Articles

Exchange between Stacking Conformers in a Four-Way DNA Junction†

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ABSTRACT: Four-way DNA junctions undergo metal ion-induced folding by means of pairwise coaxial stacking of helical arms in one of two possible conformers that depend on the choice of stacking partners. For most such junctions there is a significant bias toward one conformer over the other. In this study we have characterized a four-way DNA junction in which there is rapid exchange between equal populations of the two possible stacking conformers. Analysis of the global conformation using comparative gel electrophoresis gives results consistent with either a tetrahedral disposition of the four arms or an equilibrium between equal populations of the two alternative stacked X-structures. Protection of bases at the center of the junction against attack by osmium tetroxide indicates that base stacking is preserved through the point of strand exchange. Cleavage across the point of strand exchange by the restriction enzyme *Mbo*II is consistent with pairwise coaxial stacking of helical arms. Taken together, these indicate that the junction adopts the stacked X-structure, but unusually there appears to be little bias for one stacking conformer over the other. Complete digestion of junctions by *Mbo*II demonstrates that all the molecules in solution pass through a given conformer during the time of incubation, demonstrating that exchange between conformers must occur. This is true even for minor stacking conformers in strongly biased junctions. Comparative gel electrophoresis shows that sequence changes at the third position out from the point of strand exchange can have a marked influence on the relative stability of the stacking conformers.

The four-way junction [4H junction (*1*)] is the central DNA intermediate in a variety of recombination events. In the Holliday model for homologous genetic recombination (*2*), a four-way junction is generated upon strand invasion by single-stranded ends of DNA into a homologous duplex. The junction has remained the major intermediate in subsequent variations of this basic model (*3, 4*). There is experimental evidence for the existence of four-way junctions in both homologous (*5*) and the integrase family of site-specific (*6*−*10*) recombination events. According to the Holliday model for homologous recombination, once the four-way junction has been formed, it undergoes exchange of base pairing in a branch migration process and is ultimately resolved back into separate duplex species. The four-way junction is thus the primary substrate for structure-selective proteins that induce cleavage (reviewed in ref *11*) or branch migration (*12*−*15*).

The structure of the four-way junction has been studied intensively in the last decade (reviewed in refs *16*−*18*). In the absence of added metal ions, the junction exists in an extended 4-fold symmetrical structure where the central bases are accessible to chemical attack (*19, 20*). Upon addition of ≥100 μM magnesium ions, the four-way junction undergoes a folding process that involves the pairwise coaxial stacking of helical arms; these are rotated to form the stacked X-structure. The folding lowers the four-fold symmetry and creates two distinct types of strand. The continuous strands turn about the axis that runs through a given pair of stacked helices, while the exchanging strands cross from one pair to the other at the crossover. In free solution, the structure is predominantly antiparallel, such that the angle included between the axes of the two sections of the exchanging strands is around 60°. This structure is consistent with all experimental data concerning the global shape of the junction (*19, 21*−*25*) and recent NMR data on local stereochemical features such as coaxial stacking (*26*−*29*).

There are two stereochemically equivalent conformers of the stacked X-structure, corresponding to the two possible choices of stacking partners. The relative stability of the two conformers depends on local base sequence, and in general we have found that a junction of a given sequence will exist predominantly in one stacked form. This is generally confirmed by more recent NMR studies, where evidence for coaxial helical stacking has been obtained (*27, 29*), together with evidence of sequence-dependent stacking conformer bias (*28*). However, using the probe *Mbo*II, we previously obtained evidence that a proportion of the minor stacking conformer existed in equilibrium with the major form for one four-way junction (*30*). It is likely that both conformers coexist for most junctions, and the observed

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conformer bias reflects the Boltzmann population of the two forms, i.e., depending on the relative free energies of the two species. The magnitude of the free energy difference between conformers is not known in general, nor is the free energy barrier to their interconversion. But if the difference in free energy between the stacking conformers for a given sequence is less the thermal energy, then both forms should be detectable. In the course of our studies we have identified a junction of particular sequence, called junction 7, where both stacking conformers appear to be equally stable under some conditions.

EXPERIMENTAL PROCEDURES

Synthesis of DNA. DNA oligonucleotides were chemically synthesized using phosphoramidite chemistry implemented on Applied Biosystems 394 RNA/DNA synthesizers. Deoxynucleotide phosphoramidites (Cruachem) and 5-methylcytosine phoshoramidite (Glen Research) were coupled by standard methods (*31*). Fully deprotected oligonucleotides were purified by electrophoresis in polyacrylamide gels in buffer containing 7 M urea. Bands containing DNA were located by UV shadowing. The DNA was electroeluted from excised polyacrylamide fragments into 8 M ammonium acetate, and the DNA was then recovered by ethanol precipitation. Oligonucleotides were radioactively 5′-^{32}P labeled using [γ-^{32}P]ATP (Amersham) and T4 DNA kinase (New England Biolabs) (*32*).

Oligonucleotide Sequences. The following oligonucleotides were synthesised. All are written 5′ to 3′.

Junction 7 used for comparative gel electrophoresis. This comprised four oligonucleotides each of 80 nucleotides (nt) in length. Restriction sites are highlighted in boldface type: b-strand, CGCAAGCGACAGGAACCTCGAG**GGATCC**GTCCTAGCAAGCCGCTGCTACCGG**AAGCTT**CTCGAGGTTCCTGTCGCTTGCG; h-strand, CGCAAGCGACAGGAACCTCGAG**AAGCTT**CCGGTAGCAGCGCGAGCGGTGGTT**GAATTC**CTCGAGGTTCCTGTCGCTTGCG; r-strand, CGCAAGCGACAGGAACCTCGAG**GAATTC**AACCACCGCTCGGCTCAACTGCAG**TCTAGA**CTCGAGGTTCCTGTCGCTTGCG; x-strand, CGCAAGCGACAGGAACCTCGAG**TCTAGA**CTGCAGTTGAGCGCTTGCTAGGAC**GGATCC**CTCGAGGTTCCTGTCGCTTGCG.

Junction 1 used for comparative gel electrophoresis (*19*). This comprised four oligonucleotides each of 80 nt in length. Restriction sites are highlighted in boldface type: b-strand, CGCAAGCGACAGGAACCTCGAG**GGATCC**GTCCTAGCAAGCCGCTGCTACCGG**AAGCTT**CTCGAGGTTCCTGTCGCTTGCG; h-strand, CGCAAGCGACAGGAACCTCGAG**AAGCTT**CCGGTAGCAGCGAGAGCGGTGGTT**GAATTC**CTCGAGGTTCCTGTCGCTTGCG; r-strand, CGCAAGCGACAGGAACCTCGAG**GAATTC**AACCACCGCTCTTCTCAACTGCAG**TCTAGA**CTCGAGGTTCCTGTCGCTTGCG; x-strand, CGCAAGCGACAGGAACCTCGAG**TCTAGA**CTGCAGTTGAGAGCTTGCTAGGAC**GGATCC**CTTCGAGGTTCCTGTCGCTTGCG.

Junction 7XMbo used for *Mbo*II cleavage experiments. This comprised four oligonucleotides each of 30 nt in length. The *Mbo*II sites are highlighted in boldface type: b-strand, CCCGTCCTAGCAAGCCGCTGCTACCGGAGG; h-strand, CCTCCGGTAGCAGCGCGAGCGGTGGTTGGG; r-strand, CCCAACCACCGCTCGGC**TCTTC**TGCAGTGG; x-strand, CCACTGCA**GAAGA**GCGCTTGCTAGGACGGG.

In addition, a strand complementary to the r strand was used in order to make a perfect r strand duplex for comparison: r′ strand, CCACTGCA**GAAGA**GCCGAGCGGTGGTTGGG.

A derivative of junction 7XMbo was made for osmium tetroxide probing. This required the synthesis of a new b strand, containing a single 5-methylcytosine substitution (underlined below) hybridized to the unmodified h, r, and x strands above: b-strand (5-MeC), CCCGTCCTAGCAAG^{5m}CCGCTGCTACCGGAGG.

Junction 7B′Mbo used for *Mbo*II cleavage experiments. This comprised four oligonucleotides each of 30 nt in length. The *Mbo*II sites are highlighted in boldface type: b-strand, CCCGTCCTAGCAACGCGCTGCTACCGGAGG; h-strand, CCTCCGGTAGCAGCGGCAGCGGTGGTTGGG; r-strand, CCCAACCACCGCTGCGC**TCTTC**TGCAGTGG; x-strand, CCACTGCA**GAAGA**GCCGTTGCTAGGACGGG.

Junction 7rot used for comparative gel electrophoresis. This junction is related to junction 7 by a 90° rotation of the central two base pairs on each arm. This comprised four oligonucleotides each of 80 nt in length. Restriction sites used for electrophoretic analysis are highlighted in boldface type: b-strand, CGCAAGCGACAGGAACCTCGAG**GGATCC**GTCCTAGCAACGCGCTGCTACCGG**AAGCTT**CTCGAGGTTCCTGTCGCTTGCG; h-strand, CGCAAGCGACAGGAACCTCGAG**AAGCTT**CCGGTAGCAGCGGCAGCGGTGGTT**GAATTC**CTCGAGGTTCCTGTCGCTTGCG; r-strand, CGCAAGCGACAGGAACCTCGAG**GAATTC**AACCACCGCTGCGCTCAACTGCAG**TCTAGA**CTCGAGGTTCCTGTCGCTTGCG; x-strand, CGCAAGCGACAGGAACCTCGAG**TCTAGA**CTGCAGTTGAGCCGTTGCTAGGAC**GGATCC**CTCGAGGTTCCTGTCGCTTGCG.

Preparation of Radioactively-Labeled Four-Way DNA Junctions. A mixture containing equimolar quantities of the four strands (one or more of which were radioactively labeled) was heated at 90 °C for 5 min in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT followed by slow cooling to 4 °C. The junction species were purified by native gel electrophoresis in polyacrylamide and isolated by electroelution into 8 M ammonium acetate carried at 4 °C. Junctions were recovered by ethanol precipitation.

Native Gel Electrophoresis of Four-Way Junctions. Junction species were loaded onto 8% polyacrylamide gels (29:1 monomer:bisacrylamide) in a final concentration of 6% w/v ficoll. Electrophoresis was carried out at 5 V/cm at room temperature. The buffer system contained 90 mM Tris-borate (pH 8.3) with either 2.5 mM EDTA or the salt solutions indicated in the text. The electrophoresis buffer was recirculated between the cathodic and anodic reservoirs at a rate of 1 L/h. Polyacrylamide gels were dried onto Whatman 3MM paper and subjected to autoradiography at −70 °C using Ilford fast tungstate intensifier screens and Konika X-ray film.

MboII Restriction Enzyme Cleavage. Five picomoles of DNA junction (radioactively 5′-^{32}P-labeled on one strand) was incubated with 5 units of *Mbo*II (New England Biolabs) in 10 μL volumes containing 10 mM Tris-HCl (pH 7.9), 1 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT at 20 °C. For kinetic

studies, 2 μL aliquots were removed at various times and the reaction was stopped by addition of formamide and immediate freezing in liquid nitrogen. At the end of the incubation period, all DNA aliquots were denatured at 90 °C for 3 min, cooled on ice, and loaded onto a 15% polyacrylamide sequencing gel. Electrophoresis was carried out in 90 mM Tris-borate (pH 8.3), 10 mM EDTA, 7 M urea, and gels were run hot to the touch. Gels were exposed to storage phosphor screens, and quantification of radioactivity was performed using a phosphorimager (Fuji).

Osmium Tetroxide Modification. Osmium tetroxide was dissolved in distilled water as a 20 mM stock solution. Purified junctions were incubated in 5 mM Tris-HCl (pH 8.0) containing either 0.5 mM EDTA or 25 μM $MgCl_2$ with 1 mM osmium tetroxide, 3% pyridine (final concentration) at room temperature. Reactions were performed in a 50 μL final volume at room temperature for 15 min. After ethanol precipitation, the modified DNA was redissolved in 100 μL of 1 M piperidine, heated at 90 °C for 30 min, and then lyophilized.

Cytosine-Specific Chemical Cleavage. A cytosine-specific sequencing ladder was generated from a four-way junction radioactively 5′-^{32}P-labeled in one strand using modification by hydrazine, followed by base cleavage in piperidine (*32*).

RESULTS

Comparative Gel Electrophoretic Analysis of the Global Conformation of Four-Way DNA Junctions. We have established that comparative gel electrophoresis is a rapid yet informative way in which to analyze the global structure of four-way DNA junctions (*19*, *24*, *33*). In this method we begin with a four-way junction constructed from four oligonucleotides, each of 80 nt (called b, h, r, and x), generating a junction comprising four arms each of 40 bp in length (called B, H, R, and X in order around the center of the junction). The sequences are chosen to create nondegenerate base pairing, forming a junction that cannot undergo branch migration. In addition, each arm contains a unique restriction site approximately one turn of helix from the point of strand exchange, by which a selected arm can be specifically shortened. Restriction digests are carried out in a pairwise manner to produce the six possible species having two long and two shortened arms. These are named according to the long arms; thus the species BH has long B and H arms and shortened R and X arms. The electrophoretic mobility in polyacrylamide gels is compared for these six species, loaded in the order BH, BR, BX, HR, HX, and RX. Both theory and experiment lead us to expect that the relative mobilities will depend on the angle subtended between the two long arms, and thus the global shape may be deduced from the pattern of mobilities obtained.

In previous studies of four-way DNA junctions, we have found that we observe two kinds of pattern of electrophoretic mobility (*19*). In the absence of added metal ions, the pattern can be described by slow, fast, slow, slow, fast, slow, which is consistent with an unstacked structure where the B, H, R, and X arms extend toward the corners of a square (Figure 1) (*19*, *34*). This structure is also consistent with studies by fluorescence resonance energy transfer (FRET) under similar conditions (*20*). Upon addition of metal ions, such as 200 μM magnesium ions, a new pattern is generally observed,

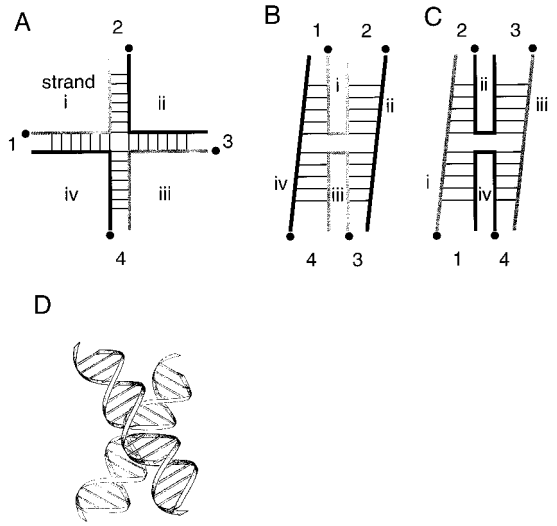

FIGURE 1: The four-way DNA junction. (A) Schematic to show the construction of a four-way junction from four separate DNA strands. (B, C) In the presence of divalent metal ions the four-way junction can fold by pairwise coaxial stacking of helical arms. There are two alternative ways in which this can occur, depending on the choice of stacking partner. One conformer (B) is formed by the stacking of arm 1 on 4 and arm 2 on 3. The alternative conformer (C) is formed by stacking arm 2 on 1 and arm 3 on 4. The two conformers are not equivalent at the level of base sequence. The formation of the stacked X-structure generates two different types of strand, exchanging and continuous. It can be seen that the bold strands are continuous in the conformer depicted in (B), but become exchanging strands in that shown in (C). (D) A ribbon model of the four-way junction (*21*). This view is rotated 90° with respect to that depicted in (B) and (C).

consisting of two slow, two fast, and two intermediate species. This is interpreted in terms of the formation of a stacked X-structure, where the arms are coaxially stacked in pairs and the axes rotated in an antiparallel manner. There are two stereochemically-equivalent conformers of this structure, depending on the choice of helical stacking partners. One conformer (called *isoI*) is based on B-on-H and R-on-X stacking. It gives a pattern of mobilities described by fast, intermediate, slow, slow, intermediate, fast (that is, the linear long-arm species are BH and RX, the acute angle species are BX and HR, and the obtuse angle species are BR and HX). The alternative conformer (*isoII*) gives an inverted pattern of mobilities described by slow, intermediate, fast, fast, intermediate, slow and is based on B-on-X and H-on-R stacking. These stacked X-structures have been confirmed by FRET studies (*21*, *35*) and are consistent with data from transient electric birefringence (*25*) and probing studies (*23*, *30*, *36*, *37*). In general, we have found that most four-way DNA junctions of different sequence exhibit one or the other of the two electrophoretic patterns, consistent with predominant formation of the stacked X-structure in either *isoI* or *isoII* conformations.

Analysis of a New Four-Way DNA Junction. In the course of our studies, we have identified one sequence that appears to depart from the above generalization. The central sequence of the junction is presented in Figure 2. All four base pairs at the point of strand exchange are C·G pairs, yet branch migration is not possible for this sequence; in fact, there is only one arrangement that fulfills this criterion. Despite the simplicity of this sequence, the potential stacking conformers are not equivalent because the *isoI* conformer

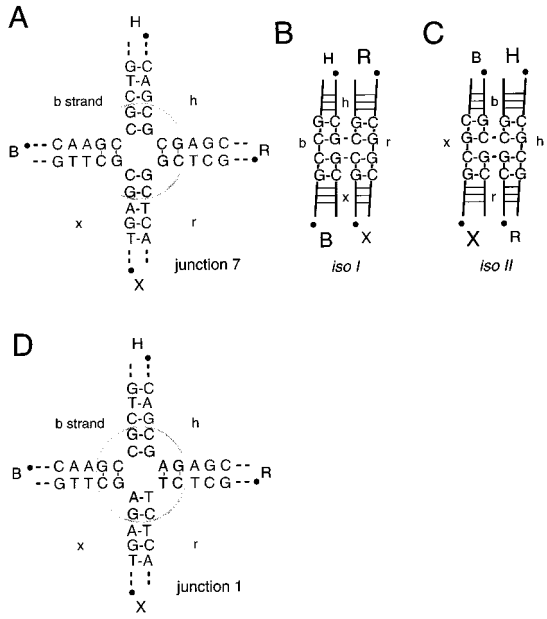

FIGURE 2: The sequence of junction 7. (A) The central base sequence of junction 7, with the novel sequence ringed. The junction comprises four strands b, h, r, and x, with 5′ ends indicated by •. These form four helical arms named B, H, R, and X; in the junction 7 used for the comparative gel electrophoretic analysis, these contain *Bam*HI, *Hin*dIII, *Eco*RI, and *Xba*I sites, respectively. (B, C) The two alternative stacking conformers, showing the different stacking partners at the center. (D) The central sequence of junction 1. The base sequence beyond the central two base pairs of each arm is identical to junction 7.

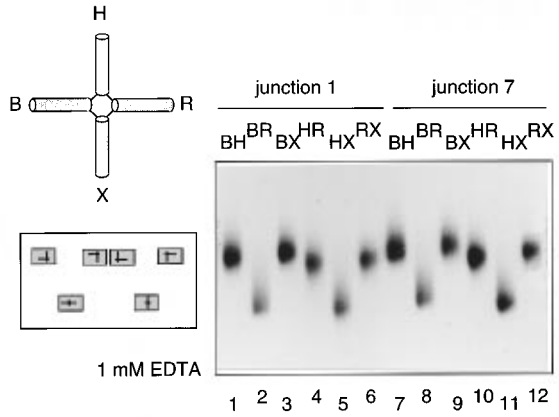

FIGURE 3: Comparative gel electrophoretic analysis of the global structure of junction 7 in the absence of added metal ions. Junctions with four arms each of 40 bp in length were subjected to pairwise restriction digests to generate the six possible species with two long and two short arms. The names of the species refer to the two long arms. These were electrophoresed in 8% polyacrylamide in the presence of 90 mM Tris borate (pH 8.3), 1 mM EDTA. Both junctions 1 and 7 give a pattern described by slow, fast, slow, slow, fast, slow. This is consistent with the four-fold extended structure indicated diagrammatically. Restriction digests of this structure lead to the six species shown on the left, where the species with long arms subtending 90° are slow, while the two species with long arms subtending 180° are faster. Tracks 1 and 7, species with long B and H arms; tracks 2 and 8, species with long B and R arms; tracks 3 and 9, species with long B and X arms; tracks 4 and 10, species with long H and R arms; tracks 5 and 11, species with long H and X arms; tracks 6 and 12, species with long R and X arms. Tracks 1−6, junction 1; tracks 7−12, junction 7.

places CpC (b strand) and GpG (r strand) sequences on the continuous strands, while these become CpG (x strand) and GpC (h strand) in the *isoII* form. We constructed a junction, called junction 7, with four arms each of 40 bp in length and the central sequence shown in Figure 2. Beyond the central region (the 2 bp of each helix adjacent to the point of strand exchange), the arms had the same sequence as those studied by us previously (including junction 1 below) (*19*) and thus contained the usual *Bam*HI, *Hin*dIII, *Eco*RI, and *Xba*I sites by which they could be selectively shortened.

An Extended Structure in the Absence of Added Metal Ions. Comparative gel electrophoresis was performed on junction 7 and in parallel on junction 1 (*19*), which adopts the *isoI* stacking isomer predominantly. The six possible two-long-arm, two-short-arm species for each junction were electrophoresed in a polyacrylamide gel under different ionic conditions. In the presence of low salt concentrations, both junctions exhibited the slow, fast, slow, slow, fast, slow pattern indicative of the extended square structure. This was true in the absence of added ions (90 mM Tris-borate (pH 8.3), 1 mM EDTA) (Figure 3), or in the presence of 20 mM Na^+ ions (data not shown). Thus, neither junction becomes folded under these low salt conditions.

Global Structure in the Presence of Mg^{2+} Ions. The comparative gel electrophoresis was repeated in the presence of 200 μM magnesium ions (Figure 4A). As expected, junction 1 exhibits a fast, intermediate, slow, slow, intermediate, fast pattern consistent with folding into the stacked X-conformation in the *isoI* (i.e., B-on-H stacking) conformation. By contrast, under the same conditions the six long−short species of junction 7 have closely similar mobility in polyacrylamide. This would be consistent with a tetrahedral disposition of the four arms, but is more likely to represent an averaging due to fast exchange between different forms. This is illustrated schematically in Figure 4C. If junction 7 were to adopt the stacked X-structure in both *isoI* and *isoII* forms, where these conformers were equally populated and exchange between the two forms was fast compared to the gel process, this would be expected to give a pattern of six equal mobilities. In this case, the averaged mobility would be expected to be intermediate between that of the fast (linear) and slow (acute angle) species; inspection of Figure 4A shows this to be true when we compare the mobility of the six species of junction 7 with the individual species of junction 1. As the magnesium concentration was raised to 1 mM, the species BH and RX of junction 7 exhibited very slightly slower mobilities (Figure 4B) which were accentuated at still higher magnesium ion concentrations (data not shown). This might reflect a bias in the equilibrium toward the *isoII* (B-on-X stacking) under these conditions.

Global Structure in the Presence of Hexammine Co(III) Ions. Hexammine cobalt(III) ions are most efficient in promoting the folding of four-way DNA junctions. We have observed that a concentration of 2 μM $[(NH_3)_6Co]^{3+}$ ions is sufficient to induce the folding of junctions 1 and 3 into their stacked X-conformations (*34*). We therefore performed the comparative gel electrophoretic analysis on junction 7 in the presence of 10 μM $[(NH_3)_6Co]Cl_3$ (Figure 5). As expected, junction 1 exhibits a well-defined fast, intermediate, slow, slow, intermediate, fast pattern consistent with the *isoI* conformation under these conditions. To our surprise, junction 7 gives rise to a quite similar pattern, where the slowest species are clearly BX and HR. This suggests that the isomer bias is displaced toward the *isoI* structure by the

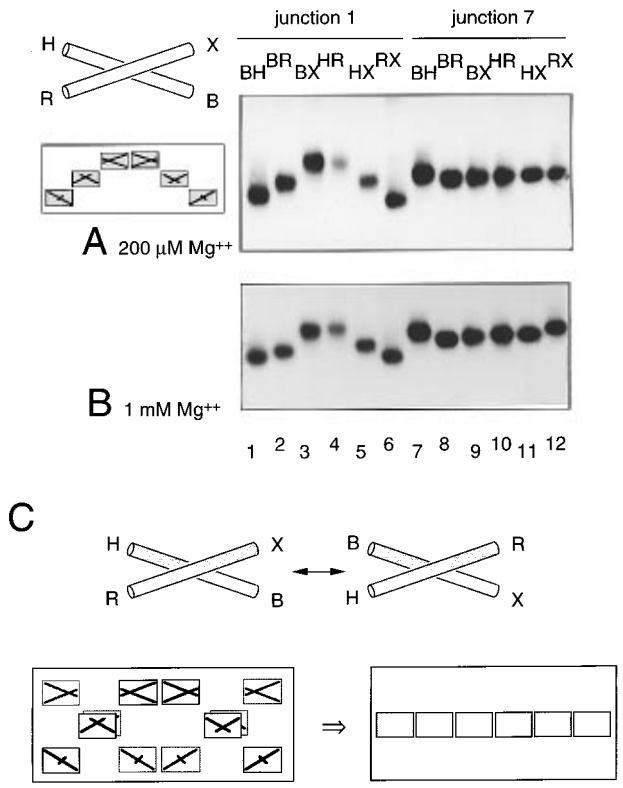

FIGURE 4: Comparative gel electrophoretic analysis of the global structure of junction 7 in the presence of magnesium ions. (A, B) The six two-long, two-short arms species of junction 1 and 7 were electrophoresed in 8% polyacrylamide in the presence of 90 mM Tris·borate (pH 8.3) plus either 200 μM (A) or 1 mM (B) $MgCl_2$. Junction 1 folds into a stacked X-structure, generating a fast, intermediate, slow, slow, intermediate, fast pattern of mobilities. This is consistent with the *isoI* conformation (B-on-H stacking), generating the six species indicated schematically at the left. By contrast, junction 7 gives a pattern of mobilities where each of the six species has the same electrophoretic mobility. At the higher magnesium ion concentration, species BH and RX exhibit a slightly retarded mobility, indicating a faint bias toward an *isoII* conformation. (C) Schematic to show how a rapid averaging of the *isoI* and *isoII* conformations would lead to six species with closely similar mobility, with a value close to that of the intermediate species for one conformer. Tracks 1 and 7, species with long B and H arms; tracks 2 and 8, species with long B and R arms; tracks 3 and 9, species with long B and X arms; tracks 4 and 10, species with long H and R arms; tracks 5 and 11, species with long H and X arms; tracks 6 and 12, species with long R and X arms. Tracks 1−6, junction 1; tracks 7−12, junction 7.

presence of hexammine cobalt(III) ions. The amplitude of the mobility pattern for junction 7 is smaller than that of junction 1, indicating that the displacement of the conformational equilibrium is not complete.

Osmium Tetroxide Probing of Junction 7: Evidence for Ion-Induced Stacking of the Junction. In principle, equal mobilities of the six long−short species of junction 7 might be interpreted in terms of an unstacked structure with tetrahedral symmetry. We have previously used osmium tetroxide probing of thymine bases immediately at the point of strand exchange of junctions as a means of reporting on the degree of stacking. In general, such bases are found to be reactive under low salt conditions where the junction is in its open conformation, but they become protected against attack as the junction folds upon addition of magnesium ions (*19*, *34*). This results from the requirement for an out-of-plane attack on the 5−6 double bond of thymine, which is

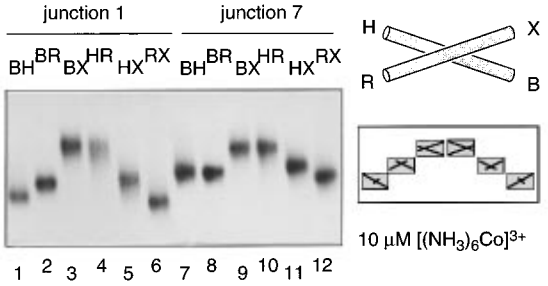

FIGURE 5: Comparative gel electrophoretic analysis of the global structure of junction 7 in the presence of hexammine cobalt ions. The six two-long, two-short arms species of junction 1 and 7 were electrophoresed in 8% polyacrylamide in the presence of 90 mM Tris·borate (pH 8.3), 10 μM $[(NH_3)_6Co]Cl_3$. Junction 1 exhibits the electrophoretic mobility pattern indicative of the *isoI* structure. Junction 7 also exhibits a similar pattern of mobilities, indicating a pronounced bias toward the *isoI* structure under these conditions. The overall amplitude of the mobility variation is lower for junction 7, however. Tracks 1 and 7, species with long B and H arms; tracks 2 and 8, species with long B and R arms; tracks 3 and 9, species with long B and X arms; tracks 4 and 10, species with long H and R arms; tracks 5 and 11, species with long H and X arms; tracks 6 and 12, species with long R and X arms. Tracks 1−6, junction 1; tracks 7−12, junction 7.

severely hindered when the base is stacked into the quasi-continuous helix in the stacked X-structure. Since junction 7 has only C·G base pairs surrounding the point of strand exchange, this method cannot be applied directly. However, we reasoned that if a cytosine base were replaced by 5-methylcytosine, the inductive effect of the methyl group could increase the reactivity to osmium addition. We have previously shown that 5-methylation of two cytosine bases in a junction had no detectable conformational consequences (*38*).

Individual substitution experiments indicated that methylation of the cytosine 5′ to the point of strand exchange on the b strand of junction 7 leads to reactivity toward osmium tetroxide under low salt conditions (Figure 6), whereas the same strand incorporated into a duplex is unreactive. Thus under these conditions the junction is sufficiently open for the probe to gain access to this base, in agreement with the results of comparative gel electrophoresis (Figure 3). Upon addition of magnesium ions, this reactivity is strongly suppressed. This is consistent with a folding into a stacked conformation and argues against an open tetrahedral form.

MboII Cleavage of Junction 7: Evidence for Coaxial Stacking. To investigate the presence of coaxial stacking of arms we have used the restriction enzyme *Mbo*II, previously used to study other four-way DNA junctions (*30*). *Mbo*II is one of the class of restriction enzymes where the cleavage occurs at a significant distance from the recognition sequence, within the contiguous duplex. It is therefore possible to arrange that the target sequence for recognition and the potential cleavage site are located in different arms, such that successful cleavage implies a coaxial alignment of the two arms to generate a quasi-continuous duplex. In a previous set of experiments (*30*), we placed the recognition sequence for *Mbo*II in the X arm of junction 1 and observed cleavage in the h and r strands within the R arm, consistent with coaxial stacking of the R and X arms. We found that the kinetics of cleavage of the h strand were considerably faster than those of the r strand, which was rationalized in

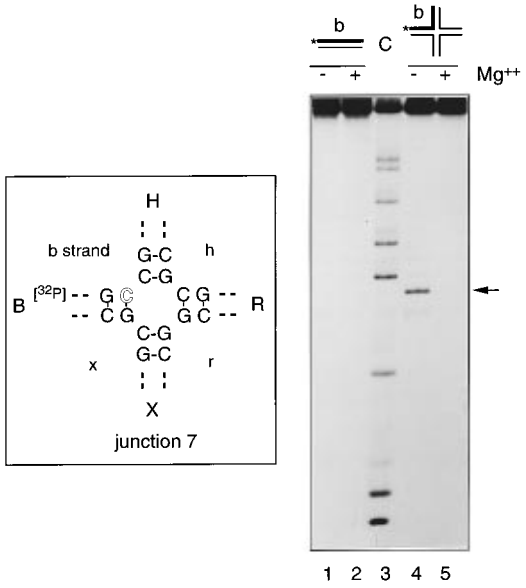

FIGURE 6: Chemical probing of base stacking at the point of strand exchange of junction 7. A modified version of junction 7 was synthesized, in which cytosine was substituted by 5-methylcytosine in the b strand at the position indicated in open type. The junction was prepared radioactively 5′-^{32}P-labeled on the b strand, and the same strand was also hybridized to its complement to generate a duplex species for comparison. Each was subjected to modification by osmium tetroxide, pyridine in the presence and absence of 25 μM $MgCl_2$. After base cleavage of osmium adducts, the products were separated by electrophoresis and visualized by autoradiography. It can be seen that the 5-methylcytosine is unreactive under all conditions when stacked into a regular duplex. However, when it is placed at the point of strand exchange in junction 7, it becomes reactive (position of cleavage arrowed on right) under conditions where the junction is known to adopt the extended structure. Upon addition of magnesium ions, it is protected against chemical attack. This is consistent with the formation of a stacked structure at the junction. To serve as a sequence marker, the same junction species was subjected to a C-specific chemical cleavage by hydrazine followed by piperidine cleavage. The 5-methylcytosine is not modified in this reaction and therefore does not appear on the ladder of bands generated. Track 1, duplex modified in the presence of 0.5 mM EDTA; track 2, duplex modified in the presence of 25 μM $MgCl_2$; track 3, C-specific chemical cleavage ladder; track 4, junction 7 modified in the presence of 0.5 mM EDTA; track 5, junction 7 modified in the presence of 25 μM $MgCl_2$.

terms of the greater accessibility of this site on the outer face of the stacked X-structure.

We constructed a new four-way DNA junction based upon the central sequence of junction 7, containing the *Mbo*II recognition sequence in the X arm (Junction 7XMbo Figure 7A). By good fortune, the *Mbo*II site can be introduced without changing any base sequence nearer than 4 bp away from the point of strand exchange. Comparative gel electrophoretic analysis confirmed that the new junction 7XMbo gave an electrophoretic pattern comprising six species of closely similar mobility, similar to the original junction 7 (data not shown). We have also shown that the ionic conditions used for *Mbo*II cleavage (1 mM Mg^{2+} and 50 mM Na^+ ions) do not alter the electrophoretic pattern of junction 7 (data not shown).

As in the corresponding *Mbo*II study of junction 1 (*30*), it is expected that formation of the *isoI* stacked X-structure will lead to coaxial alignment of R and X arms, and hence cleavage on the h strand, with perhaps weak cleavage on the r strand. Experimentally, we found that digestion of junction 7 with *Mbo*II did indeed lead to a major cleavage in the h strand, at the position expected if the X and R arms behaved like a simple uninterrupted duplex (Figure 7B). The cleavage occurred at the same position as that in a duplex comprising the r strand hybridized to its complement, at an initial rate that was close to half that on the duplex species digested under standard conditions, despite the fact that the cleaved strand is not covalently continuous with the binding target. The r strand was cleaved only very weakly in the junction, suggesting a steric protection due to the proximity of the H arm in the stacked X-structure. These results are consistent with a conformation of junction 7 based on pairwise coaxial stacking of arms in the *isoI* conformation. With extensive digestion by *Mbo*II, the h strand could be cleaved to 100% (Figure 7C), suggesting that all the molecules of junction 7 adopt the *isoI* conformation at some stage of the incubation.

It might be anticipated that the existence of a population of junction 7XMbo in the alternative *isoII* stacking conformer, where there is B-upon-X stacking (Figure 7), might lead to cleavage of the b and x strands. Yet we detected almost no cleavage of the b or x strands in this experiment, although extensive *Mbo*II cleavage did lead to a small degree of cleavage at the expected site in the x strand (data not shown). However, we have noted before (*30*) that the presentation of the binding site will be different in the two conformers. This can be seen by noting that the GAAGA strand of the *Mbo*II recognition sequence is present on the exchanging strand in the *isoI* conformer, while it is located on the continuous strand in *isoII*. Inspection of a model of the stacked X-structure suggests that the major groove at the center of the *Mbo*II site should be much more accessible in the *isoI* structure. Therefore it is not meaningful to compare rates of cleavage in the two conformers, and no conclusions about relative populations can be drawn from the relative cleavage frequencies within a single junction species.

Exchange between Stacking Conformers. We constructed a variant of junction 7XMbo, in which the central sequence comprising the 2 bp of each arm around the point of strand exchange were effectively "rotated" in the context of the remaining sequence (Figure 8A). The purpose behind this construction was to reposition the *Mbo*II site such that the cleavage might now detect the alternative stacking conformer (*isoII*), where the recognition target would now be in a stereochemical situation equivalent to the *Mbo*II site of junction 7XMbo in the *isoI* conformation. The naming of arms and strands in junctions rotated in this manner refer to the central sequence (rather than the outer arms), with primes added to distinguish these from junction 7. The rotated form of the *Mbo*II-containing junction is called 7B′Mbo because the *Mbo*II site is now present in the B′ arm.

The *Mbo*II cleavage results are presented in Figure 8B. There is strong cleavage of the r′ strand of junction 7B′Mbo, as expected if the *isoII* conformer is present. With extended *Mbo*II digestion the r′ strand is cleaved to 100%.

We carried out a control comparative gel electrophoresis analysis to examine the effect of rotating the center of junction 7 in the manner performed to generate junction 7B′Mbo. Junction 7rot is related to junction 7 by a 90° rotation of each arm beyond the central two base pairs on

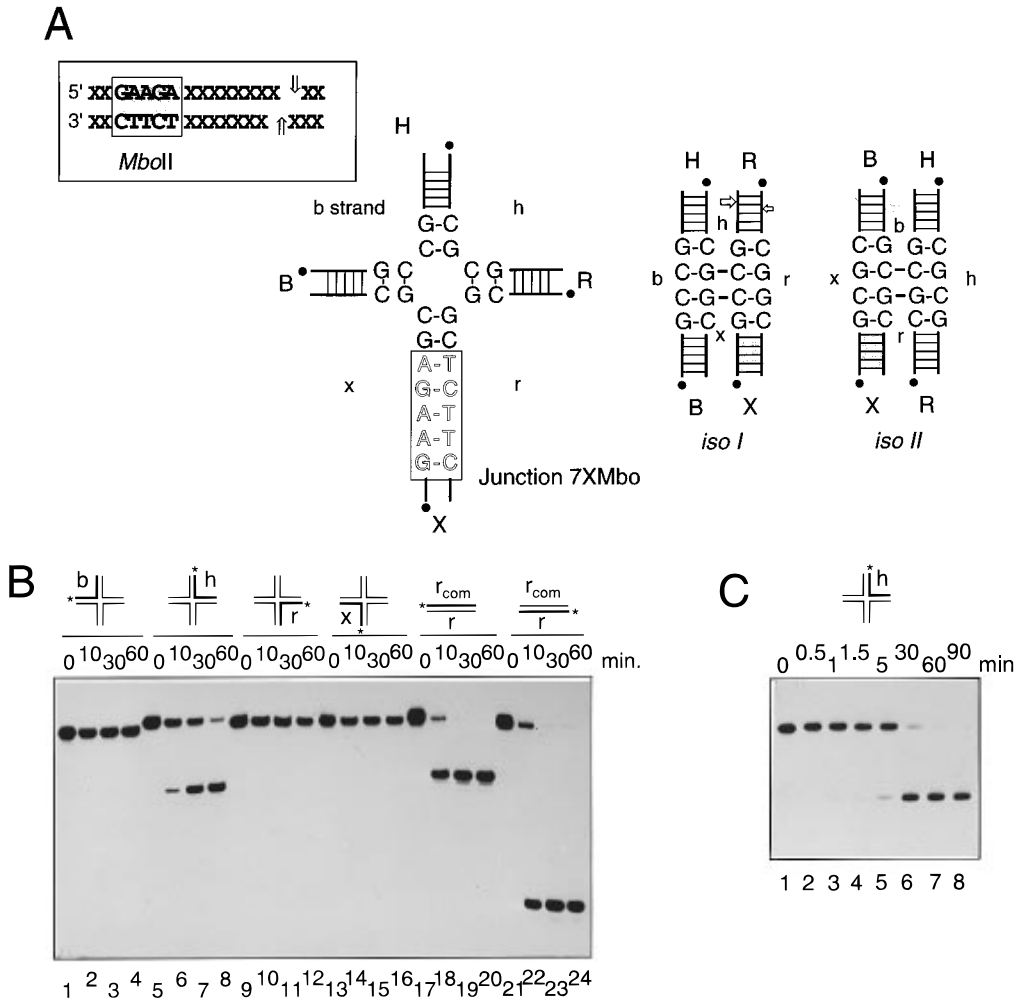

FIGURE 7: Probing coaxial helical stacking in junction 7 using *Mbo*II cleavage. (A) *Mbo*II recognizes the target sequence GAAGA (boxed) and cleaves 8/7 nucleotides away in the manner indicated by the arrows. Junction 7XMbo was constructed to introduce an *Mbo*II recognition site (boxed) into the X arm. Comparison with the sequence of junction 7 (Figure 1) shows that the first four base pairs of the X arm are unchanged by this alteration. In the *isoI* conformation of the junction, the X arm (with the *Mbo*II site stippled) is stacked with the R arm. If these arms function as a continuous duplex, *Mbo*II cleavages would be anticipated to occur in the h and r strands at the positions marked with arrows. Previous experience (*30*) shows that the accessibility of the strands is h >> r in a junction. The alternative *isoII* conformation would place the potential *Mbo*II cleavage sites in the b and x strands; however, these are expected to occur very much less efficiently due to the less favourable presentation of the recognition target. (B) *Mbo*II cleavage of junction 7XMbo and a representative duplex made by complementing the r strand. Four versions of junction 7XMbo were made, in which a single strand was radioactively 5′-^{32}P-labeled (highlighted bold, with * indicating the labeled 5′ terminus). Each junction or duplex was incubated with 5 units of *Mbo*II at 20 °C for increasing times, and the products of digestion analyzed by gel electrophoresis and autoradiography. It is clear that the h strand of junction 7XMbo is efficiently cleaved by *Mbo*II, at the same site as found in the perfect duplex. No cleavage can be seen in the r strand, although very extensive digestion with *Mbo*II does lead to a small amount of cleavage at the expected site. Tracks 1−4, cleavage of junction 7XMbo radioactively 5′-^{32}P-labeled on the b strand (incubation times 0, 10, 30, and 60 min, respectively); tracks 5−8, cleavage of junction 7XMbo radioactively 5′-^{32}P-labeled on the h strand (0, 10, 30, and 60 min.); tracks 9−12, cleavage of junction 7XMbo labeled on the r strand (0, 10, 30, and 60 min); tracks 13−16, cleavage of junction 7XMbo labeled on the x strand (0, 10, 30, and 60 min); tracks 17−20, cleavage of duplex labeled on the r-complement strand (0, 10, 30 and 60 min.); tracks 21−24, cleavage of duplex labeled on the r strand (0, 10, 30, and 60 min). (C) Extended digestion of junction 7XMbo radioactively 5′-^{32}P-labeled on the h strand, using 10 units of *Mbo*II. Note that the h strand becomes 100% cleaved in the course of the incubation. Tracks 1−8, digestion with *Mbo*II for 0, 30, 60, 90 s, and 5, 30, 60, 90 min, respectively.

each arm (Figure 8C). We made the six two-long-arm, two-short-arm species of junction 7rot and compared their electrophoretic mobilities in 8% polyacrylamide in the presence of 200 μM magnesium ions, alongside the equivalent species derived from junction 7 for comparison (Figure 8D). We found that unlike junction 7, where each of the long−short species has equal electrophoretic mobility under these conditions, junction 7rot exhibits a pronounced fast,-intermediate,slow,slow,intermediate,fast pattern of mobilities indicative of the formation of the stacked X-structure in an *isoI* conformation. Thus while the comparative gel electrophoretic analysis shows that junction 7rot exists predominantly in the *isoI* stacked conformation, the total cleavage by *Mbo*II of 7B′Mbo is consistent with a complete population in the *isoII* conformation. The only way in which to rationalize these apparently contradictory observations is to postulate an exchange process between stacking conformers such that the minor conformer is available to the restriction enzyme in all junction molecules during the time of incubation.

Next-Nearest Neighbor Influence on Junction Conformation. The comparative gel electrophoresis of Figure 8D

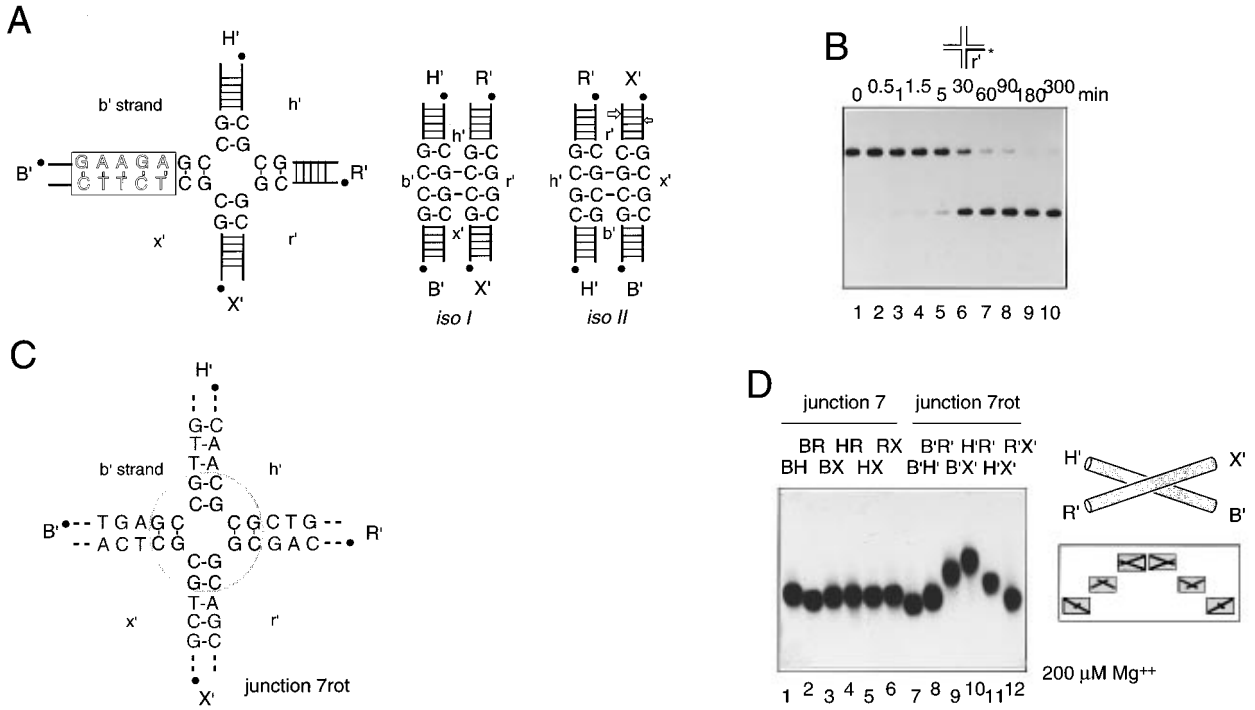

FIGURE 8: Analysis of a variant of junction 7 with a rotated base sequence. (B) A variant of junction 7XMbo was generated by effectively rotating the sequence of the four arms clockwise around the core of the junction comprising the innermost two base pairs on each arm. The arms and strands are labeled according to the inner core, with primes added to denote the difference. In this way the *Mbo*II site is now transferred to the B′ arm, and the junction is therefore named junction 7B′Mbo. The recognition target should now be optimally presented in the *isoII* conformation, when the B′ and X′ arms are coaxially stacked, and this should lead to strong cleavage on the r′ strand. (B) Junction 7B′Mbo radioactively 5′-^{32}P-labeled on the r′ strand was incubated with 10 units of *Mbo*II at 20 °C for increasing times, and the products analyzed by gel electrophoresis and autoradiography. There is a clear product of *Mbo*II cleavage on the r′ strand, and cleavage is complete beyond 90 min incubation. Tracks 1−10, digestion with *Mbo*II for 0, 30, 60, 90 s, 5, 30, 60, 90 min and 3, 5 h. respectively. (C) The central sequence of a rotated variant of junction 7. Junction 7rot was generated from junction 7 by a clockwise rotation about the central core in the same manner as the construction of junction 7B′Mbo. The arms and strands are labeled according to the inner core, distinguished by primes. Note that this junction is identical to junction 7 over the core of two base pairs on each arm (circled). This junction comprised four arms each of 40 bp, to permit analysis by comparative gel electrophoresis. (D) Analysis of the global structure of junction 7rot by comparative gel electrophoresis. The six two-long, two-short arm species of junction 7 and 7rot were electrophoresed in 8% polyacrylamide in the presence of 90 mM Tris·borate (pH 8.3), 200 μM $MgCl_2$. As before, junction 7 gives rise to six species of closely similar mobility. By contrast, junction 7rot gives a fast, intermediate, slow, slow, intermediate, fast pattern of electrophoretic mobilities, consistent with a stacking conformer population that is biased toward the *isoI* conformation (B′ on H′ stacking). This is indicated schematically on the right. Track 1, junction 7 with long B and H arms; track 2, junction 7 with long B and R arms; track 3, junction 7 with long B and X arms; track 4, junction 7 with long H and R arms; track 5, junction 7 with long H and X arms; track 6, junction 7 with long R and X arms; track 7, junction 7rot with long B′ and H′ arms; track 8, junction 7rot with long B′ and R′ arms; track 9, junction 7rot with long B′ and X′ arms; track 10, junction 7rot with long H′ and R′ arms; track 11, junction 7rot with long H′ and X′ arms; track 12, junction 7rot with long R′ and X′ arms.

reveals that the conformational equilibrium is markedly different between junction 7 and junction 7rot. Yet these two junctions differ only in the third base pairs out from the point of strand exchange. Clearly, therefore, sequences at the third position can significantly influence the relative stabilities of stacking conformers.

DISCUSSION

At low salt concentrations, junction 7 exists in a square, unstacked conformation, in common with all other four-way junctions studied in this laboratory (*19, 20*). Upon addition of magnesium ions, it undergoes a folding transition to adopt a structure(s) that results in six long−short arm species of equal mobility. This could reflect a single structure with tetrahedral symmetry, or more probably an equilibrium between the two possible stacked X-structures. The latter is consistent with the protection of 5-methylcytosine bases at the point of strand exchange against modification by osmium tetroxide and the cleavage across the junction by *Mbo*II. These would be the expected results for a mixture of stacked X-structure forms, where the helix−helix stacking protects the bases against out-of-plane attack by osmium tetroxide, and the coaxial alignment of helices generates a colinear substrate for *Mbo*II. By contrast, a tetrahedral structure precludes pairwise coaxial stacking of helices. It would be expected to leave the central bases susceptible to attack by osmium tetroxide and change the pattern of cleavage by *Mbo*II due to the disruption of colinearity of recognition and cleavage sites.

Thus the most reasonable interpretation of our data suggests that junction 7 folds into the stacked X-structure, but unlike junctions of different sequence it appears that the helical arms do not exert a well-defined bias in their choice of stacking partners. Instead, the results indicate that both stacking conformers are present in about equal proportion. The ability to obtain complete *Mbo*II cleavage of the h strand in junction 7XMbo indicates that there is a process by which the stacking conformers interconvert, such that all the molecules of the junction pass through the *isoI* stacking conformer during the time of incubation with the restriction

enzyme. This exchange must be fast with respect to the equilibration in the gel pores, because the bands seen in the comparative gel electrophoretic analysis are no broader for junction 7 than for other junctions. Even where junctions predominantly adopt a single stacking conformer, interconversion between the major and minor stacking conformers evidently occurs. This is clearly demonstrated by comparing the *Mbo*II cleavage of junction J7B′Mbo, where we can obtain 100% cleavage in the r′ strand corresponding to the *isoII* conformer, with the comparative gel electrophoretic analysis that indicates that the population is predominantly in the *isoI* conformation. In an earlier study using *Mbo*II cleavage, we also obtained evidence for a population of the alternative stacking isomer in the case of junction 1 (*30*).

Interconversion between stacking conformers has also been demonstrated in other recent biochemical experiments (*39*). While this paper was being written, we learned of a new study by Millar, Chazin, and co-workers that provides physical evidence for the coexistence of alternative stacking conformers of two four-way junctions, of different sequence from that studied here (*40*). Using deconvolution of time-resolved fluorescence spectra, they could obtain evidence for two populations of conformations with different end-to-end distances, the relative proportion of which depended upon base sequence. Selective introduction of ^{15}N-labeled thymine at the point of strand exchange provided direct evidence for two different environments, assumed to be the two stacking conformers. Some limits can be set on the rate of interconversion between the conformers. The observation of two narrow ^{1}H and ^{15}N NMR resonances indicates that exchange between the two conformers must be slow compared to the NMR time scale (in the millisecond time range), though fast compared to the processes of gel electrophoresis.

While the populations of the two stacking conformers for junction 7 appear to be closely similar, this can be biased in either direction by changes in ionic composition. Thus in elevated magnesium ion concentration, the comparative gel electrophoretic pattern is consistent with a small excess of the *iso*II conformation. By contrast, in the presence of hexammine cobalt(III) ions the population bias is significantly toward the *iso*I conformation. This difference must reflect the different character of these ions. The hydrated magnesium ion is hard, and probably occupies a binding site(s) close to the point of strand exchange where it can interact with phosphate oxygen atoms (*41*). Hexammine cobalt(III) is very efficient in folding four-way DNA junctions at a concentration two orders of magnitude lower than that required for magnesium ions (*34*), but the ammine ligands are very effective hydrogen bond donors and the ion will therefore make different interactions with the junction. The latter character will probably result in different base sequence preferences, and thus a different relative stabilization of the stacking conformers.

These results suggest that the differences in free energy between the stacking conformers can be small, especially in the case of junction 7. Indeed, since the methods used to analyze the global conformation of four-way junctions in general detect the major species present, even a difference in free energy of as little as 2 kcal mol^{-1} would probably suffice to render the less abundant conformer undetected. Perhaps the most surprising result is that sequence changes that are three base pairs from the point of strand exchange can significantly influence this conformational equilibrium. Miick et al. (*40*) found that changes that are two base pairs removed from the point of strand exchange can also influence the equilibrium. This clearly indicates that in comparing results between different studies we must examine the sequences of the junctions used out to at least the third position, and we have even detected minor effects caused by changes beyond this (unpublished data).

Exchange between alternative stacking conformers has been proposed to be an important step in the mechanism of at least one class of recombination event. In the integrase family of site-specific recombination reactions, one set of strand exchanges creates a four-way junction Holliday intermediate that becomes resolved in a second set of strand exchanges that are several base pairs distant from the first set (*6*−*10*). While this was originally thought to necessitate branch migration over the intervening duplex, a new model for integrative recombination in phage λ (*42*) has removed this apparent requirement. Instead, it is suggested that after the first strand cleavages the strands become displaced by a few nucleotides, thus locating the newly formed junction in the middle of the overlap region between cleavage sites. If this junction now undergoes an exchange of helical stacking partners, then the second set of strand exchanges are carried out in the same stereochemical environment as the first set, and the mechanism becomes symmetrical. It has recently been shown that perturbation of stacking partner choice in the junction either by local sequence changes (in the phage λ system, ref *43*) or by tethering (in the Xer system, ref *44*) can influence the outcome of the recombination reaction.

Current evidence suggests that events like the integrase-family recombination are significantly driven by DNA structure, and it is apparent we can learn about aspects of their mechanisms by studying the structural propensity of the isolated DNA intermediates. It is clear that the four-way junction has a dynamic character, and exchange between stacking conformers can be exploited in DNA rearrangement mechanisms.

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