Direct Evidence for Holliday Junction Crossover Isomerization†

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ABSTRACT: The Holliday junction is a key intermediate in genetic recombination. This is a four-stranded branched DNA structure, whose double-helical arms are stacked in two domains; two of the strands are roughly helical, and the other two cross over between domains. Switching the strands between these two roles is known as crossover isomerization; this postulated reversal is thought to be one of the key transformations that the Holliday junction can undergo, because it can lead to changing the products from patch to splice recombinants. We present direct evidence that this reaction can indeed occur in Holliday junctions in solution. We have constructed a double-crossover molecule containing a branched junction, constrained not to be in its favored conformation. This junction is released from the double-crossover molecule by digestion with restriction endonucleases. We demonstrate by means of hydroxyl radical autofootprinting that the junction changes its crossover isomer spontaneously when released from the double crossover. We control for the possibility that the experimental protocol causes the isomerization. We also exclude dissociation and interaction with other molecules in solution as contributing to the phenomenon. Thus, crossover isomerization is an authentic spontaneous transformation of Holliday junctions.

Genetic recombination occurs universally in living organisms, from viruses to humans. A great deal of DNA metabolism is clearly devoted to ensuring the stability required for its role as genetic material, but recombination is a process that affords the flexibility necessary for the adaptation of species to a changing environment. The basic molecular feature of recombination is the interaction of two pieces of DNA to yield new genetic material that may physically incorporate segments of both interacting molecules. The resulting DNA may show such alterations as insertions, deletions, changes of sequence, rearrangements, or exchanges of flanking markers. The Holliday (1964) junction intermediate is a central paradigm in the molecular mechanism of this process. It has been shown to be an authentic intermediate in site specific recombination (Hoess *et al.*, 1987; Kitts & Nash, 1987; Nunes-Duby *et al.*, 1987), and it is believed to be involved in homologous recombination, as well (Kowalczykowski *et al.*, 1994).

Figure 1 illustrates the way in which the Holliday junction can participate in genetic recombination. At the first stage (**I**, in Figure 1), DNA strands containing homologous sequences, but different flanking markers, are aligned. The allelic flanking markers are A and α and B and β. The Holliday junction is a four-stranded intermediate (**II**) in which two strands have (effectively but not mechanistically) been nicked and religated together so that they fuse the sequences of two strands and form a branch point; the other two strands remain intact and in the equilibrium structure are not involved directly in branching (Lilley & Clegg, 1993; Seeman & Kallenbach, 1994). The strands forming the branch point are called the "crossover strands", and the other two strands are called the "helical strands". Homology permits the Holliday junction to undergo branch migration, an isomerization that relocates the branch point (**III**) [e.g., Hsieh and Panyutin (1995)]. In addition to branch migration, another isomerization appears possible, the crossover isomerization reaction, which is the focus of the work reported here. In crossover isomerization, the crossover strands and the helical strands switch functions (**IV**). Thus, the fused strands now take on the role of the helical strands, and the intact strands become the crossover strands. This is a key point in the Holliday paradigm. Resolution of the unisomerized Holliday junction (**III**) by a resolvase that cleaves the crossover strands (**v**), such as endonuclease VII (Mueller *et al.*, 1988), yields patch recombinants (**vi**) upon ligation; patch recombinants can lead to gene conversion. However, resolution of the isomerized Holliday junction (**IV**) by the same resolvase (**V**) produces splice recombinants (**VI**), equivalent to the exchange of flanking markers. Resolvases that cleave the helical strands, such as endonuclease I from T7 (Duckett *et al.*, 1988), would produce an opposite set of products: patch recombinants from **IV** and splice recombinants from **III**.

Thus, the ability of the Holliday junction to undergo crossover isomerization is at the heart of Holliday junction-mediated recombination. Nevertheless, this isomerization has remained a mechanistic postulate, because no direct evidence for the isomerization reaction has been presented yet. The presence of two crossover isomers in synthetic systems *in vitro* (Zhang & Seeman, 1994; Carlström & Chazin, 1996) can be ascribed to the formation of different isomers when strands are heated and cooled during the annealing process. Likewise, the presence of both possible resolvase products in *in vitro* reactions (Mueller *et al.*, 1990) could be a complicated function of the sequence specificity of the enzyme. Crossover isomers are the consequence of stacking dominance (Wang *et al.*, 1991), in which one pair of stacking isomers is preferred to another pair. Thus, the stacking shown in **III** would dominate if the free energy

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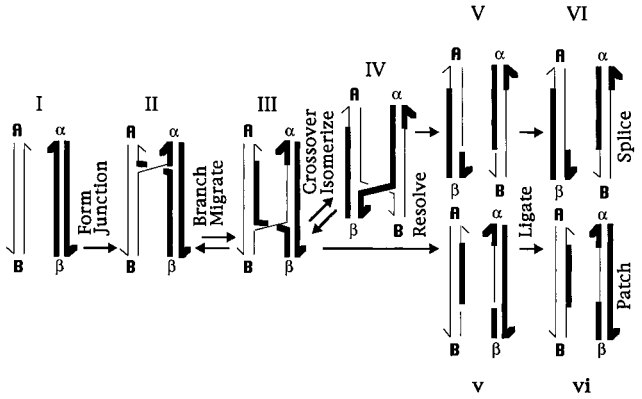

FIGURE 1: Formation and resolution of the Holliday structure in genetic recombination. The process is shown proceeding from the left to the right. Each of the possible stages is labeled with capital or lowercase Roman numerals. In the first stage, **I**, two homologous double helices of DNA align with each other. The two strands of each duplex are indicated by the two pairs of lines terminated by half-arrows, which indicate the 3′ ends of the strands. Strands are distinguished by their thickness. Each of these two homologous regions carries a flanking marker, A and B in the strands on the left and α and β on the right. After the first step, the homologous pairs have formed a Holliday intermediate, **II**, by exchanging strands. Note that the two crossover strands are composite strands with both a thick and a thin portion formed through any number of possibilities. The parallel representation of the Holliday junction is shown. The homologous 2-fold sequence symmetry of this structure permits it to undergo the iterative isomerization process, branch migration; movement in the direction indicated results in structure **III**. The Holliday intermediate may or may not undergo the crossover isomerization process to produce structure **IV**, in which the crossover and noncrossover strands are switched. Note that this process only has meaning if the Holliday intermediate is 2-fold symmetric, rather than 4-fold symmetric. Although indicated as separate, the crossover isomerization process could be a feature of branch migration (Mueller *et al.*, 1988). If crossover isomerization occurs an odd number of times, resolution by cleavage of the crossover strands yields structure **V**, but structure **v** results if crossover isomerization occurs an even number of times (including 0) before cleavage. Ligation of **v** generates a patch recombinant, **vi**; this is a pair of linear duplex DNA molecules containing heteroduplex DNA because of branch migration, but which have retained the same flanking markers. Ligation of **VI** yields splice recombinant molecules, which have exchanged flanking markers.

resulting from the stacking of arms A on B and α on β were lower than the free energy from stacking A on β and α on B. The free energy differences are low in molecules with homologous symmetry flanking the branch point (Zhang & Seeman, 1994).

Here, we present direct evidence that crossover isomerization can occur spontaneously in Holliday junctions. We demonstrate this fact by using the double-crossover system illustrated in Figure 2. There are five isomers of double crossovers (Fu & Seeman, 1993), three of which contain parallel helical domains and two of which contain antiparallel domains. The upper left diagram in Figure 2 illustrates a DAO molecule, a double-crossover molecule with antiparallel helical domains, containing an odd number of helical half-turns between the two crossover positions (Fu & Seeman, 1993); a DAO molecule showing the helical turns is sketched in Figure 3. Each of the two crossovers in Figure 2 is flanked by asymmetric sequences, so they are immobile junctions (Seeman, 1982), unable to undergo branch migration. It is well-known that the crossover isomer of an immobile junction is determined by the nucleotide pairs that flank it (Chen *et al.*, 1988). We have surrounded each of these branch points with four or more nucleotides corresponding to the well-characterized junction, J1 (Seeman & Kallenbach, 1983; Kallenbach *et al.*, 1983). Its preferred crossover isomer was determined in the late 1980s by hydroxyl radical autofootprinting (Churchill *et al.*, 1988) and reporter arm gel mobility (Cooper & Hagerman, 1987) experiments; more recently, its preferred crossover isomers have been confirmed by fluorescence measurements (Eis & Millar, 1993), by competition experiments (Zhang & Seeman, 1994), and by NMR spectroscopy (Carlström & Chazin, 1996).

The experiment that we have performed is illustrated in Figure 2. We have constructed a double-crossover molecule, called DX, in which one junction is in its preferred crossover isomer (labeled J1 in Figure 2) and the other junction is in its unfavorable crossover isomer (labeled Anti-J1 in Figure 2). Upon restriction, the junction in its unfavorable isomer is free to isomerize. The structural requirements of antiparallel double-crossover molecules are determined by the base pairing; these requirements outweigh any stacking preferences that determine preferred crossover isomers in single Holliday junctions (Fu & Seeman, 1993; Zhang *et al.*, 1993; Zhang & Seeman, 1994).

The lower junction in Figure 2 (called JB) is flanked by the J1 sequence, in its favorable crossover isomer, but the upper junction (called JT) is flanked by the J1 sequence in its unfavorable crossover isomer. Figure 2 shows that we have included (blunt-cutting) restriction sites at the center of the double-crossover molecule. Upon cleavage, both liberated halves of the molecule are free to isomerize to form their favored crossover isomer. The JB junction contains a biotin group so that both it and incompletely digested DX molecules can be removed by treatment with streptavidin beads. The crossover isomer of the remaining JT junction can be ascertained by hydroxyl radical autofootprinting analysis (Churchill *et al*., 1988). We demonstrate by means of this experiment that the crossover isomerization reaction can indeed occur spontaneously in Holliday junctions.

MATERIALS AND METHODS

Strand and Sequence Design. The double-crossover molecule, DX, shown in Figure 2, is composed of two junctions, JT and JB. JT is composed of four 16-mers, leading to eight nucleotide pairs per arm, and its sequence is identical to the well-characterized junction J1 (Seeman & Kallenbach, 1983; Kallenbach *et al.*, 1983) except near the restriction sites (Figure 2), where the sequence has been changed to accommodate them. JB is similar to J1 near the branch point, but its sequence has been changed in the region away from the branch point so that the strands would not be identical upon cleavage. The arms of JB farthest from the restriction sites have been lengthened to contain 16 nucleotide pairs per arm; in this way, the two molecules could be distinguished on nondenaturing gels, following cleavage. The two nucleotide pairs closest to its branch point are the same as in J1. However, the crossover isomers in a DAO molecule are determined by the base pairing, not the sequence preference (Fu & Seeman, 1993; Zhang *et al.*, 1993; Zhang & Seeman, 1994). Sequences were designed with the program SEQUIN (Seeman, 1990). The restriction endonucleases chosen were *Stu*I and *Pvu*II, which leave blunt ends; blunt ends minimize cohesion between the two product junctions. This is important, because the entire experiment

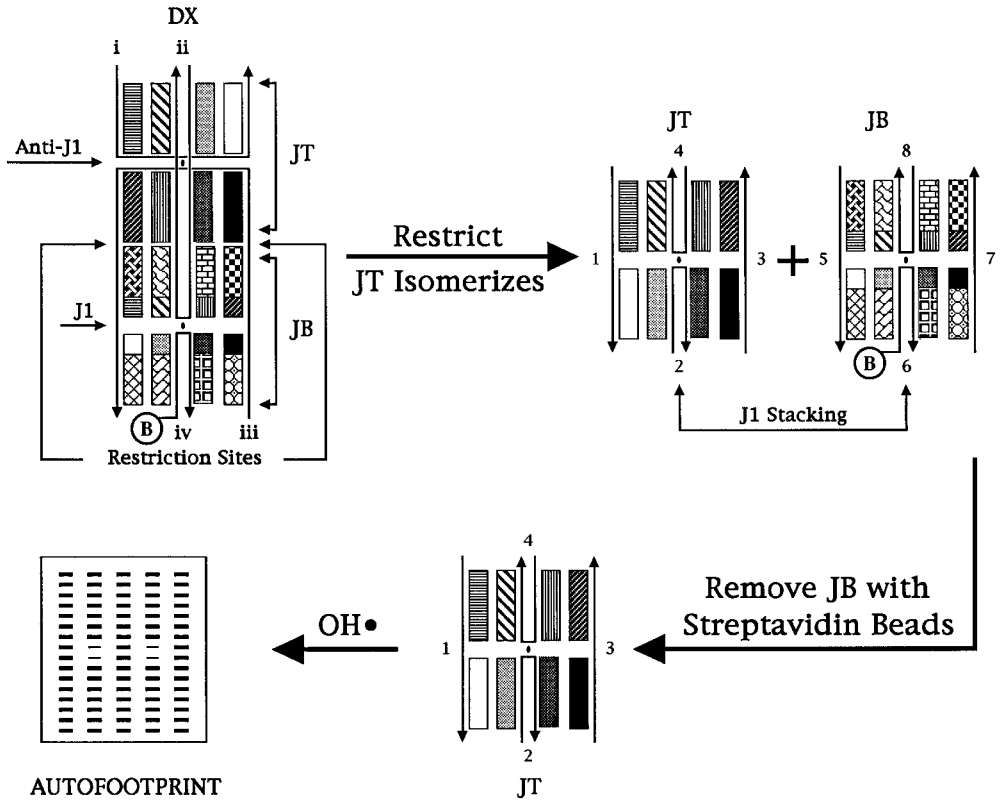

FIGURE 2: Experiment to demonstrate crossover isomerization. The initial molecule, DX, is shown in the upper left. Its four strands are labeled with lowercase Roman numerals, i−iv, and the 3′ ends of strands are indicated by arrowheads on the strands. The double-crossover molecule is the fusion of two single-crossover molecules, JT and JB. Although not indicated on the figure, strand iv and strand iii are longer than strand i and strand ii, so two arms of JB are longer than the arms of JT (see Figure 3). At the fusion point of JT and JB are two restriction sites (*Pvu*II and *Stu*I), whereby DX can be cleaved to form JT and JB; JB contains a biotinylated hexanucleotide attached to strand iv, indicated by a B in a circle, so that JB and incompletely restricted DX can be removed from solution, following restriction. The sequences of the molecule are shown schematically as filled rectangles with individual patterns. The junction JT is virtually the same as the well-characterized junction J1 (Seeman & Kallenbach, 1983; Kallenbach *et al.*, 1983). The junction-flanking region of JB is identical to the junction-flanking sequence of J1, as indicated by the same shading in that region. The JB junction is designed to assume the preferred J1 crossover isomer, but the JT junction is forced to assume the unfavorable J1 crossover isomer. After restriction, JT is free to isomerize, as indicated at the upper right portion of the drawing. Note that the strand numbers have been switched to Arabic numerals, which are used throughout the text to describe the strands of JT and JB, when they are treated as single junctions. The next step in the analysis is indicated at the lower right, where JB is removed from solution by treatment with streptavidin beads, leaving purified JT. The experiment is completed by hydroxyl radical autofootprinting analysis of JT, as indicated at the lower left.

is performed at 4 °C, to minimize the contributions of other processes, such as strand dissociation.

The strand names are shown in Figure 2, lowercase Roman numerals for the strands of DX and Arabic numerals for the strands of JT and JB; because they are not cleaved, strand i is identical to strand 1 and strand iv is identical to strand 6. The strand structure of the DAO molecule, the sequence of the DX molecule, and the nucleotide numbering are shown in Figure 3. The numbering of individual nucleotides in the DX molecule is retained in JT and JB for clarity; for example, the 16 nucleotides of strand 8 are numbered from 17 at the 5′ end to 32 at the 3′ end, corresponding to their positions in strand ii. An extra hexanucleotide with the sequence BTTBTT has been added, as indicated (see below), to the 5′ ends of strands i and ii or strand iv, where B stands for a biotinylated residue.

Synthesis and Purification of DNA. All DNA molecules used in this study were synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected using routine phosphoramidite procedures (Caruthers, 1985). Biotinylated "virtual nucleotide" phosphoramidites (Clontech) were included in the synthesis where appropriate. All strands were purified by polyacrylamide gel electrophoresis. When strand ii or iii required an internal radioactive label, the two components were synthesized separately; the 3′ portion was labeled at the 5′ end, and then the two strands were ligated to form an intact molecule.

Formation of Hydrogen-Bonded Complexes Complexes are produced by mixing a stoichiometric quantity of each strand, as estimated by OD_{260}. The complexes are formed by heating the samples to 90 °C and cooling slowly to 4 °C in NEBuffer 2 (New England Biolabs), which contains 50 mM NaCl, 10 mM Tris-HCl (pH 7.9 at 25 °C), 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Exact stoichiometry is determined, if necessary, by titrating pairs of strands designed to hydrogen bond together and visualizing them by nondenaturing gel electrophoresis; the absence of monomer is taken to indicate the end point.

Polyacrylamide Gel Electrophoresis. Denaturing Gels. These gels contain 8.3 M urea and are run at 55 °C. Gels contain 10−20% acrylamide (19:1 acrylamide:bisacrylamide). The running buffer consists of 89 mM Tris-borate (pH 8.3) and 2 mM EDTA (TBE). The sample buffer consists of 10 mM NaOH and 1 mM EDTA containing 0.1% xylene cyanol FF tracking dye. Gels are run on an IBI model STS 45 electrophoresis unit at 70 W (50 V/cm, constant power) or on a Hoefer SE 600 electrophoresis unit at 55 °C

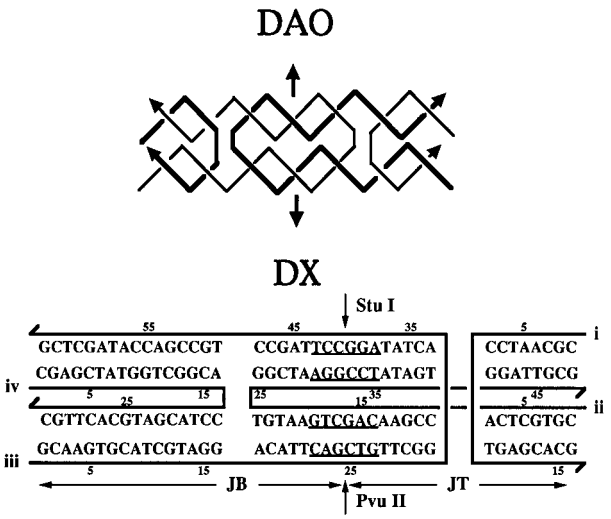

FIGURE 3: Structure and sequence of the DX molecule. At the top of the drawing is a DAO molecule, a double-crossover molecule, with antiparallel helix axes, and an odd number of double-helical half-turns between crossover points. The arrowheads indicate 3′ ends of the strands, and the arrows above and below the molecule indicate the axis of backbone symmetry in the central portion of the molecule; symmetrically related strands are drawn with lines of different thickness. The DAO molecule used here, DX, is composed of four strands, i−iv, as indicated in the lower panel; half-arrowheads indicate the 3′ ends of strands. The junctions are indicated in two different ways in this picture, in which the strands are "unwound". The crossover on the left, formed by strands iv and ii, is drawn as a normal junction, with the crossover strands on the inside and the helical strands on the outside. The crossover in the junction on the right is formed by strands i and iii, which pass from the top to the bottom of the drawing, whereas the helical strands are on the inside. The restriction sites for *Pvu*II and *Stu*I are underlined, and the cleavage sites are indicated by arrows. The nucleotide numbering shown is used through the paper, even when the junctions JT and JB are separated.

(31 V/cm, constant voltage). They are then dried onto Whatman 3MM paper and exposed to X-ray film for up to 15 h.

Nondenaturing Gels. These gels contain 6−14% acrylamide (19:1 acrylamide:bisacrylamide). DNA is suspended in 6−40 μL of NEBuffer 2 from New England Biolabs, containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM DTT, and 10 mM $MgCl_2$; the quantities loaded vary as noted below. The solution is boiled and allowed to cool slowly to 4 °C. One to four microliters of nondenaturing tracking dye containing 40 mM Tris (pH 7.5 at 25 °C), 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate (TAEMg), 50% glycerol, and bromophenol blue and xylene cyanol FF tracking dyes (0.02% each) are added to the samples prior to loading onto gels. Gels are run on a Hoefer SE-600 gel electrophoresis unit at 11 V/cm at 4 °C and exposed to X-ray film for up to 15 h or stained with Stainsall dye.

Enzymatic Reactions. (A) Kinase Labeling. DNA (1−13 μM) is phosphorylated in a solution containing 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, and 10 mM DTT and mixed with 5 μL of 2.2 μM [γ-^{32}P]ATP (10 mCi/mL) and 6 units of polynucleotide kinase (U.S. Biochemical) for 2 h at 37 °C. The reaction is stopped by heat inactivation, followed by gel purification.

(B) Ligations. Ten units of T4 polynucleotide ligase (U.S. Biochemical) in 30 μL of a buffer supplied by the manufacturer is added to 10 pmol of each strand, and the reaction is allowed to proceed at 16 °C for 10−16 h. The reaction is stopped by heat inactivation, followed by gel purification.

(C) Restriction Endonuclease Digestions. Restriction enzymes are purchased from New England Biolabs and used in buffers suggested by the supplier. The DX complex (100 nM) is digested at 4 °C for 16 h with 60 units of *Stu*I and 100 units of *Pvu*II in 40 μL of NEBuffer 2.

Streptavidin Bead Treatment. Streptavidin beads (50−100 μL) (Promega), stored at 4 °C in a solution containing phosphate-buffered saline (PBS), 1 mg/mL BSA, and 0.02% sodium azide, are put into a siliconized Eppendorf tube on a magnetic stand and allowed to settle for 5−10 min. The buffer is then removed, and the beads are rinsed three times with 50−100 μL of fresh PBS buffer. The beads are then rinsed twice with a solution containing NEBuffer 2. The biotinylated DNA, which has been restricted, is added to the beads, mixed well, and allowed to sit at 4 °C for 30 min. The solution is separated from beads, by allowing it to settle on the magnetic stand. Streptavidin particles are supplied as a 1 mg/mL suspension with a binding capacity of 0.75−1.25 nmol/mg. The amount used in this protocol is 5 times greater than that recommended by the manufacturer.

Hydroxyl Radical Analysis. Individual strands of the complexes are radioactively labeled and are additionally gel purified from a 10−20% denaturing polyacrylamide gel. Each of the labeled strands [approximately 1 pmol in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$] is derived from a complex, complexed with its linear duplex or junction-forming complement, left untreated as a control, or treated with sequencing reagents (Maxam & Gilbert, 1977) for a sizing ladder. Hydroxyl radical cleavage of the double-strand, junction, and double-crossover complex samples for all strands takes place at 4 °C for 2 min and 40 s (Tullius & Dombroski, 1985), with modifications noted by Churchill *et al.* (1988), but the final concentrations of L-ascorbic acid, Fe(II)EDTA^{2-}, and thiourea are 10 times higher (10 mM L-ascorbic acid, 1 mM Fe(II)EDTA^{2-}, and 10 mM thiourea). The reaction is stopped by addition of thiourea. The sample is dried, precipitated with ethanol, dissolved in a formamide/dye mixture, and loaded directly onto a 10−20% polyacrylamide/8.3 M urea sequencing gel. Autoradiograms are analyzed on a Bio-Rad model GS-525 molecular imager.

Strand Exchange Assay. Sample Preparation. The target complexes are formed as described. Radioactively labeled strand i is mixed with a stoichiometric amount of unlabeled strand i. This mixture is annealed to an equimolar amount of strands ii−iv, to form the double-crossover complex. The same procedure is used to construct the junction, called JTL (see below). Unlabeled versions of both complexes are also assembled in the same manner, except that no labeled strand i is used. The target complexes are isolated by excising the corresponding bands from 8% nondenaturing polyacrylamide gels containing TAEMg. The gel slices are eluted in NEBuffer 2 at 4 °C. The recovered samples are dissolved in NEBuffer 2 and quantitated using an SL 30 liquid scintillation counter. Two batches of each set of samples are prepared, one at a concentration of 1 μM and the other at 50 nM.

The strand exchange assay is performed by incubating a labeled and an unlabeled complex as described below. The complexes are mixed at 4 °C in NEBuffer 2. The final volume of each reaction is 10 μL. The incubation is performed both in the presence and in the absence of

restriction enzymes. For the assays containing restriction enzymes, 50 units of *Pvu*II (New England Biolabs) and 50 units of *Stu*I (New England Biolabs) are present in the incubation mixture. The incubation is carried out at 4 °C for 22 h. The species are quantitated with a Bio-Rad model GS-525 molecular imager.

Quantitation of Unlabeled Complexes. Radioactively labeled strand i is added to each of the isolated unlabeled complexes in separate vials. Each mixture is then heated and re-annealed either in the presence or in the absence of an equimolar amount of the three other strands of the complex. The same procedure is used with a set of radioactively labeled complexes that have been previously quantitated. Radioactive strand i is added to labeled complexes either in the presence or in the absence of an equimolar amount of the remaining three DNA molecules of the particular species. Each of the re-annealed samples is then electrophoresed on 8% nondenaturing polyacrylamide gels. The product distribution resulting from re-annealing the initially unlabeled complexes is compared with the corresponding labeled complexes for each assay condition. The species are quantitated with a Bio-Rad model GS-525 molecular imager.

RESULTS

Formation of the Double-Crossover Complex. The first step in this analysis is the demonstration that the double-crossover molecule, DX, and its component junctions, JT and JB, form unique, stable complexes. Figure 4a shows a nondenaturing gel that demonstrates this fact. Each of the three complete species forms a clear band on the gel, with neither dissociation nor multimerization evident. This result is to be expected for immobile four-arm junctions (Kallenbach *et al.*, 1983), and for antiparallel double-crossover molecules (Fu & Seeman, 1993). However, it is in contrast to the patterns seen for parallel double-crossover molecules (Fu & Seeman, 1993), mesojunctions, and antijunctions (Du *et al.*, 1992; Wang & Seeman, 1995), all of which multimerize; likewise, these results differ from the dissociation patterns seen for five-arm and six-arm junctions when the arms are too short (Wang *et al.*, 1991).

Restriction of Junctions and Purification by Streptavidin Beads. The key to this experiment is the restriction of the double-crossover molecule, DX, into the two individual junctions, JT and JB. All operations are performed at 4 °C, to minimize dissociation of hydrogen-bonded species. This is far from the thermal optima of the restriction enzymes, but longer incubation periods and high concentrations permit enough restriction to perform the experiment. Figure 4b shows the results of the restriction treatment on a nondenaturing gel. The left panel (lanes 1−5) contains individually prepared junctions, J1, JT, JB, and DX, along with double-stranded markers. The middle panel (lanes 6−9) shows the JB molecule after restriction and treatment with streptavidin beads; the different lanes correspond to different labeled strands. Strands i and ii were biotinylated in the DX molecule, from which these junctions were derived. Only the target digestion product (JB) remains, and it is seen to be intact. The rightmost panel contains the JT molecule, prepared by restriction of DX. Lanes 10−13 correspond to labels in each strand, following treatment with streptavidin beads; strand iv was biotinylated in this case. Again, only the target digestion product (JT) remains, and it, too, is undissociated. For comparison, lane 14 shows digestion that is not followed by streptavidin treatment can lead to residual DX in solution. Thus, the data in this gel support the notion that it is possible to restrict the DX molecule and to purify away unrestricted and partially restricted material by streptavidin bead treatment, while leaving the junction of interest intact.

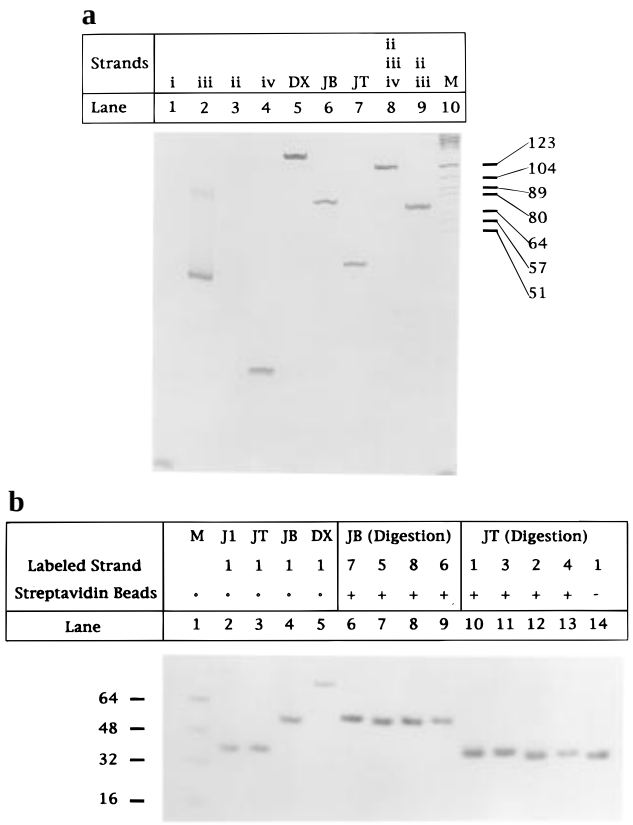

FIGURE 4: Nondenaturing gels showing the molecules used in this work. (a) The components of DX. This is a 14% nondenaturing gel. Lanes 1−4 contain the individual component strands of DX, i−iv, respectively. Strand iv has been biotinylated (see Materials and Methods) in all lanes here. Lanes 8 and 9 contain partial complexes, and lane 10 contains a group of double-helical markers, whose lengths are indicated on the right; the blotch at the bottom of lane 10 is due to running dye, not DNA. Lanes 5−7 contain DX, JB, and JT, respectively. Although the individual strands (lane 2) or partial complexes (lane 8) contain multiple bands, the junctions are all clean, intact bands. DX runs very slowly here, because high acrylamide concentrations were used to display all species on a single gel. (b) The digestion products of DX. This is an autoradiogram of a 6% nondenaturing gel. Lane 1 contains a group of double-helical markers, whose lengths are indicated on the left. In addition, the left panel of the gel contains the junctions used in this study, J1, JT, JB, and DX, respectively. The ° above each of these lanes indicates that (except for J1) they contain biotinylated complexes, but because they are unrestricted, there was no need to purify them by the streptavidin bead treatment. Whereas JT is the same size and contains virtually the same sequence as J1, it is not surprising that their mobilities are similar. The middle panel illustrates the products of DX restriction for producing JB, labeled in each of the strands of the molecule. The molecules have been purified by streptavidin beads; strands i and ii are biotinylated. The panel on the right shows the same experiment with JT, strand iv having been biotinylated. Lane 14 contains JT, labeled in strand 1, which has not been treated with streptavidin beads (indicated by the − above the lane). A faint band with the mobility of DX is visible in this lane, on the original of the gel.

Hydroxyl Radical Analysis. We have used hydroxyl radical autofootprinting previously to characterize unusual

DNA molecules, including branched junctions (Churchill *et al.*, 1988; Chen *et al.*, 1988; Wang *et al.*, 1991), antijunctions and mesojunctions (Du *et al.*, 1992; Wang & Seeman, 1995), and double crossovers (Fu & Seeman, 1993; Zhang *et al.*, 1993; Zhang & Seeman, 1994; Fu *et al.*, 1994a). These experiments are performed by labeling a component strand of the complex and exposing it to hydroxyl radicals. The key feature noted in these analyses is the decreased susceptibility to attack when comparing the pattern of the strand as part of the complex, relative to the pattern derived from linear duplex DNA. Decreased susceptibility is interpreted to suggest that access to the hydroxyl radical may be limited by steric factors at the sites where it is detected. Likewise, similarity to the duplex pattern at points of potential flexure is assumed to indicate that the strand has adopted a helical structure in the complex, whether it is required by the secondary structure or not. In previous studies of junctions, double crossovers, and mesojunctions, protection has been seen particularly at the crossover sites, but also at noncrossover sites where strands from the two domains appear to occlude each other's surfaces from access by hydroxyl radicals (Churchill *et al.*, 1988; Fu & Seeman, 1993; Fu *et al.*, 1994a; Du *et al.*, 1992; Wang & Seeman, 1995). Hence, protection is a reliable indicator of the crossover isomer; strands seen to be protected relative to duplex are the crossover strands of the junction.

We have performed hydroxyl radical autofootprinting experiments on each of the strands of the junction JT (shown in the top panel of Figure 5) in four different complexes: [1] complexed with its Watson−Crick complement ("DS" in Figure 5), [2] complexed with the other strands of the free JT junction ("JT Junction" in Figure 5), [3] in the DX complex ("DX" in Figure 5), and [4] in the liberated JT junction derived by digesting the DX complex with *Pvu*II and *Stu*I ("JT Digestion" in Figure 5). Comparison of the free JT junction with DS reveals protection on strands 2 and 4, and none on strands 1 and 3, suggesting that in the favored crossover isomer for this junction strands 1 and 3 assume the role of helical strands and strands 2 and 4 cross over between helical domains. By contrast, the opposite crossover isomer is seen in the same residues of the JT junction when it is constrained to be part of the DX double crossover. Here strands 1 and 3 show protection at the junction, and strands 2 and 4 are unprotected. Nevertheless, the data show that the liberated JT junction reverts to its original crossover isomer. Thus, the data present direct evidence that the Holliday junction can undergo crossover isomerization.

Is it possible that the protocol we have devised to demonstrate crossover isomerization is itself the causative factor in the isomerization? In order to control for this possibility, we have performed the same experiment with the other junction in the DX molecule, JB; JB is designed to be in its favored crossover isomer when it is part of the DX molecule. For this purpose, we have attached biotin molecules to strands i and ii, performed the same operations, under the same conditions, and analyzed the JB section liberated from DX, rather than the JT section. Strands 5−8, respectively, have the same junction-flanking sequences in JB as strands 1−4 in JT (see Figures 2 and 3). The results of these experiments are shown in the bottom panel of Figure 5. In each of the three autofootprints of the strands of JB, strands 5 and 7 are the helical strands and strands 6 and 8 are the crossover strands. Thus, the experiment itself does not cause the crossover isomerization seen in JT to occur in JB.

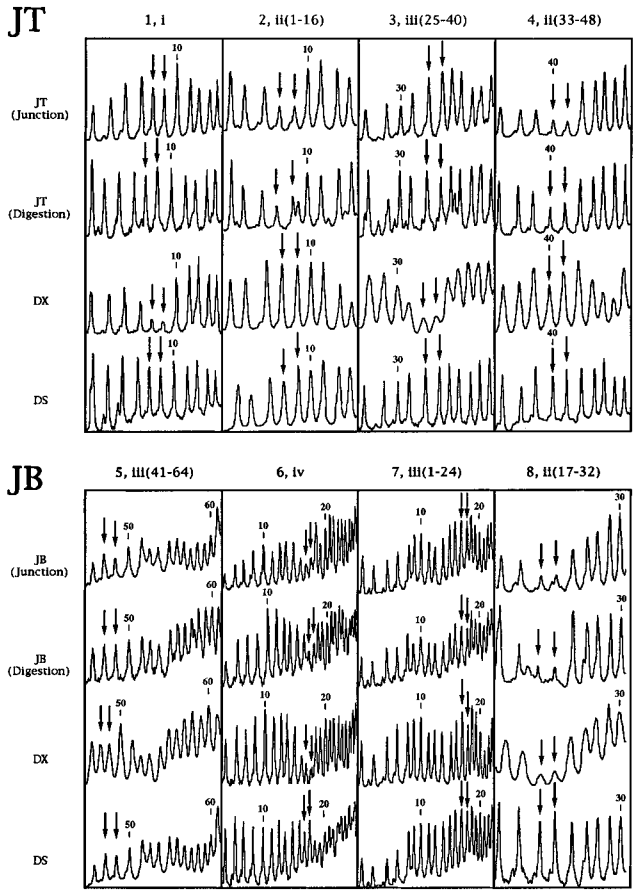

FIGURE 5: Hydroxyl radical autofootprinting of the species in this experiment. Each panel contains scans of autoradiograms quantitated by a phosphorimager; they illustrate the cleavage pattern due to attack by hydroxyl radicals on a particular strand in a particular strand environment. (top) JT and the relevant portions of DX. The synonyms for the strands are shown at the top of each panel. Strand 1 corresponds to strand i, strand 2 to nucleotides 1−16 of strand ii, strand 3 to nucleotides 25−40 of strand iii, and strand 4 to nucleotides 33−48 of strand ii; see the numbering scheme in Figure 3. These numbers are indicated on the patterns for the orientation of the reader. The arrows pointing down indicate the nucleotides that flank branch points, either on crossover or on helical strands. The four rows correspond to four different environments for the strand. "JT (Junction)" is the isolated JT junction, "JT (Digestion)" the JT junction derived by digesting DX, "DX" the double crossover, and "DS" the strand complexed with its Watson−Crick complement to form a linear double-helical molecule. It is clear from the strong protection seen that the crossover in JT occurs *via* strands 1 and 3 when it is contained within DX but that it reverts to strands 2 and 4 when it is freed from those constraints. (bottom) JB and the relevant portions of DX. The same conventions apply as in the top panel. Strand 5 corresponds to nucleotides 41−64 of strand iii, and strands 6 and iv are identical; strand 7 corresponds to nucleotides 1−24 of strand iii, and strand 8 corresponds to nucleotides 17−32 of strand ii. In contrast to JT, there is no change of the protection pattern when JB is constrained to be part of DX and when it is free.

Strand Exchange Analysis. We are trying to establish that a single branched DNA molecule containing long arms within a cell undergoes the crossover isomerization reaction. The experiments available to do this entail using $10^{12}-10^{14}$ short-armed molecules in a single reaction vessel. How do we know that the molecules are not themselves dissociating or exchanging and that these processes do not provide intermediates for the isomerization that would not be

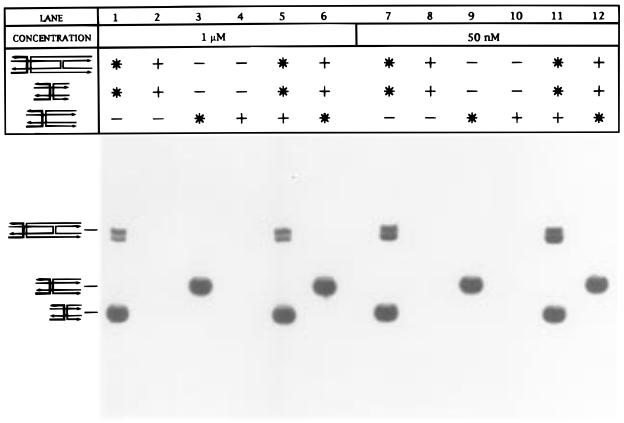

FIGURE 6: Determination of the extent of strand exchange during the experiment. This is an autoradiogram of an 8% nondenaturing gel. The two portions of this gel indicate experiments at two different strand concentrations, 1 μM (lanes 1−6) and 50 nM (lanes 7−12). Fifty units of *PvuII* and *StuI* are also present in each preparation. The three species present are indicated on the left of the box at the top: DX, JT, and JTL, respectively, from the top to the bottom. The individual species are indicated on the left of the gel, at positions corresponding to their mobilities. Strand i (identical to strand 1) is the common strand in each of these species; if the species is labeled, it is labeled in strand i, which has been drawn slightly darker than the other strands. The reader should be aware that no JT has been added to the gel; its presence is derived from the digestion of DX by the two restriction enzymes. The explanation in the box is as follows. A species that is labeled is indicated by an asterisk; a species that is present is labeled by a plus sign, and a species that is absent is indicated by a minus sign. Lanes 2, 4, 8, and 10 were run to establish background levels. The doublets at the DX positions in lanes 1, 5, 7, and 11 result from partial digestion of the double-crossover molecule. The key lanes are 5, 6, 11, and 12, where exchange would be visible if it had occurred. Insofar as we can establish, it is undetectable.

available in the cell? It is not possible to exclude this possibility entirely, but we have sought evidence for transient dissociation under our experimental conditions by monitoring strand exchange in the following experiment. Two types of complexes were constructed. One was the double-crossover molecule (DX), and the other was a traditional four-arm junction (JTL). Two opposite strands of JTL are strands i and iv of DX, and the other two strands complement them in the sense that the set of four strands forms a four-arm junction. JTL was designed to share at least one strand with DX, yet be larger than JT, so that the two could be distinguished on nondenaturing polyacrylamide gels by their distinct electrophoretic mobilities.

Strand i was radioactively labeled in one complex, but not in the other, and then the two complexes were incubated together under our experimental conditions. The extent of strand exchange would be reflected by the amount of strand i that migrated from the originally labeled complex to the unlabeled complex, after incubating at 4 °C for 22 h, the time period of the experiments reported above. The assay was performed at DNA strand concentrations of both 50 nM and 1 μM in four combinations: (1) labeled DX with unlabeled JTL at 1 μM, (2) labeled JTL and unlabeled DX at 1 μM, (3) labeled DX and unlabeled JTL at 50 nM, and (4) labeled JTL and unlabeled DX at 50 nM. Experiments were performed both in the presence and in the absence of the restriction enzymes, *Pvu*II and *Stu*I.

The results of the four experiments performed in the presence of the restriction enzymes are shown in Figure 6. Lane 1 contains labeled DX, and lane 3 contains labeled JTL at 1 μM strand concentrations. Lanes 2 and 4 contain unlabeled controls used to establish a baseline for the phosphorimager. Lane 5 contains labeled DX, but unlabeled JTL, and lane 6 contains labeled JTL, but unlabeled DX. No exchange is seen in either lane. The short junction present, JT, is derived exclusively from the restriction of DX by *Pvu*II and *Stu*I; it is not added separately to the gel. Lanes 7−12 contain, respectively, the same components as lanes 1−6, but now at 50 nM strand concentrations. No exchange is seen in lanes 11 or 12. The two concentrations shown on this gel bracket the 100 nM strand concentrations used in the isomerization experiments. The results of the four experiments performed in the absence of the restriction enzymes show no exchange either (data not shown). Thus, we exclude the contribution of exchange and dissociation reactions to the crossover isomerization that we have observed.

DISCUSSION

We have demonstrated unambiguously that it is possible for a Holliday junction analog to undergo crossover isomerization at 4 °C, in our experimental system. We have shown this by demonstrating that the JT junction is capable of undergoing this transformation spontaneously. We have excluded the experimental protocol from contributing to crossover isomerization by observing that the JB junction does not crossover isomerize under the same conditions where JT does isomerize. Furthermore, we have excluded the participation of strand exchange and dissociation in this process. We feel that the elimination of strand exchange excludes both mechanisms involving strand dissociation and mechanisms in which molecules collide and then undergo branch migration. It is important to point out that it was necessary to eliminate ethidium from strand purification protocols in order to obtain the clean results depicted in Figure 6; otherwise, as much as 4% strand exchange could be observed.

We have biased this experiment intentionally by choosing a branched junction whose crossover isomer was known (Churchill *et al.*, 1988) and for which the bias was known to be significant (Zhang & Seeman, 1994; Carlström & Chazin, 1996). Holliday junctions in which significant amounts of symmetry flank the branch point are likely to have lower biases (Zhang & Seeman, 1994). Nevertheless, there is no reason to believe that the lack of sequence symmetry in this experiment decreases the value of the evidence we have presented. The backbone structures involved in immobile junctions are expected to be similar to those seen in symmetric Holliday junctions (Lilley & Clegg, 1993; Seeman & Kallenbach, 1994); therefore, if a crossover bias is present, the junction is capable of undergoing the isomerization. However, it is important to emphasize that the ability of a single Holliday junction to crossover isomerize in no way suggests that a double-crossover molecule can also undergo this isomerization.

We have pointed out previously that, if crossover isomerization occurs in parallel double-crossover molecules, it would appear to require the assistance of topoisomerases (Fu & Seeman, 1993). Likewise, the Sigal-Alberts (1972) mechanism of crossover isomerization in Holliday junctions constrained to adopt a parallel conformation leads to braiding of the crossover (Sobell, 1974), which is apparently not a

favorable structure (Fu *et al.*, 1994b). The braiding problem also could be solved by strand passage catalyzed by topoisomerases. In contrast to these systems, the conformations of the free junctions in the experiments above are likely to be antiparallel (Lilley & Clegg, 1993; Seeman & Kallenbach, 1994), so there are no topological impediments to their crossover isomerizations.

It is useful to compare crossover isomerization with branch migration, another spontaneous transformation of the Holliday junction. Within the context of a given resolvase, both of these isomerizations can change the ultimate products of recombination. It is well-known that branch migration can also be catalyzed by proteins, such as RuvAB (Tsaneva *et al.*, 1992) or RecG (Lloyd & Sharples, 1993), that utilize an energy source. It is apparently to the cell's advantage to catalyze branch migration, most probably to overcome the energy barriers arising from the lack of homology (Panyutin & Hsieh, 1993). Likewise, it may be to the cell's advantage to catalyze crossover isomerization, in order to equalize the small biases (Zhang & Seeman, 1994) between crossover isomers. However, such activities are unknown, and the enzymatic catalysis of crossover isomerization in Holliday junctions remains an open question.

In order for single-Holliday junction-mediated recombination to yield the splice recombinants illustrated in Figure 1, the original helical strands must be cleaved. This can happen in two different ways. (1) Resolution cleavage can occur on the helical strands in the absence of crossover isomerization. (2) Resolution cleavage can occur on the original helical strands that have become the crossover strands by means of crossover isomerization. A 4-fold symmetric Holliday junction, containing structurally equivalent strands, as suggested by the RuvA crystal structure (Rafferty *et al.*, 1996), falls under the first pathway. The work presented provides direct evidence for the fact that the second pathway cannot be excluded arbitrarily from recombination mechanisms.

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