each capable of binding a recombinase monomer, separated by a 6–8 bp strand exchange region (spacer). Initiation of recombination requires strand cutting by the active-site tyrosine (Tyr343 in Flp) at one end of the spacer in each substrate to produce a 3′-phosphotyrosine linkage and a DNA 5′-hydroxyl group. During the strand-joining step, the 5′-hydroxyl groups attack the phosphotyrosine bonds across partner substrates, forming a Holliday junction intermediate. By repeating the stand cleavage and exchange steps at the other end of the spacer, the junction can be resolved into two reciprocal recombinant DNA products.

Within the family, there appears to be some discrepancy in the mechanism of the tyrosine-mediated cleavage reaction: the so-called *cis–trans* paradox (Jayaram and Lee, 1995; Stark and Boocock, 1995; Jayaram, 1997). The recombination reaction requires the controlled breakage and reformation of four phosphodiester bonds, mediated by the cooperative action of four recombinase monomers. Does each monomer contribute the catalytic tyrosine *in cis* (for attacking the scissile phosphate adjacent to it) or *in trans* (for attacking one of the other three scissile phosphates) (Figure 2). For the Flp protein, a large body of evidence favors *trans* cleavage and disfavors *cis* cleavage (Chen *et al*., 1992a, 1993; Lee and Jayaram, 1993,1995; Lee *et al*., 1994). On the contrary, the *Escherichia coli* XerC and XerD recombinases appear to cleave DNA exclusively *in cis* (Arciszewska and Sherratt, 1995). For lambda Int, experiments based on the distinct binding specificities of the Int proteins of lambda and the

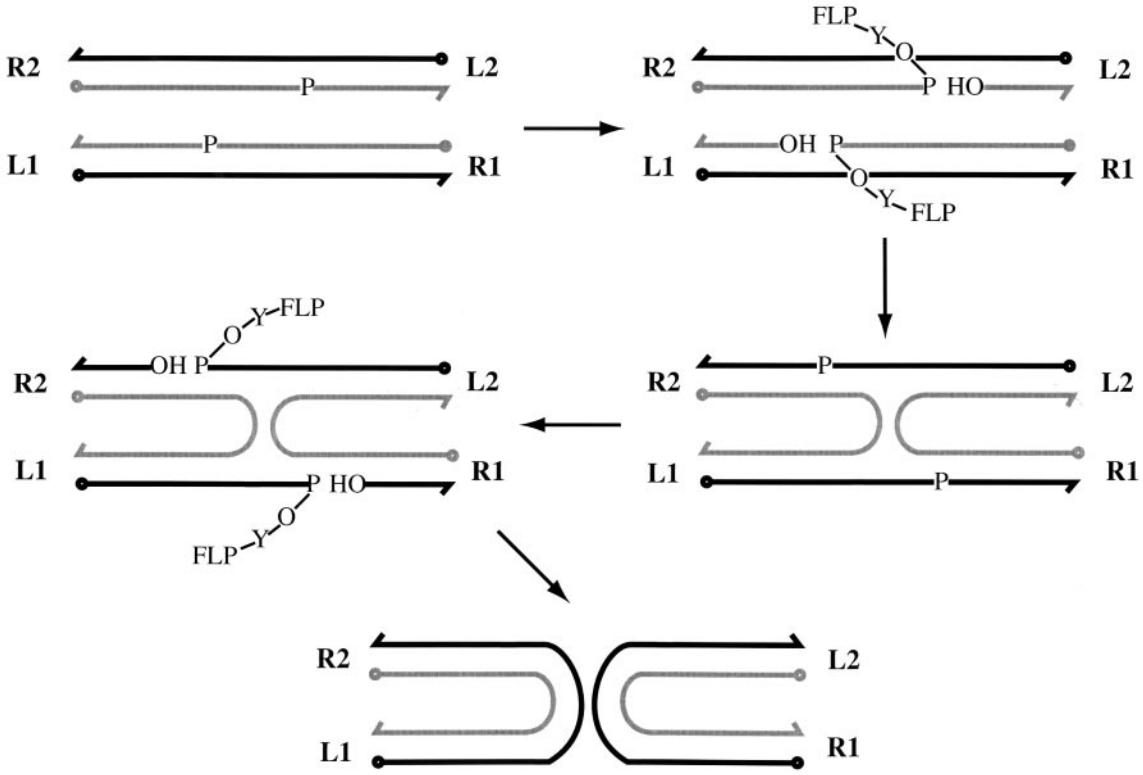

Fig. 1. The pathway for Integrase family recombination. The core recombination sites are shown in the antiparallel orientation. Each one harbors two recombinase-binding elements on either side of the strand-exchange region (spacer) in a head to head orientation. The scissile phosphodiester bonds on the top and bottom strands, denoted by P, define the borders of the spacer. The strand polarities are indicated by circles at the 5′ ends and split arrows at the 3′ ends. Strand cutting results in the covalent linkage of Flp, via its active site tyrosine, to the 3′-phosphate end of the broken strand. The first strand cleavage/exchange event at one end of the spacer (say, left or L end) results in the Holliday junction intermediate. The chemical steps are repeated at the other end of the spacer (right or R end) to yield the final recombinants.

related phage HK022 are consistent with *cis* cleavage (Nunes-Düby *et al*., 1994). In contrast, the apparent catalytic complementation between two active-site mutants of lambda Int on suitably designed DNA substrates is more readily accommodated by *trans* cleavage (Han *et al*., 1993). In the crystal structure of the Int catalytic domain (Kwon *et al*., 1997), the position of the active-site tyrosine (substituted by phenylalanine in the crystallized protein) corresponds to the *trans* cleavage configuration. However, by a minor perturbation of a loop at its base, the peptide segment harboring the tyrosine can be folded back to establish the *cis* cleavage configuration. The most recent contradictions surfaced when solution studies with the Cre recombinase from phage P1 indicated *trans* cleavage (Shaikh and Sadowski, 1997), whereas the crystal structure of the Cre tetramer complexed with the DNA substrate clearly demonstrated *cis* cleavage (Guo *et al*., 1997).

The apparently non-uniform modes of DNA cleavage (*cis* versus *trans*) by the Int family recombinases despite their nearly identical three dimensional peptide folds (suggested by structures and sequence alignments) and their common mechanistic pathway pose an annoying contradiction. Furthermore, how does one reconcile the dichotomy of both *cis* and *trans* DNA cleavages by the same protein? The *cis* cleavage mode directly observed in the Cre–DNA crystals has caused the *trans* cleavage mode inferred from reactions with Flp to be viewed with renewed, though guarded skepticism. Hence we have subjected Flp to as rigorous a *cis–trans* test as we could design, and summarize the results in this report: Flp cleaves DNA *in trans*.

Results and discussion

Is trans DNA cleavage by Flp an artefact?

The original definition of *trans* DNA cleavage (Chen *et al*., 1992a) is schematically outlined in Figure 2A. The three potential modes of *trans* cleavage refer to the directions in which the active-site tyrosine (Tyr343) of a Flp monomer may be donated to a second monomer (which provides the RHR triad) to effect strand breakage. The scheme on the left illustrates the operation of *trans* cleavage in a pair of DNA substrates, L1R1 and L2R2, synapsed in a parallel orientation (from left to right in both cases). The diagram on the right represents *trans* cleavage as it applies to a Holliday junction with its arms arranged in the antiparallel configuration. The junction could be imagined to have arisen as a result of the first strand exchange step (at the left end) during recombination between L1R1 and L2R2.

The first evidence of DNA cleavage *in trans* by Flp was provided by *in vitro* catalytic complementation between a triad mutant and a Tyr343 mutant of Flp (each being inactive by itself) in reactions with half-site substrates (Chen *et al*., 1992a). A half-site reaction is a simplified mimic of the recombination reaction that requires the breakage of only one phosphodiester bond, and the formation of one recombinant DNA strand (Nunes-Düby

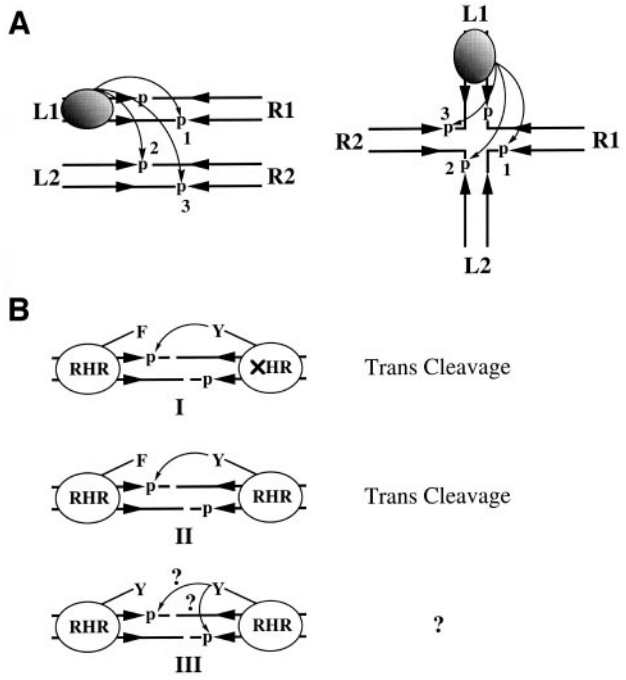

Fig. 2. *Trans* mode of DNA cleavage. (**A**) The three potential modes of *trans* DNA cleavage are shown for a pair of linear substrates (left) and a Holliday junction intermediate (right). The substrates on the left are arranged in the parallel configuration according to the convention of Chen *et al*. (1992a). Each pair of parallel arrows represents a recombinase-binding element. L and R refer to the left and right DNA arms. The labile phosphates are indicated by 'p'. *Trans* horizontal denotes the donation of the active site tyrosine from left to right within the same substrate (1). *Trans* vertical represents the action of tyrosine across substrates, but in a left to left direction (2). *Trans* diagonal refers to tyrosine donation from left to right, but across substrate partners (3). The same definitions hold for the square planar Holliday junction shown on the right. *Cis* cleavage, which refers to tyrosine cleavage at the adjacent phosphodiester bond, is not shown here. (**B**) *Trans* cleavage by Flp has been demonstrated by using a triad mutant (denoted XHR) and a Tyr343 mutant (shown as F in place of Y) of Flp bound to half-site substrates (I). *Trans* cleavage has also been observed in a combination of wild-type Flp and the Tyr343 mutant (II). The experiments in this study were aimed at deciphering the cleavage mode in a Flp dimer which is fully wild-type with respect to the triad and Tyr343 (III).

et al., 1989; Qian *et al*., 1990; Amin *et al*., 1991; Serre and Jayaram, 1992). It was inferred that a single active site required for the reaction is assembled by two Flp monomers acting in concert, one providing the RHR domain, and the other providing Tyr343. Furthermore, the products of the reaction demonstrated that the Tyr343 attack occurs on the scissile phosphate of the half-site bound by the Tyr343 mutant, and not by the RHR mutant (Figure 2B, I; Chen *et al*., 1992a). Later experiments, designed to assemble one active site from a Flp dimer constituted by a wild-type monomer and a Flp(Y343F) monomer, also yielded results that support *trans* cleavage (Figure 2B, II; Lee *et al*., 1994; Whang *et al*., 1994).

Could the presence of the mutant proteins in the reactive heterodimers (Figure 2B, I and II) have forced the recombinase to cleave *in trans* by masking a normal *cis* cleavage mode by wild-type Flp? Since, in the triad mutant–tyrosine mutant pair (Figure 2B, I), the single intact RHR moiety and the single Tyr343 residue are harbored by separate Flp monomers, *cis* cleavage would have been impossible. However, in the wild-type Flp–Flp(Y343F) heterodimer (Figure 2B, II), there are two intact triads, ensuring that the single Tyr343 could have, in principle, cleaved either one of the 'triad-activated' phosphodiesters. Yet, the concern that the absence of the active-site tyrosine in one Flp monomer could have altered the protein interactions in the dimer to yield a false mode of cleavage cannot be completely allayed. The only recourse then is to test cleavage by a Flp dimer containing an intact triad and an intact Tyr343 in each of the two monomers (Figure 2B, III).

The experimental design for testing cleavage mode in a 'wild-type' Flp dimer

The reaction components are: (i) a right half-site labeled on the cleavage strand at the 5′ end (asterisk in Figure 3); (ii) an unlabeled left half-site whose spacer nucleotides are perfectly complementary to those of the right half-site; (iii) wild-type Flp protein; and (iv) wild-type Flp protein fused at its N-terminal end to glutathione *S*-transferase protein (GST) (Figure 3). Strand cleavage by Flp or by GST–Flp within the labeled half-site can be distinguished by the difference in the sizes, and therefore the electrophoretic mobilities of the radioactive covalent DNA complexes formed by the two proteins. The 5′ ends of the 'non-cleaved' strands are phosphorylated in order to block them from carrying out the strand-joining reaction. It should be clarified that, throughout the text, the term 'dimeric half-site complex' refers to a dimer formed by two recombinase bound half-site molecules. Therefore, this complex contains two half-site molecules and two recombinase monomers.

The labeled half-site cannot be cleaved, or is cleaved quite inefficiently by Flp (Figure 3, row 1) or by GST–Flp (row 2). The poor reactivity of a half-site containing only a single spacer nucleotide (T) following the scissile phosphate position results from its failure to establish a functional homodimeric assembly from two recombinase bound half-site molecules (Amin *et al*., 1991; Chen *et al*., 1992a, 1993). However, such a half-site can be rescued by a second half-site (which also contains a single spacer nucleotide on the cleavage strand), provided their single-stranded spacer segments are mutually complementary. Perhaps the base pairing between the spacer strands promotes the dimerization step. In our experiments, this helper function is served by the unlabeled left half-site (Figure 3, rows 3–6). When the two complementary half-sites harbor Flp or GST–Flp on both the left and right partners (that is, homo-dimers with respect to the proteins), distinction between *cis* and *trans* modes of cleavage is not possible (Figure 3, rows 3 and 4). However, when the protein composition of the reactive complex is heterodimeric, the radiolabeled protein–DNA complex resulting from *cis* cleavage can be distinguished from that produced by *trans* cleavage (Figure 3, rows 5 and 6).

It should be pointed out that the data contained in Figures 4–6 are most easily accommodated by the interpretation that the GST–Flp hybrid protein binds as a monomer to a single Flp-binding element (or to a half-site) under the ionic strength employed in our assays. The binding assays have not revealed patterns that would be indicative of the presence of a mixture of GST–Flp monomers as well as GST–Flp homodimers. These results are fully consistent

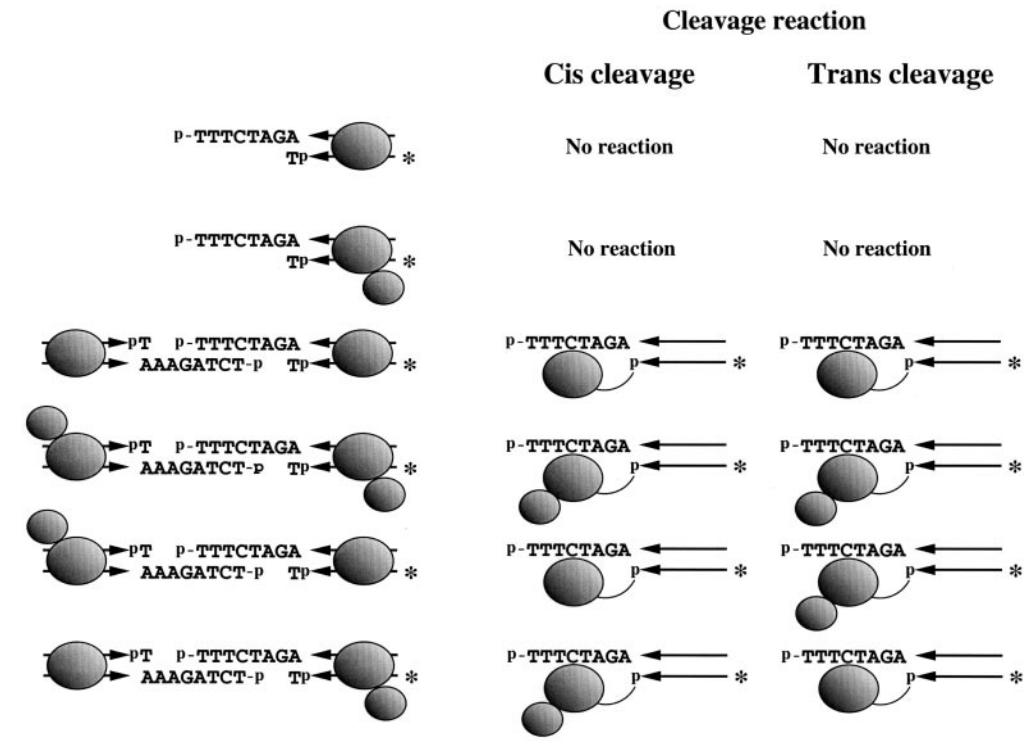

Fig. 3. *Cis* versus *trans* DNA cleavage in Flp-half-site dimers. The labeled right half-site alone (rows 1 and 2; asterisk indicates the labeled 5′ end) cannot form a productive dimeric complex in association with Flp (row 1) or GST–Flp (row 2). However, the left and right half-sites bound to Flp (or GST–Flp) can establish cleavage-competent interactions (rows 3–6). Specific association of Flp or GST–Flp with either the left or the right half-site (rows 5 and 6) permits the distinction between *cis* and *trans* DNA cleavage. In this figure and elsewhere, Flp is drawn as an oval, and GST–Flp as an oval with an attached lobe. The expected results of *cis* and *trans* cleavage are shown to the right of each combination of half-site/protein complexes.

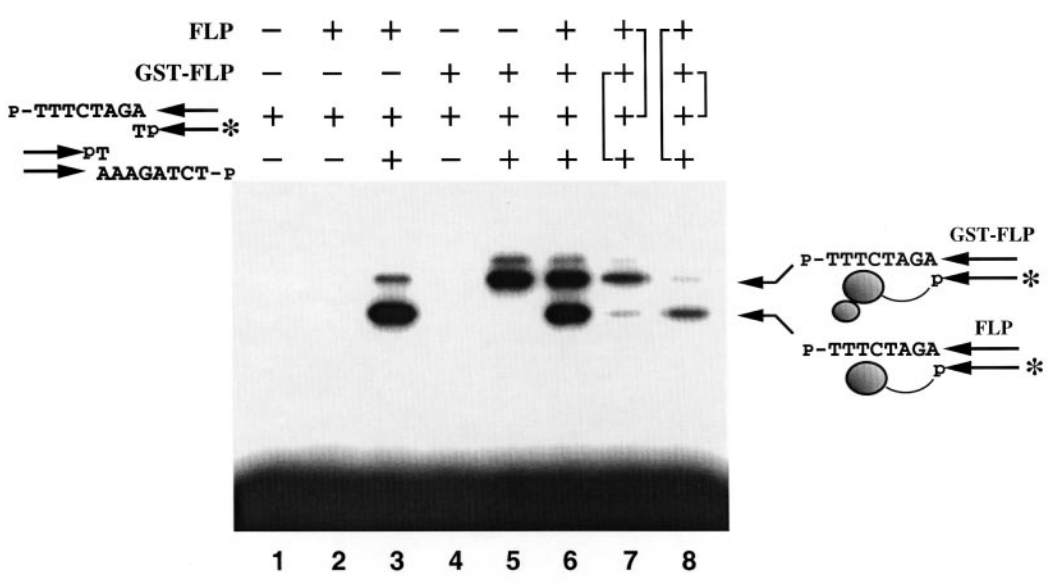

Fig. 4. Cleavage by Flp or GST–Flp in half-sites during random and targeted recombinase association. All reactions contained ~0.01 pmol of the end-labeled right half-site (lanes 1–8); those in lanes 3 and 5–8 contained, in addition, 0.01–0.02 pmol of the unlabeled half-site. Reactions with Flp alone or GST–Flp alone contained ~5 pmol protein per pmol of half-site (lanes 2–5). The same protein to DNA molar ratio was maintained in reactions containing roughly equimolar amounts of the two proteins (lanes 6–8). In the reaction in lane 6, the proteins were added to a mixture of the left and right half-sites. In the reactions in lanes 7 and 8, the half-sites were pre-bound to the indicated proteins (parentheses) on ice and then mixed to initiate strand cleavage.

with the earlier observations of Lee and Jayaram (1995) regarding the binding of GST–Flp to DNA molecules containing two Flp-binding elements (full sites). Note that the deduction of the cleavage mode by Flp, *cis* or *trans*, is independent of whether a half-site is complexed with a monomer or dimer of the GST–Flp protein.

DNA cleavage mode by wild-type Flp pre-associated with a half-site

The results of a solution assay to test the predictions laid out in Figure 3 are shown in Figure 4. The labeled right half-site alone (~0.01–0.02 pmol per reaction) yielded no detectable cleaved DNA–protein complex with either Flp (Figure 4, lane 2) or with GST–Flp (lane 4). In the presence of the unlabeled helper left half-site (~0.02 pmol per reaction), strand cleavage on the labeled half-site was observed with Flp (Figure 4, lane 3) as well as with GST–Flp (lane 5). When both Flp and GST–Flp were present in the reaction, the cleavage complex formed by each protein was seen at roughly equal intensity (Figure 4, lane 6). When the labeled half-site was pre-bound by Flp and the unlabeled half-site by GST–Flp (see Materials and methods), and the two were mixed, the radioactive product resulted almost exclusively from GST–Flp cleavage (Figure 4, lane 7). When the reaction was repeated by switching the half-site protein association (with Flp on the unlabeled half-site), the radioactive product was almost entirely due to Flp cleavage. Since the half-life of the Flp–DNA complex is long (Qian *et al*., 1990; Chen *et al*., 1992a) relative to the half-life for the cleavage reaction, little or no recycling of the bound proteins is expected to occur during the assays. The predominance of one type of cleavage over the other in Figure 4, lanes 7 and 8 (~10:1, or greater) is consistent with this notion.

The cleavage patterns observed with the pre-associated half-site complexes demonstrate that strand cleavage by Flp occurs *in trans*, even when each of the partner monomers contains a complete RHR triad set and an active site tyrosine. The weak bands migrating above the expected half-site cleavage products (for example, Figure 4, lanes 3 and 5) may be explained as follows. The 'non-cleaved' strand of the labeled right half-site might not have been exhaustively blocked at the 5′-hydroxyl end (due to incomplete phosphorylation). These free ends could mediate strand joining within the cleaved left half-site molecules (Whang *et al*., 1994). The resulting full-site mimics, when cleaved within the labeled strand, would give rise to the slower migrating cleavage product. If the DNA strands are completely denatured by boiling the reaction mixture and rapidly chilling it prior to electrophoresis, the larger product can be eliminated (data not shown). In this gel system, DNA fragments that are not covalently linked to protein migrate together as one unresolved band (S). We noted that the yield of the covalent complex from the pre-bound half-sites is less than that from half-sites allowed to bind protein randomly (Figure 4, compare lanes 7 and 8 with lane 6). We suspect that there is some interaction, though non-productive, between two protein-bound right half-site molecules that impedes sterically or kinetically the interactions between a protein-bound left half-site and a protein-bound right half-site.

DNA cleavage by wild-type Flp in isolated dimeric half-site complexes

To verify the results supporting *trans* cleavage by a wild-type Flp dimer, we isolated specific DNA–protein complexes from reactions corresponding to those described in Figure 4, and probed them for the type of DNA cleavage they harbored (Figures 5 and 6). Lanes 1–6 in Figure 5

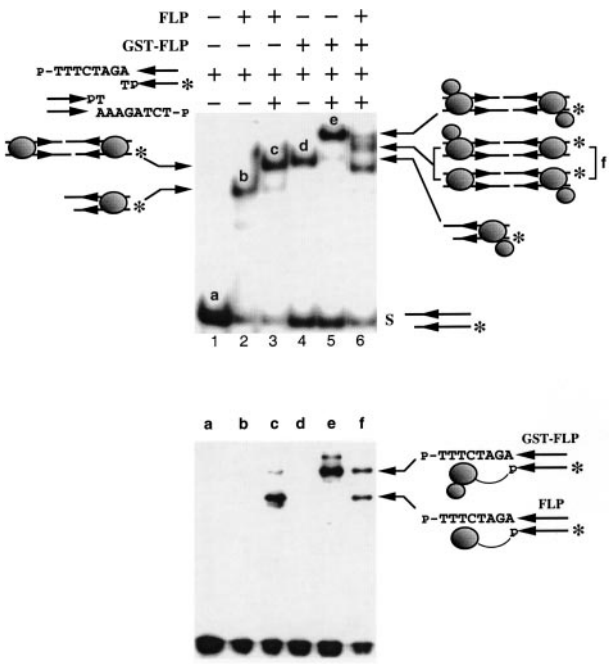

Fig. 5. Monitoring cleavage by Flp or GST–Flp in isolated half-site complexes. Top: reactions were set up as in 1–6 of Figure 3, and were fractionated by electrophoresis in a 10% non-denaturing polyacrylamide gel. The individual DNA–protein complexes b–f are schematically diagrammed. a, unbound substrate; b, Flp-right half-site complex; c, Flp-half-site dimeric complex; d, GST–Flp-right half-site complex; e, GST–Flp-half-site dimeric complex; f, the heterodimeric complex containing Flp and GST–Flp. Bottom: the indicated complexes were excised from the gel, and were rerun in a 12% SDS–polyacrylamide gel. The radiolabeled, covalent cleavage complex derived from Flp or GST–Flp are schematically represented.

are representative of lanes 1–6 in Figure 4. The samples were first run in a native polyacrylamide gel to separate the monomeric and dimeric complexes (Figure 5, top panel). The cleavage products from complexes of interest were then revealed by electrophoresis in SDS–polyacrylamide (Figure 5, bottom panel; lanes a–f). The monomeric Flp-half-site complex (Figure 5, lane b) and the monomeric GST–Flp-half-site complex (Figure 5, lane d) formed with the labeled right half-site alone were inactive in cleavage as seen previously in solution (Figure 4). In contrast, the dimeric complex formed by Flp (Figure 5, lane c) and by GST–Flp (Figure 5, lane e) in the presence of the unlabeled left half-site contained, in each instance, the expected cleavage product. The heterodimeric complex f (Figure 5, lane f) from a reaction in which roughly equimolar amounts of Flp and GST–Flp were added to a mixture of the left and right half-sites (random mixing of proteins and substrates) yielded both cleavage products at approximately equal levels.

In Figure 6, lane 2 is equivalent to lane 6 of Figure 5; lanes 1 and 3 of Figure 6 are equivalent to reactions 7 and 8 of Figure 4. Note that the radiolabeled complexes in the random mixing reaction (Figure 6, lane 2, top) consisted almost exclusively of the three possible dimeric forms: Flp/Flp homodimer (c), Flp/GST–Flp heterodimer (f), GST–Flp/GST–Flp homodimer (e) (Figure 5). Analysis of the heterodimer complex f in SDS–polyacrylamide (Figure 6, lane f, bottom) is reproduced

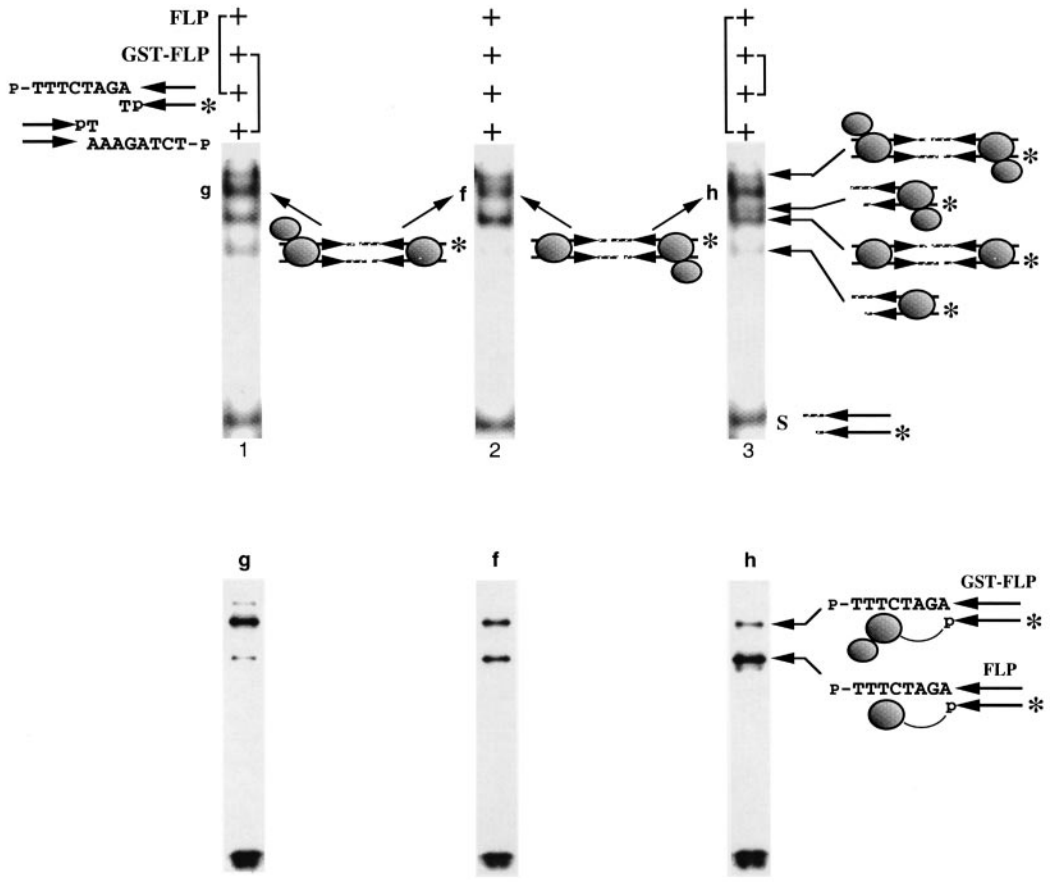

Fig. 6. DNA cleavage in dimeric half-site complexes containing Flp and GST–Flp. Reactions corresponding to 6, 7 and 8 of Figure 4 (in parentheses) were first fractionated in lanes 2, 1 and 3, respectively, of a non-denaturing polyacrylamide gel. The heterodimeric complexes f, g and h (schematically drawn to represent their protein and half-site compositions) were subjected to electrophoresis in a 12% SDS–polyacrylamide gel. The complex f obtained from the random mixing reaction is expected to contain g and h forms in approximately equal proportions.

here from Figure 5 for reference. Note that f contained Flp as well as GST–Flp cleaved products in nearly equal proportions. From this cleavage equivalence, we surmise that roughly half the molecules in the f population contained Flp on the left half-site, the other half contained Flp on the right half-site. We know, from the extent of cleavage observed in the Flp/Flp or GST–Flp/GST–Flp dimeric complexes (Figure 5, c and e), that the cleavage efficiencies per se of Flp and GST–Flp are not significantly different. On the other hand, the Flp/GST–Flp heterodimeric complexes g and h from the pre-bound reactions (Figure 6, lanes g and h, top panel) contained predominantly either the Flp cleavage product, or the GST–Flp cleavage product, respectively (Figure 6, lanes g and h, bottom panel). In the complex g, obtained from Flp bound to the labeled half-site and GST–Flp bound to the unlabeled one, nearly all of the labeled cleavage product was yielded by GST–Flp (Figure 6, lane g, bottom panel). In the complex h, resulting from the opposite binding pattern, Flp produced almost all of the labeled cleavage product (Figure 6, lane h, bottom panel).

If the pre-binding of Flp or GST–Flp to the labeled half-site had been absolute, one should have observed only two protein–DNA complexes each in lanes 1 and 3 of the binding gel shown in Figure 6: the monomeric complex and the heterodimer. However, there was a low background from other complexes, resulting from some protein 'recycling' on the substrates. It is plausible that the recycling is facilitated during electrophoresis, prior to the entry of the samples into the gel. The sum of the unexpected complexes accounted for only ~25% or less of the total complexes, and only the expected heterodimeric complex was excised and examined for cleavage. Furthermore, the monomeric protein-half-site complexes are likely to be more prone to this destabilization effect than the dimeric complexes, so this effect should not cloud the interpretation of our results. Otherwise, we should not have obtained the large cleavage bias seen in g and h relative to f (Figure 6). The ratio of the prominent cleavage signal to the lesser band showed a slight reduction in the isolated complexes (~7:1) compared with the solution reaction (~10:1) (compare lanes g and h of Figure 6 with lanes 7 and 8 of Figure 4).

The congruence between the results obtained with pre-bound half-sites in solution and those observed within isolated half-site complexes indicates that wild-type Flp donates its active-site tyrosine *in trans* during DNA cleavage. The small amounts of cleavage product (10–12% at the most) that might be interpreted as due to *cis* cleavage are most easily accommodated by deviations from the intended protein–DNA pre-associations under our experimental conditions. Thus, the overwhelming body of evidence from this study and from previous work argues against *cis* cleavage by Flp.

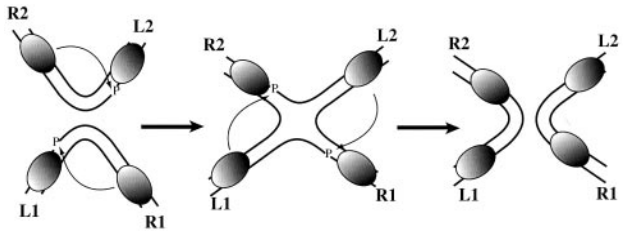

Fig. 7. Relevance of *trans* DNA cleavage by Flp to the recombination pathway. Reaction is initiated by the interactions between Flp monomers bound to the left and right arms of each of the two substrates L1R1 and L2R2. The resultant cleavage modes are *trans* horizontal. The phosphates taking part in the reaction are denoted by P. The Holliday intermediate is resolved by dimeric interactions between a Flp monomer bound to each of the two right arms with a monomer bound to each of the left arms derived from the partner substrate (R1 and L2, R2 and L1). The cleavage modes during resolution are *trans* diagonal.

Implications of trans cleavage for the Flp recombination pathway

Currently available information suggests that *trans* cleavage mode may be unique to Flp and other yeast site-specific recombinases which form a subfamily within the larger Int family (Lee *et al*., 1994; Yang and Jayaram, 1994; Whang *et al*., 1994; Blakely and Sherratt, 1996). We propose that, regardless of *cis* or *trans* cleavage, the active species is a recombinase dimer constituted by monomers bound to the left and right binding arms of a DNA substrate. Solution studies with Flp (Lee *et al*., 1996; Voziyanov *et al*., 1996) and the protein contacts observed in the structure of the DNA-bound Cre tetramer (Guo *et al*., 1997; Gopaul *et al*., 1998) are consistent with this generalization. Experiments with Flp have shown that, although a dimer is capable of assembling two active sites at the left and right ends of the spacer, it cannot do so simultaneously. The steric constraints imposed by the geometry of the spacer DNA permits only one of the two active-site tyrosines to assume the reactive orientation, even though both of the scissile phosphodiester bonds are activated by the Flp dimer (Lee *et al*., 1997). Thus, only one of the two functional active sites can be arranged at a time, as would be consistent with the two-step, single-strand exchange reaction mechanism.

Earlier experiments which address the types of *trans* cleavage that Flp is capable of performing (Lee *et al*., 1994, 1996) have provided the following answers. In single DNA substrates, the mode of cleavage is *trans* horizontal (cleavage type 1 in Figure 2). In synthetic Holliday structures, the cleavage is either *trans* horizontal or *trans* diagonal (cleavage types 1 and 2 in Figure 2). *Trans* vertical cleavage (cleavage type 3 in Figure 2) has never been observed in linear or branched substrates. These results imply that the recombinase dimer interactions relevant to the Int family reaction are always between a left arm and a right arm, never between two left arms or two right arms. In the Flp-recombination pathway illustrated in Figure 7, the reaction is initiated by *trans* horizontal cleavages at the left end of the spacer. In contrast, the resolution of the Holliday junction intermediate (the termination step in recombination) is mediated by *trans* diagonal cleavages at the right end of the spacer. This scheme, in which the left–right interaction within the same substrate arms and that between partner substrate arms are equally important for the reaction, agrees well with the protein–DNA and protein–protein interactions observed in the crystal structure of the Cre synaptic complex (Guo *et al*., 1997). We believe that recombination reactions catalyzed by all Int family members can be accommodated by this general model, regardless of whether they cleave *in cis* or *in trans* (Voziyanov *et al*., 1998). One basic feature of the model is that it utilizes all four recombinase monomers (as a dimer of dimers) at each of the two strand cleavage/exchange steps of recombination (Lee and Jayaram, 1997). Another important feature of the model is that the initiation and termination events of recombination are mediated by functionally equivalent, but geometrically distinct recombinase dimers. Within the framework of this model, *trans* DNA cleavage by Flp represents a special case of the catalytically relevant monomer–monomer interactions which are uniform within the family.

Materials and methods

Purification of Flp and GST–Flp

Flp and GST–Flp proteins used in these experiments were purified by previously published procedures (Lee *et al*., 1994; Yang and Jayaram, 1994; Lee and Jayaram, 1995).

Synthetic half-site substrates

The half-sites were assembled by hybridization between pairs of synthetic oligodeoxynucleotides under conditions standardized previously (Lee *et al*., 1994). The 5′ end of an oligodeoxynucleotide was labeled using [γ-^{32}P]ATP in a T4 polynucleotide kinase reaction. The unreacted ATP was removed by spin-dialysis on a Sephadex G-25 column.

Half-site cleavage reactions

The reactions were performed essentially according to the protocol described for Flp-recombination assays by Chen *et al*. (1992a). Each reaction contained ~0.01–0.02 pmol of the labeled half-site; in addition, 0.02 pmol of an unlabeled half-site complement (see Results and discussion) were present in some of the reactions. The molar ratio of Flp, GST–Flp, or the two combined (in reactions containing both proteins) to half-site was ~5:1. Incubations were carried out at 30°C for 5 min. For some reactions, the labeled and unlabeled half-sites were incubated separately with the desired form of Flp (either native Flp or GST–Flp) for 10 min on ice, and combined at time zero in a tube maintained at 30°C. In some cases, reactions were terminated by the addition of SDS (0.2% final concentration), and the samples were analyzed in a 12% SDS–polyacrylamide gel (acrylamide to bis-acrylamide, 30:1). In other cases, the incubation mixtures were loaded, without addition of SDS, on a 10% non-denaturing polyacrylamide gel (acrylamide to bis-acrylamide, 30:1), and subjected to electrophoresis to separate DNA–protein complexes. Following autoradiography, gel slices containing complexes of interest were excised, placed in wells of a 12% SDS gel and fractionated.

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