Directed protein replacement in recombination full sites reveals *trans*-horizontal DNA cleavage by Flp recombinase

Jehee Lee, Ilson Whang, Joonsoo Lee and Makkuni Jayaram¹

Department of Microbiology, University of Texas at Austin, Austin, TX 78712, USA

¹Corresponding author

Communicated by R.Kahman

One round of site-specific recombination between two DNA partners mediated by the Flp recombinase requires the breakage and reformation of four phosphodiester bonds. The reaction is accomplished by the combined action of four Flp monomers. Within the recombination complex, what is the relative disposition of a Flp monomer with respect to the target diester that it cleaves? To address this question, we have devised a strategy for the targeted orientation of Flp monomers within full-site recombination substrates. Our experimental design is not dependent on 'altered binding specificity' of the recombinase. Analysis of the pattern of DNA cleavage by this method reveals no evidence for DNA cleavage *in cis*. A Flp monomer bound to its recognition element within the full site does not cleave the scissile phosphodiester bond adjacent to it. Our results are most consistent with '*trans*-horizontal cleavage'. Cleavage by Flp occurs at the scissile phosphodiester distal to it, but within the same full site. The general experimental design employed here will be of widespread utility in mechanistic analyses of nucleic acid transactions involving multimeric DNA–protein assemblies.

Key words: catalytic complementation/directed protein replacement/site-specific recombination/*trans* DNA cleavage

Introduction

Conservative site-specific DNA recombination between two double-stranded DNA molecules requires the breakage and joining of four specific phosphodiester bonds. The Int family of recombinases (Argos *et al.*, 1986; Abremski and Hoess, 1992) executes this reaction in two sequential steps. The first pair of strand cleavage/joining reactions produce a Holliday intermediate which, following branch migration, is resolved into recombinants by the second pair of cleavage/joining reactions.

Strand cleavage and strand joining during recombination are accomplished by a transesterification mechanism. In the cleavage step, the nucleophile derived from the active site tyrosine of the recombinase attacks the phosphodiester at the exchange site. As a result, a 3'-phosphotyrosyl bond between the recombinase and DNA and a free 5'-hydroxyl group are formed. Nucleophilic attack by the 5'-hydroxyl

from the nicked strand of one DNA substrate on the phosphotyrosyl bond formed in the partner substrate produces a recombinant strand. Were this chemistry to be performed within a nicked DNA substrate, rather than between nicked partner substrates, strand joining will regenerate the parental DNA strand. In a general sense, the overall recombination reaction is a 4-fold repetition of the same breakage/joining chemistry. In order that the reaction be carried out efficiently to mediate a physiologically relevant outcome, the recombination machinery must produce coordinated cleavages in partner substrates and must facilitate strand joining in the recombinant, rather than parental, mode. Since these are not mutually exclusive problems, a unified solution to them seemed plausible.

Recent experiments using half recombination sites and step-arrest mutants of the yeast recombinases Flp and R, two members of the Int family, suggest that such a solution might reside in the architectural design of Flp and R active sites (Chen *et al.*, 1992, 1993; Lee and Jayaram, 1993; Yang and Jayaram, 1994). In these reactions a half site is not cleaved by the Flp monomer bound to it; rather it is cleaved by a Flp monomer bound to a second half site. This mode of cleavage is called *trans* DNA cleavage (see Figure 1). *Trans* cleavage can be neatly accommodated by a model in which a recombinase monomer harbors a partial active site. A complete active site is assembled by the contribution of residues from more than one recombinase monomer. In the shared active site, three of the invariant Int family residues, two arginines and a histidine (the RHR triad) are derived from one monomer; the active site tyrosine, the fourth invariant family residue, is provided by a second monomer (Chen *et al.*, 1992; Pan *et al.*, 1993; Yang and Jayaram, 1994). Thus *trans* DNA cleavage is a necessary consequence of the shared active site. The active site configuration of Flp and its mode of DNA cleavage suggest potential strategies for postponing the chemistry of recombination until the recombination complex is fully organized and for simultaneously assembling two active sites for coordinated strand cleavages in the two substrate partners.

Are the catalytic strategies displayed by Flp universal within the Int family? Complementation between an RHR mutant and an active site tyrosine mutant has been observed with λ Int as well (Han *et al.*, 1993). This result is strongly suggestive of *trans* DNA cleavage by Int. However, other results with Int are more parsimoniously explained by *cis* DNA cleavage (Kim *et al.*, 1990; Nunes-Duby *et al.*, 1994). Is there a *cis*–*trans* duality in the Int mechanism? If so, does this extend to the Flp mechanism as well? Could the failure to observe *cis* cleavage by Flp have been a peculiarity of the half site substrates used?

Here we address the mode of strand cleavage by Flp in full sites by using step-arrest mutants of Flp and a site-specific protein replacement strategy. We demonstrate that

Fig. 1. Possible modes of cleavage during Flp site-specific recombination (redrawn from Chen *et al.*, 1992). The two DNA substrates are aligned in parallel (left to right alignment). The Flp binding elements are shown by the parallel arrows. The sequence of the strand exchange region (spacer) is spelt out. For a Flp monomer bound to the left binding element of the top substrate the four potential modes of cleavage are indicated by the curved arrows: *cis* (A); *trans-horizontal* (B); *trans-vertical* (C) and *trans-diagonal* (D). In the λ Int reaction, the cleavage modes A–D are named *cis* cleavage, cross-core cleavage, partner cleavage and partner cross-core cleavage respectively (Nunes-Duby *et al.*, 1994).

the mode of DNA cleavage is indeed *trans*. Our results are most consistent with the *trans-horizontal* cleavage mechanism.

Results

Catalytic complementation between a triad mutant and Flp(Y343F) has not been observed in full sites. This is not surprising. For a full site recombination event, four breakage–union reactions must occur. This requires the operation of four functional active sites. In a tetrameric protein assembly between a triad mutant and Flp(Y343F) one can derive, at best, only two functional active sites. A Holliday junction may be formed, however, its resolution into recombinants is impossible. Hence, reverse resolution to parents obscures catalytic complementation. Unmasking complementation would require trapping of the reaction intermediate by using a suicide substrate. We discovered that mismatches at certain positions within the strand exchange (spacer) region of a full site can strongly inhibit the strand joining step of recombination, thus effectively transforming the target sites harboring such mismatches into suicide substrates (J.Lee and M.Jayaram, unpublished data). We also devised a strategy to direct a Flp monomer to a specific binding element within a full site substrate. A combination of substrates harboring spacer mismatches and targeted recombinase replacement has enabled us to map cleavage patterns within full sites.

Directed replacement of recombinase monomers supports the *trans* DNA cleavage model

Directing a protein monomer to a specific DNA site among a set of identical sites can be accomplished if a protein variant that specifically recognizes a variant site is available. We have not yet obtained an altered specificity variant of Flp, hence, an alternative experimental strategy was devised. The nucleotide contact points within the Flp binding elements of the recombination site have been mapped by mutational and biochemical analyses (Andrews

et al., 1986; Bruckner and Cox, 1986; Prasad *et al.*, 1986; Senecoff *et al.*, 1988; Panigrahi *et al.*, 1992). In particular, the alteration of a CG pair to a TA pair at position 7 within the Flp binding element of a full site substrate (Figure 2) decreases recombination efficiency by a factor of ~50. This large decrease in reactivity is a direct consequence of the drop in binding affinity of the Flp monomer to the mutated recognition sequence. However, when the substrate contains one wild-type and one mutant binding element, the effect of the mutation is ameliorated. Protein–protein cooperativity mediates partial restoration of the recombination potential. We argued that if such a hybrid site occupied by a Flp variant were to be challenged by an excess of a second variant, the latter would preferentially replace the former at the mutant binding element. If so, one would have, in effect, achieved altered target specificity.

The expected pattern of recombinase replacement was verified by the experiments depicted in Figure 3. The three full site substrates (Figure 2) used contained a spacer with a double bubble (two adjacent mismatches each at the left and right cleavage points) and were 3' end-labeled on both strands. Products of cleavage of the top strand (left cleavage) and the bottom strand (right cleavage) could thus be monitored (marked CL and CR in Figure 3). One of the three substrates contained two wild-type binding elements (S1). Of the other two, one contained a mutant left binding element 1a (S2); the second one contained a defective right binding element 1'a (S3). These substrates were bound, in separate experiments, by Flp(Y343F) and then challenged with an excess of wild-type Flp. Samples were withdrawn at various times to assay strand cleavage and thereby indirectly monitor replacement of Flp(Y343F) by Flp on the DNA substrate. In the bubbled substrate there was an inherent asymmetry in strand cleavage by wild-type Flp. There was approximately twice as much cleavage on the bottom strand (right cleavage) as there was on the top strand (left cleavage) (compare CL and CR in the lane marked WT in Figure

Fig. 2. Flp full-sites containing spacer mismatches and mutant binding elements. The substrates used for the experiments in Figure 3 are schematically represented at the left. The parallel arrows represent the Flp binding elements (1a at the left and 1'a at the right). The mismatches within the spacer are represented by the bubbles. The points of top strand and bottom strand cleavage are indicated by the short vertical arrows. The radioactive label at the 3' ends are denoted by the asterisks. The wavy line adjacent to 1a and 1'a are sequences not directly relevant to Flp binding. The X within a binding element symbolizes a mutation within it. The sequences of the Flp recombination sites within each substrate are shown at the right. The Flp binding elements and the spacer are spelt in bold uppercase and normal upper case letters respectively. The mutant position within 1a of S2 and 1B of S3 is shown in *italics*.

Fig. 3. Selective replacement of Flp(Y343F) by Flp in a full site substrate. Each indicated substrate (refer to Figure 2) was incubated with Flp(Y343F) to virtually saturate all of the Flp binding elements. At time zero, an ~3- to 4-fold molar excess of wild-type Flp was added. Reaction mixtures were sampled at the indicated times to assay strand cleavage. The substrate band is marked S. The left and right cleavage bands are indicated by CL and CR respectively. For each reaction set, the first three lanes are controls. Lane C is a reaction without added Flp. Lanes marked WT and Y343F refer to 10 min reactions with Flp alone and Flp(Y343F) alone respectively. The asterisk represents the 3' end-label.

3). In assessing the left and right cleavage levels in the Flp challenge experiments, this asymmetry must be factored in. No detectable cleavage occurred in a reaction with Flp(Y343F) alone (lane marked Y343F in Figure 3).

The data assembled in Figure 3 permit certain significant inferences. First, following addition of Flp to the reaction, strand cleavage was detected in the mutant substrates at

early time points (reaction sets S2 and S3, Figure 3). Very little strand cleavage was detected in the wild-type substrate, even at late time points (as late as 10 min; reaction set S1, Figure 3). Second, cleavage in the mutant substrates was biased towards one of the two strands: the bottom strand for S2 (right cleavage; Figure 3) and the top strand for S3 (left cleavage; Figure 3). For example,

Fig. 4. Pattern of strand cleavage by selective replacement of Flp(Y343F) by the RHR triad mutants of Flp within the left binding element. Reactions were done with substrate S2 (see Figure 2). Protein replacement was done as described for Figure 3. For each reaction set, the left-most lane represents a 10 min control reaction with wild-type alone (WT) or the triad mutant alone. The time points at which cleavage was monitored following start of selective protein replacement are shown. A reaction without added Flp (lane C) and with Flp(Y343F) alone (lane marked Y343F) are also displayed. The ^{32}P label at the 3' ends is symbolized by the asterisk. The substrate band is denoted by S2. The cleavage bands are labeled CL and CR. The CL band in the 1 min reaction of the Y343F→R308Q panel is an experimental error. Replications of the experiment have confirmed this surmise.

at the 10 min time point the ratio of CL to CR was $\sim 1:15$ for S2 and $>10:1$ for S3. With the S1 substrate (containing two wild-type binding elements) the CR:CL bias in a normal Flp reaction (lane marked WT in Figure 3) or in a Flp(Y343F) to Flp substitution reaction was the same ($\sim 2:1$). Although this inference is not clear from the data shown in Figure 3 (reaction set S1), it was supported by strand cleavage patterns at later time points (results not shown). Together, these results demonstrate that it is the Flp(Y343F) monomer bound to the mutant binding element that was preferentially replaced by Flp. Thus the expectation that recombinase monomers can be directionally replaced on the DNA target is fully realized. Finally, the bias in strand cleavage in the mutant substrates upon protein replacement is significant. When the left element was mutant (and was therefore more readily occupied by the wild-type Flp added in excess), the reaction was strongly biased towards right cleavage (compare CL and CR in S2, Figure 3). When the right binding element was mutant, the bias was reversed towards left cleavage (S3, Figure 3). The direction of the cleavage asymmetry in the mutant substrates argues against *cis* cleavage and strongly favors *trans* cleavage.

The shared active site configuration of Flp (Chen *et al.*, 1992, 1993) accounts for the *trans* mode of DNA cleavage in half sites and for the patterns of complementation observed in half site strand transfer with pairwise combinations between Flp(Y343F) and the RHR triad mutants. Provided these rules apply to full site reactions as well, one would expect the triad mutants to mimic wild-type Flp during directed protein replacement, analogous to that presented in Figure 3. In these reactions, the substrate contained the impaired binding element on the left (Figure 4) or on the right (Figure 5). In each reaction, the substrate

was bound by Flp(Y343F) and the bound complex was then challenged with an excess of a triad point mutant, a triad double mutant or a triad triple mutant. A challenge reaction with wild-type Flp was included for reference. As controls, reactions with wild-type Flp alone (WT, Figures 4 and 5) and Flp(Y343F) alone (Y343F, Figures 4 and 5) were also done.

The most obvious result is that in reactions with S2 (mutant binding element on the left), cleavage was distinctly biased to the right ($\text{CR} \gg \text{CL}$; Figure 4); similarly, in reactions with S3 (mutant binding element at the right), the bias was strongly to the left ($\text{CL} \gg \text{CR}$; Figure 5). In reactions with Flp(R191Q) or with the double and triple triad variants containing the Arg308Gln mutation, the bias in left or right cleavage was less pronounced, especially at later time points, than in reactions with Flp, Flp(R191S) or Flp(H305L). This effect was particularly noticeable in the assays shown in Figure 5. The Arg308Gln mutation may affect protein–protein cooperativity, thus increasing the rate of protein recycling and randomization within the substrates. Hence, for Flp variants harboring this mutation, only the earlier time points (1 and 2 min) may represent true directed replacement at the mutant binding element. Note, however, that even at the latest time point in the assay [10 min lanes in reactions with Flp(R308Q) and Flp(R191S, R308Q) in Figure 5, where the CL and CR bands are roughly equal in intensity], cleavage was still biased to the left by a factor of two. Recall that the right cleavage site is normally two-fold more reactive than the left cleavage site (see the normal wild-type Flp reaction shown in lane 3 from the left, Figure 5).

In summary, the assays with the triad variants of Flp paired with Flp(Y343F) reveal a large bias towards cleavage adjacent to the normal binding element [tightly

Fig. 5. Pattern of strand cleavage by selective replacement of Flp(Y343F) by the RHR triad mutants of Flp within the right binding element. Reactions were done with the substrate S3 (Figure 2). The reaction protocol was exactly as described for Figure 4. The substrate is denoted by S3. The cleavage bands are labeled CL and CR.

bound Flp(Y343F)] and away from the mutant binding element [where replacement of Flp(Y343F) by the triad mutants occurs]. These results are most easily interpreted by the following rules. Flp(Y343F) is competent in activating the scissile phosphodiester adjacent to it. The triad mutant can deliver its active site tyrosine in *trans* to Flp(Y343F). Hence, during complementation, cleavage occurs adjacent to Flp(Y343F) and away from the recombinase monomer containing the active site tyrosine (cleavage *in trans*).

Flp follows the *trans*-horizontal mode of cleavage

There are three possible modes of *trans* DNA cleavage (Figure 1): *trans*-vertical (partner cleavage); *trans*-horizontal (cross-core cleavage); *trans*-diagonal (cross-core partner cleavage). Unambiguous identification of the particular mode of *trans* cleavage was impossible from previous studies with half sites. While *trans*-vertical cleavage seemed unlikely, no clear distinction between *trans*-horizontal cleavage and *trans*-diagonal cleavage could be made (Chen *et al.*, 1992; Jayaram, 1994a).

By using two substrate partners and modifying the experimental paradigm depicted in Figures 3–5, we attempted to identify the mode of *trans* cleavage used by Flp. The substrates were assembled by hybridizing two pairs of appropriate oligodeoxynucleotides. Each strand was labeled at the 3' end with ^{32}P . The lengths of the four strands were so arranged that each of the four strand cleavage products (CL1, CR1, CL2 and CR2) could be unambiguously identified. The left and right cleavage products are represented by CL and CR respectively in Figures 6 and 7. The numbers 1 and 2 refer to substrate 1 and substrate 2 respectively. A binding element harboring the 'X' was weakened by the CG→TA mutation and was therefore the locale of protein replacement. Thus in reactions A–G in Figure 6, the ovoids carrying 'X' were equivalent to wild-type Flp monomers; those without 'X' represented Flp(Y343F).

Figure 6 summarizes the seven experimental crosses A–G (substrate 1 with substrate 2) and the expected outcome in a cross for each of the four types of strand cleavages. The additional cleavage products expected to result from self synopsis of the substrates (substrate 1 with substrate 1 and substrate 2 with substrate 2) are listed at the lower bottom corners of the appropriate boxes in Figure 6. The experimental results for A–G are compiled in Figure 7. These outcomes are represented by shaded boxes in Figure 6. Reactions in which a single Flp(Y343F) monomer is replaced by Flp (B and C) make unique predictions for each cleavage mode. The cleavage patterns from B and C (Figure 7) could only fit the *trans*-horizontal type and excluded the other three types. Reactions in which two and three monomers are substituted make predictions that fit more than one type of cleavage. Double replacements, depending on their relative dispositions, cannot distinguish *trans*-horizontal from *cis* and *trans*-vertical from *trans*-diagonal (D) or *trans*-vertical from *trans*-diagonal (E). The triple replacement also fails to differentiate between *trans*-vertical and *trans*-diagonal (F). The results from D (Figure 7) could be accommodated by *cis* or *trans*-horizontal cleavage; those from E and F were consistent only with *trans*-horizontal cleavage. Quadruple replacements are expected to make the outcome completely degenerate (G); the profile in G (Figure 7) revealed all possible cleavages. When Flp(Y343F) is not replaced by Flp at any of the four binding elements (A), no cleavage is expected; none was observed (A in Figure 7).

It must be pointed out that in deducing the mode of cleavage by comparison of Figures 6 and 7, the predicted absence of a cleavage product was as critical as the predicted presence of one. For example, it was the absence of CL1 in F and of CL1 and CR2 in E that rules in *trans*-horizontal cleavage and rules out the other three types of cleavage. Similarly, it was the absence of CR1 in B and of CL1 in C that permits *trans*-horizontal cleavage to be distinguished from *trans*-diagonal cleavage.

		CIS	TRANS- VERTICAL	TRANS- DIAGONAL	TRANS- HORIZONTAL
A					
B		CL2	CL1 CL2	CR1 CR2	CR2
C		CR2	CR1 CR2	CL1 CL2	CL2
D		CL2 CR2	CL1 CR1 CL2 CR2	CR1 CL1 CR2 CL2	CR2 CL2
E		CL1 CR2	CL2 CR1 CL1 CR2	CR2 CL1 CR1 CL2	CR1 CL2
F		CL1 CL2 CR2	CL2 CL1 CR1 CR2	CR2 CR1 CL1 CL2	CR1 CR2 CL2
G		CL1 CR1 CL2 CR2	CL2 CR2 CL1 CR1	CR2 CL2 CR1 CL1	CR1 CL1 CR2 CL2

Fig. 6. Predicted strand cleavage outcomes for crosses between two full sites upon targeted replacement of Flp(Y343F) by Flp. Pairs of full sites containing the indicated mutant Flp binding elements (X) were crossed as shown schematically in rows A–G. The positions of strand cleavage are denoted by the vertical arrows. The asterisks stand for the 3' end-label on the two strands. The top strand cleavages in the two substrates are represented by CL1 and CL2; the bottom strand cleavages by CR1 and CR2. For each substrate pair, the predicted cleavage products for the four potential modes of strand cleavage are tabulated. The cleavage products shown in large bold letters correspond to synapsis between substrate 1 and substrate 2, as diagrammed in A–G. Additional cleavage products resulting from self synapsis (substrate 1 against substrate 1 or substrate 2 against substrate 2) are indicated in smaller letters. The shaded boxes depict the experimental outcome of crosses A–G as deduced from Figure 7.

There are some aspects of the results in Figure 7 that may interest the specialist. The data from the earliest time points (the 1 and 2 min lanes) provided the cleanest interpretations. At later time points (5–20 min) there was a low level randomization of protein arrangement within a full site. Even when the two substrates harbored only wild-type binding elements (and hence were resistant to protein substitution), background cleavage bands could be detected (panel A in Figure 7, 5–20 min lanes). In reactions with substrates containing two weakened binding elements (D, F and G), it is clear that the bias was towards left cleavage (CL1 and CL2) at the earliest time points. This result indicates that replacement by wild-type Flp at the right binding element preceded that at the left binding element. There was a progressive increase in right cleavages (CR1 and CR2) with time. At late time points, as the inequality in recombinase replacement at left and right decreased, the left to right cleavage ratio tended to approach that seen in a regular wild-type Flp reaction. This can be appreciated by comparing the CL2 and CR2 bands in the 20 min lane of the replacement reaction in

F with the corresponding bands in the lane marked WT. At late times there was a slight decrease in the left cleavages (for example compare the CL2 band at 1 and 20 min in F) resulting, probably, from the processing of the cleaved ends via strand joining (cleavage reversal).

While our results clearly establish *trans*-horizontal cleavage, can they rule out *trans*-diagonal cleavage unequivocally? Whereas *trans*-horizontal cleavage is not necessarily dependent upon synapsis between two substrates, *trans*-diagonal cleavage would be contingent upon synapsis. If our assay conditions preclude or inhibit synapsis, *trans*-diagonal cleavage could have been missed. However, this appears to be rather unlikely. Reaction efficiencies of normal full sites and those containing spacer mismatches in recombination assays with wild-type Flp were comparable. In addition, during catalytic complementation between step-arrest mutants of Flp in bubbled full sites (Whang *et al.*, 1994), not only strand cleavage but also strand joining between two substrates could be detected. The level of strand transfer observed in the complementing reactions did not suggest impairment of synapsis. In a reaction containing roughly equimolar amounts of two DNA substrates (one of which was end-labeled on one strand) and two complementing Flp variants (in an approximate molar ratio of 1:1), one would expect 6/16 synaptic complexes to contain two monomers each of the two Flp variants. Out of these, only half (3/16) would be recombinationally productive (non-self synapsis). Since the radioactive substrate was labeled only on one strand, out of the six possible protein configurations in this set of complexes only one would be strand transfer competent. Note that a single exchange (that would be assayed as a radioactive recombinant strand) requires two matched cleavages within the substrate partners. Thus, one would predict ~3% (1/32) of the reaction seen with wild-type Flp. This would be an overestimate, since some reversal of exchange is to be expected for lack of the second pair of exchanges (resolution of the Holliday junction) in the complementing reaction. The reaction efficiencies observed with various pairwise combinations of complementing Flp variants were within this expected range (Whang *et al.*, 1994).

In sum, our results are most consistent with the *trans*-horizontal mode of cleavage in full site substrates. However, because of uncertainties in the level of synapsis in these assays, cleavage by Flp across partner DNA molecules within a synaptic complex cannot be absolutely ruled out.

Discussion

This study explores the mode of DNA cleavage during Flp recombination in full site substrates. The results are generally consistent with those obtained from reactions using half site substrates (Chen *et al.*, 1992) and strongly support DNA cleavage *in trans*. The preponderance of evidence supports the *trans*-horizontal cleavage mechanism.

Site-specific replacement of protein monomers within the Flp recombination complex

In principle, one may distinguish between *cis* and *trans* cleavage by directing a tagged recombinase monomer to

Fig. 7. Strand cleavage profile upon single or multiple replacements of Flp(Y343F) by Flp in a pair of full sites. The reaction sets labeled A–G represent the corresponding substrate configurations depicted in Figure 6. For each reaction set, the first three lanes are controls: without added Flp (C); with Flp(Y343F) alone (Y343F); and with wild-type Flp alone (WT). Strand cleavage was monitored at the indicated times following initiation of protein replacement. The cleavage bands derived from substrate 1 are CL1 and CR1; those derived from substrate 2 are CL2 and CR2. The two substrate bands are jointly represented by S.

a particular binding site within the recombination substrate and mapping the phosphodiester targeted for cleavage by this monomer. Recombinase variants with altered binding specificity would be ideal for this purpose. Such a variant has been exploited to analyze the mechanism of the $\gamma\delta$ resolvase reaction (Grindley, 1993). The strategy of orienting partner monomers of the *Escherichia coli* CAP protein dimer based on binding selectivity has identified the monomer of CAP that directly contacts the RNA polymerase (Zhou *et al.*, 1993). Similarly, the distinct binding specificities of λ Int and λ HK022 Int provide the basis for an experimental design to distinguish between *cis* and *trans* DNA cleavage in chimeric substrates harboring dual specificities (Nunes-Duby *et al.*, 1994). The outcome of these experiments tend to strongly favor the *cis* mode of cleavage for Int and to discount the *trans* mode of cleavage.

The lack of Flp variants with altered target specificity has necessitated an alternative experimental design for directed replacement of Flp monomers within the recombination complex. The technique of filling a set of binding elements with a particular Flp variant and then replacing the protein monomers associated with weak binding elements by a second Flp variant offers an effective solution. Application of this technique to full sites carrying

spacer mismatches (and hence suicide substrates) allows precise mapping of Flp cleavage patterns in full sites. Site-directed protein replacement is expected to provide a general and effective tool for mechanistic analysis in multi-component DNA–protein systems analogous to the Flp system.

From earlier results with half sites (Chen *et al.*, 1992) we had preferred the *trans*-diagonal mode of cleavage to the *trans*-horizontal mode, for two reasons. First, we could demonstrate cleavage of a half site by a Flp monomer bound on a full site (suggestive of cleavage across partners). Second, in the context of recombination between two DNA molecules, *trans*-diagonal cleavage seemed to be functionally more relevant. We had suggested that ‘across the partner cleavage’ might represent the coupling of strand cleavage to the conformational switch required for strand joining in the recombinant mode. However, we were wary of the potential flexibility in solution of a half site in its stacking interactions with a second half site or a full site that would tend to make the distinction between *trans*-horizontal and *trans*-diagonal quite tenuous (Jayaram, 1994a; Whang *et al.*, 1994). Targeted replacement of recombinase monomers at individual binding elements within full sites has now unequivocally demonstrated *trans*-horizontal cleavage; no *trans*-diagonal cleav-

age has been detected under these conditions. The question whether, under appropriate conditions, Flp bound to one full site can cleave a partner full site remains open.

Cleavage before or after synapsis?

If Flp executes strand cutting only within a substrate and not across substrates, a central issue would be whether cleavage requires the assembly of the full recombination complex (four Flp monomers and two DNA partners) or merely the occupancy of two Flp monomers within one DNA substrate. If synapsis is a prerequisite for *trans*-horizontal cleavage, it will have almost all the functional virtues of the *trans*-diagonal mode of cleavage. If not, we must conclude that the chemistry of recombination can be triggered even when only one half of the recombination complex is organized. Protein asymmetry, originally inferred from Flp half site associations (Qian *et al.*, 1990), within the complex may permit only one of the two possible DNA cleavages. The prevention of double breaks could be a safety mechanism to protect DNA from damage or to prevent potential 'contrary' recombination events (Nash and Robertson, 1989) when a recombination-competent partner is absent. The single break can, of course, be healed by reversal of cleavage. Our initial enthusiasm for *trans*-diagonal cleavage stemmed from the superior solutions it can offer to the problems of coordinated phosphoryl transfers during recombination. Our present findings indicate that the Flp system may have devised what appears to be a less optimal solution. If *trans*-horizontal cleavage does have a mechanistic or physiological advantage over *trans*-diagonal cleavage, it is not apparent to us what this might be.

DNA cleavage in *trans* is not global to the Int family?

Our finding that DNA cleavage by Flp in full sites follows the *trans* paradigm raises questions regarding the generality of *trans* DNA cleavage within the Int family recombinases. In the case of λ Int, evidence strongly suggestive of *trans* cleavage has been obtained using suicide *attL* substrates (Han *et al.*, 1993). In contrast, analyses using suicide *attB* and Holliday substrates discredit *trans* cleavage and support *cis* cleavage (Nunes-Duby *et al.*, 1994). It is not clear how the apparent λ Int paradox can be resolved.

In Flp, *trans* DNA cleavage follows directly from its active site configuration. The Flp active site is assembled by sharing of catalytic residues from more than one monomer of Flp (Chen *et al.*, 1992, 1993). *A priori*, there is no reason to suspect that a recombinase from a bacteriophage and a recombinase from yeast should share a common active site assembly, even though they execute the same chemistry of recombination. Since the number of biochemical solutions to the problem of phosphoryl transfer in a nucleic acid chain is finite, it would not be surprising if the same solutions were independently arrived at in phage and in yeasts. In this view, the only unifying feature among the set of proteins grouped together as the Int family may be a common catalytic module co-opted by them. The high degree of global diversity among the Int family proteins would be consistent with this notion. As has been pointed out by Nunes-Duby *et al.* (1994), here may be a genuine instance of mechanistic convergence

(Doolittle, 1994). It is likely that the active site configuration within the recombination assembly consisting of two DNA substrates and four protein monomers is similar for Flp and Int and other members of the Int family. However, the distribution within protein monomers of amino acid residues that congregate to generate the full active site complement may vary from recombinase to recombinase (Jayaram, 1994b).

The Xer site-specific recombinase, an Int family recombinase responsible for stable chromosome partitioning in *E. coli*, appears to follow the λ Int cleavage rather than the Flp cleavage paradigm (Blakely *et al.*, 1993). This system has evolved a natural dual binding specificity and requires the combined action of two recombinases, XerC and XerD. The cleavage pattern observed with Xer is consistent only with *cis* cleavage or *trans*-vertical (partner *trans*) cleavage.

The λ Int and Xer examples, contrasted with Flp, would argue against a unified set of rules by which the Int family recombinases build their active sites and execute DNA cleavage.

***Cis-trans* duality in DNA cleavage?**

The finding that λ Int appears to be capable of *cis* or *trans* cleavage raises an interesting question: is *cis-trans* duality in cleavage a normal feature of Int family recombination or is it a special attribute of λ Int? Nunes-Duby *et al.* (1994) raise the intriguing possibility that *trans* cleavage may operate during the first pair of exchanges (a bimolecular reaction) and *cis* cleavage may come into play during the resolution of the Holliday junction (a unimolecular reaction). The experiments described here with Flp and suicide full site substrates of Flp give no indication of *cis* cleavage.

From a purely enzymological perspective, it is hard to imagine how a key active site residue can be delivered to the catalytic pocket in *cis* or in *trans*. One will have to imagine that the interactions among the tetrameric protein assembly within the recombination complex are sufficiently different at the cleavage and strand exchange steps to confer the *cis/trans* selectivity. The real explanation may have to wait until the three-dimensional structure of Int protein (and/or of Int-DNA complexes) can be solved.

Parting of ways between λ Int and Flp

Is there some means by which the Flp and λ Int results can be accommodated by a unitary mechanism? Note that the proof for *cis* cleavage is never absolute. For example, reciprocal *trans* cleavages performed by two recombinase monomers bound to the left binding elements of a synapsed pair of substrates (as in partner *trans* or *trans*-vertical; see Figure 1C) would in effect also be scored as *cis* cleavage executed by each monomer within the substrate occupied by it. Given this caveat, can we reinterpret the Int result to bring Int into the Flp fold? Imagine that λ Int and λ HK022 Int can satisfy the allosteric interactions required for recombination, but cannot swap their catalytic tyrosines. Imagine, further, that two allosterically primed λ Int monomers (or λ HK022 Int monomers) on separate DNA substrates can donate their catalytic tyrosines to each other. Thus cleavage would only be possible by λ Int- λ Int or HK022 Int-HK022 Int interactions. Under this set of rules, the results of Nunes-Duby *et al.* (1994)

would be consistent with *trans* cleavage. The number of rather cumbersome assumptions involved in this interpretation makes it unattractive at present. We must conclude, therefore, that Flp and Int follow different molecular paths to reach the same chemical goal.

Materials and methods

Purification of Flp

Wild-type Flp and Flp variants were 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). Purity of the final Flp preparations ranged from ~75 to 90%.

Synthetic recombination sites

Oligodeoxynucleotides for construction of full sites were synthesized in an Applied Biosystems DNA synthesizer (model 380A) using phosphoramidite chemistry (Beaucage and Caruthers, 1981). Normally, 10–20 pmol each of two appropriate oligodeoxynucleotide pairs were mixed in TE (10 mM Tris–HCl, pH 7.8, at 23°C, 1 mM EDTA, pH 8.0), heated to 65°C for 10 min and cooled slowly to room temperature. The relevant features of these sites are described in the Results section and displayed in the figures. The complete sequences of the substrates are available upon request.

The 3' end of a deoxyoligonucleotide was labeled with α -³²P-labeled cordycepin phosphate. The unreacted cordycepin phosphate was removed by spin-dialysis on a G-25 column. Hybridization to the partner oligodeoxynucleotide was done in TE.

Strand cleavage assays

The strand cleavage reactions were done under standard recombination conditions (Chen *et al.*, 1992). Normally, 0.025–0.05 pmol 3' end-labeled substrate was reacted with approximately 0.5 pmol Flp or Flp variant (roughly 5 pmol Flp/pmol binding element) in a reaction volume of 30 μ l. Incubations were done at 30°C for 30 min. Reactions were stopped by immersing samples in a boiling water bath for 5 min. After addition of SDS (0.1% final concentration) and proteinase K treatment (100 μ g/sample for 1 h at 37°C), samples were phenol–chloroform extracted and DNA was precipitated with ethanol. The DNA pellet was recovered by centrifugation, washed twice with 80% ethanol and dried *in vacuo*. Strands were denatured in 95% formamide at 95°C and samples were fractionated by electrophoresis in 10% denaturing polyacrylamide gels (acrylamide:bis-acrylamide, 19:1). Cleavage products were identified following autoradiography.

Targeting of Flp to specific binding elements within full site substrates

To approximately 0.02 pmol labeled full site, roughly 0.5 pmol Flp(Y343F) (a molar ratio of protein monomer to binding element of 12.5) was added in the standard recombination medium (Chen *et al.*, 1992) and incubated at 30°C for 10 min. At time zero, approximately 2 pmol Flp or a Flp variant were added to the assay mixtures and incubation was continued at 30°C. At various time points samples were processed as described under strand cleavage assays.

General methods

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

Acknowledgements

We thank A.Landy and S.Nunes-Duby for communicating results prior to publication. We acknowledge useful suggestions by Bob Weisberg and David Sherratt for improving the manuscript. The design of several experiments was helped by thoughtful input from R.M.Harshey. This work was supported by a grant from the NIH. Partial support was received from the Council for Tobacco Research (USA) and the Robert F.Welch foundation.

References

- Abremski, K. and Hoess, R.H. (1992) *Protein Engng*, **5**, 87–91.
- Andrews, B.J., McLeod, M., Broach, J.R. and Sadowski, P.D. (1986) *Mol. Cell. Biol.*, **6**, 2482–2489.

- Argos, P., Landy, A., Abremski, K., Egan, J.B., Haggard-Ljungquist, E., Hoess, R.H., Kahn, M.L., Kalionis, B., Narayana, S.V.L., Pierson, L.S., III, Sternberg, N. and Leong, J.M. (1986) *EMBO J.*, **5**, 1433–1440.
- Beaucage, S.L. and Caruthers, M.H. (1981) *Tetrahedron Lett.*, **22**, 1859–1862.
- Blakely, G., May, G., McCulloch, R., Arciszewska, L.K., Burke, M., Lovett, S.T. and Sherratt, D.J. (1993) *Cell*, **75**, 351–361.
- Bruckner, R.C. and Cox, M.M. (1986) *J. Biol. Chem.*, **261**, 11798–11807.
- Chen, J.W., Lee, J. and Jayaram, M. (1992) *Cell*, **69**, 647–658.
- Chen, J.W., Yang, S.H. and Jayaram, M. (1993) *J. Biol. Chem.*, **268**, 14417–14425.
- Doolittle, R.F. (1994) *Trends Biochem. Sci.*, **19**, 15–18.
- Grindley, N.D.F. (1993) *Science*, **262**, 739–740.
- Han, Y.W., Gumpert, R.I. and Gardner, J.F. (1993) *EMBO J.*, **12**, 4577–4584.
- Jayaram, M. (1994a) In Eckstein, F. and Lilley, D.M.J. (eds), *Nucleic Acid Molecular Biology*. Springer-Verlag, Vol. 8, pp. 268–286.
- Jayaram, M. (1994b) *Trends Biochem. Sci.*, **19**, 78–82.
- Kim, S., Moitso de Vargas, L., Nunes-Duby, S.E. and Landy, A. (1990) *Cell*, **63**, 773–781.
- Lee, J. and Jayaram, M. (1993) *J. Biol. Chem.*, **268**, 17564–17579.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Nash, H. and Robertson, C.A. (1989) *EMBO J.*, **8**, 3523–3533.
- Nunes-Duby, S.E., Tirumalai, R.S., Dorgai, L., Yagil, E., Weisberg, R.A. and Landy, A. (1994) *EMBO J.*, in press.
- Pan, G., Luetke, K. and Sadowski, P.D. (1993) *Mol. Cell. Biol.*, **13**, 3167–3175.
- Panigrahi, G.B., Beatty, L.G. and Sadowski, P.D. (1992) *Nucleic Acids Res.*, **20**, 5927–5935.
- Parsons, R.L., Evans, B.R., Zheng, L. and Jayaram, M. (1990) *J. Biol. Chem.*, **265**, 4527–4533.
- Prasad, P.V., Horensky, D., Young, L.J. and Jayaram, M. (1986) *Mol. Cell. Biol.*, **5**, 4329–4334.
- Prasad, P.V., Young, L.J. and Jayaram, M. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 2189–2193.
- Qian, X.H., Inman, R.B. and Cox, M.M. (1990) *J. Biol. Chem.*, **265**, 21779–21788.
- Senecoff, J.F., Rossmeissl, P.J. and Cox, M.M. (1988) *J. Mol. Biol.*, **201**, 405–421.
- Wang, I., Lee, J., Lee, J. and Jayaram, M. (1994) *Mol. Cell. Biol.*, in press.
- Yang, S.H. and Jayaram, M. (1994) *J. Biol. Chem.*, **269**, 12789–12796.
- Zhou, Y., Busby, S. and Ebright, R.H. (1993) *Cell*, **73**, 375–379.

Received on July 21, 1994; revised on August 18, 1994