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The isomeric preference of Holliday junctions influences resolution bias by λ integrase

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λ site-specific recombination proceeds by a pair of sequential strand exchanges that first generate and then resolve a Holliday junction intermediate. A family of synthetic Holliday junctions with the branch point constrained to the center of the 7 bp overlap region was used to show that resolution of the top strands and resolution of the bottom strands are symmetrical but stereochemically distinct processes. λ integrase is sensitive to isomeric structure, preferentially resolving the pair of strands that are crossed in the protein-free Holliday junction. At the branch point of stacked immobile Holliday junctions, the number of purines is preferentially maximized in the crossed (versus continuous) strands if there is an inequality of purines between strands of opposite polarity. This stacking preference was used to anticipate the resolution bias of freely mobile junctions and thereby to reinforce the conclusions with monomobile junctions. The results provide a strong indication that in the complete recombination reaction a restacking of helices occurs between the top and bottom strand exchanges.

Keywords: Holliday junctions/integrase/λ/site-specific recombination

Introduction

The site-specific recombination system of bacteriophage λ mediates insertion of the circular phage DNA into the *Escherichia coli* host chromosome, as first proposed by Allan Campbell (Campbell, 1962; reviewed in Landy, 1989; Stark *et al.*, 1992). This is a highly programmed transaction that occurs between two loci, the 240 base pair (bp) phage *att*P and the 25 bp bacterial target *att*B. The products of this recombination are two hybrid sites, *att*R and *att*L, that form the boundaries of the integrated prophage. An excisive recombination can occur between these sites to regenerate the 'substrates', *att*P and *att*B. Both of these reactions are characterized by two pairs of temporally distinct strand exchanges, separated by 7 bp (overlap region), that proceed via an obligatory Holliday junction intermediate (Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987). In essence, this reaction can be said to convert continuous (i.e. stacked) parental helices into continuous recombinant helices; thus, the substrates are not only reshuffled but restacked. We wished to determine at what stage of the recombination reaction this restacking occurred and to test a previously proposed model for strand exchange (Nunes-Düby *et al.*, 1995).

The catalytic steps are executed by the phage-encoded λ integrase (Int, a type I topoisomerase) in concert with other protein factors (IHF for integrative recombination; IHF, Xis and Fis for excisive recombination). Two λ Int monomers introduce a nick into the top strand of each recombining partner at the 5′-boundary of the 7 bp overlap and in the process become covalently attached via a 3′-phosphotyrosyl bond (Mizuuchi *et al.*, 1981; Craig and Nash, 1983; Pargellis *et al.*, 1988). It has been proposed that, once the 5′-ends are freed by the nicks, the top strands of each overlap region swap 2–3 nt with the partner duplex (Nunes-Düby *et al.*, 1995). If proper base pairing is achieved, the newly exchanged strands become ligated to the receiving strands, disconnecting the two λ Int monomers in the process. According to this model, a Holliday junction intermediate is created with its crossover sitting near the center of the overlap region. The conformation of the newly generated Holliday junction most closely reflects the helical axes of the parental duplexes. The model calls for a limited net movement of the branch point of ~1–3 bp and for the Holliday junction to assume a different isomer, that now anticipates the helical axes of the recombinant helices. This isomerization (which constitutes the restacking step) prepares the Holliday junction for resolution by cleavage of the bottom strands.

This model of λ site-specific recombination is based on studies of synthetic Holliday junctions that have their branch migration constrained by the incorporation of sequence heterologies (Figure 1; Nunes-Düby *et al.*, 1995). It challenges the long-standing notion that the requirement for overlap sequence homology (Weisberg *et al.*, 1983) is to permit branch migration across the full 7 bp of the overlap region. A critical conclusion drawn from that study is that the Holliday junctions are optimally resolved by λ Int when the branch point is located in a 1–3 bp region at the center of the 7 bp overlap region. Most strikingly, the DNA junctions are efficiently and exclusively resolved to 'substrate' helices (top strand resolution) when the branch point is fixed at position 3/4, immediately left of the center of the overlap region, and to 'product' helices (bottom strand resolution) when the branch point is fixed at position 4/5, immediately right of the center. In order to postulate that these reactions are symmetrical (i.e. that the geometries of top strand resolution and bottom strand resolution are identical), the authors invoked an isomerization step between the first and second pairs of strand exchanges.

The structure of immobile four-way junctions in solution has been extensively characterized (reviewed in Lilley and Clegg, 1994; Seeman and Kallenbach, 1994) and has been shown to depend both on the ionic conditions and on the sequence at the branch point (Duckett *et al.*, 1988, 1990). In the absence of metal ions the Holliday junction assumes an extended structure, in which the four helices

are unstacked and point towards the corners of a square. Addition of micromolar concentrations of divalent cations, such as Mg^{2+}, promotes pairwise stacking of the helical arms and rotation into an antiparallel X-structure. Millimolar concentrations of monovalent cations, such as Na^{+}, can also stimulate this transition, albeit less efficiently. A consequence of this structural transition is to generate two strands that are 'continuous' with each helical axis and two strands that are sharply bent and 'exchange' between the two stacked helices. In this study we shall refer to the latter pair of strands as 'crossed', to avoid any confusion with strand exchange during resolution. It has also been shown that immobile four-way junctions choose one of two possible isomers of the stacked structure, based on their relative stability (Duckett *et al.*, 1988). This stability is dictated, in an unknown manner, by the sequence of the base pairs at the branch point. The two possible stacking isomers are relevant to our recombination model in the following way: we had predicted that a Holliday junction with its top strands crossed will be resolved preferentially to parental helices and one with its bottom strands crossed will be resolved preferentially to product helices (Nunes-Düby *et al.*, 1995).

In this study we demonstrate a correlation between the isomeric structure of the naked Holliday junction substrate and the ability of λ Int to preferentially cleave, exchange and ligate the crossed pair of strands. We believe that these results have important implications not only for the geometry of the resolution reaction, but also for the complete recombination reaction. Furthermore, we have expanded upon an observation made by David Lilley's group (von Kitzing *et al.*, 1990; Duckett *et al.*, 1995): the stacking preference of certain branch point sequences that possess an inequality of purines and pyrimidines between opposing pairs of strands tends to maximize the number of purines in the crossed strands.

Results

Construction of central mobility Holliday junctions

Spontaneous branch migration of a Holliday junction is an isoenergetic process that involves sequential dissoci-

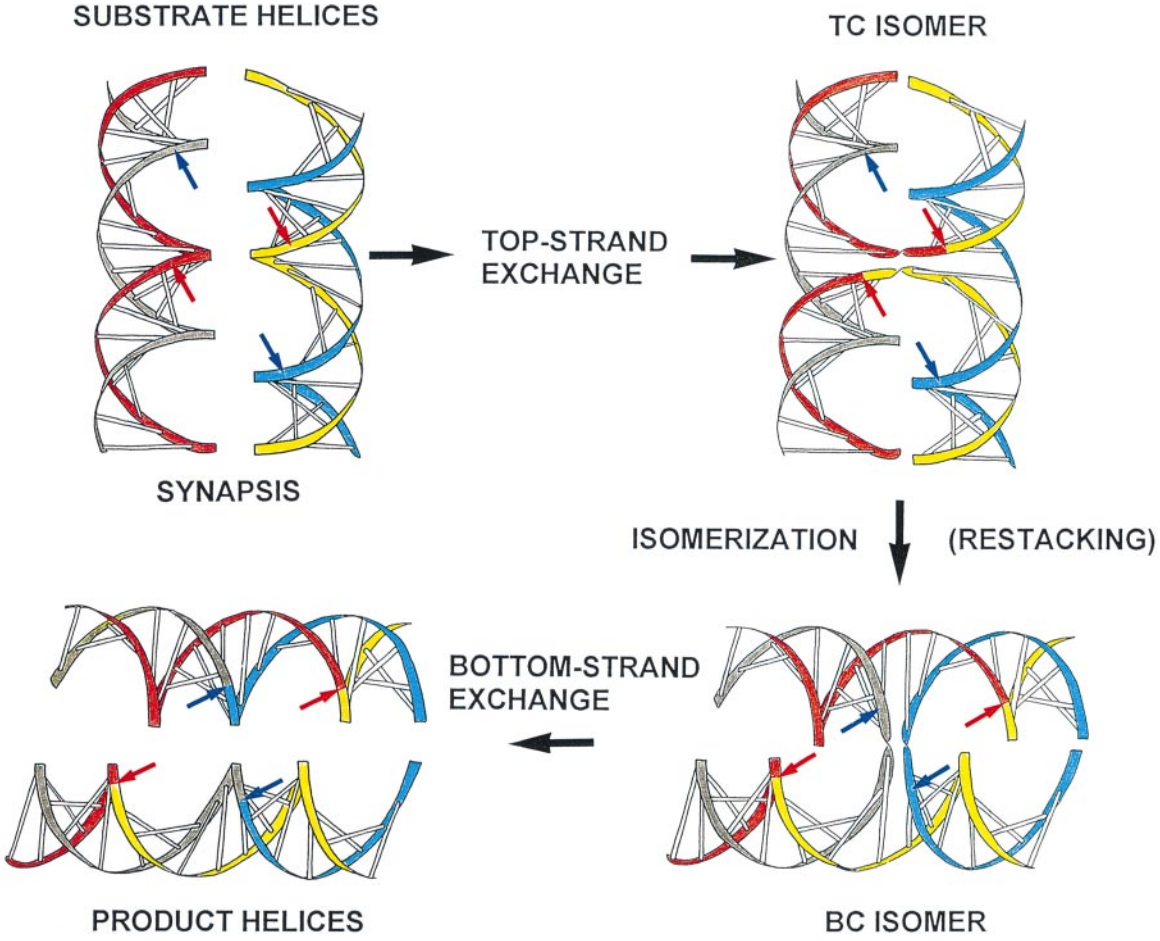

Fig. 1. Strand swapping model of recombination as proposed by Nunes-Düby *et al.* (1995). In this representation the substrate helices (*att*P and *att*B or *att*L and *att*R) are aligned in an approximately antiparallel orientation (synapsis). λ Int nicks the top strands (red and yellow ribbons) at the top strand cleavage sites (red arrows). A short top strand segment (~3 nt) disanneals from each original helix, exchanges and becomes ligated to the partner helix. A Holliday junction intermediate, in which the top strands are sharply bent at the branch point (TC isomer), is generated. This species isomerizes to a conformation that resembles a BC isomer and, in the process, the substrate helices are unstacked and then restacked into an orientation that anticipates the product helices. Notice that in this structure the bottom strands (gray and blue ribbons) are now sharply bent at the branch point. λ Int now cleaves, exchanges and ligates the bottom strands at the sites indicated by blue arrows to generate a pair of product helices (*att*L and *att*R or *att*P and *att*B).

ation and reassociation of hydrogen bonds between homologous (matched) base pairs. It is possible to block this process by the formation of even a single mismatched base pair (Quartin *et al.*, 1989; Panyutin and Hsieh, 1993). A Holliday junction will consequently prefer to occupy regions of sequence homology, where full base pairing is permitted. It will refrain from entering regions of sequence heterology (where the DNA helices differ in sequence), since this would entail the formation of energetically unfavorable mismatched base pairs. This phenomenon has been widely exploited in the design of Holliday junction substrates with constrained mobility (de Massy *et al.*, 1989; Dixon and Sadowski, 1994; Kho and Landy, 1994; Arciszewska *et al.*, 1995; Lee and Jayaram, 1995; Nunes-Düby *et al.*, 1995). Bilateral blocks to branch migration can be imposed by placing heterologous base pairs around the desired region of branch mobility. When the branch point is properly contained, the 'barrier' base pairs are fully matched; if the branch point moves beyond either one of these barriers, two of the four heterologous base pairs will become mismatched, depending upon whether the branch moves towards the C or C′ site. As stated above, this is an energetically unfavorable situation that is rapidly reversed.

We had observed previously that immobile Holliday junctions with the branch point positioned 3 bp away from the top and bottom strand cleavage sites were exclusively resolved by λ Int at the top and bottom strands respectively. Monomobile Holliday junctions with access to both positions (3/4 and 4/5, Figure 2A and B) could be resolved in either direction (Nunes-Düby *et al.*, 1995). However, the latter often displayed a bias of resolution that was sequence dependent. Since our model suggests an isomerization step at the center of the overlap region, we hypothesized that the resolution bias was linked to a preferred isomerization state of a monomobile Holliday junction.

In order to study such a correlation, a set of monomobile Holliday junctions was constructed (see Materials and methods; Figure 2A) where the branch point was confined to the center of the 7 bp overlap region with access to positions 3/4 and 4/5 (Figure 2B). These substrates needed to meet two requirements. First, each Holliday junction must have a unique and demonstrable isomerization state; it should predominantly assume a top strands-crossed isomer (TC isomer) or a bottom strands-crossed isomer

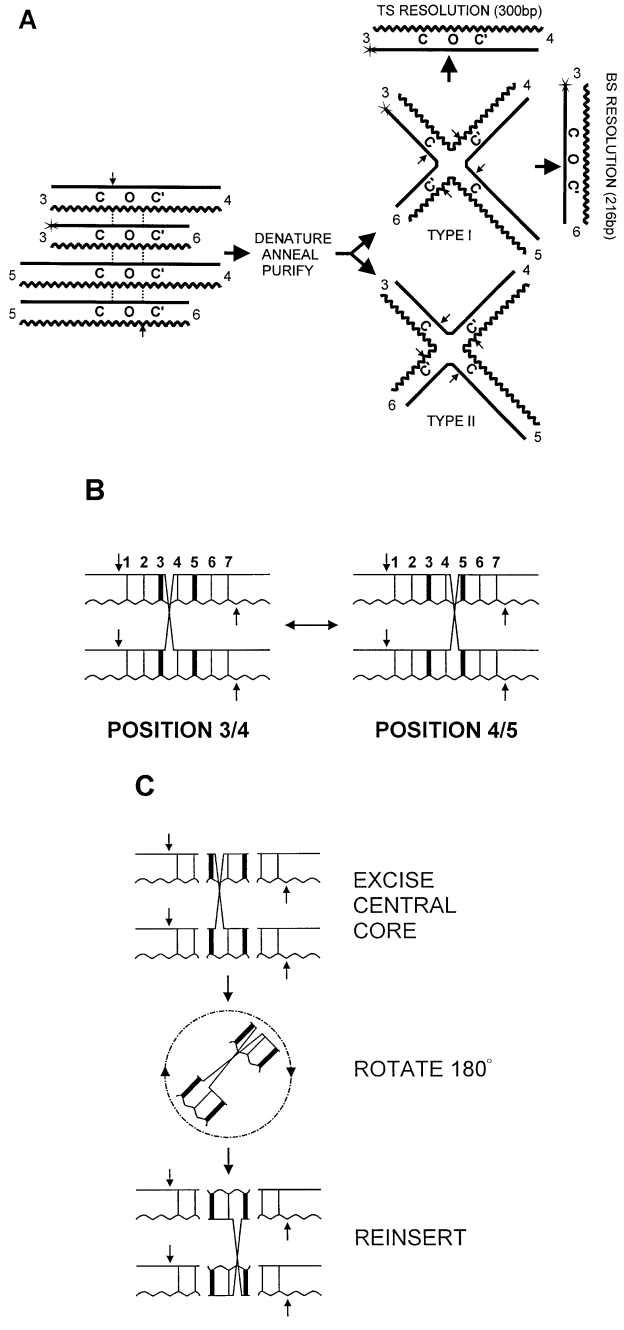

Fig. 2. (**A**) Protocol to generate radiolabeled Holliday junction substrates. The substrates are assembled from four DNA duplexes generated by PCR, each containing a different pair of heterologous arm sequences: 3–4, 300 bp; 3–6, 216 bp; 5–4, 483 bp; 5–6, 399 bp. They each contain C and C′ λ Int core binding sites that surround a 7 bp overlap region. The 3 arm of the 3–6 duplex was labeled by performing the PCR reaction with a 5′-^{32}P-end-labeled primer and a second unlabeled primer. These four duplexes are mixed in equimolar amounts, denatured and reannealed (see Materials and methods). Besides regenerating the original duplexes, two exchange forms of Holliday junctions are formed: types I and II. In this example only the type I Holliday junction is labeled. By convention, the 'top strands' are depicted by straight lines and the 'bottom strands' are depicted by wavy lines. The arrows indicate the positions of Int cleavage sites. Resolution at the top strands of the type I Holliday junction produces a 300 bp labeled duplex and resolution at the bottom strands produces a 216 bp labeled duplex. These products are fractionated by gel electrophoresis and are subsequently identified and analyzed by autoradiography (see Materials and methods). (**B**) Accessible branch point positions in the central mobility Holliday junctions. The base pairs in the overlap region are numbered from 1 to 7, starting immediately after the top strand cleavage sites and ending immediately before the bottom strand cleavage sites. The heterologous base pairs that impose the bilateral constraints to branch migration are depicted by bold slashes at positions 3 and 5. In the state shown to the left, the branch point sits at position 3/4 and in the state shown to the right, the branch point sits at position 4/5. Only the top strands-crossed isomer (TC isomer) is shown here; the bottom strands-crossed isomer has precisely the same junction mobility. (**C**) The logic used to construct central mobility Holliday junctions with opposite isomer preferences. Starting with a central mobility junction that has a unique isomer preference (TC isomer in this example) the central core of 6 bp is 'excised', rotated about 180° and replaced between the flanking arm sequences. This concept was not executed as diagramed, but was performed according to standard cloning procedures (see Materials and methods). It can be seen that the top strands have now become continuous with the bottom strands and vice versa. This construction will now prefer the BC isomer.

(BC isomer). Second, each Holliday junction should permit analysis of the resolution bias (i.e. the relative proportion of top strand to bottom strand resolution).

Our strategy to design Holliday junction substrates with opposite isomer preferences was based on the observation that the 4 bp that flank the branch point of an immobile Holliday junction are the exclusive determinants of which pair of strands are crossed and which pair of strands are continuous (Duckett *et al.*, 1988). Since the branch point of a monomobile junction has access to two positions, 6 bp (3 bp from each duplex) now influence the global structure. We reasoned that an existing central mobility junction with an arbitrary but unique isomer preference could be used to rationally generate another central mobility junction with the opposite isomer preference. This could be accomplished by changing the orientation of the central core of 6 bp relative to the four distal arms of the Holliday junction (Figure 2C). As shown below, this proved to be a successful approach. A central mobility Holliday junction, HJ 10, of unknown stacking preference was selected (Figure 3A). Assuming that this construct preferred a unique isomer, another Holliday junction was constructed, HJ 11 (Figure 3A), in which the orientation of the base pairs that circumscribe the two possible branch point positions was altered according to the concept described above (Figure 2C). The sequences of these two Holliday junctions are identical except for the central 3 bp of the overlap region.

Isomer preference of central mobility junctions

Gel electrophoretic methods have been extensively utilized to determine the structure of the Holliday junction (Cooper and Hagerman, 1987; Duckett *et al.*, 1988, 1990). The validity of these approaches as a tool to infer the structure of the four-way junction has been independently confirmed by fluorescence resonance energy transfer studies (Murchie *et al.*, 1989; Clegg *et al.*, 1992, 1994), chemical probing studies (Chen *et al.*, 1988; Churchill *et al.*, 1988; Lu *et al.*, 1989; Murchie *et al.*, 1990, 1991), a molecular modeling exercise (von Kitzing *et al.*, 1990) and other physical methods (Cooper and Hagerman, 1989). The experimental design of Duckett *et al.* (1988) employs immobile Holliday junctions with four arms of equal length (40 bp) that each have a unique restriction site located 12 bp from the branch point. All four arms are radioactively end-labeled, making it possible to identify the six permutations of doubly restricted Holliday junctions. These six species are electrophoresed through a high composition polyacrylamide gel in the presence or absence of divalent cations. The pattern of shifts that are observed reflect the disposition of the two long (unrestricted) arms in space. They determined that the rate of migration through the gel matrix is approximately proportional to the angle displayed by the two long arms. Since all of these species are the same size (i.e. they have the same number of base pairs), one is able to make qualitative comparisons between the relative mobilities of identical, or different, pairs of digests.

When permuted sets of immobile Holliday junctions that prefer opposite isomers are electrophoresed under ionic conditions that either promote complete unstacking (2 mM EDTA) or efficient stacking (1 mM Mg^{2+}) of the helical arms, characteristic patterns are obtained. Under the former condition the patterns of mobility shifts between the two sets are virtually identical, whereas in the latter condition the patterns of shifts between the two sets are dramatically different.

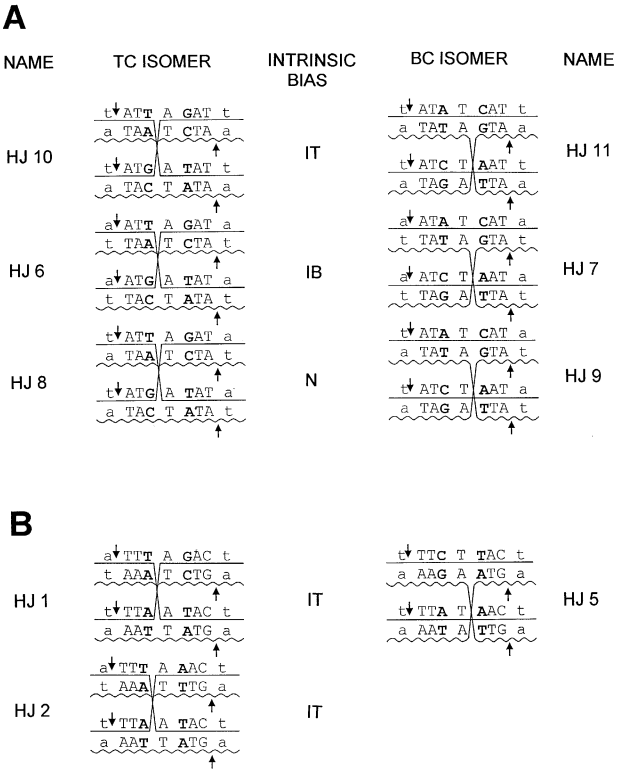

Fig. 3. Central mobility Holliday junctions. Holliday junctions are arranged into columns according to their isomer preference (TC or BC isomer), as determined by mobility shift assays, and are labeled according to their intrinsic bias [intrinsic top strand bias (IT), intrinsic bottom strand bias (IB) and no intrinsic bias (N)], as determined by resolution assays. The TC isomers are shown with their branch points at 3/4 and the BC isomers are shown with their branch points at 4/5, since these have been demonstrated to be the positions that favor top strand and bottom strand resolution respectively (Nunes-Düby *et al.*, 1995). However, in every case the branch point has access to both positions 3/4 and 4/5. Heterologous base pairs that delimit the range of branch migration are in bold type; base pairs that affect the intrinsic bias are lower case. Top and bottom strand Int cleavage sites are indicated with downward or upward arrows respectively. 'New' constructs (**A**) and 'old' constructs (**B**) are listed separately.

To demonstrate which isomer prevailed (i.e. which pairs of arms were stacked on each other), our Holliday junctions were subjected to a gel permutation analysis modified from the method described above (Figure 4). Since it was essential for our Holliday junction substrates to have four arms of different lengths, in order to identify top and bottom strand resolution products (see below), mobility comparisons were made only between identically digested TC and BC isomers. Unique restriction enzyme cleavage sites were engineered into arms 4, 5 and 6. These cleavage sites are located 18–22 bp away from the branch point on their respective arms. Radioactively labeling arm 3 and performing sets of double digests allowed us to identify three of the six possible permuted species and to unambiguously differentiate their respective isomeric forms.

To establish which isomer the two Holliday junctions (HJ 10 and HJ 11) adopted, they were doubly digested with either *Bam*HI and *Bgl*II, *Bam*HI and *Sal*I or *Bgl*II and *Sal*I. Pairs of undigested and digested Holliday junc-

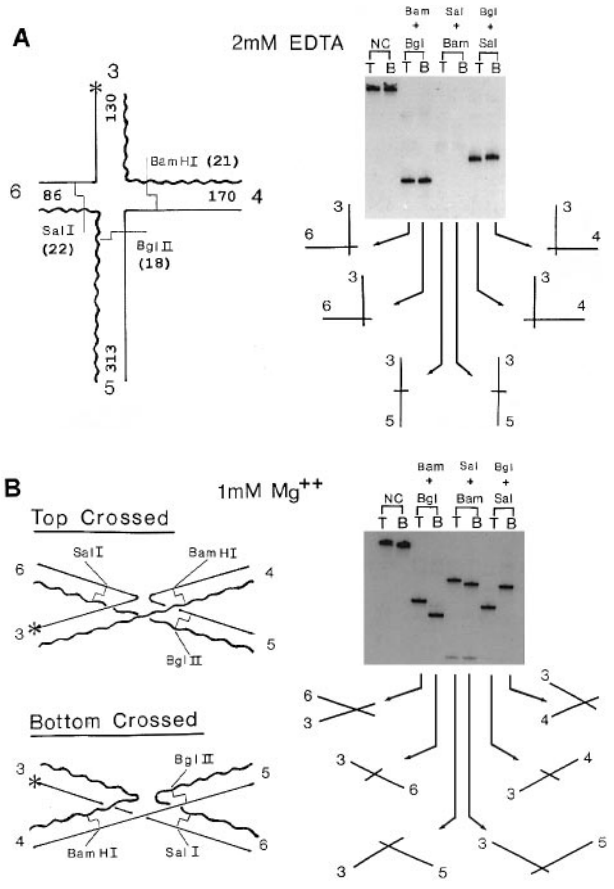

Fig. 4. Mobility shift assay to determine isomer preference. The Holliday junctions were uniquely labeled on the 3 arm with ^{32}P (indicated by an asterisk). They were doubly restricted with either *Bam*HI and *Bgl*II, *Bam*HI and *Sal*I or *Bgl*II and *Sal*I to generate modified junctions with two long arms and two short arms. Pairs of these digested and undigested Holliday junctions were electrophoresed (see Materials and methods) either in 2 mM EDTA (**A**) or in 1 mM Mg^{2+} (**B**). The conditions used in (A) have been shown to promote complete unstacking of Holliday junctions into an extended structure, in which the four arms point towards the corners of a square (Duckett *et al.*, 1990). The conditions used in (B) have been shown to promote complete stacking of Holliday junctions into one of two possible isomeric forms (Duckett *et al.*, 1990): in the top strands-crossed isomer (HJ 10) (T) the 3 and 4 arms and the 5 and 6 arms are stacked on each other; in the bottom strands-crossed isomer (HJ 11) (B) the 3 and 6 arms and the 5 and 4 arms are stacked on each other. The stacked Holliday junction representations are shown in side view, in which it can be seen that the helical axes are related by an ~60° angle (Duckett *et al.*, 1988). The predicted conformations of the migrating species are symbolized by stick figures under each lane. The lengths of the arms and the positions of the unique restriction enzyme cleavage sites are indicated in (A).

tions were electrophoresed in 4% polyacrylamide containing either 2 mM EDTA or 1 mM Mg^{2+} (Figure 4). In 2 mM EDTA the mobilities were very similar between identical double digests of HJ 10 and HJ 11 (Figure 4A), suggesting that the molecules had similar conformations in this ionic environment. The variation in mobility between different types of digests is due to the different arm lengths that remain after restriction enzyme treatment. When the Holliday junctions were electrophoresed through the gels containing 1 mM Mg^{2+}, the difference in mobilities between identical double digests of HJ 10 and HJ 11 was striking (Figure 4B).

*Bam*HI/*Bgl*II doubly-restricted HJ 10 had a retarded mobility compared with similarly digested HJ 11, as evidenced by an upward shift of the former and a downward shift of the latter species in the gel mobility shift assay. This suggests that 1 mM Mg^{2+} induced a (structural) transition that either facilitates or hampers migration through the polyacrylamide matrix respectively. The *Bgl*II/*Sal*I-restricted HJ 10 and HJ 11 constructs showed the opposite trend. In this case, 1 mM Mg^{2+} ionic conditions induced a transition that allows the former species to migrate more rapidly than the latter. *Bam*HI/*Sal*I-restricted HJ 10 and HJ 11 constructs displayed virtually identical shifts, suggesting that these molecules exhibit similar characteristics under this ionic condition. The slight discrepancy in mobility between these species might be due to a minor variation in conformation. It should be noted that the *Bam*HI/*Sal*I doubly restricted Holliday junctions (with two opposing short arms) are less stable in 2 mM EDTA, where they tend to fall apart during the course of the electrophoretic run, as compared with 1 mM Mg^{2+}. A small fraction of this species remains intact and can be seen on a darker exposure.

All of the shifts can be explained in terms of HJ 10 existing as a TC isomer and HJ 11 existing as a BC isomer. Based on the models of Holliday junction migration through high composition gels (Cooper and Hagerman, 1987; Duckett *et al.*, 1988), we interpret that the uncleaved long arms of *Bam*HI/*Bgl*II-restricted HJ 10 (arms 3 and 6) describe an acute angled species, whereas the remaining long arms of similarly restricted HJ 11 describe an extended species. The migration pattern of the *Bgl*II/*Sal*I doubly digested fragments is also consistent with the isomer prediction. This time we expect the faster moving HJ 10 digest to resemble a more linear, extended form and the retarded HJ 11 digest to resemble an acute angled conformation. The slight discrepancy in mobility between the uncleaved Holliday junctions is expected because of the slight pre-existing difference in the lengths of the arms. Indeed, this difference in mobility is consistent with the theory that the rate of migration is approximately proportional to the angle disposed between the two longest arms [in this case arms 4 (170 bp) and 5 (313 bp)], i.e. that the TC isomer will be retarded relative to the BC isomer, as observed.

Resolution bias of central mobility Holliday junctions

The Holliday junctions were designed with four arms of varying length so that placing a unique label on one of the arms (3 arm) would make it possible to distinguish between top strand and bottom strand resolution products, based on the relative sizes of the resulting radiolabeled duplexes (Figure 2A). Top strand resolution produces two linear duplexes: a 300 bp radiolabeled fragment and a 399 bp unlabeled fragment. Bottom strand resolution also produces two linear duplexes: a 216 bp radiolabeled fragment and a 483 bp unlabeled fragment. Following a resolution reaction, the DNA species can be separated by gel electrophoresis and subsequently identified and quantitated by phosphorimager analysis (see Materials and methods).

All of the experiments reported here have been carried out with λ C65 Int, a C-terminal fragment that comprises the catalytic domain. This cloned peptide carries out

topoisomerase function, forms a covalent complex with a suicide substrate and resolves Holliday junctions with the same efficiency as full-length λ Int (Pargellis *et al.*, 1988; Tirumalai *et al.*, 1996). In resolution reactions it has the advantage of giving linear reaction rates over a wider concentration of protein than intact λ Int and, in every instance tested, it gave identical results to intact λ Int (data not shown). Int (C65) resolution of the HJ 10 and HJ 11 Holliday junction constructs was assayed in 50 mM Na^+, which has been previously shown to induce partial stacking (Duckett *et al.*, 1990). HJ 11 displayed a weak top strand resolution bias (60–65%), whereas HJ 10 exhibited a 50% enhancement of this bias to give an almost exclusive top strand resolution (90–95%).

It is therefore feasible to manufacture central mobility Holliday junctions with opposite isomers and, in the process, to dramatically alter their relative resolution bias. However, even the Holliday junction with the lower top strand bias still favors top strand resolution. We shall now show that the difference in bias is a consequence of the two different isomeric forms and that the intrinsic top strand bias is due to sequence effects at the site of strand cleavage.

Intrinsic bias

Suspecting that the DNA sequence at the sites of Int cleavage might significantly affect the efficiency of the cleavage and/or ligation reactions, it was noted that the top and bottom strand cleavage sites have a different base (Figure 3A). Specifically, at the position where Int cleaves and forms a transient covalent 3′-phosphotyrosine linkage there is a thymine at the top strand cleavage sites and an adenine at the bottom strand sites, i.e. adjacent to the overlap region on the left and right respectively. If this difference is the source of the intrinsic bias, then switching the arrangement of these two positions (i.e. placing A at the top strand cleavage sites and T at the bottom strand cleavage sites) would create a pair of Holliday junctions with an intrinsic bias to resolve their bottom strands. Accordingly, a second pair of Holliday junctions were constructed (HJ 6 and HJ 7) that were identical to the previous set except for having the sequences at positions –1 and 8 reversed (Figure 3A). HJ 6 resolved with a slight top strand bias (60–65%) in 50 mM Na^+ and was shown to be a TC isomer in the gel permutation assay. HJ 7 resolved with a strong bottom strand bias (90–95%) and was shown to exist as a BC isomer (Figure 5). In other words, these two Holliday junctions show the same dramatic relative difference in resolution bias observed for HJ 10 and HJ 11, but now the intrinsic bias has been reversed to favor the bottom strands, as predicted.

If the successful reversal of the intrinsic bias was due to reversal of the base pairs flanking the overlap region, it should be possible to make these positions equivalent (i.e. placing a T 5′ to both the top and bottom strand cleavage sites) and thereby create a pair of central mobility Holliday junctions without any intrinsic bias (neutral). For this purpose, a third pair of Holliday junctions was created. They were identical to the previous two pairs except that both constructs had a T at both the top and bottom strand cleavage sites (Figure 3A). HJ 8 resolved with a strong top strand bias (90–95%) in 50 mM Na^+ (Figure 6A) and was a TC isomer in the gel permutation assay. HJ 9

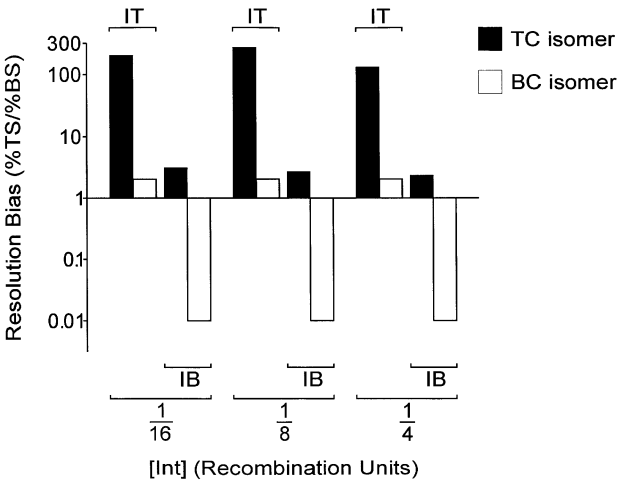

Fig. 5. Quantitation of resolution assays for HJ 10, HJ 11, HJ 6 and HJ 7. The quantitation of Holliday junction resolution by gel electrophoresis and phosphorimager analysis is described (see Materials and methods; see also Figure 6). The resolution bias is the ratio of the percentage of top strand resolution to the percentage of bottom strand resolution and is plotted on a logarithmic scale. The constructs that display an intrinsic bias for top strand resolution (IT) (HJ 10 and HJ 11) versus an intrinsic bias for bottom strand resolution (IB) (HJ 6 and HJ 7) are bracketed in pairs. The isomeric preferences of the Holliday junctions are indicated by black bars for a TC isomer (HJ 10 and HJ 6) and by white bars for a BC isomer (HJ 11 and HJ 7). The results for three different concentrations of Int are shown.

resolved with an almost equally strong bottom strand bias (80–85%) (Figure 6A) and was a BC isomer. Thus, with this set of Holliday junctions virtually all of the intrinsic biases had been eliminated (Figure 6B). This indicates that with each matching pair of isomers the strongly contrasting relative biases are a consequence of their alternative stacking preferences.

The experiments described above identified two factors that affect the resolution bias of these core-type Holliday junctions: the stacking preference and the sequence of the base pairs at the Int cleavage sites. To strengthen these conclusions the analyses were applied to three additional, unrelated central mobility Holliday junctions in our collection (Figure 3B). First, HJ 1 was selected and subjected to the gel permutation assay. The pattern of mobilities of the doubly restricted fragments in 2 mM EDTA and 1 mM Mg^{2+} indicated that HJ 1 was preferentially a TC isomer. The arrangement of base pairs at positions –1 and 8 were inspected to predict whether this construct might have a residual intrinsic bias. Based on the findings outlined above it was predicted that this Holliday junction should exhibit an intrinsic bias to resolve its top strands. Therefore, HJ 1 should resolve with a bias comparable with HJ 10. Consistent with these expectations, HJ 1 resolved with a strong top strand bias (90–95%) (data not shown). Next, HJ 2 was selected and subjected to the same treatment. The results of the gel permutation assay indicated that this construct was also predominantly a TC isomer and the sequences at positions –1 and 8 suggested that this construct would also have an intrinsic bias to resolve its top strands. As predicted, this Holliday junction resolved with a strong top strand bias (90–95%) (data not shown), comparable with HJ 10. Finally, HJ 5 was selected and was shown to be preferentially a BC isomer. The sequences at positions –1 and 8 indicated that this construct

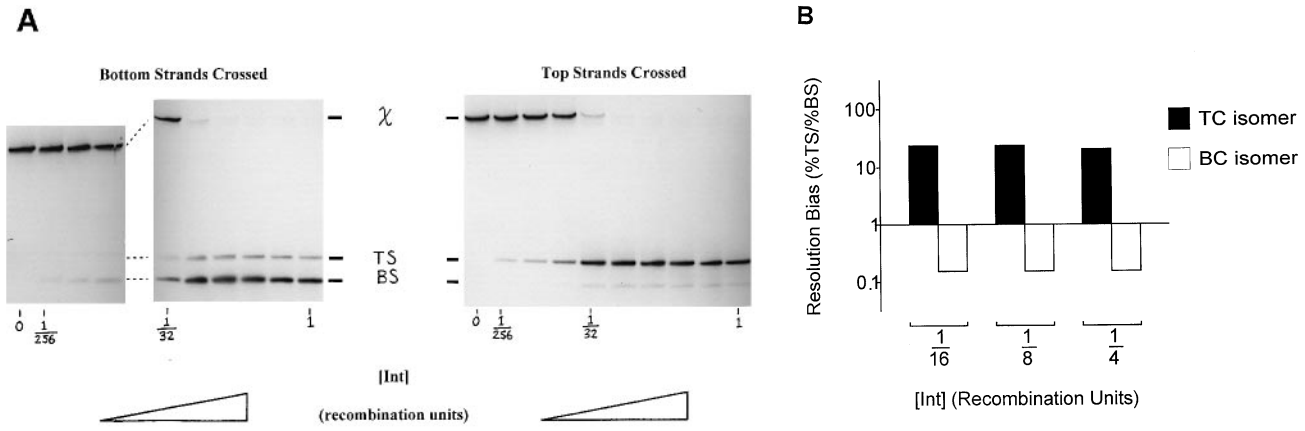

Fig. 6. Resolution of central mobility Holliday junctions with no intrinsic bias. (**A**) Labeled Holliday junctions were incubated with varying concentrations of Int (1/256–1 U) in resolution buffer and analyzed by gel electrophoresis (see Materials and methods). The resolution of HJ 9 and HJ 8 are shown in the left and right panels respectively. The positions of the Holliday junction (X), the 300 bp top strand resolution product (TS) and the 216 bp bottom strand resolution product (BS) are indicated. (**B**) The gels shown in (A) were quantitated by phosphorimager analysis (see Materials and methods). The resolution bias (plotted on a logarithmic scale) is the ratio of the percentage of top strand resolution to the percentage of bottom strand resolution for HJ 8 (black bars) and HJ 9 (white bars). A 4-fold range of Int concentrations is shown.

should have an intrinsic bias to resolve its top strands. Therefore, it should resolve with a bias comparable with HJ 11 and, consistent with this prediction, this Holliday junction resolved with a slight top strand bias (60–65%) (data not shown).

Holliday junctions preferentially maximize the number of purines in the crossed strands

We would now like to draw a correlation between the resolution bias and isomer preference of Holliday junction substrates that are not amenable to our gel permutation analysis, such as freely mobile junctions. If our model for strand exchange is correct (Nunes-Düby *et al.*, 1995), the complexity of the problem can be simplified to determining the isomer preferences of only a centrally located branch point; in particular, how it will stack at the positions where the molecule's top and bottom strands are resolved (i.e. at positions 3/4 and 4/5 respectively) (Figure 1B). To do this one needs a way to 'read' the base pairs that flank a branch point position and to anticipate how they might influence stacking. In searching for such a map, we decided to pursue an observation by von Kitzing *et al.* (1990) and Duckett *et al.* (1995). They noted that in three of the junction sequences they studied the preferred stacking isomers maximize the number of purine bases in the crossed strands. To determine whether this phenomenon was indicative of a general pattern, we constructed a collection of immobile junctions. These were designed to have an inequality of purines and pyrimidines at the branch point between crossed and continuous pairs of strands (this definition is irrespective of which pair of strands are crossed). The resulting collection was analyzed by the same gel permutation assay as outlined above and the stacking preference for each Holliday junction was determined (Figure 7).

These six Holliday junctions comprise six of the 18 theoretically possible immobile branch point sequences that create an inequality of purines and pyrimidines at the junction. We have determined that all of these constructs assume the isomer that maximizes the number of purines in the crossed strands. Furthermore, all of the monomobile junctions considered in this study showed an identical preference. According to a recently proposed nomenclature for branched DNA junctions (Altona, 1996) (for an explanation see Figure 7) the unique immobile junction branch point sequences we have considered are: class 2–5, TAAG; 2–7, TGAA; 4–1, CATG; 4–2, CGTA; 5–3, CTTA; 5–7, TTCA. It should be noted that 4–1, CATG, and 4–2, CGTA, correspond to J3 and J1 of Duckett *et al.* (1988) and J6 and J5 of Carpenter *et al.* (1996). We observed the same stacking preference with our gel permutation assay as in both of these studies. Although we have demonstrated a robust stacking pattern, we have considered only a minority of the theoretically possible immobile and monomobile branch point sequences and must make allowance for the existence of junctions that display the opposite preference.

Resolution of freely mobile Holliday junctions

All of the above experiments were, by necessity, carried out with constrained branch points. However, the ultimate goal is to be able to project these results onto the natural situation, where the freely mobile branch point has access to all 7 bp of the overlap region. Unfortunately, there is no simple way to predict the global conformation of a Holliday junction at specific branch point positions within a domain of unrestrained mobility. For example, it would be difficult to interpret the results of a gel permutation assay performed on one of these substrates. Any observed shifts would represent the combined contributions of a diverse population of molecules; i.e. their branch points would be at different positions and, perhaps, could even be changing during gel electrophoresis.

According to our model for Int-catalyzed strand exchange, Int will bind more favorably to Holliday junctions when their strands are crossed in the middle of the overlap region. In other words, even though the junction is freely mobile, Int will preferentially sample those Holliday junctions that transiently resemble a central mobility junction. Consequently, the base pairs at positions 3/4 and 4/5 should determine the relevant isomeric forms. Theoretically there are four possible freely mobile branch point sequences with an inequality of purines between the crossed and continuous pairs of strands. In order to permit

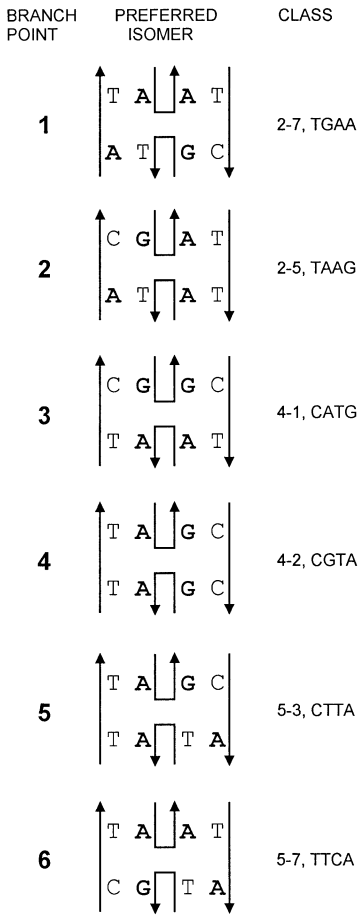

Fig. 7. Stacking preference of immobile branch point sequences. They are represented in their preferred isomeric form, as determined by gel permutation assays. The arrows indicate the 5′→3′ orientation of the DNA strands. The straight lines and the sharply bent lines depict the 'continuous' and 'crossed' strands respectively. Note that in every case the branch point sequence stimulates formation of the isomer that maximizes the number of purines (in bold type) in the crossed strands. The designation for each unique branch point sequence, according to the nomenclature proposed by Altona (1996), is indicated to the right (class number, sequence). To obtain the sequence code for each construction follow these simplified instructions: (i) represent the Holliday junction such that the 5′→3′ polarities of the strands follow a clockwise route; (ii) list the bases on the 5′-side of the branch point from each strand in a clockwise direction; (iii) write the string of residues from left to right.

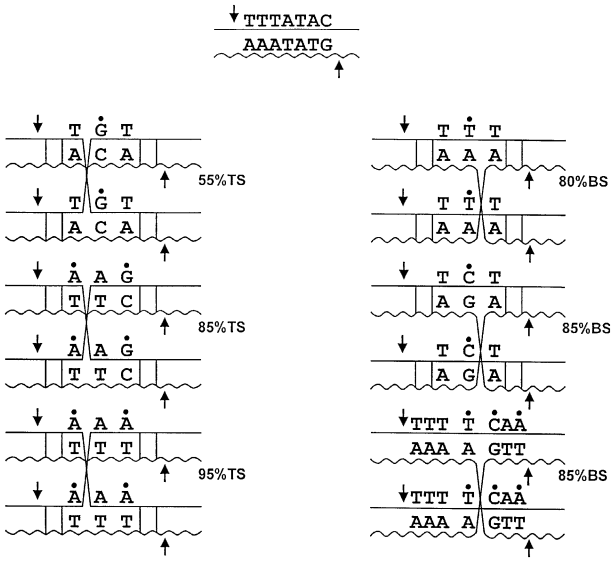

Fig. 8. Resolution bias of freely mobile Holliday junctions. Freely mobile junctions are represented in the isomer that maximizes the number of purines in the crossed strands at positions 3/4 or 4/5, according to the results reported here. The wild-type overlap sequence is shown at the top. Only the base pairs that are relevant to isomer determination (base pairs 3, 4 and 5) are displayed explicitly, except in the bottom right construction, which is the safG mutant Holliday junction (de Massy *et al.*, 1989). Positions that deviate from the wild-type overlap sequence are indicated with dots. Top and bottom strand Int cleavage sites are indicated by downward and upward arrows respectively. The percentages of top strand (TS) or bottom strand (BS) resolution are indicated next to their respective constructions.

free mobility these junctions necessarily have a disparity of four purines to zero purines between strands of opposite polarity. Consequently, with respect to the distribution of purines and pyrimidines at these junctions they would most closely resemble 4–1, CATG, and 4–2, CGTA. We expect these freely mobile branch point sequences to select a similar arrangement and to place their four purines on the crossed strands.

The wild-type freely mobile Holliday junction has a core sequence 5′-TTTATAC-3′. This means that if the branch point sits at either 3/4 or 4/5 there will be an equivalent number of purines in the top and bottom strands. Therefore, one cannot infer the preferred isomeric structure at these positions. However, if the central base pair of the core sequence is changed to either a T or a C, it can be seen that an imbalance of purines at both 3/4 and 4/5 is created (Figure 8). More precisely, there are four purines on the bottom strands and no purines on the top strands at both of these branch point positions. Based on the observed stacking preference, the Holliday junction should adopt an isomer that maximizes the number of purines in the crossed strands, namely a BC isomer. We have demonstrated that in monomobile junctions this conformation should favor resolution of the bottom strands and, indeed, both of these freely mobile junctions show a strong bias in this direction (80% bottom strand resolution for the 'central T' Holliday junction and 85% bottom strand resolution for the 'central C' Holliday junction) (Figure 8). A third freely mobile Holliday junction generated in another laboratory (de Massy *et al.*, 1989), which should be a BC isomer at 3/4 and 4/5 according to the reasoning outlined above, also gives a strong bottom strand resolution bias (85%) (Figure 8).

Conversely, a freely mobile Holliday junction with four purines in the top strands at 3/4 and 4/5 should be a TC isomer and should preferentially resolve its top strands. This was demonstrated with two different constructs (Figure 8), the 'central AAG' and the 'central AAA' Holliday junctions (80–85 and 95% top strand resolution respectively). The results obtained with these five freely mobile λ *att* Holliday junctions serve to validate the extrapolations made from experiments with constrained Holliday junctions concerning the mechanisms of strand exchange during normal recombination.

Discussion

This study has focused on the structure and resolution of Holliday junctions by λ Int. Since the Holliday junction

intermediate is a very transient species in the complete recombination reaction (Nunes-Düby *et al.*, 1987), it is difficult to observe individual strand exchanges or to characterize the global structure of the junction by starting with linear reactants. In a previous study we showed that Holliday junction branch point positions 3/4 and 4/5 of the 7 bp overlap region favored top strand and bottom strand resolution respectively (Nunes-Düby *et al.*, 1995). The present study was designed to investigate how λ Int would catalyze the strand exchange of a Holliday junction that was permitted access to both of these positions but was predisposed to a unique isomer. We have shown that λ Int preferentially resolves the top strands of a TC isomer and the bottom strands of a BC isomer. This means that λ Int is sensitive to the global structure of the Holliday junction and can distinguish between the two possible stacking isomers. It appears to favor cleavage and exchange of the strands that are crossed or sharply bent at the branch point of the Holliday junction.

Our data are consistent with a model of λ site-specific recombination that places the restacking of the helices between the top and bottom strand exchanges (Figure 1). Top strand resolution of a Holliday junction is a reversal of the first pair of strand exchanges, whereas bottom strand resolution constitutes a forward progress to products. Since a TC isomer strongly favors exchange of the top strands, then exchange of the top strands in the forward direction must favor creation of a structure similar to the TC isomer. Similarly, a BC isomer strongly favors exchange of the bottom strands. This suggests that between the step that immediately follows top strand exchange and the step that immediately precedes bottom strand exchange there must occur a rearrangement or restacking of the helices.

Another implication of these results is that the top and bottom strand exchanges are globally symmetrical. To observe this symmetry, compare a TC isomer with its branch point 3 bp away from the top strand cleavage site to a BC isomer with its branch point 3 bp away from the bottom strand cleavage site (Figure 1). In both of these molecules the orientation of the preferred cleavage sites with respect to the branch point position, helical phasing and λ Int binding sites are identical. This suggests that the geometry of the protein–DNA complex between the catalytic portion of λ Int and the Holliday junction may be identical for both strand exchanges. Although the execution of strand cleavage, exchange and ligation may be similar at the top and bottom strand sites, the directionality and chronology of the complete recombination reaction is likely to be influenced by accessory DNA bending factors, supercoiling and by interaction of the arm binding domain of λ Int with its cognate sites.

Although we have not studied the structure of the λ Int–Holliday junction complex, there is ample precedent for a protein-stimulated deformation of the fully stacked DNA junction. This deformation could manifest itself as a rotation of the helical arms, an unstacking of the junction and an unpairing of the base pairs at the branch point. Nevertheless, we believe that there must be stereochemical characteristics that distinguish a λ Int–Holliday junction complex that favors top strand resolution from a complex that favors bottom strand resolution, i.e. in the former state the Holliday junction would most closely resemble a TC isomer and in the latter state the Holliday junction would most closely resemble a BC isomer.

A number of studies have characterized the different ways in which Holliday junction binding/resolving enzymes perturb the structure of the four-way junction (Lilley, 1995). In studies on the RuvC–Holliday junction complex using DNase I footprinting, gel electrophoretic analysis and $KMnO_4$ modification it was shown that the Holliday junction is 2-fold symmetrical, partially unstacked and has several of the base pairs at the branch point pulled apart (Bennett and West, 1995). Although this structure differs from both the extended and stacked X-structures (Lilley and Clegg, 1993, 1994), there is still a distinction between continuous and crossed strands in the complex and the enzyme has a clear preference for nicking the continuous strands at the point of strand exchange (Bennett and West, 1996). A resolution-defective mutant of T4 endonuclease VII has also been shown to both select and alter the structure of an immobile Holliday junction (Pöhler *et al.*, 1996). The protein–DNA complex assumes one of two stereochemically equivalent forms, depending on the isomeric preference of the Holliday junction substrate (Pöhler *et al.*, 1996), and the cleavage-competent form of T4 endonuclease VII preferentially nicks the crossed strands of a four-way junction (Bhattacharyya *et al.*, 1991). Most recently, and most relevant to the λ Int experiments, is the accompanying paper by Arciszewska *et al.* (1997). They have probed the structure of the XerC/XerD–Holliday junction complex by chemical modification and found that the bound recombinases also induce a perturbation of the Holliday junction from a fully stacked form and also show isomer selectivity (see below).

It is apparent, and expected, that in addition to branch point location and isomeric state other parameters can also affect the direction of Int-catalyzed resolution. One of these has been identified in the present experiments and is termed ‘intrinsic bias’, because it is determined by the DNA sequence at the sites of Int cleavage. Specifically, at the position where λ Int cleaves and forms a transient covalent 3′-phosphotyrosine linkage (adjacent to the overlap region) a thymine is favored over an adenine (Figure 3). We do not know whether the bias in favor of thymine over adenine at this position is exerted at the level of Int cleavage and/or ligation. An effect on Int binding cannot be ruled out, but is less likely since this position has not previously been identified as contributing to the consensus Int recognition sequence (Ross and Landy, 1983). To neutralize the intrinsic bias of the cleavage/ligation reaction we have used Holliday junctions containing the same base (a thymine) at both the top and bottom strand cleavage sites (Figure 3). In this case, the resolution bias is almost entirely due to the isomeric state, giving ~90% top strand resolution for the TC isomer and ~90% bottom strand resolution for the BC isomer (Figure 6).

In a Holliday junction with an intrinsic bias in favor of top strand cleavage/ligation a substantial amount of top strand resolution is observed even in the BC isomer (Figure 5, IT). We consider two explanations. According to the first, this is due to cleavage of the continuous strands, as has been proposed to occur with reduced efficiency of XerC under conditions of reduced stringency (see also below; Arciszewska *et al.*, 1997). This unfavorable reaction would be due to the higher frequency of

cleavage at the thymine in the top strands compared with the adenine in the bottom strands. Occasionally the cleaved continuous strands would carry out an aberrant exchange and ligation. The second explanation, which we favor, is based on the view that the monomobile Holliday junctions are almost certainly not isomerically homogeneous. A small fraction of the population of molecules in the resolution mixture exist in the opposite isomer, and perhaps in other intermediate states. If there is an active equilibrium between these isomeric states, the less predominant species (a small amount of TC isomer in this example) may be recognized, trapped and resolved as rapidly as it appears. Over a period of time this could lead to a substantial accumulation of product material.

Another factor that has been shown to influence the bias of Holliday junction resolution is the higher order structure formed in a full recombination system involving accessory proteins and binding sites distant from the site of strand exchange (Franz and Landy, 1995). Those experiments incorporated complete or truncated arm-type sequences (P or P′), either singly or in pairs, to create Holliday junction substrates that more closely resemble the canonical integrative and excisive recombination intermediates. With these substrates the effect of accessory DNA bending proteins, IHF and Xis, on the resolution reaction was studied. Proteins binding to the P arm influenced the bias and proteins binding to the P′ arm affected the overall efficiency of resolution by full-length λ Int. Interestingly, the effect of Xis on the directionality of resolution was consistent with a role in promoting second strand exchange during excision. It was suggested that programmed delivery of the λ Int catalytic core binding domain to particular core sites leads to preferential resolution at those sites. For example, delivery to C and/or B would favor top strand resolution and delivery to C′ and/or B′ would favor bottom strand resolution. In light of our study, we can propose an alternative explanation that does not exclude these interpretations: higher order structure formation may lead to a deformation of the Holliday junction that favors one pair of strand exchanges over another. We might predict then that the enhancement of bottom strand exchange in the excisive intermediate, seen in the presence of Xis and IHF, may be due to stabilization of the BC isomer.

Based on the above results and the potential for energy input from DNA supercoiling, protein–DNA and protein–protein interactions we think it is unlikely that in full recombination reactions isomerization from the TC to the BC isomer would be driven solely by the DNA sequence at the branch point. It is possible, however, that branch point DNA sequences might influence the overall efficiency of recombination. If the arrangement of purines and pyrimidines at the branch point of a Holliday junction is an accurate predictor of preferred isomerization state, as it appears to be with our monomobile and immobile constructs, then a recombination overlap region composed of mostly purines in the top strands should favor formation of a TC isomer. In this case, progression to the BC isomer would be impeded, resulting in Holliday junction accumulation and/or reversal of the reaction to initial substrates. This may be the explanation for why Xer-mediated site-specific recombination at *cer* sites arrests after the first pair of strand exchanges, leading to an accumulation of Holliday junction-containing products (Colloms *et al.*, 1996). The *cer* overlap sequence contains a preponderance of purines in the top strands and, based upon the results reported here, would be expected to favor a TC isomer. If isomerization to a BC isomer is essential for resolution of the bottom strands, a possibility that the authors propose, then stabilization of the TC isomer might preclude this rearrangement. Interestingly, a Holliday junction containing the *cer*6 core sequence (also rich in top strand purines) resolves exclusively at its top strand cleavage sites (Arciszewska and Sherratt, 1995), suggesting that this molecule may prefer the TC isomeric state at the branch point positions that permit resolution.

The data presented here suggest that Holliday junction branch points have a robust preference for maximizing the number of purines in the crossed strands; all of the immobile and monomobile junctions considered in this study displayed this bias. However, we have considered only a minority of the theoretically possible immobile and monomobile branch point sequences and cannot discount the existence of junctions that show the opposite preference. But even if we catalogued the stacking preference of all the theoretically possible immobile and monomobile junctions (there are 38 that create an inequality of purines and pyrimidines), the sequence of a freely mobile branch point cannot be incorporated into this list (i.e. it cannot be inserted into an immobile or monomobile junction). Therefore, we have used the observed stacking preferences to test our predictions about the specific location (Nunes-Düby *et al.*, 1995) and global structure of the Holliday junction in the context of normal fully homologous overlap regions. Based on our previous finding that positions 3/4 and 4/5 of the overlap region are the positions where Int resolves the top and bottom strands respectively (Nunes-Düby *et al.*, 1995), five freely mobile *att* site Holliday junctions with an inequality of purines at both of these positions were examined (Figure 8). In each case the preference for resolution of the top versus the bottom strands conformed to the predictions from *att* site Holliday junctions with constrained branch points. We therefore suggest that experiments with constrained Holliday junctions afford useful insights about the mechanisms of strand exchange in the normal recombination pathway. Specifically, we suggest that in λ recombination: (i) the two pairs of strand exchanges are symmetrical but stereochemically distinct reactions that are executed entirely within the central portion of the overlap region (Figure 1); (ii) that λ Int preferentially catalyzes the formation and resolution of those strands most closely resembling the crossed strands of a particular Holliday junction isomer; (iii) that in a complete recombination reaction, a restacking of the helices must occur between the top and bottom strand exchanges.

Arciszewska *et al.* (1997) also investigated the structure of Int family recombination intermediates, but in a different system, XerC/XerD, and using a different experimental approach. In their work specific isomeric forms of the Holliday junctions were established by incorporating a nine thymine oligonucleotide tether far from the branch point. They found that at the most discriminating reaction temperature (20°C) both XerC and XerD resolve the crossed strands with >10-fold preference over the continuous strands. Furthermore, XerD cleavage of an unconstrained Holliday junction is undetectable (<0.2%) and is

stimulated >10-fold when the XerD substrate strands are constrained in the crossed conformation. Notwithstanding the more complex results obtained at a higher temperature (37°C), we believe that their experiments, which are based upon structural tethering at a distance, and our experiments, which depend upon micromanaging the sequence at the branch point, strongly complement and reinforce each other. Taken together, the two experiments suggest that these conclusions may be generally applicable to other members of the Int family.

Materials and methods

Plasmids

To generate immobile (IM), central mobility (CM) and freely mobile (FM) Holliday junctions, plasmid templates were constructed with 38 bp core-type cassettes comprising a 21 bp COC′ site embedded between unique restriction enzyme sites. The wild-type COC′ sequence is given by 5′-cagcttT↓TTTATAC↑Taagttg-3′. The arrows indicate λ Int cleavage sites and the lower case letters denote the C and C′ core binding sites at the 5′- and 3′-ends respectively. Capital letters denote the 7 bp core overlap region and the 2 bp immediately flanking it; these positions were changed by PCR site-directed mutagenesis, either singly or multiply. Mutated core-type cassettes were incorporated into four different plasmid vectors in between four different pairs of flanking DNA sequences, 3–4, 3–6, 5–4 and 5–6, according to standard techniques. The mutations were confirmed by sequencing the entire core-type cassette with the US Biochemical Sequenase Kit versions 1.0 and 2.0. Below are listed all the core-type sequences utilized in this study.

BF: 5′-T↓TTTATAC↑T-3′; BF21: T↓TTTTTAC↑T; BF22: T↓TTTCTAC↑T; JB1: A↓TTTATAC↑T; JB2: A↓TTAATAC↑T; JB3: A↓TCAATAC↑T; JB4: A↓CTAATAC↑T; JB5: A↓CTTATAC↑T; JB6: A↓TCTATAC↑T; JB7: A↓CCTATAC↑T; KHO3: T↓TTAATAC↑T; KHO5: T↓CTTATAC↑T; MA7: A↓TTTAGAC↑T; MA8: T↓TTAAGAC↑T; MA11: A↓TTTAAAC↑T; MA12: T↓TTAAAAC↑T; MA15: T↓TTTGTAC↑T; MA18: T↓TTCTTAC↑T; MA21: T↓TTATTAC↑T; MA22: T↓TTATAAC↑T; MA23: T↓TTCTAAC↑T; MA25: A↓ATTATAT↑A; MA26: A↓ATGAGAT↑A; MA27: A↓ATTAGAT↑A; MA28: A↓ATGATAT↑A; MA29: A↓ATATCAT↑A; MA30: A↓ATCTAAT↑A; MA31: A↓ATATAAT↑A; MA32: A↓ATCTCAT↑A; MA33: T↓ATTATAT↑A; MA34: T↓ATGAGAT↑A; MA35: T↓ATTAGAT↑A; MA36: T↓ATGATAT↑A; MA37: T↓ATATCAT↑A; MA38: T↓ATCTAAT↑A; MA39: T↓ATATAAT↑A; MA40: T↓ATCTCAT↑A; MA41: T↓ATTATAT↑T; MA42: T↓ATGAGAT↑T; MA43: T↓ATTAGAT↑T; MA44: T↓ATGATAT↑T; MA45: T↓ATATCAT↑T; MA46: T↓ATCTAAT↑T; MA47: T↓ATATAAT↑T; MA48: T↓ATCTCAT↑T.

Construction of Holliday junctions

The plasmids were used as templates to amplify the core-type cassette region and portions of the flanking sequences by PCR. To create a Holliday junction, four of these PCR duplexes (each one containing the desired core sequence and a different pair of flanking arm sequences, either 3 and 4, 3 and 6, 5 and 4 or 5 and 6) were gel purified, mixed at equimolar concentrations, denatured at pH 12 at room temperature for 5 min, neutralized and quickly transferred to a 90°C water bath. Annealing was allowed to proceed as the water bath slowly cooled to room temperature over an ~4 h duration. The annealed Holliday junctions were ethanol precipitated, resuspended in 10 mM Tris–HCl, 1 mM EDTA buffer and gel purified on a 1.5% polyacrylamide–1.1% agarose composite gel. The band corresponding to the Holliday junction was excised, cut into fine slivers with a scalpel and submerged in 200 µl 10 mM Tris–HCl buffer. The Holliday junction was allowed to elute passively into the buffer overnight. The annealed Holliday junctions consist of a mixture of two chemically distinct species referred to as exchange type I and exchange type II. To visualize selectively one of these species, a single primer used to generate one of the four substrate PCR duplexes was 5′-^{32}P-end-labeled. Below are listed all of the sets of plasmids used to generate each Holliday junction. For example, pMA7-34 is a plasmid with an MA7 core-type cassette sequence flanked by arm 3 and 4 sequences on the left and right respectively.

Central mobility (monomobile) Holliday junctions. HJ 1: pMA7-34, pJB1-36, pMA8-54, pKHO3-56. HJ 2: pMA11-34, pJB1-36, pMA12-54, pKHO3-56. HJ 5: pMA23-34, pMA18-36, pMA22-54, pMA21-56. HJ 6: pMA27-34, pMA25-36, pMA26-54, pMA28-56. HJ 7: pMA31-34, pMA29-36, pMA30-54, pMA32-56. HJ 8: pMA35-34, pMA33-36, pMA34-54, pMA36-56. HJ 9: pMA39-34, pMA37-36, pMA38-54, pMA40-56. HJ 10: pMA43-34, pMA41-36, pMA42-54, pMA44-56. HJ 11: pMA47-34, pMA45-36, pMA46-54, pMA48-56.

Immobile Holliday junctions. IM0: pJB5-34, pJB1-36, pKHO5-54, pBF506. IM1 (no. 1): pJB6-34, pJB1-36, pJB7-54, pJB5-56. IM1 (no. 2): pJB6-34, pJB2-36, pJB7-54, pJB4-56. IM2: pJB6-34, pJB3-36, pBF504, pKHO3-56.

Freely mobile Holliday junctions. FM (wild type): pBF304, pBF306, pBF504, pBF506. FM (central G): pMA15-34, pMA15-36, pMA15-54, pMA15-56. FM (central C): pBF22-34, pBF22-36, pBF22-54, pBF22-56. FM (central T): pBF21-34, pBF21-36, pBF21-54, pBF-56. FM (central AAG): pMA8-34, pMA8-36, pMA8-54, pMA8-56. FM (central AAA): pMA12-34, pMA12-36, pMA12-54, pMA12-56.

Resolution of Holliday junctions

Resolution reactions were carried out with 0.1 pmol radiolabeled Holliday junction DNA in a 20 µl mix of 50 mM Tris–HCl, pH 7.8, 50 mM NaCl, 10 mM DTT and 1 mg/ml bovine serum albumin. The reaction was initiated by addition of varying concentrations of λ C65 Int and was incubated at 25°C for 40 min. One recombination unit of λ C65 Int is defined as the minimum concentration needed to produce maximal recombination in a standard reaction. The reaction was stopped by the addition of 0.2% SDS. Resolution products were fractionated on a 1.5% polyacrylamide–1.1% agarose composite gel in 0.5× TBE buffer at 100 V for ~4 h. The gels were dried and exposed on a phosphorimager plate and also autoradiographed on Fuji X-ray film. The phosphorimager plate was scanned with a Fujix BAS 1000 Scanner and the image was visualized and quantitated with the Fuji MacBAS software package.

Gel permutation assays

Aliquots of 0.1 pmol radiolabeled Holliday junction DNA was incubated in 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT with either *Bam*HI and *Bgl*II, *Bam*HI and *Sal*I or *Bgl*II and *Sal*I in a 20 µl reaction mix for 2 h at 25°C. An aliquot of 10 µl each reaction was loaded onto a 4% polyacrylamide gel in a buffer system of 1× Tris–borate and 1 mM Mg^{2+}; the other 10 µl were supplemented with 20 mM EDTA and loaded onto a 4% polyacrylamide gel in a buffer system of 1× Tris–borate and 2 mM EDTA. Both gels were run at 120 V for 6 h at 4°C. The gels were dried and autoradiographed on Fuji X-ray film.

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