Symmetric Immobile DNA Branched Junctions†

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ABSTRACT: Branch migration is an isomerization of Holliday recombination intermediates that arises from their homologous (2-fold) sequence symmetry. This isomerization relocates the branch point in an apparently random fashion and thereby complicates the study of the physical and structural properties of these structures. For the past decade, these properties have been studied in low-symmetry immobile junctions, whose sequence asymmetry eliminates branch migration. The asymmetric findings of many of these studies suggest the need for a system combining both immobility and symmetry. Double-crossover DNA molecules have been used to create molecules with both these properties. Immobility is achieved by flanking one crossover with a symmetric junction and the other crossover with an asymmetric junction. Close torsional coupling between the two junctions renders the symmetric junction immobile. These molecules will enable the characterization of thermodynamic, structural, dynamic, liganding, and substrate properties of symmetric branched DNA molecules in a sequence-specific fashion.

The Holliday (1964) junction is a four-stranded branched DNA structure that is known to be a central intermediate in site-specific recombination (Hoess *et al.*, 1987; Kitts & Nash, 1987; Nunes-Duby *etal.*, 1987) and that is thought to be involved in general homologous recombination (Dressler & Potter, 1982). When formed from sequences of DNA containing homology, this structure is inherently unstable: The homology results in 2-fold sequence symmetry in the nucleotides flanking the branch point; this symmetry in turn enables the junction to undergo an iterative isomerization known as branch migration (Lee *et al.*, 1970). Conversely, the mobility of the branch point may be eliminated in synthetic analogs of the Holliday junction by destroying the homologous symmetry (Seeman, 1982). This strategy has been employed to design and construct immobile DNA junctions (Kallenbach *et al.*, 1983). The structural and thermodynamic properties of immobile junctions have been characterized extensively over the course of the past decade (Kallenbach *et al.*, 1983; Seeman *et al.*, 1985; Wemmer *et al.*, 1985; Marky *et al.*, 1987; Cooper & Hagerman, 1987, 1989; Churchill *et al.*, 1988; Chen *et al.*, 1988; Duckett *et al.*, 1988; Murchie *et al.*, 1989; Chen *et al.*, 1991; Lu *et al.*, 1991, 1992).

One of the key structural features of the immobile DNA branched junction is that its four double-helical arms are organized into two helical domains (Cooper & Hagerman, 1987, 1989; Churchill *et al.*, 1988; Duckett *et al.*, 1988). Thus, in the well-characterized junction J1 (Seeman & Kallenbach, 1983; Kallenbach *et al.*, 1983), arm I stacks on arm II and arm III stacks on arm IV (Churchill *et al.*, 1988), rendering strands 2 and 4 the crossover strands of the junction (Figure 1). The alternative stacking arrangement (arm I stacked on arm IV and arm II stacked on arm III, with strands 1 and 3 being the crossover strands) has not been detected. The various experiments that have established the stacking structures of four-arm DNA branched junctions (Cooper & Hagerman, 1987, 1989; Churchill *et al.*, 1988: Chen *et al.*, 1988; Duckett *et al.*, 1988; Murchie *et al.*, 1989) are likely to be sensitive to the presence of the other conformer if it were present as a 10–20% component. Thus, the observed isomers are favored by a factor of 5 or greater over the alternative isomers. The bases directly flanking the branch point appear to determine the dominant stacking arrangement (Chen *et al.*, 1988; Duckett *et al.*, 1988).

The implications of this biased stacking preference for the products of recombination are significant, particularly if the junction rearranges itself to assume the favored stacking structure after every step of branch migration (Mueller *et al.*, 1988). It is known that junction resolvases cleave one particular pair of strands to yield two duplex molecules (Mueller *et al.*, 1988; Duckett *et al.*, 1988; Bhattacharyya *et al.*, 1991). For example, the junction resolvase endonuclease VII cleaves the crossover strands of a junction, so the exchange of flanking markers appears to require that the crossover strands and the noncrossover strands exchange identities (crossover isomerize) an odd number of times. Therefore, if the stacking arrangement is not random, but is instead a function of the position to which the junction has migrated, the products of recombination will depend on the sequence that happens to flank the junction when it is cleaved (Chen *et al.*, 1988; Mueller *et al.*, 1988; Seeman *et al.*, 1990).

As noted above, the primary way in which immobile junctions differ from naturally occurring Holliday junctions is their lack of sequence symmetry. It would be desirable to devise a system in which symmetric Holliday junctions can be assembled, yet held immobile. The development of such a system would permit the characterization of symmetric branched junctions in a sequence-specific fashion. Molecules with this property would enable us to address the key question involving the stacking biases noted above: Are they retained in molecules possessing homologous symmetry? Furthermore, detailed structures bearing more closely on the natural situation could be determined by X-ray crystallography or by NMR. In addition, the sequence specificity of enzymatic resolution (Picksley *et al.*, 1990, Pottmeyer & Kemper, 1992), liganding properties (Guo *et al.*, 1989), or local dynamic properties [e.g., Hustedt *et al.* (1993)] could be studied without having the experiment complicated by branch migration or by asymmetric features.

We have devised a system that holds a four-arm branched junction immobile, even though it has the symmetry to migrate. We do this by utilizing a double-crossover DNA molecule.

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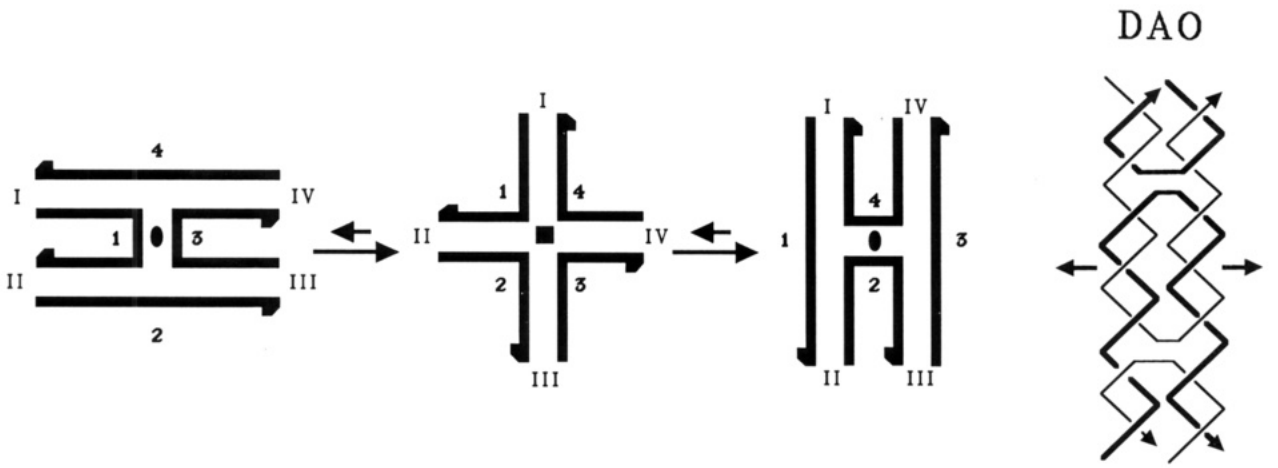


FIGURE 1: Domain structures in molecules containing four-arm branched junctions. (a, left) The domain structure of the four-arm immobile junction J1. Each strand is indicated by Arabic numerals, and double-helical arms are indicated by Roman numerals. Helical regions are indicated by parallel strands; half-arrowheads represent the 3′ ends of strands. The implicit symmetry of the structure is shown in the middle, with the square representing a potential 4-fold symmetric structure. The dyad symmetry of the known structure of J1 (Cooper & Hagerman, 1987, 1989; Churchill *et al.*, 1988) is indicated by the lens-shaped object at the center of the two structures that flank the central structure. The dominant structure is the one on the right, in which arm I stacks on arm II and arm III stacks on arm IV. The experiments performed so far have not detected the presence of the structure on the left. (b, right) The DAO arrangement of strands in a double-crossover molecule. The four strands are potentially related by a dyad symmetry axis in the plane of the page, indicated by the arrows. The thin strands are symmetrically related to the thick strands. The helicity is shown explicitly by having one strand pass in front of another, which is indicated as broken. Note that because there are an odd number of half-turns between crossovers, only four strands are needed to construct this complex.

We have described the five different possible isomers of DNA molecules containing double crossovers; three of these isomers contain parallel helical domains, and two contain antiparallel domains (Fu & Seeman, 1993). Holliday junctions preferentially assume antiparallel conformations (Cooper & Hagerman, 1987, 1989; Duckett *et al.*, 1988; Murchie *et al.*, 1989; Lu *et al.*, 1991), and this preference is heightened in double-crossover molecules (Fu & Seeman, 1993). Therefore, we have used an antiparallel motif for this construction. Double-crossover molecules containing an odd number of half-turns between crossovers require only four strands, whereas those with an even number require five strands, including a cyclic molecule whose synthesis and hybridization are both difficult. Accordingly, we have built our immobile symmetric branched junction from the antiparallel, odd class of double-crossover molecules, acronymized as DAO (Fu & Seeman, 1993). This isomer is shown in Figure 1b.

The rationale behind employing a double-crossover molecule to immobilize a symmetric branched junction is that tight torsional coupling exists between two crossover points separated by a short distance: Whether the helical domains are parallel or antiparallel, they must branch migrate together or the DNA between them will become overwound or underwound. Therefore, if one of the crossover points is flanked by an immobile junction, the other junction is prevented from migrating, regardless of its symmetry. Here we describe the construction of a molecule containing six mobile nucleotide pairs that flank the junction and demonstrate by means of $Fe(II)EDTA^{2-}$ autofootprinting that the junction they surround is immobile.

MATERIALS AND METHODS

Sequence Design. The sequence of the symmetric immobile junction (SIJ) is derived directly from the sequence of the DAO molecule already studied. The only change that we have made is to replace the six residues that flank one junction on both crossover strands by the sequence dT_6. The residues complementary to them are correspondingly replaced by dA_6. We term this molecule SIJ01. Whereas interpretation of the $Fe(II)EDTA^{2-}$ autofootprinting analysis utilizes the complements to each strand (Churchill *et al.*, 1988), this complementary set defines a second molecule of the same form, SIJ01C, but with the sequence dA_6 flanking the crossover. For comparison, we have used a tetramobile junction, JTM01, whose sequence is derived elsewhere (S. Zhang and N. C. Seeman, manuscript in preparation).

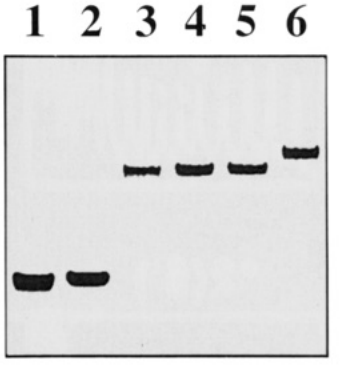
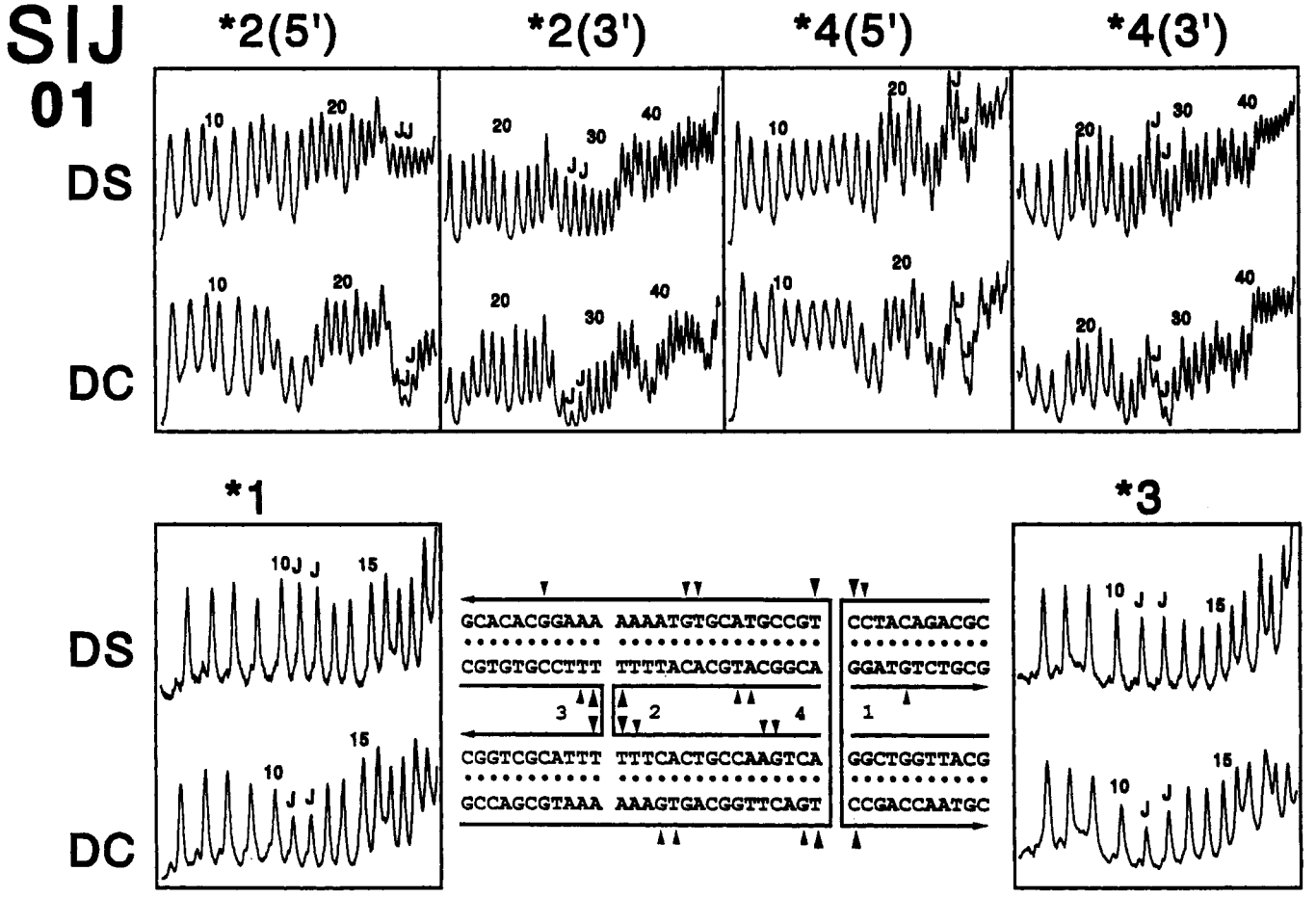


FIGURE 2: Nondenaturing gel of the molecules used in this study. This is a 12% polyacrylamide gel containing stoichiometric complexes of the symmetric immobile junctions and appropriate reference standards. Each strand is present at a concentration of 10 μM. Lane 1 contains the well-characterized four-arm junction J1 (Kallenbach *et al.*, 1983). Lane 2 contains the tetramobile junction JTM01. Lane 3 contains the previously characterized (Fu & Seeman, 1993) completely immobile double-crossover molecule, DAO, on which the symmetric immobile junctions were based. Lane 4 contains SIJ01 and lane 5 contains SIJ01C. Lane 6 contains a four-arm branched junction with the same domain structure as DAO, but lacking the second crossover (Fu & Seeman, 1993). Note that the new molecules, JTM01, SIJ01, and SIJ01C, form clean structures, with very little material migrating either as multimers or as breakdown products.

Synthesis and Purification of DNA. All DNA molecules in this study have been synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected, using routine phosphoramidite procedures (Caruthers, 1982). DNA strands less than 20 nucleotides long have been purified by HPLC, utilizng a Du Pont Zorbax Bio Series oligonucleotide column using a gradient of NaCl in a solvent system containing 20% acetronitrile and 80% 0.02 M sodium phosphate, as described previously (Wang *et al.*,


SIJ
01
*2(5')
*2(3')
*4(5')
*4(3')
DS
DC
*1
*3
GCACACGGAAA AAAATGTGCATGCCGT CCTACAGACGC
CGTGTGCCTTT TTTTACACGTACGGCA GGATGTCTGCG
CGGTCGCATTT TTTCACTGCCAAGTCA GGCTGGTTACG
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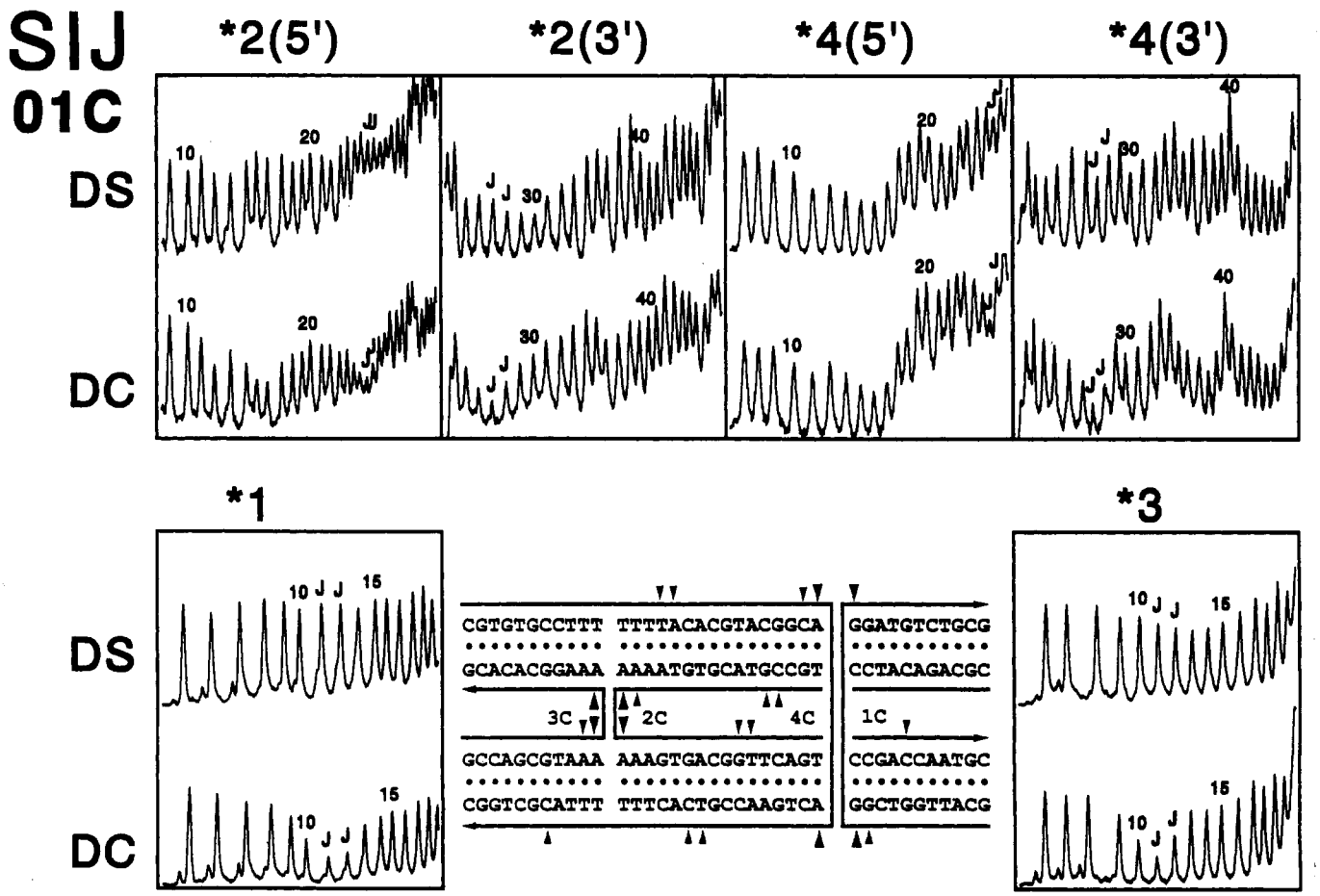
SIJ
01C
*2(5')
*2(3')
*4(5')
*4(3')
DS
DC
*1
*3
CGTGTGCCTTT TTTTACACGTACGGCA GGATGTCTGCG
GCACACGGAAA AAAATGTGCATGCCGT CCTACAGACGC
CGGTCGCATTT TTTCACTGCCAAGTCA GGCTGGTTACG
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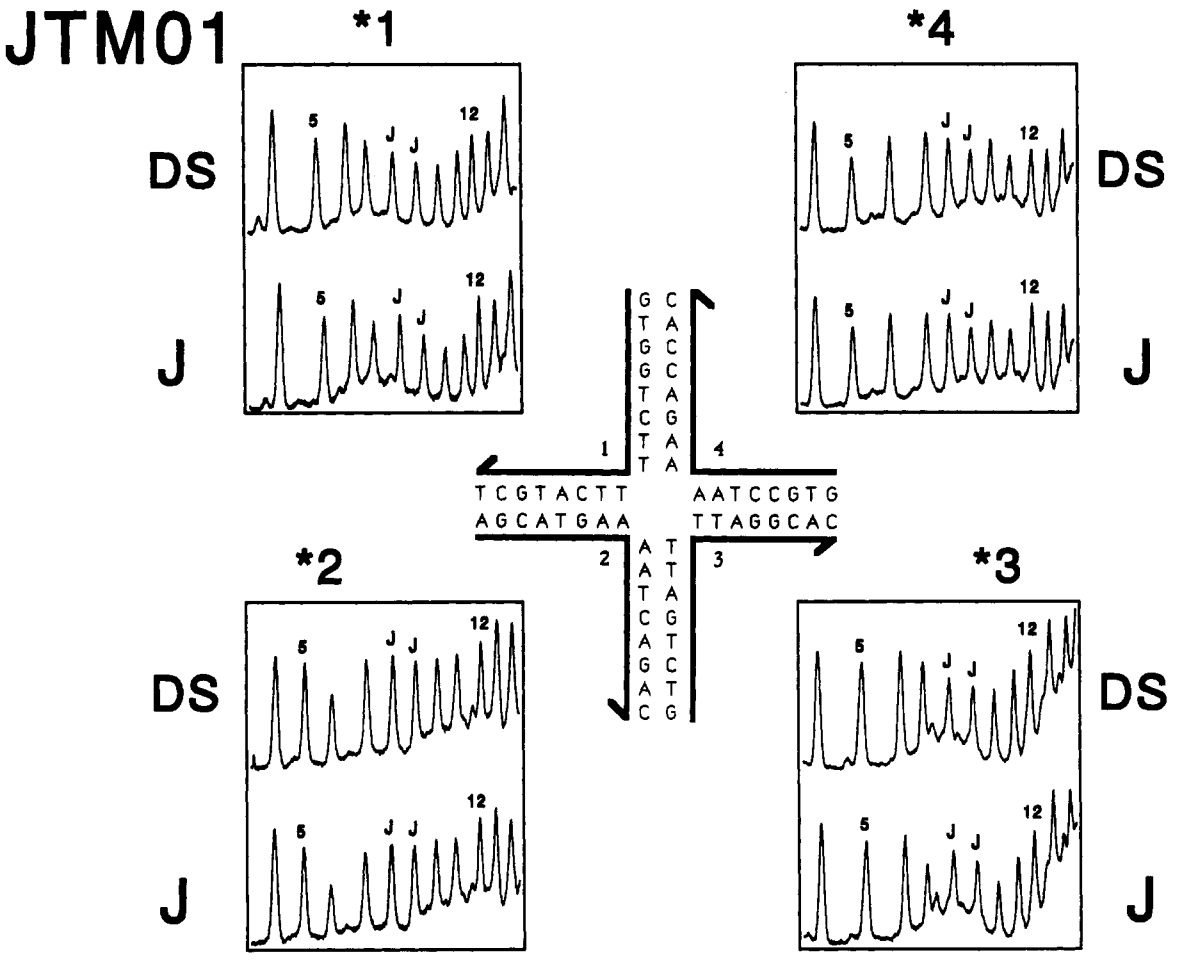

FIGURE 3: Hydroxyl radical cleavage patterns for the symmetric immobile junctions and the control tetramobile junction. In each case, we present scans of the denaturing gels surrounding a summary of the results on a schematic of the molecule. Numbers in the scans are from the 5′ end of the strand. The nominal site of bends, as directed by the sequence, is indicated in the scans as a J. The intensity of a peak indicates cleavage, so a low peak indicates protection. DS indicates duplex, DC represents double-crossover structures, and J represents junctions. Arrowheads represent the 3′ ends of strands in the schematic. The 5′ and 3′ ends of the longer strands (2 and 4) of the double-crossover molecules are shown separately to increase the resolution of the picture. The relative intensity of protection is indicated by the size of the triangles pointing at the sites of protection. In the symmetric immobile molecules, protection is expected at residues 11 and 12 on the odd-numbered strands and at residues 27 and 28 on the even-numbered strands. The sites of weaker protection noted are likely to derive from occlusion a turn away from the junction (Fu & Seeman, 1993). (a, top, left page) The protection pattern of SIJ01. (b, bottom, left page) The protection pattern of SIJ01C. (c, above) The protection pattern of a positive control, the tetramobile junction JTM01. Note the similarities of the protection patterns for the immobile junctions (strands 4 and 1, strands 4C and 1C) and the potentially mobile junctions (strands 2 and 3, strands 2C and 3C). Compare the dramatic protection in these complexes with that in the tetramobile complex, JTM01.

1991). Longer molecules have been purified by denaturing gel electrophoresis.

Formation of Hydrogen-Bonded Complexes. Complexes are formed by mixing a stoichiometric quantity of each strand, as estimated by OD_{260}. This mixture is then heated to 90 °C for 3 min and slowly cooled to the desired temperature. A single band on a native gel is taken to indicate a homogeneous stoichiometric complex.

Thermal Transition Profiles. Response to increased temperature is measured on a Gilford Response II UV–vis spectrophotometer at 268 nm. Complexes are formed at a concentration of 224 nM for each strand in a solution containing 40 mM sodium cacodylate, pH 7.0, and 10 mM $MgCl_2$. Temperature is incremented at the rate of 0.1 °C/min. The data have been smoothed by using a 21-point interpolation routine.

Hydroxyl Radical Analysis. Individual strands of the complexes are radioactively labeled and are additionally gel purified from a 15% 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 annealed to a 100-fold excess of the unlabeled complementary strands, or it is annealed to a 100-fold excess of a mixture of the other strands forming the complex, or it is left untreated as a control, or it is treated with sequencing reagents (Maxam & Gilbert, 1977) for a sizing ladder. The samples are annealed by heating to 90 °C for 3 min and then cooled slowly to 4 °C. Hydroxyl radical cleavage of the double-strand and junction samples for all strands takes place at 4 °C for 2 min (Tullius & Dombroski, 1985), with modifications noted by Churchill *et al.* (1988). The reaction is stopped by addition of thiourea. The sample is dried, dissolved in a formamide/dye mixture, and loaded directly onto a 15% polyacrylamide/8.3 M urea sequencing gel. Autoradiograms are scanned with a Hoefer GS300 densitometer in transmission mode.

Polyacrylamide Gel Electrophoresis. Denaturing and nondenaturing gels are run as described previously (Du *et al.*, 1992).

RESULTS

Formation of the Complexes. Nondenaturing gel electrophoresis is a technique that has been used frequently to demonstrate the formation of unusual DNA structures

containing multiple strands (Kallenbach *et al.*, 1983; Ma *et al.*, 1986; Wang *et al.*, 1991; Du *et al.*, 1992; Fu & Seeman, 1993). The formation of the target complex is shown by the presence of a single band in a lane containing an equimolar mixture of its components. Bands that correspond to multimers of the target complex have revealed the instability of antijunctions, mesojunctions, and parallel double-crossover structures (Du *et al.*, 1992; Fu & Seeman, 1993). Likewise, breakdown of the complex has been seen in five-arm and six-arm junctions containing a small number of nucleotide pairs in their arms (Wang *et al.*, 1991). Figure 2 shows that the symmetric immobile junctions form as closed stable complexes. Their mobilities are similar to that of a previously characterized DAO molecule (Fu & Seeman, 1993) containing the same number of nucleotides. Note that their mobilities are different from that of a molecule with the same domain structure but lacking a second crossover. The stability of the tetramobile control junction is also evident from inspection of Figure 2. Its mobility is very similar to that of the previously characterized junction, J1.

Hydroxyl Radical Analysis. Previous experiments with hydroxyl radical autofootprinting of structures containing DNA branched junctions have yielded valuable information about the molecules under investigation (Churchill *et al.*, 1988; Chen *et al.*, 1988; Kimball *et al.*, 1990; Lu *et al.*, 1990; Wang *et al.*, 1991; Du *et al.*, 1992; Fu & Seeman, 1993). Every experiment involves a two-part comparison: (1) Each strand is labeled individually, hybridized to the other strands in the complex, and then exposed to hydroxyl radicals. (2) The strand is complexed with its Watson–Crick complement, followed by exposure to hydroxyl radicals. Thus, the comparison made is between the strand in the complex and the strand in linear duplex. Previous studies have all revealed a dramatic protection, relative to duplex, at the site of an immobile junction. This protection is accentuated in double-crossover molecules, where some degrees of freedom have been eliminated (Fu & Seeman, 1993). In those studies in which a limited amount of mobility is possible, the protection pattern is less dramatic, because of the multiplicity of migratory isomers (Chen *et al.*, 1988; Lu *et al.*, 1990).

Thus, the hydroxyl radical autofootprinting experiment is an excellent probe of branch migration on a small scale. In this system, each molecule contains an internal standard of mobility, because one of the junctions is designed to be immobile. The data comparing each double-crossover strand with its duplex standard are shown in Figure 3. We show comparisons for both SIJ01 and SIJ01C. The duplex standard is the same molecule in each instance, although opposite strands are labeled for the two sets of experiments. The immobile junction in SIJ01 is flanked by residues 11 and 12 of strand 1 and residues 27 and 28 of strand 4. The pattern in Figure 3 shows the characteristic strong protection, relative to duplex, at those positions, with lesser protection one nucleotide 5′ to them (Churchill *et al.*, 1988). Residues 11 and 12 on strand 3 and residues 27 and 28 on strand 2 flank the nominally mobile junction. Strong protection is again visible at these sites, relative to duplex, suggesting that these positions are immobile. Protection is also evident on the long strands (more on 2 than on 4), one turn 5′ and one turn 3′ to the crossover point, as noted previously (Fu & Seeman, 1993). Similar results are evident for SIJ01C: The protection at the crossover points on strands 2C and 3C is as pronounced as that on strands 1C and 4C, which constitute the internal standards.

We also demonstrate by means of a positive control that the presence of branch migration eliminates the well-localized protection characteristic of an immobile junction. For this purpose, we have used the tetramobile junction JTM01, whose cleavage pattern is also shown in Figure 3. This molecule can exist as five different migratory isomers, so the crossover point could fall anywhere between residues 6 and 11 on each strand. None of the strands shows the characteristic strong protection pattern seen in SIJ01 or SIJ01C at the crossover points. A slight protection may be noted on strand 1 on residues 7 and 9–11 and likewise on strand 3 on residues 7 and 10–11. The differential pattern is clearly less dramatic than that seen for either junction in the double-crossover molecules.

Thermal Transition Profiles. The thermal transition profiles of the symmetric immobile junction and its complement are shown in Figure 4. T_{max} (the inflection point temperature of the thermal transition profile) for SIJ01 = 63.8 °C, and T_{max} for SIJ01C = 64.1 °C; T_{max} for the duplex complexes are 80.4 °C (strand 2) and 79.6 °C (strand 4). The higher melting temperature of SIJ01C is in agreement with more sensitive quantitative thermodynamic experiments that demonstrate that dApdA is slightly preferred to dTpdT as the sequence on the crossover strand (S. Zhang and N. C. Seeman, manuscript in preparation).

DISCUSSION

We have demonstrated clearly the construction of a symmetric immobile DNA branched junction. In order to do this, we have had to sacrifice certain features of the branched junction. In particular, we have relinquished the ability of the junction to crossover isomerize, and we have fixed the relative orientation of the helical domains in this system. The crossover isomer is determined by the sequence in the DAO class of double-crossover molecules (Fu & Seeman, 1993). By selection of the double-crossover system, one automatically foregoes the freedom of helical domain reorientation.

The earliest and most convenient system for studying the Holliday junction was the immobile junction (Seeman, 1982). By eliminating its sequence symmetry, the Holliday junction can be "projected" into this tractable system, where it retains all of its structural degrees of freedom except branch migration: It can assort its four double-helical aroms into two double-helical domains (or not), it can crossover isomerize to change domain components, and it can form parallel, antiparallel, and oblique isomers that change domain relationships. Here, we have presented a different projection of the Holliday junction, one in which we forfeit structural degrees of freedom. In this system, the structure, constituents, and orientation of the double-helical domains are all prescribed by sequence selection. By paying this price, we can achieve symmetry and immobility simultaneously. We expect that this system will complement immobile junctions in the effort to learn more about the thermodynamic, structural, dynamic, liganding, and substrate properties of the crucial Holliday recombination intermediate.

We have selected the antiparallel double-crossover system for creating symmetric immobile junctions. In principle, one could also construct parallel symmetric immobile junctions. These structures have been shown to be less well-behaved than antiparallel molecules, but those with a turn or less between crossovers appear tractable (Fu & Seeman, 1993). These molecules would offer a different window on the properties of the Holliday junction.

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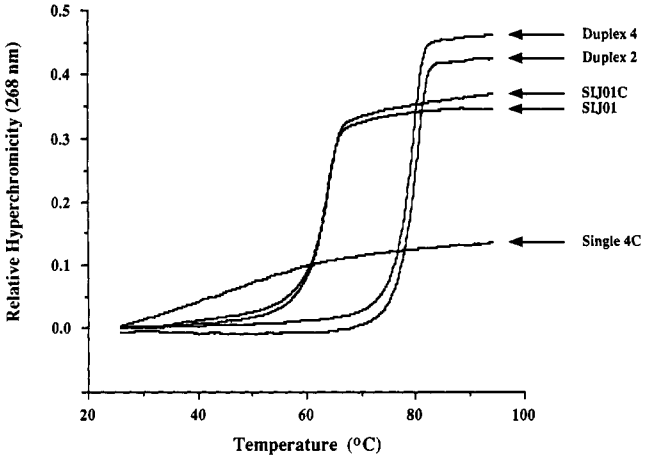

FIGURE 4: Thermal denaturation profiles for the symmetric immobile junctions. The relative hyperchromicity $\{[(A(T)/A(T_0)] - 1\}$, where $T_0 = 25$ °C, is shown. SIJ01 and SIJ01C, the two double-crossover molecules (76 nucleotide pairs each), melt cooperatively but at lower temperatures than the two duplexes (54 nucleotide pairs each) corresponding to the long strands (2 and 4) of the complexes. The individual strand 4C is shown for comparison, indicating a noncooperative transition.

of thermal denaturation profiles on his spectrophotometer. We thank Drs. Junghuei Chen, Yuwen Zhang, Yinli Wang, and Bing Liu for performing experiments that convinced us to seek an immobile symmetric system. We also thank Dr. Rodney Rothstein for valuable discussions on the topology of double-crossover systems. The support of Biomolecular Imaging on the NYU campus by the W. M. Keck Foundation is gratefully acknowledged.

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