

Construction and Analysis of Parallel and Antiparallel Holliday Junctions*

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The Holliday junction is a four-stranded DNA intermediate that arises during recombination reactions. We have designed and constructed a set of Holliday junction analogs that model each of the ideal conformations available to a 2-fold symmetric four-arm junction. The strategy used is to connect two arms of a junction molecule with a short tether of thymidines. These DNA molecules share a common core sequence but have different arms that are connected so that each molecule is constrained in either an antiparallel or a parallel structure. For tethered antiparallel molecules the identity of the crossover strands is determined by which arms are connected. Different arm connections gave molecules representing each of the two antiparallel crossover isomers. Two parallel molecules that differ in the length and position of the tether exhibit opposite biases in their choice of crossover strands. Thus, a physical constraint applied at a distance from the branch point can determine the conformation of a junction.

A current challenge in biochemistry is to understand multistep recombination pathways. Many recombination reactions proceed through a four-stranded DNA intermediate, the Holliday junction (1-6). A detailed description of the molecular pathways of recombination will require an understanding of the properties of four-stranded DNA molecules.

Enzymatically generated Holliday junctions are unsuitable for structural analysis because strand crossovers are made in regions of DNA homology. Sequence homology allows strands to exchange pairing partners via the mobile process known as branch migration (7, 8). Members of a population have different branch positions. As a stable alternative to natural Holliday junctions, synthetic four-stranded DNA molecules of lower sequence symmetry have been used to investigate structural details of the Holliday junction (9). A stable immobile

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Holliday junction, J1, is formed (10) by synthesizing and annealing four 16-nucleotide-long strands of DNA that base pair to form a four-arm junction with a unique branch point and 8-base pair arms. The core structure of this molecule has been characterized extensively (11-13). In particular, cleavage of this molecule by the hydroxyl radical (14) reveals 2-fold structural symmetry, suggesting that J1 is made up of two helical stacking domains, as was proposed by Sigal and Alberts (15) for the Holliday junction.

A structure with a unique crossover point and two helical domains can be drawn in four idealized isomeric forms; either set of two strands can cross over, and the non-crossover strands can run either parallel or antiparallel to one another (Fig. 1). The non-tethered synthetic junctions that have been studied to date have been free to undergo these isomerizations. In spite of this freedom, hydroxyl radical protection patterns show that J1 exhibits a strong bias as to which strands cross over (14). Hydroxyl radical cleavage experiments have also

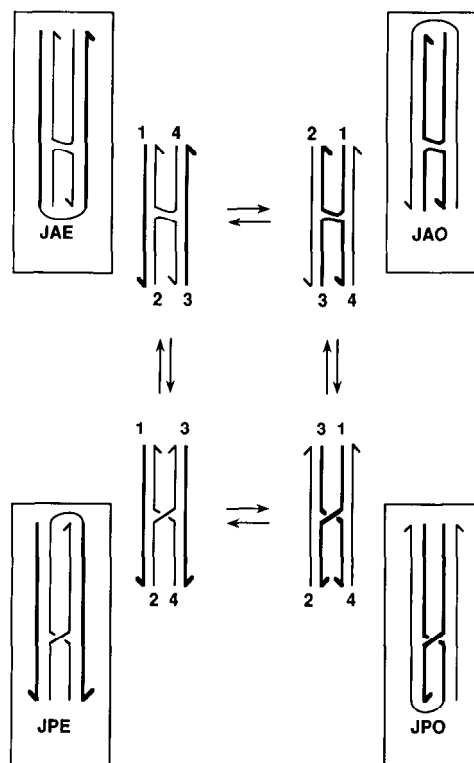


FIG. 1. The four conformational isomers available to an immobile 2-fold symmetric four-arm DNA junction. In the central part of the diagram are shown the four isomers that a junction without branch migratory freedom can assume. Flanking each of these ideal isomers are the tethered molecules we used to characterize the conformational isomers. The second letter of a conformer's name, A or P, indicates the relative orientation of the helical strands, antiparallel or parallel, respectively; the third letter (E or O) indicates whether the even or odd strands form the crossover. Strands 1 and 3 are drawn in boldface. The half-arrowheads indicate the 3' ends of the strands. The arcs represent the thymidine tethers, and relative arm lengths are illustrated. The diagrams at the top depict antiparallel arrangements of the helix axes. The structures differ by which strands are involved in the crossover; the even numbered strands (thin lines) cross over in the structures on the left, while the odd numbered strands (thick lines) cross over in the structures on the right. The lower diagrams show the two possible parallel conformations.

demonstrated that a local change in sequence at the branch point can change the strands that cross over (16). Electrophoretic mobilities of synthetic junctions with pairs of elongated arms (17, 18) and covalent tethers (19) confirm the principle that individual junctions preferentially adopt a single crossover conformation that is determined by the branch point sequence and also indicate that the molecules are in the antiparallel conformation (Fig. 1, *upper structures*). Solution studies verify that synthetic junctions containing the J1 core sequence (20) and an unrelated core sequence (21) exist in single crossover versions of an antiparallel conformation.

While unconstrained junction molecules seem to prefer the antiparallel conformation, homologous recombination is most readily imagined to involve parallel Holliday junctions (22). Models for site-specific recombination involve both parallel (23) and antiparallel (24) Holliday junctions. DNA molecules constrained in parallel or antiparallel Holliday conformations would provide useful bench marks for investigating the properties of branched structures and are a first step toward understanding the nature of the biological intermediates. We report here the design, construction, and structural characteristics of such a set of conformationally defined four-arm junctions (Table I). Each junction contains at its core the 32-base pair sequence of J1. This sequence is then elaborated by elongating two arms to 15 base pairs and by incorporating a run of thymidine residues that tethers two of the four arms in four different ways (Fig. 1, *boxed structures*). Hydroxyl radical cleavage is used to determine the crossover structure of the four junctions in solution (14).

EXPERIMENTAL PROCEDURES

Each DNA strand (Table I) is synthesized by standard phosphoramidite techniques (25), purified as described previously (26), and annealed (10). The procedure of Churchill *et al.* (14) for junction formation and hydroxyl radical cleavage and analysis is followed generally. However, in order to ensure incorporation of radioactive strands into complexes that migrate as single bands on nondenaturing gels, for the junctions JPE and JPO we raised the ratio of radioactivity labeled to unlabeled strands to 1:4.

RESULTS AND DISCUSSION

When one strand from any of the tethered junctions described here is radioactively labeled and combined with an

excess of the other two strands, heated, and cooled, the radioactivity is entirely incorporated into a structure that runs on a non-denaturing gel with the same (JAE and JAO) or similar (JPO and JPE) mobility as control unconstrained junctions. (A note on our nomenclature: the second letter of a junction's name indicates whether it is designed to be Antiparallel or Parallel, and the third letter indicates whether the Even or Odd numbered stands cross over.) Strand titrations and lack of reaction with osmium tetroxide indicate that the junction arms are fully base-paired. The maximum distance that could be spanned by the unpaired thymidine residues is about 40 Å for JAE and JAO (Fig. 1, *top structures*). For these molecules to adopt a parallel Sigal-Alberts conformation (Fig. 1, *bottom structures*), the thymidine tethers would have to span about 80 Å. Therefore we assume that annealed JAO and JAE are antiparallel junctions and JPO and JPE are parallel junctions.

Hydroxyl radical cleavage, which has been successfully used before to identify the crossover strands of junction molecules (14, 16), is used here to characterize the structures of the tethered junctions. The cleavage pattern of a junction is compared with the cleavage pattern for the same sequence in a normal duplex. Differences reveal structural features of the junction. The sequences of all of the junction strands are numbered so that nucleotide positions 8 and 9 flank the branch point, as they do in J1 (10, 14). All junction cleavage patterns are compared with the reference pattern of the same strand incorporated into a normal DNA duplex (Fig. 2, *row 1*). Protection from cleavage at the branch point (positions 8 and 9), the signature of the crossover strands (14, 16), is seen on strands 2 and 4 of an unconstrained junction (Fig. 2, *row 6*). An unconstrained junction also shows protections on the non-crossover strands at positions 12 and 13 (Fig. 2, *row 6*). These protections arise from the helical nature of DNA; the backbones of these strands are fully exposed to solution at the crossover positions 8 and 9 but wind around the helical axis and are protected by a nearby junction arm about a half-turn away from the branch point (Fig. 3).

When strands 2 and 4 are incorporated into the tethered antiparallel junction JAE, strong minima in the cleavage patterns of these strands occur at positions 8 and 9 (Fig. 2, *row 2*). A 2,4-strand crossover is built into the design of JAE

TABLE I
Sequences of the four tethered DNA junctions

Strand no.	Equivalent strand no.	Sequence ^a
JAE		
1(T ₆) ₃	1	5'-GCCGACGCGCAATCCTGAGCACG (T ₆ to strand 3)
2	2	5'-CGTGCTCACC ^u GAATGC-3'
1(T ₆) ₃	3	(from strand 1) GCATTGGGACTATGGCGGACCTC-3'
4	4	5'-GAGGTCCGCCATAGTGGATTGCGAGTCGGC-3'
JAO		
1	1	5'-GCCGACTCGCAATCCTGAGGCCACGACCTC-3'
4(T ₆) ₂	2	(from strand 4) GAGGTCTGGCCTCACCACCATGC-3'
3	3	5'-GCATGGTCGGACTACCTGGC-3'
4(T ₆) ₂	4	5'-GCCAGGTAGTGGATTGCGAGTCGGC (T ₆ to strand 2)
JPO		
1	1	5'-GCCGACGCGCAATCCTGAGCACG-3'
3(T ₆) ₂	2	(from strand 3) CGTGCTCACC ^u GAATGGCGGACCTC-3'
3(T ₆) ₂	3	5'-GAGGTCCGCATTCGGACTATGGC (T ₆ to strand 2)
4	4	5'-GCCATAGTGGATTGCGAGTCGGC-3'
JPE		
1	1	5'-GCCGACTCGCAATCCTGAGCACG-3'
2	2	5'-CGATCGCACCGAATGCGGACCTC-3'
4(T ₉) ₃	3	(from strand 4) GAGGTCCGCATTCGGACTATGGC-3'
4(T ₉) ₃	4	5'-GCCATAGTGGATTGCGAGTCGGC (T ₉ to strand 3)

^a Nucleotides at the branch point (positions 8 and 9, Fig. 2) are underlined.

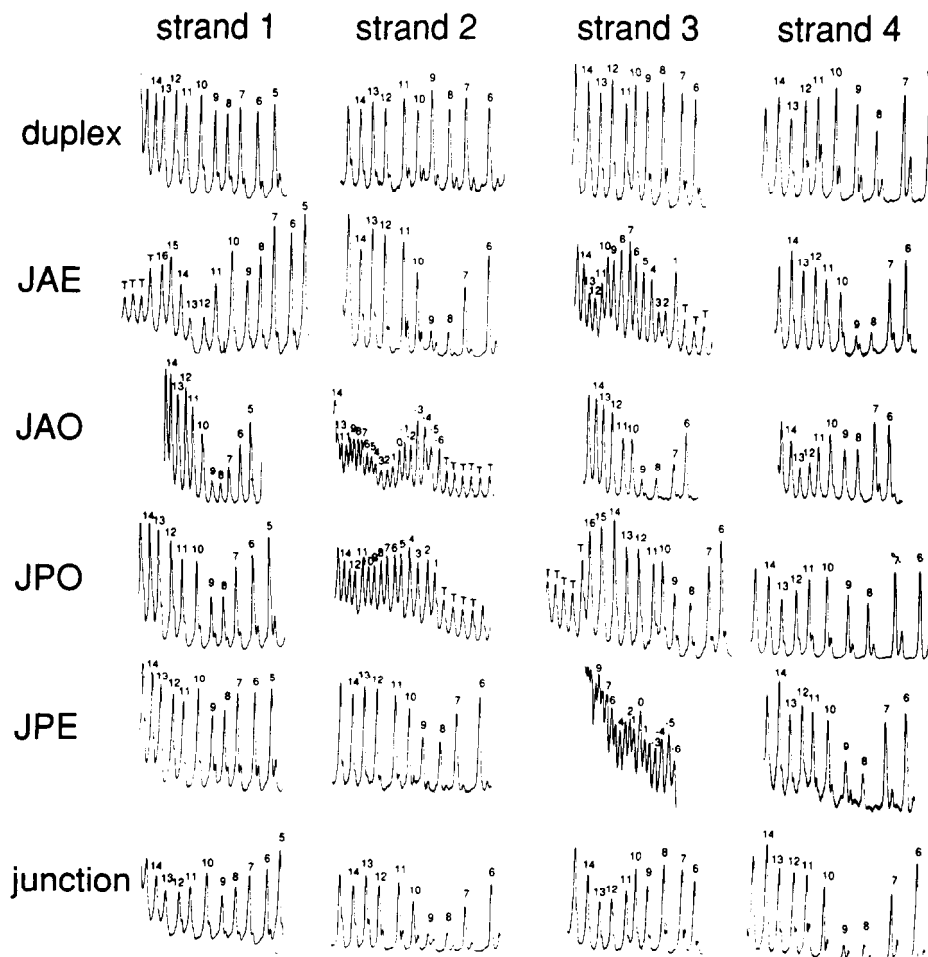


FIG. 2. Densitometer traces of the hydroxyl radical cleavage patterns around the branch points of the DNA junctions. Nucleotides were numbered (from 5' to 3') so that the branch point would occur between residues 8 and 9 of each segment. Some of the strands with tethers may contain more than one such numbered region. Row 1, the region of interest in control duplex DNA; row 2, the same region in the junction constrained to be antiparallel with even strand crossover (JAE); row 3, antiparallel with odd strand crossover (JAO); row 4, parallel with odd strand crossover (JPO); row 5, parallel with even strand crossover (JPE); row 6, the untethered junction. Split bands reflect the presence of two different 3' termini upon hydroxyl radical cleavage (28) that are visible here because the strands are labeled at their 5' ends and highly resolved. The resolution of the cleavage pattern of one strand of each constrained junction (strand 2 of JAO and JPO, and strand 3 of JAE and JPE) is poor due to the design of the molecules; these cleavages occur near the end of the strand that is distant from the radioactive label. Note that the unpaired thymidine residues in the tether are cleaved at a lower rate than adjacent paired sequences; we and others (27) have observed that a decreased rate of hydroxyl radical cleavage is a general property of single-stranded DNA.

(see Fig. 1), and so these protections are not unexpected. It is important to note that while the rate of hydroxyl radical cleavage is lower for single-stranded than for base-paired DNA (Fig. 2) (27), the protections described here are best attributed to shielding of a nucleotide by the junction instead of to unpairing of the strands at the junction. This is true because any reduction in cleavage due to unpairing at the branch point positions on strands 2 and 4 would also be seen at the complementary positions, 8 and 9, of strands 1 and 3. This is not observed. In addition to protections at the branch point of the crossover strands, protections are seen on the non-crossover strands of JAE at positions four nucleotides away from the branch point (positions 12 and 13 of strands 1 and 3). Corresponding, but weaker, protections are seen in the unconstrained junction (Fig. 2, row 6) (14).

The junction JAO was designed to anneal into an antiparallel junction with strands 1 and 3 crossing over. Hydroxyl radical cleavage shows strong protections at the branch point positions of these strands (Fig. 2, row 3). In a number of

earlier experiments on untethered junctions with the J1 core sequence (14, 17, 20, 26), the 1,3 crossover isomer was never observed to predominate. However, despite existing in the less favored crossover isomerization state, the junction JAO appears to be stable. It anneals cleanly, runs on a native gel with mobility identical to JAE and unconstrained control junctions (19) (data not shown), and shows characteristic protections on the crossover strands.

Our strategy for tethering junction arms makes use of a run of thymidine residues to connect the 3' end of one strand to the 5' end of another strand. It can be seen in Figs. 1 and 3 that, in contrast to the case for antiparallel junctions, the distance to be spanned by the tether in a planar parallel junction is equal for either crossover isomer. For parallel molecules, therefore, the junction design in no obvious way favors a particular crossover isomer. In the case of the tethered parallel junction JPO, in which the arms are connected by six thymidines, we observe the surprising result that branch point protections occur on strands 1 and 3 (Fig. 2, row 4). While

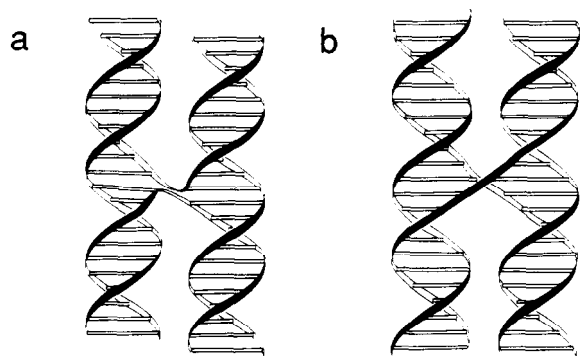


FIG. 3. Helical representations of Holliday structures. *a*, an antiparallel Holliday structure. Although this model shows a planar structure, recent work demonstrates that the helical domains of antiparallel junctions actually deviate from coplanarity (20, 21). *b*, a parallel Holliday structure. For a parallel structure with coplanar helical domains, as shown here, the distance is equal between any two ends that are spanned with a thymidine tether to restrict the molecule in a parallel configuration.

the 8,9 protections here are weaker than in the antiparallel junctions (Fig. 2, rows 2 and 3), they can be interpreted as reporting local steric protection from cleavage because the adjacent nucleotides, at positions 7 and 10, also are cut at lower frequency in the junction than in duplex controls. Since strands 2 and 4 of this junction are not protected at the branch point positions, strands 1 and 3 are clearly the crossover strands of JPO. We do not know why the branch point protections of a parallel structure are less pronounced than in an antiparallel structure.

Experiments on unconstrained junctions with the J1 (14, 17, 20, 26) and other (16, 18, 21) core sequences suggest that the sequence at the branch point is the dominant factor governing crossover bias, so it is important to determine what directs the crossover choice of JPO. The crossover structure of JPO may be due to the junction being strained by the length of the thymidine tether or might reflect a new crossover preference of this DNA sequence when in a parallel structure. To address this question we have examined an alternative version of a constrained parallel junction, the molecule JPE. The junction JPE is constrained in a parallel configuration by a tether of nine thymidines rather than the six in JPO. Hydroxyl radical reactivity of this molecule now shows branch point protections on strands 2 and 4, demonstrating that a parallel junction can adopt the crossover state that predominates for free junctions.

We have constructed and analyzed four tethered versions of the immobile four-arm junction J1. By introducing covalent tethers, we have applied the most powerful forces at our disposal to generate the four possible structural isomers of a 2-fold symmetric junction. Hydroxyl radical analysis is well suited for determining which strands of a junction cross over. Using this method, we demonstrate that the common DNA sequence at the core of these junctions can adopt any of the four canonical isomers illustrated in Fig. 1. It is noteworthy that a junction without the sequence symmetry of a natural

Holliday junction can form with parallel or antiparallel helical domains in either of two crossover isomers. We also demonstrate that a change in tension is capable of reversing the crossover preference of a parallel junction. Proteins associated with recombination phenomena perhaps could use this or other interactions to produce variations in the structure of a Holliday junction.

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