

Structural Alterations and Conformational Dynamics in Holliday Junctions Induced by Binding of a Site-Specific Recombinase

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Summary

Binding of a cleavage-incompetent mutant of the Flp recombinase induces a roughly square-planar geometry in synthetic immobile Holliday junctions. The branch points, which are rigidly fixed in these junctions in their free forms, tend to be more flexible in their protein-bound forms. Our results (1) suggest a plausible mechanism for the switching of the recombination complex from the Holliday-forming mode to the Holliday-resolving mode, (2) provide a rationale for previous observations that Flp resolves preformed immobile Holliday structures in the parental or in the recombinant mode in a relatively unbiased manner, and (3) accommodate two modes of DNA cleavage by Flp (transhorizontal or transdiagonal) in Holliday substrates.

Introduction

The four-way DNA junction, the Holliday structure, is a key intermediate in homologous recombination and in site-specific recombination mediated by the Integrase family of recombinases (Craig, 1988; Landy, 1993; Sadowski, 1993). During homologous recombination, the mode of resolution of a Holliday junction determines whether the markers flanking the region of genetic exchange will be in the parental or in the recombinant configuration. In site-specific recombination (since the strand exchange region is identical between the recombining partners), the equivalent consequence is either the reversal of the initial exchange or the production of reciprocal recombinants. In principle, this choice can be influenced by a combination of junction configuration and the cleavage propensity of a given resolution enzyme.

In the presence of divalent metal ions, synthetic Holliday junctions adopt a right-handed, antiparallel "X structure" in which pairs of helical arms are coaxially stacked about the point of strand crossing (Figure 1; reviewed in Duckett et al., 1995a). In this structure, 2 of the 4 DNA strands have continuous axes ("continuous," "nonexchanging," or "noncrossed" strands), while the other two have sharply bent axes ("discontinuous," "exchanging," or "crossed" strands). To be consistent with the

terminology used in two recent papers on Int family site-specific recombination (Arciszewska et al., 1997; Azaro and Landy, 1997), we will also use the terms "continuous" and "crossed" to describe strand configurations within Holliday junctions. It should be emphasized that the crossed strands need not necessarily correspond to the strands that are broken and exchanged by the recombinase to initiate recombination. The primary determinant of which strands will be crossed and which ones will be continuous is the sequence of the bases immediately flanking the junction point (Duckett et al., 1988; von Kitzing et al., 1990; Duckett et al., 1995b; Azaro and Landy, 1997). For a freely mobile junction, a resolution enzyme with a strong bias for cleavage of the continuous strands can yield crossover or noncrossover products depending on the sequence context of the junction at the time of resolution. Alternatively, within the same sequence context, two enzymes with opposite cleavage bias (for the crossed strands in one case, and the continuous strands in the other) can resolve a junction in opposite ways, one to parental and the other to recombinant configuration.

The Flp recombinase is a member of the Integrase family. Consistent with the common mechanistic path of recombination exhibited by this family, the Flp protein can resolve synthetic Holliday junctions into linear products (Jayaram et al., 1988; Dixon and Sadowski, 1993; Dixon and Sadowski, 1994; Dixon et al., 1995; Lee et al., 1995). In this paper, we examine how a Flp variant that cannot mediate strand cutting manipulates the structure of preformed immobile Holliday junctions. The observed conformational alterations have important implications in the assembly and orientation of Flp active sites, the mode of DNA cleavage by Flp, the isomerization of the recombination complex following the exchange of the first pair of strands, and the consequent directionality of Holliday resolution. The dynamics of branch migration within the Flp–Holliday junction complex inferred from our results share similarities with the proposed model for branch migration and resolution mediated by the general recombination proteins RuvABC (Parsons et al., 1995; Rafferty et al., 1996). We discuss our findings in the light of recent observations that the lambda integrase and the *Escherichia coli* XerC/XerD recombinases preferentially cleave the crossed strands of a Holliday structure (Arciszewska et al., 1997; Azaro and Landy, 1997).

Results

Rationale for Deducing the Global Conformation of a Holliday Junction from Electrophoretic Mobilities

Our analyses have utilized electrophoretic mobility in polyacrylamide gels as the assay for arriving at the global conformation of a given Holliday structure (Cooper and Hagerman, 1987; Duckett et al., 1988). Briefly, the rate of migration of a four-way junction with two short arms and two long arms through the gel under an

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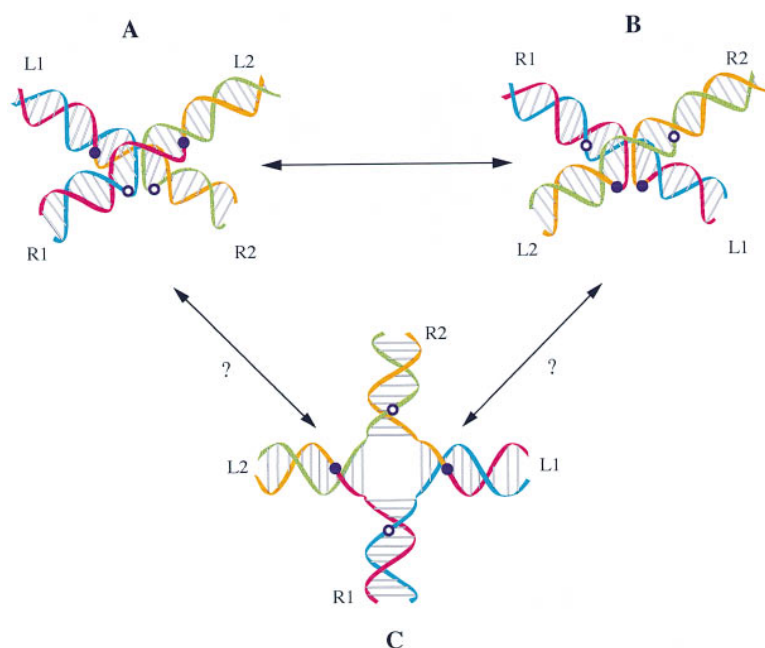


Figure 1. Potential Holliday Junction Configurations during Flp Site-Specific Recombination

Three possible conformations of a Holliday junction intermediate formed during Flp site-specific recombination are shown. (A) and (B) represent the right-handed antiparallel stacked X forms, while (C) represents the unstacked square-planar form. The L and R designations refer to the left and right arms of the two DNA substrates, L1R1 and L2R2, from which the Holliday intermediate is formed by exchange between red and orange strands (say, the top strands). In (A), the top strands, which have already been recombined, are continuous; the parental bottom strands (shown in blue and green) are discontinuous (or crossed). The opposite is true in (B). The scissile phosphates at the left and right ends of the spacer are shown by the closed and open circles, respectively. Experiments with the lambda Int protein and the *E. coli* XerD and XerC proteins have revealed a large cleavage bias toward the crossed strands (Arciszewska et al., 1997; Azaro and Landy, 1997). If this rule holds for Flp as well, resolution of the X-form isomer in (A) would yield the recombinants L1R2 and L2R1. On the other hand, resolution of the X-form isomer in (B) would reverse the initial

strand exchange to yield the parents L1R1 and L2R2. It has been proposed that A→B isomerization may be an integral step in the Int family recombination pathway (Arciszewska et al., 1997; Azaro and Landy, 1997). The planar, four-fold symmetric Holliday isomer in (C) could, in principle, be an intermediate during the isomerization step. Note that, in this isomer, all four scissile phosphates are in an equivalent but noncleavable state. Based on the results with Flp, we suggest that cleavage configuration at the left or at the right end of the spacer can be obtained by limited, directional branch migration coupled with modest reconfiguration of the Flp monomers (see Figure 8). This model does not postulate the restacking of helical arms in an X-form Holliday structure. The arrangement of the DNA arms and the recombinase subunits within the recently solved structure of the “cleaved Cre-loxP intermediate” (Guo et al., 1997) would be generally consistent with the Flp model.

electric field is determined by the spatial disposition of the long arms. The shorter the end-to-end distance between the arms (or more acute the angle subtended by them at the junction), the slower the migration of the junction. Thus, in principle, the mobility profile of a combination of the six possible two short-armed/two long-armed species (of identical mass and charge) derived from a single junction structure should define its global configuration: the angle and end-to-end distance between each pair of arms (Figure 1). The general validity of this analytical method has been substantiated by FRET (fluorescence resonance energy transfer) analyses, chemical probing, and model building (Chen et al., 1988; Cooper and Hagerman, 1989; Lu et al., 1989; Murchie et al., 1989; von Kitzing et al., 1990; Clegg et al., 1992; Eis and Millar, 1993).

To analyze each of the Flp-site-containing Holliday junctions, we generated the corresponding sextuplet combination by chemically synthesizing 54 bp oligonucleotides, joining them by ligation, and hybridizing the four requisite strands (see Experimental Procedures). The arms were designated as L1, L2, R1, and R2 to identify them with the left (L) and right (R) arms of the two DNA molecules (L1R1 and L2R2, for example) partaking in a recombination reaction (see Figure 1). These synthetic junctions mimicked an exchange event within the 8 bp spacer regions of two 54 bp recombination targets (each harboring two 13 bp Flp binding elements bordering the spacer along with a 10 bp extension at either end); 2 of the 4 arms contained additional extensions of 54 bp each. Thus, for a junction immobilized at

the center of the spacer, the two short arms would be 27 bp long (23 plus 4), and the two long arms would be 81 bp long (4 plus 23 plus 54). In Figures 2–7, the top strands (contributing the phosphodiester at the left border of strand exchange; this designation is arbitrary) are represented by the thick lines, and the bottom strands (contributing the phosphodiester at the right border) are represented by the thin lines.

Analysis and Interpretation of Holliday Junction Mobilities: Simplifying Rules

In evaluating the data presented in Figures 2–6, it is useful to keep in mind the following conventions that were employed in displaying them. The ordering of the gel tracks was uniform for all of the figures. The six species for each junction were arranged from left to right in the sequence L2R1, L1R2, R1R2, L1L2, L2R2, and L1R1, where L and R stand for the two long arms. For example, in the species L2R1, the two long arms would be L2 and R1, and the two short arms would be L1 and R2. Given this scheme, a set of simple rules can be formulated for deducing the pattern of strand crossing in a given X-form junction in the protein-unbound state. For this purpose, we need to focus only on the set of the lower three pairs of adjacent bands in panels (A). If they formed the steps of a descending stairway from left to right (as in J1; Figure 2), the top strands (thick lines in all figures) were in the crossed configuration. If they formed the steps of an ascending stairway (as in J2; Figure 3), the bottom strands (thin lines in all figures) were in the crossed configuration. If they did not form

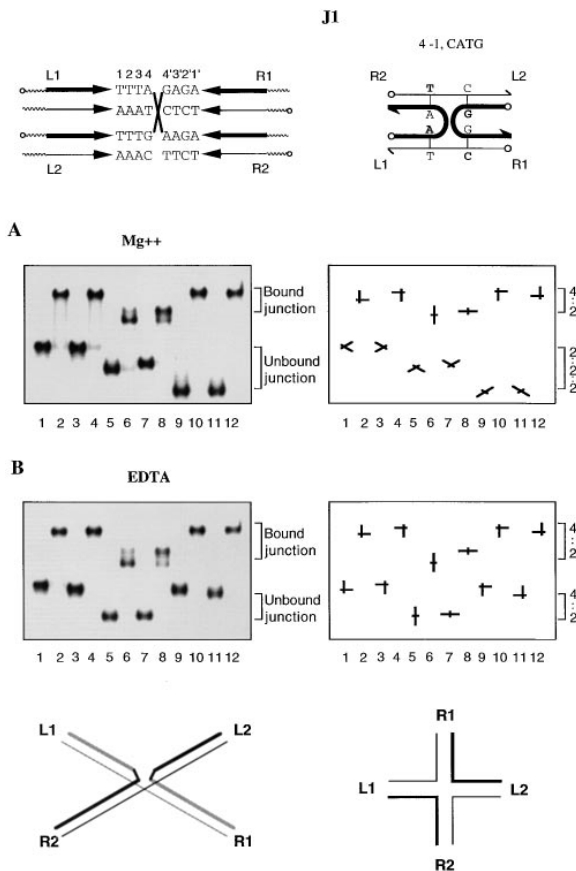


Figure 2. Conformations of a Holliday Junction Immobilized at the Center of the Spacer in Its Free and Flp-Bound Form

The junction, J1, is schematically represented at the top left with the arms arranged in the conventional parallel orientation. The left arms are designated as L1 and L2, the right arms as R1 and R2. The parallel arrows are the Flp binding elements. The wavy lines represent DNA sequences that are not part of the Flp target. The 5' end of each strand is indicated by the open circle. The top and bottom strands are drawn in thick and thin lines, respectively. The point of immobilization of the branch point in J1 is between 4 and 4' positions of the spacer. Positions 0 and 0' correspond to the bases immediately to the left and to the right of the spacer, respectively. At the top right is a representation of J1 in the antiparallel form, with the branch point assigned its class number and sequence designation according to Table 1 in Altona (1996). Note that the junction is named by reading the string of four bases to the 5' side of the branch point on each strand (shown in bold letters) in a clockwise direction. This nomenclature facilitates comparisons of this junction (as well as others used in this study) with the Int Holliday junctions described in Azaro and Landy (1997). The electrophoretic mobilities of the six permuted Holliday species with two long arms and two short arms in the presence of Mg^{2+} (A) or in the presence of EDTA (B) are displayed at the left. The inferred disposition of the DNA arms are schematically shown at the right. The protein-free junctions were run in the odd-numbered lanes; the Flp(Y343F)-bound junctions were run in the even-numbered lanes. The data from (A) was used to deduce whether the top or the bottom strands assumed the crossed configuration in the stacked X form of the junction. The X form and the square-planar form are diagrammed at the bottom so that the reader can easily see how these structures satisfy the correspondence between the experimentally obtained gel mobilities shown in the left panels and the Holliday species symbolically represented in the right panels. Note that in this figure and the subsequent ones, the junctions were run as follows: L2R1 (lanes 1 and 2); L1R2 (lanes 3 and 4); R1R2 (lanes 5 and 6); L1L2 (lanes 7 and 8); L2R2 (lanes 9 and 10); and L1R1 (lanes 11 and 12). By convention, the name of a junction is denoted by its two long arms.

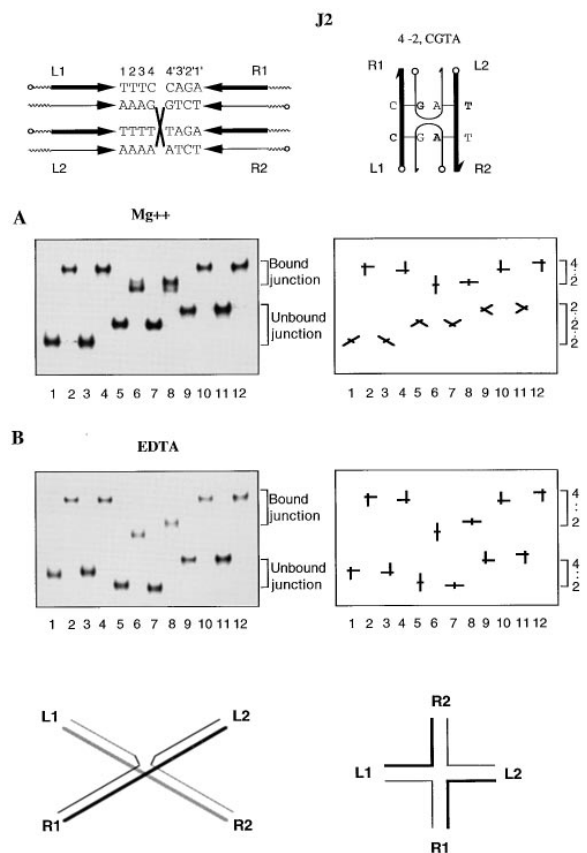


Figure 3. Analysis of a Centrally Immobilized Holliday Structure with Purines Flanking the Junction Being Concentrated in the Bottom Strand

The details are as described for Figure 2. Note that in the stacked X form of J2, the bottom strands (thin lines) were in the crossed configuration.

steps, but were arranged in a line (as in J3; Figure 4), there was no preference for one mode of strand crossing over the other. When the branch point was not positioned at the center of the spacer (as in J4 and J5; Figures 5 and 6, respectively), the middle pair of bands would no longer run in tandem, but would be staggered. If the branch point displacement was to the left of center, the right band would be shifted up (as in J4; Figure 5); if the displacement was to the right of center, the left band would be shifted up (as in J5; Figure 6). As a result, it is the pair of central bands for each junction that is important in revealing protein-induced flexibility in branch points (see below). At the bottom of Figures 2, 3, 5, and 6, we have drawn the X and square-planar forms of the individual junctions that are consistent with the respective mobility profiles (discussed in detail in the sections to follow). These representations together with the schematic drawings in the right-hand panels should help the reader to identify each of the experimental bands in the left panels and correctly interpret its relative mobility.

Conformations of Free and Flp-Bound Holliday Junctions Immobilized at the Center of the Strand Exchange Region

The "comparative gel mobility" analyses of Holliday structures with the junction immobilized at the center

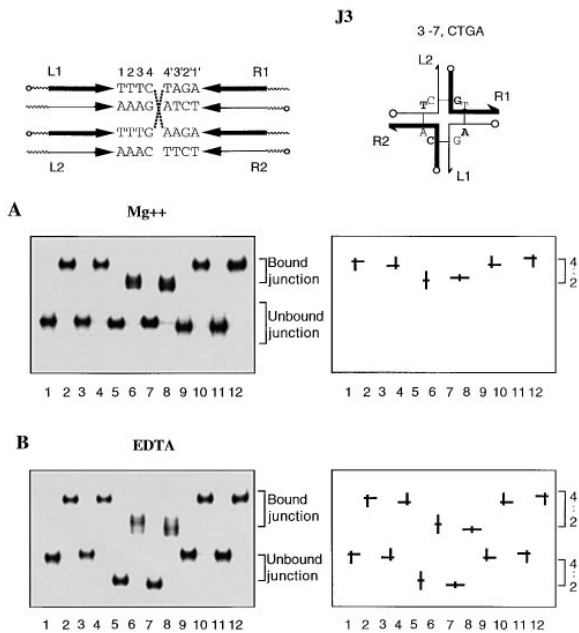


Figure 4. Analysis of a Centrally Immobilized Holliday Structure with Equimolar Purine-Pyrimidine Distribution of Flanking Bases in the Top and Bottom Strands

Since the six permuted species of J3 in the presence of Mg^{2+} had virtually identical mobility, it is not possible to determine which strands were continuous, and which ones were crossed. This is indicated by using dashed lines for the strand crossing in the parallel representation of J3 (top left). Similarly, all four strands are shown as being equivalent under the Altona (1996) classification (top right).

of the spacer, J1–J3, are shown in Figures 2–4. As expected, J1 and J2 gave the 2:2:2 (slow:intermediate:fast) pattern characteristic of the antiparallel stacked X form in the presence of Mg^{2+} , and the 4:2 (slow:fast) pattern characteristic of the open square-planar form in the presence of EDTA (odd-numbered lanes, Figure 2 and 3). Upon binding the cleavage-incompetent Flp variant, Flp(Y343F), the conformation of the junctions was noticeably altered from the stacked X form. The bound forms acquired a conformation that approximates square planarity regardless of the presence or absence of magnesium ions (even-numbered lanes, Figure 2 and 3).

Thus, Flp(Y343F) and, by inference, Flp can actively manipulate the structure of a preformed four-way junction. Since the gel analysis is not sensitive enough to detect slight deviations from planarity, the term “square-planar,” as used in this paper, is not strictly correct. In the context of the present experiments, we define a square-planar junction as an approximately four-fold symmetric junction in which the four DNA arms are roughly confined to one plane, and the angle between any two arms approaches 90° .

Certain interesting features of the individual junctions and of their structural transitions upon binding Flp(Y343F) need to be pointed out. Consistent with the observations of von Kitzing et al. (1990) and Duckett et al. (1995b), recently extended and generalized by Azaro and Landy (1997), the X-form junctions tended to maximize purines from the four base pairs immediately flanking the intersection in their crossed strands. Thus, in presence of Mg^{2+} , it was the top strands (two As and two Gs) that

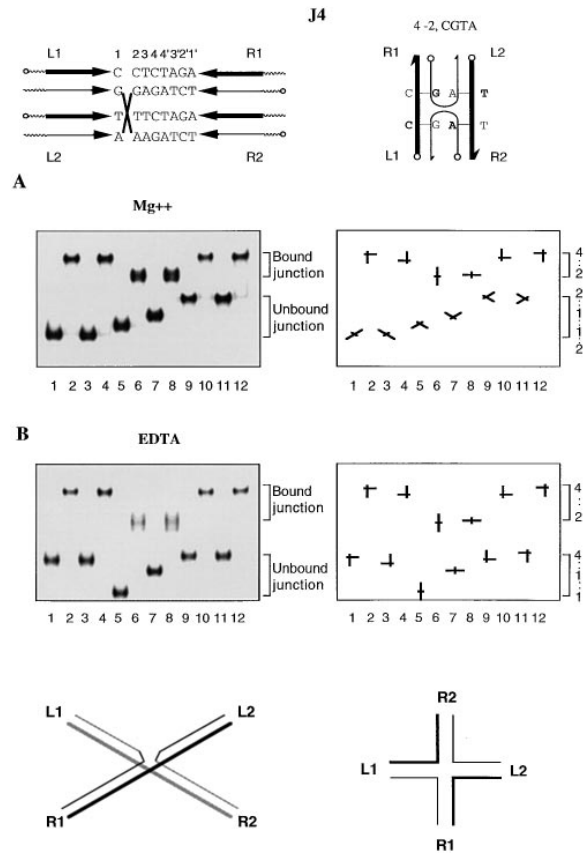


Figure 5. Mobility Patterns of a Holliday Junction with the Branch Point Immobilized near the Left End of the Spacer

The asymmetric location of the crossover point in J4 (between spacer positions 1 and 2) is responsible for the stagger between the bands in lanes 5 and 7 of (A) and (B). Note that only the two species with two short left arms or two short right arms showed the aberrant migration.

were crossed in J1 (odd-numbered lanes, Figure 2A), whereas it was the bottom strands (two As and two Gs) that were crossed in J2 (odd-numbered lanes, Figure 3A). Similarly, while protein-free J1 was almost perfectly four-fold symmetric under the metal-free conditions (concordance with the 4:2 rule; odd-numbered lanes, Figure 2B), J2 showed slight departure from this configuration (tending toward rectangular character; odd-numbered lanes, Figure 3B). The reason for this is not clear. It is possible that the track of five consecutive A-T base pairs spanning the branch point in J2 had some influence on the Holliday structure. Finally, when J1 and J2 were bound by Flp(Y343F), the junctions appeared to be structurally anisotropic, as indicated by a pair of diffused bands among the Holliday–protein complexes (lanes 6 and 8 of Figure 2 and 3). The weak additional bands in lanes 6 and 8 of Figure 3B were not readily apparent due to the relatively low radioactivity present in these complexes. However, they were revealed by longer phosphorimaging of the same gel, and were also seen in other electrophoretic runs not shown here. The corresponding free junctions, by contrast, migrated as sharp compact bands (lanes 5 and 7 of Figures 2 and 3). We believe that the dynamic nature of the interactions

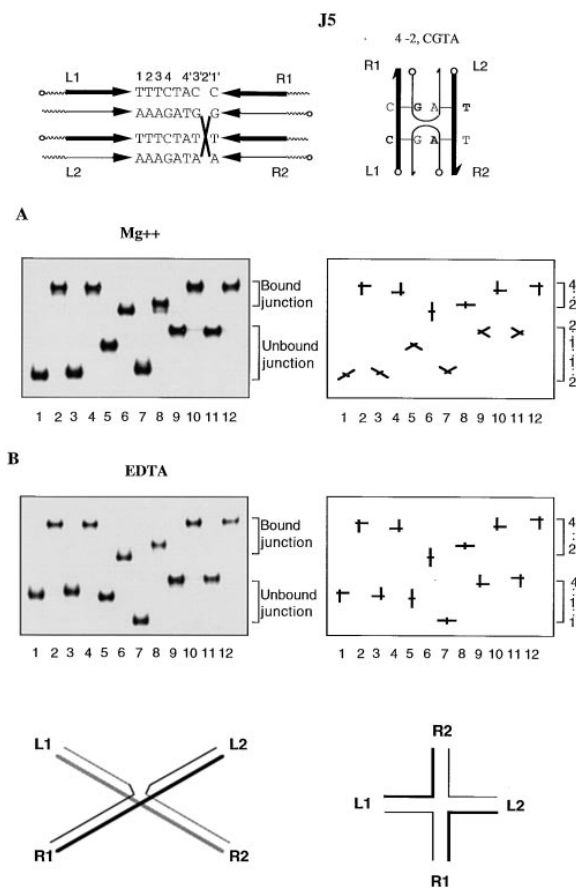


Figure 6. Mobility Patterns of a Holliday Junction with the Branch Point Immobilized near the Right End of the Spacer

As explained under Figure 5, the stagger between the bands in lanes 5 and 7 is due to the asymmetry in the position of the crossover point in J5 (between spacer positions 1' and 2'). Note that the direction of the stagger for J5 is opposite to that of J4 (immobilized close to the left end of the spacer; Figure 5).

among the Flp(Y343F) subunits within the tetramer causes the branch point to be at least partially mobile even though it was rigidly fixed in the protein-free state (for details, see below; Figure 7).

The junction J3 contained equivalent purine-pyrimidine composition ($G = A = T = C$) at the cross-point in the top or the bottom strands. The nearly identical mobilities of all the six permuted forms of this junction in the presence of Mg^{2+} suggests either that the four DNA arms of J3 were arranged in approximately tetrahedral geometry, or that the two crossed-strand isoforms rapidly interconverted during electrophoresis (odd-numbered lanes, Figure 4A). In the absence of Mg^{2+} , or upon binding by Flp(Y343F), J3 conformed to a nearly square-planar conformation (odd-numbered lanes of Figure 4B; even-numbered lanes of Figures 4A and 4B). Once again, the diffused bands in lanes 6 and 8 of Figure 4 were suggestive of junction mobility in the Flp(Y343F)-junction complexes.

Conformational Analysis of Two Junctions Immobilized near the Spacer Extremities

Previous experiments had suggested that, unlike the case with lambda Integrase system (Nunes-Duby et al.,

1995), the position of the crossover point within the spacer in immobilized Flp-Holliday junctions did not significantly affect the directionality of their resolution (Dixon and Sadowski, 1994; Lee et al., 1995). Only when the point of exchange was positioned at or close to the far left or right ends of the spacer was a clear-cut bias in resolution observed (Lee and Jayaram, 1995). Even in this case, the magnitude of the bias was not as large as that seen with lambda Int; it was approximately 4- to 6-fold for junctions immobilized at the very end of the spacer (positions 0 and 1 or 0' and 1'; see Figure 2 for numbering of spacer nucleotides), and approximately 2-fold for those immobilized at the penultimate spacer positions (between 1 and 2 or 1' and 2').

Does the location of the cross within the spacer affect the conformation of the Flp(Y343F)-occupied Holliday junction? To examine this issue, the conformations of J4 and J5, immobilized between spacer positions 1 and 2 and 1' and 2', respectively, were probed (Figures 5 and 6). These junctions behaved very much like those in which the cross was confined to the midpoint of the spacer. They adopted the stacked X form when Mg^{2+} was present (odd-numbered lanes, Figures 5A and 6A), and switched to the square-planar form when no metal was bound to it (odd-numbered lanes, Figures 5B and 6B), or when it was associated with Flp(Y343F) (even-numbered lanes, Figures 5 and 6). The apparent deviation from the 2:2:2 or 4:2 mobility pattern of J4 and J5 in the absence of bound protein to yield the 2:1:1:2 pattern (odd-numbered lanes, Figures 5A and 6A) or 4:1:1 pattern (odd-numbered lanes, Figures 5B and 6B) is easily accommodated by the asymmetric disposition of the cross in these two junctions, effectively making the left arms 7 bp longer than the right arms for J4, and 7 bp shorter for J5. As would be expected from this premise, the aberrant electrophoretic mobilities were only exhibited by the Holliday pairs in which two left arms or two right arms were shortened (lanes 5 and 7 of Figures 5 and 6 representing the free junctions in their X form or square-planar form). In these junction species, the asymmetry in arm length would be additive. On the other hand, this asymmetry would be balanced out for junctions in which one left arm and one right arm were shortened. This reasoning would also be consistent with the opposite relative mobilities of the 1:1 pair from J4 (cross near the left end and therefore shorter left arms) and J5 (cross near the right end and therefore shorter right arms) (compare lanes 5 and 7 of Figures 5B and 6B, for example).

The adoption of the square-planar geometry upon binding of Flp(Y343F) was also observed for two junctions that were immobilized at the left and right extremities of the spacer (between the two base pairs that flank the cleavage positions; 0 and 1 at the left and 0' and 1' at the right), as well as for a junction that was freely mobile through the spacer (data not shown). Thus, the sum of our results indicates that the Flp protein actively manipulates the structure of Holliday junctions by binding to the four arms, at least partially unstacking them and forcing them toward the four corners of a square. This structural alteration is not affected by the degree of freedom for branch migration through the spacer or by the location of the immobilization point (partner heterology) within the spacer. Furthermore, all of the junctions

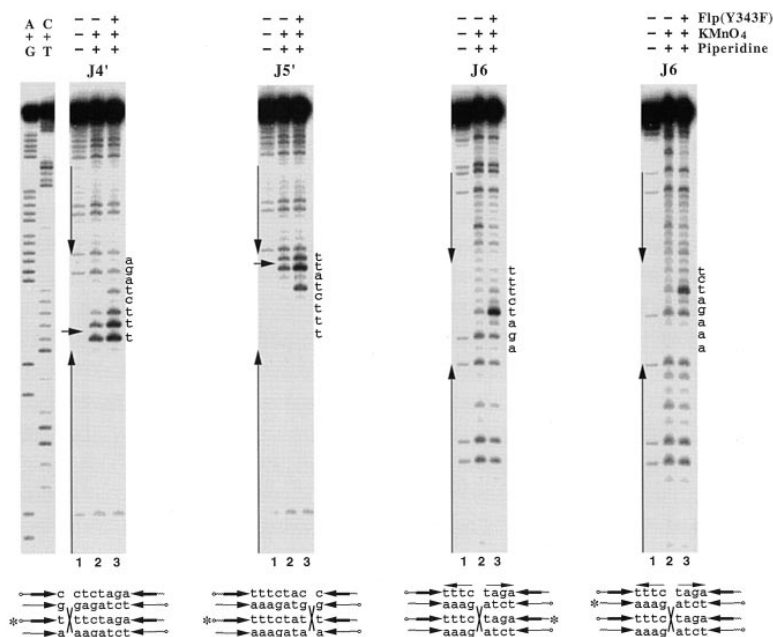


Figure 7. Probing of Immobilized Junctions with Potassium Permanganate for Unstacked/Unpaired Thymine Residues

Two of the junctions tested, J4' and J5', were derivatives of J4 and J5 (see Figures 5 and 6) with the crossover points locked in between spacer positions 1 and 2 or 1' and 2', respectively. J6 was a mobile junction with the branch point free to migrate through the spacer as well as the Flp binding elements bordering it. After binding Flp(Y343F), the junctions were subjected to electrophoresis in the presence of EDTA. The bands corresponding to the free and bound junction were excised and treated with KMnO_4 in situ in the gel. After extraction from the gel, the DNA was subjected to piperidine cleavage. Lanes 2 and 3 represent the strand cleavage profiles from the permanganate-treated free junction and Flp(Y343F)-associated junction, respectively. Lane 1 represents the free junction subjected to the piperidine reaction without prior KMnO_4 treatment. The pattern in lane 1 results primarily from background bands due to some cleavage at the G positions during piperidine treatment. The G+A and C+T Maxam-Gilbert sequence ladder at the left

was run for reference. The position of the radioactive label is denoted by the asterisk. The label was placed at 5' ends in J4' and J5', and at 3' ends in J6. One strand each of J4' and J5' and two strands of J6 were probed for permanganate sensitivity. The horizontal arrows indicate the positions of the branch point in J4' and J5' in the absence of Flp(Y343F).

tested here that were immobile in the absence of bound protein showed branch point mobility when occupied by Flp(Y343F) (see below).

Branch Point Dynamics within the Flp(Y343F)-Occupied Holliday Junctions

In the square-planar configuration, the opening of the Holliday structure at the crosspoint renders the C5–C6 double bond of neighboring unstacked/unpaired thymine residues susceptible to oxidation by potassium permanganate (Duckett et al., 1988; Bennett and West, 1995). Permanganate sensitivity of thymines can therefore reveal the location of the strand crossing. We have used permanganate probing to verify our surmise that the apparent structural anisotropy in Flp-bound junctions (diffused bands in lanes 6 and 8 of Figures 2–6) was due to protein-induced perturbations of the spacer DNA, and the possible movement of the junction despite the block imposed by flanking heterology.

The assay was first performed on the two junctions J4' and J5' in which the cross was positioned close to the left and right spacer ends, respectively (just as in J4 and J5; see Figures 5 and 6). A control was also done using a junction, J6, quite similar to J4' and J5', but in which the branch point was completely mobile through the spacer. J4' and J5' were obtained by trimming the terminal 54 bp from the two long arms of J4 and J5, respectively, reducing the appendage at the end of each Flp binding element to 10 bp. The relative mobilities of the two short left-arm and the two short right-arm species of J4 in the free (lanes 5 and 7, Figure 5) and Flp(Y343F)-associated forms (lanes 6 and 8, Figure 5) had suggested that the protein was pushing the cross

toward the center of the spacer in a left-to-right direction. Similar mobility comparisons for J5 (lanes 5 and 7 versus lanes 6 and 8 of Figure 6) had suggested that, in this case, the forced movement of the cross was from right to left.

In the chemical probing assays, the free junctions (J4' and J5') and the corresponding complexes with Flp(Y343F) were first separated by gel electrophoresis. The gel slices harboring them were excised and treated with permanganate in situ. Following piperidine cleavage, samples were analyzed by electrophoresis in a denaturing gel against a Maxam-Gilbert sequence ladder as control. For each junction, lane 1 was a piperidine reaction in the absence of prior permanganate treatment; lanes 2 and 3 were the permanganate-treated free junction and Flp(Y343F)-junction complex, respectively. In J4', the junction was stationary between spacer positions 1 and 2 as indicated by the sensitivity of the thymines at these positions, the weak sensitivity of thymine at 3, and the lack of sensitivity of thymine at position 5 (lane 2, Figure 7). In the J4'–Flp(Y343F) complex, the thymines at 3 and 5 became permanganate sensitive, as were those at 1 and 2 (lane 3, Figure 7). This result would be consistent with the suspected escape of the cross from the 1, 2 heterology trap and its migration to the right. So also, the sensitivity of thymines only at positions 1' and 2' in J5' (lane 2, Figure 7) and the additional sensitivity of the thymine at position 4' in the J5'–Flp(Y343F) complex (lane 3, Figure 7) would be consistent with the movement of the cross toward its left. Note also that the two T's flanking the crosspoint in unbound J4' and J5' became hypersensitive to permanganate after they were complexed with Flp(Y343F) (compare lanes 3 to lanes 2 in [A] and [B]). This result

agrees with the notion that the branch point does migrate, since the resulting mismatch at these positions is likely to render the T's more reactive.

The thymine sensitivity to permanganate in the mobile junction, J6, was probed on the top as well as the bottom strands (left and right panels, respectively; Figure 7). In this molecule, the junction would be free to branch-migrate not only through the spacer, but also through the adjacent Flp binding elements on either side of it. The cleavage patterns for the unbound junction were consistent with this unrestricted, relatively long-range (34 bp) branch migration. Thus, in contrast to the immobile junctions (J4' and J5'), no marked cleavage enhancements at specific spacer positions were observed (lanes 2 of J6). However, the pattern was quite distinct for the Flp(Y343F)-bound form of J6. Now the T at the 4' position (lane 3, J6 left panel) and that at the 3' position on the bottom strand (lane 3, J6 right panel) became hypersensitive to permanganate. A slight increase in T sensitivity was also observed at spacer position 3 on the top strand (lane 3, J6 left panel). Neither the T residues at positions 1 and 2 nor the one at position 1' showed differential permanganate reactivity in the free versus bound form of J6. These results are accommodated by the interpretation that the bound Flp(Y343F) restricts the branch point to the central core of the spacer and limits its mobility to no more than 2–3 bp. Our data are quite similar to the results of permanganate probing of a mobile Holliday junction bound by the XerC and XerD recombinases (Arciszewska et al., 1997). Thus, the observed displacements in permanganate-sensitive spacer positions in J4' and J5' upon binding to Flp(Y343F) are consistent with the tendency of the protein to localize the branch point toward the center of the spacer. It is interesting to note that the limited branch migration of 2–3 bp suggested by these experiments agrees well with a proposed model for Flp recombination (Lee and Jayaram, 1995; Y. V. et al., unpublished data).

There is some concern that the changes in the T-cleavage patterns obtained with bound Flp(Y343F) may be due to some deformation of the spacer (as a result of protein-induced DNA bending, perhaps), and not directly due to a change in the branch-point position. However, the correlation between the permanganate sensitivity of J4' and J5' (Figure 7) and the mobility-shift data on J4 and J5 (junctions with branch points immobilized at the same two positions as J4' and J5', respectively; Figures 5 and 6) cannot be ignored. In the unbound form, the junctions with two short right arms or two short left arms migrated at different positions because the branch point was not central to the spacer (lanes 5 and 7 of Figures 6 and 7). For J4, in the bound form, these two species became somewhat diffuse, and the asymmetry in their migration was suppressed (lanes 6 and 8, Figure 6). Thus, the branch point appeared to be no longer constrained to a single position away from the center of the spacer. For the bound form of J5, the mobility of the sharpest bands corresponding to the two junctions showed an opposite asymmetry from that of the free junction bands (compare lanes 6 and 8 to lanes 5 and 7 in Figure 7). It is possible that association of Flp(Y343F) with the Holliday junction could result in local DNA distortions that would favor

the branch point being located at certain positions within the spacer. The apparent delocalization of the branch point may thus be the indirect result of the protein–DNA interactions within the bound Holliday junction. Overall, the results of permanganate probing in conjunction with the gel mobility assays indicate that the binding of Flp to Holliday junctions is a dynamic event that could facilitate short-range “branch migration.”

Discussion

The Flp Protein Manipulates the Conformation of Holliday Junctions

In this study, we have demonstrated that binding of the Flp(Y343F) tetramer imparts an approximate square-planar configuration on synthetic Holliday junctions. This geometry is established regardless of the initial state of the free junction: the stacked X, tetrahedral (?), or square-planar form. In this respect, Flp is similar to other Holliday-binding or Holliday-resolving proteins such as RuvA, T4 endonuclease VII, CCEI, and RuvC that actively distort the structures of DNA junctions (reviewed in White et al., 1997). The opening of the DNA at the junction by unstacking of bases, which is a common theme in all of these structural distortions, may serve to facilitate branch migration, or promote access of the enzyme active sites to the labile phosphodiester bonds that are normally located at or close to the branch point. Our analyses could be performed only with a Flp variant that is incapable of DNA cleavage, since resolution by wild-type Flp would result in the breakdown of the Holliday structure. Nevertheless, several attractive aspects of the Flp(Y343F)-bound Holliday junction suggest that its structural features are likely to be relevant to the conformational dynamics and the chemical steps of the recombination pathway (see below). For example, the establishment by Flp of a Holliday conformation conducive to branch migration would be a pertinent first step for effecting junction mobility within the spacer as suggested by this study. Coordinated, but limited protein–DNA movements can, in principle, bring about the juxtaposition of alternative pairs of active sites against the phosphates at the top or the bottom strand-exchange points. Thus, protein reconfiguration coupled to branch migration can provide the basis for selectivity and orientation of Flp active-site assembly, and account for Holliday resolution either to parental or to recombinant duplexes. Furthermore, relatively unbiased resolution by Flp of even those Holliday junctions that were immobilized within the spacer (Lee et al., 1995) would be consistent with the freedom from immobilization accorded to them as a result of Flp binding (this study).

Holliday Junction Structure and Strand Bias during Resolution

The rather unbiased resolution by Flp of immobile Holliday junctions frozen at different positions within the spacer region to parentals or to recombinants (Dixon and Sadowski, 1994; Lee et al., 1995) contrasts with the highly biased, position-dependent resolution of similar junctions by lambda Int (Nunes-Duby et al., 1995). Assuming that the top- and bottom-strand resolution

reactions are symmetric with respect to protein-DNA geometry, the normal Int recombination reaction can be accommodated by an isomerization step involving a restacking of helical arms between the first and second pair of strand exchanges and a 1 bp branch migration. Consistent with this model, Azaro and Landy (1997) have established a strong correlation between the isomeric state of a Holliday junction (in its protein-free form) and the direction of its resolution by Int. The crossed strands are almost invariably the targets for cleavage and exchange by the Int protein. A similar situation has been encountered in the XerC/XerD system as well. In this case, a nearly exclusive XerD preference, and a strong, though not absolute, XerC preference for cleavage of the crossed strands has been demonstrated by using conformationally constrained antiparallel Holliday junctions obtained by tethering of strands (Arciszewska et al., 1997). On the other hand, unidirectional resolution of tethered, antiparallel junctions has not been observed with Flp (J. L. and M. J., unpublished data).

Is the proposed changeover in the Holliday isoform (the swapping of configurations between the continuous and crossed strands by restacking of helical arms) prior to the resolution step of recombination a common feature of the Int family? The structure of the Flp(Y343F)-bound Holliday junctions observed in this study (which have an approximately four-fold symmetry), together with the propensity of Flp to resolve preformed, tethered or untethered Holliday junctions without large bias (Lee et al., 1995; J. L. and M. J., data not shown), is not readily accommodated by the lambda Int model. To bring the Flp results into conformity with the Holliday restacking paradigm, one must suppose that the square-planar Holliday form is an intermediate in the structural transition and is enriched in our assays by the use of the noncleaving Flp variant. However, it is worthwhile to consider an alternative model for recombination (discussed below) that does not involve the conventional, stacked X-form Holliday junction and its isomerization via swapping of helical partners.

Models for Flp Recombination: Contributions of DNA Cleavage Mode and Branch Migration to the Reaction Path

In Figure 8, we present two possible schemes for Flp recombination. Imagine that the reaction between two linear duplexes, L1R1 and L2R2, is initiated by two "transhorizontal" cleavages at the left end as indicated in Figure 8A. According to the Chen et al. (1992a) convention, the transhorizontal mode is defined as the donation of the active site tyrosine by a Flp monomer to cleave the labile phosphate across the spacer (from left to right or right to left), but within the same DNA molecule. Similarly, donation of tyrosine across the spacer to effect cleavage in the partner DNA molecule defines the transdiagonal mode. The convention is based on two DNA substrates synapsed in parallel alignment; if they are placed in anti-parallel alignment (as in Figure 8), the apparent cleavage directionality must be suitably modified to account for the change in the reference frame. In the example in Figure 8, Tyr-343 residues for the first two cleavages are donated in the R1-to-L1 and R2-to-L2 directions. Subsequent strand exchange produces

a Holliday structure in which the branch point is positioned 3 nt away from the left cleavage site (3, 5; Figure 8B). This placement is consistent with the results of Lee and Jayaram (1995) and Zhu et al. (1995). Two bp of branch migration (Figure 8B→C→D or D') would move the branch point to the 5, 3 position in preparation for the cleavage/exchange at the right end. This transition (or isomerization) is shown to proceed via an approximately four-fold symmetric structure (4, 4), as suggested by the present analyses (Figure 8C). During the C→D transition, the L1-R1 and L2-R2 pairs become so disposed as to accomplish cleavage at the right ends and produce recombinants by the transhorizontal mode (C→D→E). During the C→D' transition, it is the L1-R2 and L2-R1 arms that assume the cleavage-competent geometry. The resulting cleavages at the right ends, with Tyr-343 being donated from L2 to R1 and from L1 to R2, would be "transdiagonal" (according to the original DNA reference frame).

Currently available experimental data do not permit a clear-cut choice between the two alternative pathways of recombination outlined here. Tests using linear full-site substrates have revealed only transhorizontal cleavage by Flp (Lee et al., 1994). Opposite directions of transhorizontal cleavage, right-to-left and then left-to-right, may potentially be established in a linear substrate by two functionally equivalent but asymmetric geometries adopted by a Flp dimer bound to the DNA arms across the spacer (Luetke and Sadowski, 1995; Lee et al., 1997). However, it is not clear whether the protein-DNA movements required for switching between these alternative geometries would be permissible in a four-armed junction bound by a Flp tetramer. Assays using synthetic Holliday structures suggest that either transhorizontal or transdiagonal cleavage by Flp is equally likely (Lee et al., 1994, 1996). Since this cleavage duality can, in principle, be accounted for by the presence of two crossover isoforms of a Holliday junction (see Figure 1), its potential significance to the recombination pathway has not been seriously considered. The relative simplicity of the C-D'-E' pathway for recombination (Figure 8) suggests that transdiagonal cleavage may indeed be functionally relevant. It would appear that the Holliday junction formed by the initial transhorizontal cleavage (3, 5; Figure 8B) can isomerize to the D' form (5, 3) conducive to transdiagonal cleavage without large DNA-protein distortions. The relative merits of unimodal cleavage (transhorizontal alone) versus bimodal cleavage (transhorizontal and transdiagonal) in the context of the DNA-protein mechanics of recombination will be considered elsewhere (Y. V. and M. J., unpublished data). Note that, in the bimodal scheme, the recombination event can also be accomplished by an initial pair of transdiagonal cleavages (across partner substrates) within the synaptic complex, and a subsequent pair of transhorizontal cleavages within the Holliday junction. It should be pointed out that the transhorizontal to transdiagonal switch has functional parallels to the swapping of recombinase subunit interactions during the proposed isomerization step in Int and in XerC/XerD recombination (Nunes-Duby et al., 1995; Arciszewska et al., 1997; Azaro and Landy, 1997).

The location of the substrate arms and of the cleaved DNA ends in a recently solved crystal structure of the

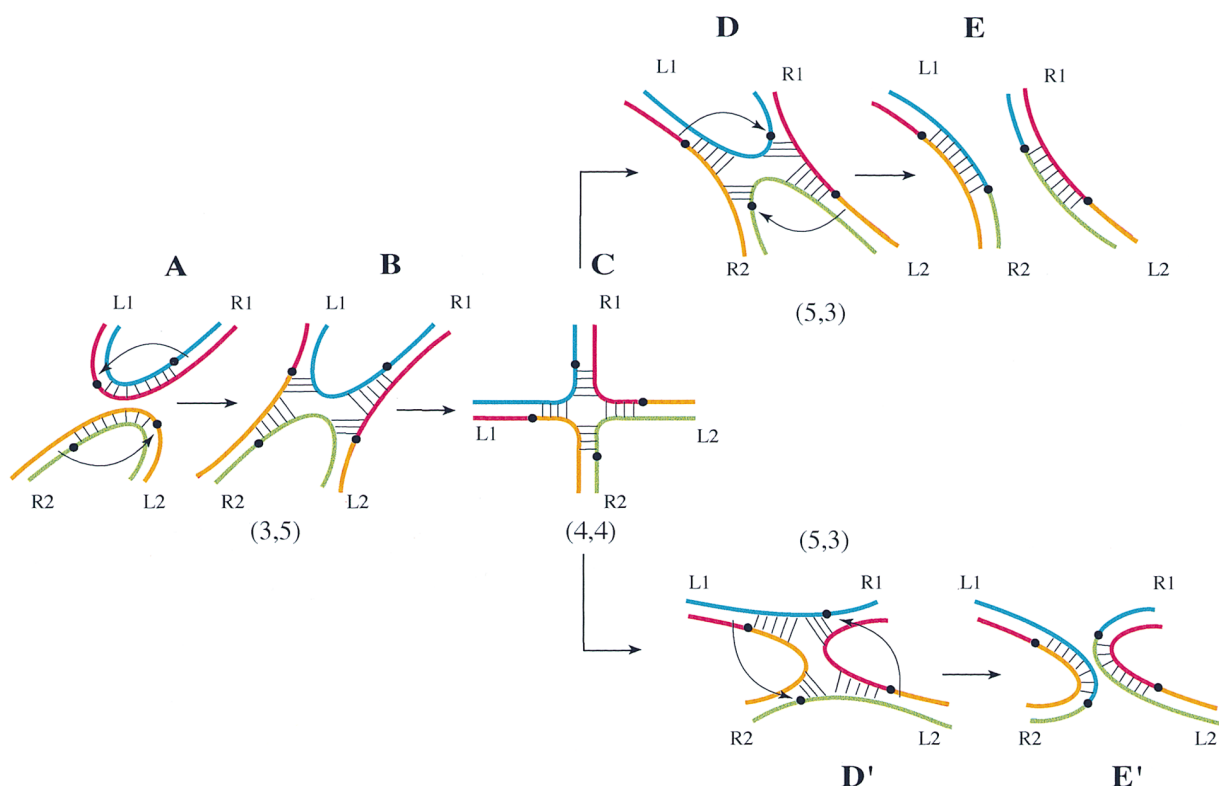


Figure 8. Models for the Flp Recombination Reaction that Utilize Unimodal or Bimodal Cleavage by Flp

In the initial step, (A), the two cleavages (donation of active site tyrosine) are directed from R1 to L1 and R2 to L2, and correspond to the transhorizontal mode (Chen et al., 1992a). The tilt in the DNA arms is intended to schematically represent the Flp-induced DNA bend (Schwartz and Sadowski, 1990; Chen et al., 1992b) as well as the selective strand cleavage observed in substrates with strand-specific nucleotide bulges (Lee et al., 1997). In the resultant Holliday junction (B), the branch point is placed 3 nt away from the left cleavage positions (3, 5; Lee and Jayaram, 1995; Zhu et al., 1995). This junction is shown to assume a square-planar configuration (C) in accordance with the results of this study. The branch point (4, 4) in this four-fold symmetric state is equidistant from all four cleavage positions. The reaction path diverges at this point between the two models. The path along C→D→E resolves the junction by transhorizontal cleavages (L1 to R1 and L2 to R2), whereas that along C→D'→E' resolves the junction by transdiagonal cleavages (L1 to R2 and L2 to R1). In both models, the branch point at the time of resolution is shown to be situated 3 nt away from the right end of the spacer (5, 3; D and D'). For details, see text. The mode of "discontinuous" exchange of the spacer DNA between the recombining partners proposed here (in steps of 3, 1, 1, and 3 nt) is compatible with the "strand swapping" model for λ Int recombination arrived at by Nunes-Duby et al. (1995). Results from XerC/XerD-mediated resolution of Holliday junctions also agree with this model (Arciszewska et al., 1995).

Cre recombinase (an Int family member) complexed with DNA (Guo et al., 1997) is consistent with a Holliday intermediate whose geometry is close to square-planar. In this pre-Holliday state, the four Cre subunits show a cyclical connectivity. In the context of Flp recombination, this structure would more easily accommodate the transhorizontal/transdiagonal mechanism than the transhorizontal/transhorizontal mechanism.

Branch Migration of Holliday Intermediates in General and in Site-Specific Recombination

The approximate square planarity of the Holliday junction bound by Flp(Y343F), as well as the observed conformational dynamics within it, agree with the notion that branch migration is intrinsic to the reconfiguration of Flp subunits during the assembly of the active sites for the resolution step. The parallels to the proposed models for branch migration and Holliday junction resolution mediated by the RuvABC proteins during homologous recombination in *E. coli* cannot be overlooked (Parsons et al., 1995; Rafferty et al., 1996). In the latter

system, the RuvA protein contributes to the square-planar architecture of the Holliday junction, and recruits the RuvB helicase that actively drives branch migration using the energy of ATP hydrolysis. The resolution reaction is then carried out by RuvC. In our Flp model, the Flp tetramer dictates the open Holliday conformation, acts as a "passive helicase" to promote short-range mobility of the junction, and mediates the resolution event. Thus, from the standpoint of mechanism and mechanics, Int family site-specific recombination has interesting similarities to homologous recombination (Lee and Jayaram, 1995).

Experimental Procedures

Purification of Flp(Y343F)

Flp(Y343F) used in these experiments was approximately 90% pure, and was obtained by published procedures that included a final affinity purification step (Lee et al., 1996). Estimation of protein concentrations in the preparation has been described previously (Lee and Jayaram, 1995).

Synthetic Holliday Junctions

Each Holliday junction was assembled from four DNA strands by hybridization. Conditions for hybridization were the same as detailed in Lee et al. (1996). 5 of the 8 junctions described in this paper, J1-J5, were reconstituted from 108 nt single-stranded DNAs. The other 3 were derived from 54 nt single strands. The relevant features of the various substrates used are described in the Results, displayed in the figures, and explained in their legends. Each of the two DNA partners generating a Holliday junction contained a pair of Flp binding elements flanking the 8 bp spacer placed in head-to-head orientation. The binding element had the sequence: 5'-GAAGTTCCTA TAC-3'/3'-CTTCAAGGATATG-5'. The sequence of the spacer was not constant from junction to junction, and is spelled out in the figures. The complete sequences of the substrates used in these experiments are available upon request.

The 5' end of a deoxyoligonucleotide was labeled using [γ - 32 P]-ATP in a T4 polynucleotide kinase reaction. The 3' end was labeled by the terminal deoxynucleotide transferase reaction in the presence of 3'-[α - 32 P]-cordycepin 5'-triphosphate. The unreacted ATP or cordycepin phosphate was removed by spin dialysis on a Sephadex G-25 column.

Electrophoretic Assays of Permuted Sets of Holliday Junctions

The rationales of the assays and the experimental approaches were essentially the same as those on which the original analysis of permuted Holliday junctions (for example, Duckett et al., 1988) was based. In our assays, though, the short arms of the junctions were not obtained by restriction enzyme digestion. Instead, each of the six junctions was separately assembled so as to mimic the products obtained by pairwise digestion of arms of a single junction with four equal arms. Each of the four 108 nt strands required for a junction was obtained by first synthesizing two 54 nt deoxyoligonucleotides and ligating them in the presence of a short complementary anchor strand. The ligated products were gel-purified prior to hybridization. All experimental analyses were done using Holliday junctions that were gel-purified to separate them from minor aberrant hybridization products. Binding of Flp(Y343F) to the end-labeled junctions was carried out as described previously (Lee et al., 1996) at a molar ratio of approximately 10 Flp(Y343F) molecules per Flp binding arm. Under these conditions, virtually all of the junctions in the substrate population were occupied by a "tetramer" of Flp(Y343F). Electrophoresis of the bound and unbound junctions was done at 4°C or at room temperature in 1× Tris-borate buffer containing either NaCl or EDTA (Duckett et al., 1988). The mobility patterns were visualized by autoradiography or by phosphorimaging.

KMNO₄ Probing of Flp(Y343F)-Bound Junctions

The protein-bound and unbound junctions were separated by electrophoresis. Excised gel slices were subjected to permanganate modifications under conditions described by Bennett and West (1995) for the reaction in solution. DNA was then extracted from the gels by standard procedures, digested with piperidine, and analyzed by electrophoresis in 10% polyacrylamide sequencing gels.

General Methods

Restriction enzyme digestions, isolation of plasmid DNA, and other miscellaneous procedures were done as described by Sambrook et al. (1989).

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