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Effect of Sequence on the Structure of Three-Arm DNA Junctions†

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ABSTRACT: We have investigated the geometry of a number of three-arm branched DNA molecules by measuring the relative electrophoretic mobilities of analogues of each junction in which one pair of arms is extended. In general, the mobilities of three species of three-arm junctions in which the duplex arms are extended pairwise differ in the presence of Mg^{2+}. This effect is eliminated by the absence of Mg^{2+} or by an increase in temperature, leading us to conclude that the three-arm DNA junctions are not 3-fold symmetric, because of either preferential stacking or asymmetric kinking of the arms at the branch that occurs in the presence of Mg^{2+}. The geometry of the junction is governed by the base sequence at the branch and 1 bp removed from the branch. The pairwise elongated analogues of junctions that contain identical base pairs at the branch or 1 bp from the branch show mobility differences; when both positions have the same sequence no mobility differences are detected even in the presence of Mg^{2+}. Formation of a branch in three-arm DNA junctions can be seen to produce a strain or deformation that propagates about one turn of the helix from the branch, leading thymines in this region to become hyperreactive to osmium tetraoxide. Surprisingly, the effect is independent of the presence or absence of metal cations. The structure of the three-arm junction is thus quite different in character from that of four-arm junctions both in the presence and absence of high concentrations of metal cations.

Certain states of DNA, such as Holliday branched recombination junctions (Holliday, 1964), are stable only transiently; these intermediates need to be trapped in order to avoid resolution of the system into two duplexes by the process of branch migration (Kim et al., 1972; Meselson & Radding, 1975; Warner et al., 1978). By use of symmetry-breaking sequences that are thermodynamically stable, the branch point in a four-arm model Holliday intermediate can be fully or partially immobilized, making it possible to investigate recombination intermediates formed from oligonucleotides (Seeman et al., 1989). Most attention has been directed so far at four-arm models of Holliday intermediates formed from two DNA duplexes (Kallenbach & Seeman, 1986; Cooper & Hagerman, 1987, 1989; Duckett et al., 1988; Murchie et al., 1989). However, three-arm structures have been found to arise as intermediates in general recombination in the absence of replication (Broker & Doermann, 1975; Minagawa et al., 1983; Jensch & Kemper, 1986), and one is naturally interested in the structural and substrate properties (Jensch & Kemper, 1986; Dickie et al., 1987) of these species as well. We have recently observed that the three-arm branched DNA molecule JL 1 forms an asymmetric structure at low temperature in the presence of Mg^{2+} (Guo et al., 1990a), using gel electrophoresis and footprinting experiments.

Gel electrophoresis has been widely used to investigate the properties of stable DNA junctions (Cooper & Hagerman, 1987; Duckett et al., 1988; Seeman et al., 1989). Cooper and Hagerman (1987) studied a four-arm synthetic junction by gel electrophoresis under native conditions, utilizing the fact that bent DNA molecules have reduced mobilities on polyacrylamide gels to an extent that is roughly proportional to the apparent bend angle (Koo et al., 1986). Cooper and Hagerman (1987) appended pairs of long duplex arms to a four-arm junction and observed different mobilities in the presence of Mg^{2+} depending on which pairs of arms were extended. These electrophoretic experiments suggest that the junction geometry is consistent with a stacked structure, roughly like a Sigal–Alberts model (Sigal & Alberts, 1972).

Chemical modification of DNA followed by strand scission at the modified sites is an effective way to detect unusual DNA conformations at the nucleotide level (Maxam & Gilbert, 1977; Peattie & Gilbert, 1980). Chemical probing of four-arm DNA

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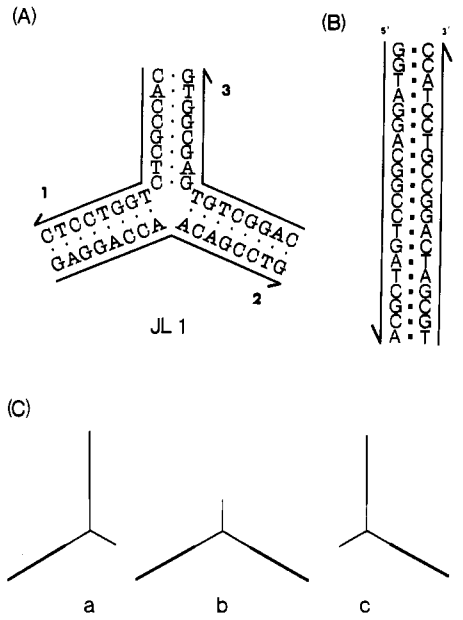

FIGURE 1: Analysis of a three-arm DNA junction JL 1. (A) Sequence of the three-arm junction JL 1. The junction is shown schematically and consists of three 16-mer strands of DNA. The 3′ ends of the strands are indicated by half-arrowheads. The strand numbering indicated is used throughout the text. (B) Sequence of the 20-bp molecule used for elongation of two arms of JL 1 in turn in electrophoretic mobility experiment. The top of the molecule indicated by 5′ and 3′ is the free end in the arm-extended junctions. (C) Outline of the construction of three possible species for analysis by polyacrylamide gel electrophoresis. Each species was assembled by the hybridization of one 56-mer and two 36-mer oligonucleotides, respectively, in which two of the three arms are each elongated by 20 bp.

junctions suggests that the base pairs flanking the branch remain paired in the presence of Mg^{2+} (Duckett et al., 1988, 1990; Lu et al., 1990a,b), consistent with the conclusion from nuclear magnetic resonance experiments (Wemmer et al., 1985). By contrast, the bases located at the branch of a three-arm DNA junction are more reactive to diethyl pyrocarbonate and osmium tetraoxide than duplex controls, indicating that weakening of base pairs or local opening occurs at this point (Duckett & Lilley, 1990; Guo et al., 1990a).

The question we address in this study is how the geometry of a three-arm junction depends on the sequence of base pairs at the branch. In four-arm junctions, the specific choice of double-helical partners for stacking in the two helical domains of a four-arm DNA junction is found to be governed only by the sequence of pairs flanking the branch (Chen et al., 1988; Duckett et al., 1988; Guo et al., 1991). Substitutions at other sites do not influence the structure (Duckett et al., 1988). Since a three-arm branched DNA molecule (JL 1) is found to form an asymmetric structure in the presence of Mg^{2+} (Guo et al., 1990a), we would like to know if and how the sequence flanking the branch of JL 1 influences its molecular geometry. We show here that the structure of three-arm DNA junctions is determined by both the base pairs that flank the branch and the pairs that are 1 bp removed from the branch. To study the structure we have measured the relative electrophoretic mobilities of three complexes for each sequence (Figure 1C), with a different pair of duplex arms extended in each complex (Cooper & Hagerman, 1987). These three complexes have different mobilities, an effect that is eliminated by the absence of Mg^{2+} or by raising the temperature, unless the two base pairs abutting the branch have the same sequence in each arm. Formation of a three-arm junction introduces some kind of strain or deformation at the branch that propagates one turn of helix from the branch, as detected by hyperreactivity of thymines in this region with osmium tetraoxide; this effect is observed both in the presence and absence of Mg^{2+}. On the other hand, the purine reagent diethyl pyrocarbonate is found to become reactive only to purines at the branch itself.

Materials and Methods

Synthesis and Purification of Oligonucleotides. Oligonucleotides used in these experiments were synthesized on an ABI 380B automated synthesizer and deprotected by routine phosphoramidite procedures (Caruthers, 1982). All strands 30 nucleotides long or less were purified by preparative HPLC on a Du Pont Zorbax Bio Series oligonucleotide column, following the manufacturer's recommended elution protocol. Longer strands were purified by polyacrylamide gel electrophoresis. Oligonucleotides were labeled at their 5′ termini by using T4 polynucleotide kinase (Boehringer); the labeled strands were purified by polyacrylamide gel electrophoresis. DNA concentrations in each solution were determined spectrophotometrically, with single-strand, 260-nm extinction (ϵ) at 80 °C, by using the nearest-neighbor values of Cantor et al. (1970).

Annealing Reactions. Annealing reactions were carried out by incubating stoichiometric amounts of the appropriate DNA strands for 10 min at 80 °C in 15 μL of 50 mM Tris·HCl (pH 7.5) with or without 5 mM $MgCl_2$ and then allowing them to cool slowly. In each annealing reaction only one of the oligonucleotides was radioactively labeled, giving three different species for each junction.

Gel Electrophoresis. Native gels (20%, 19:1 monomer:bis ratio) were run for 70 h at 80 V. The electrophoresis plates were jacketed and cooled with circulating water to provide a running temperature of 4 ± 0.1 °C in the gel throughout the electrophoresis. The buffer system contained 40 mM Tris, 20 mM acetic acid, pH 8.1, and 1 mM EDTA (TAE) or the same buffer with 5 mM $MgCl_2$ (TAE-Mg). The gels were exposed for 1 h without an intensifying screen. For denaturing gels, the products of cleavage reaction were taken up in formamide loading buffer, heated briefly to 90 °C, cooled, and then run on a denaturing polyacrylamide gel (16%, 19:1 monomer:bis ratio) for 2.5 h at 2000 V (ca. 50 V/cm) and 40 °C. No dyes were added in these runs. The gel was dried immediately on a vacuum drying apparatus (Hoefer) and exposed at room temperature to film without an intensifier screen.

Osmium Tetraoxide Modification Reactions. Junctions (10 μM) were incubated with 1 mM osmium tetraoxide and 3% pyridine in 50 mM Tris·HCl (pH 7.5), with or without 5 mM $MgCl_2$ in 15 μL final volume at 4 °C for 15 min (Lilley & Palecek, 1984). The reactions were stopped by two sequential ethanol precipitations and then lyophilized. The DNAs were cleaved at the sites of osmate adducts by treatment with 100 μL of 1 M piperidine at 90 °C for 30 min, followed by extensive lyophilization.

Diethyl Pyrocarbonate Modification. Branched DNAs were modified by diethyl pyrocarbonate essentially as described by Herr (1985). DNAs (10 μM) were suspended in 15 μL of 50 mM Tris·HCl (pH 7.5), with or without 5 mM $MgCl_2$. The Eppendorf tubes were placed on ice, and 1 μL of diethyl pyrocarbonate (DEPC) was added to each sample. The samples were incubated for 60 min at 4 °C. Because DEPC is relatively insoluble in water, the samples were thoroughly mixed at the beginning as well as halfway through the 60-min incubation. The reactions were terminated by two sequential rapid ethanol precipitations and then lyophilized. The DNAs were cleaved at the sites of DEPC modification by treatment with 100 μL of 1 M piperidine at 90 °C for 30 min.

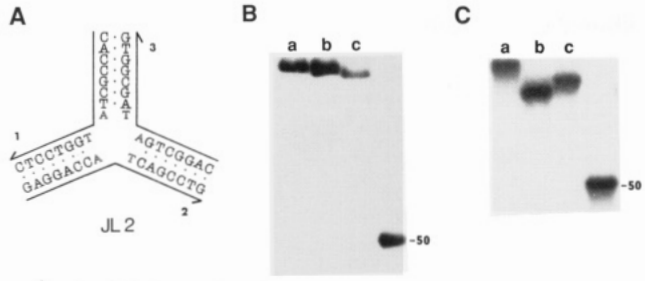

FIGURE 2: Gel electrophoretic analysis of a second three-arm junction (JL 2), with sequence changes at the branch. (A) Sequence of the three-arm junction JL 2. (B) Autoradiograph of a 20% polyacrylamide gel of three pairwise elongated species in the absence of Mg^{2+}. The numbering convention refers to that of Figure 1. Gel marker is 50-bp linear duplex DNA. (C) Autoradiograph of a 20% polyacrylamide gel of three pairwise elongated species in the presence of Mg^{2+}.

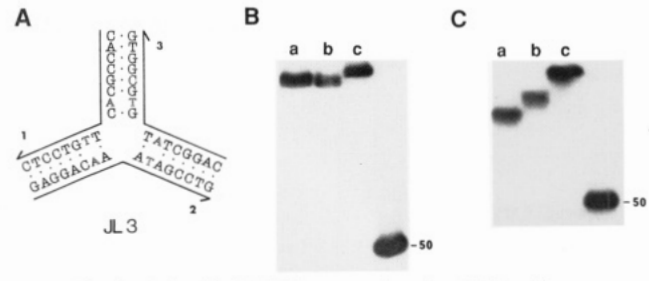

FIGURE 3: Analysis of a third three-arm junction JL 3, with sequence changes 1 bp removed from the branch. (A) Sequence of the three-arm junction JL 3. (B) Autoradiograph of a 20% polyacrylamide gel of three pairwise elongated species in the absence of Mg^{2+}. The numbering convention refers to that of Figure 1. The gel marker is 50-bp linear duplex DNA. (C) Autoradiograph of a 20% polyacrylamide gel of three pairwise elongated species in the presence of Mg^{2+}.

UV Melting Curves. All DNA solutions were prepared in a 50 mM Tris·HCl (pH 7.5) buffer containing 10 mM NaCl, with or without $MgCl_2$. Absorbance vs temperature profiles were measured on a Perkin-Elmer 575 spectrophotometer interfaced to a computer. The temperature of the cell holder was thermoelectrically controlled and programmed. Samples were heated at a rate of 0.5 °C/min. Absorbance and the temperature were recorded every 30 s.

Sequencing Reaction. Purine-specific (A + G) sequencing ladders were generated from each 5′-^{32}P-labeled oligonucleotide by using the piperidine–formate reaction (Maxam & Gilbert, 1977).

Results

Geometry of the Three-Arm Junctions Is Determined by the Sequences at the Branch and 1 bp Removed from the Branch. The oligonucleotides used to construct the junction JL 1 were resynthesized, first with each of the base pairs flanking the branch an A-T (Figure 2A). If three-arm junctions behave similarly to four-arm junctions, the three complexes of JL 2 with extended arms should have equal migrational mobility with or without magnesium ion. The argument is the following. If the effective angle between the helix axes of each pair of extended arms is the same, we expect the mobilities of each of the three complexes with one pair of arms extended at a time to be the same. This assumes that the mobility of each arm is roughly the same and that the mobility depends on the angle between the axes of the pair of helices that have been elongated. Following Cooper and Hagerman (1987), it can be anticipated that the more acute this angle, the greater the retardation in mobility will be.

Gel electrophoretic analysis of the three-arm junction in which each pair flanking the branch is AT shows that the mobilities of the three pairwise elongated complexes (a, b, and c) differ in the presence of Mg^{2+} (Figure 2). In the absence of Mg^{2+}, the electrophoretic mobilities of the three extended complexes of the junction are similar (Figure 2B), consistent with a trigonal planar or a pyramidal arrangement of the arms about the branch with the angles between each pair of helix axes equal. We take these mobility values as a reference for interpreting the data in the presence of Mg^{2+}. When Mg^{2+} is present, the three pairwise elongated complexes of JL 2 migrate differently: complex a is the slowest, complex b the fastest, and complex c has an intermediate mobility (Figure 2C). By the argument stated, this suggests that the effective angle between the helix axes of arms 1 and 3 is most acute, and that between the arms 1 and 2 least acute. In any case, the conformation of a three-arm junction is governed not just by the sequence at the branch but also by stacking interactions and/or pairing within the arms themselves, possibly even the global stability of each of the arms. Comparison of the mobility pattern seen for JL 2 with that of JL 1 shows that there is no general structure for three-arm DNA junctions, as is seen in the four-arm case. We also observe that the mobilities of the complexes a, b, and c are lower in the absence than in the presence of Mg^{2+} (Figure 2, panel B vs panel C). This would be consistent with a model in which the angles subtended by the helix axes in the three-arm junction structure are smaller in the presence than in the absence of Mg^{2+} (Guo et al., 1990a; Duckett & Lilley, 1990), but it does not prove this.

In order to determine the effect of sequence on the geometry of three-arm junctions, we have assumed that bases closer to the branch play a stronger role in determining its conformation than ones at more remote positions. Accordingly, we constructed a third junction, JL 3, in which the penultimate base pair from the branch is A-T in each of the arms (Figure 3A). If, as in the four-arm case, this second position from the branch exerts no influence on the junction conformation, then the electrophoretic pattern of the pairwise elongated complexes of JL 3 should match those of JL 1; if this position is also a determinant of junction geometry, a different pattern from that of JL 1 might be seen. The results of the electrophoretic analysis are shown in Figure 3. In the presence of Mg^{2+}, complex a is the fastest species, complex b is intermediate, and complex c is slowest. The electrophoretic pattern of the pairwise elongated complexes of JL 3 differs from that of JL 1 or JL 2 in the presence of Mg^{2+}, indicating that the second position from the branch of a three-arm junction indeed exerts some influence.

It might reasonably be suspected that the identity of the base pairs per se is perhaps less important than the stacking interactions between next-neighbor pairs, which influence the thermodynamic stability of the double helix (Breslauer et al., 1986). If so, sequences that are identical at both the flanking and penultimate positions might lead to a three-fold symmetric junction geometry and eliminate the differences in mobility among the pairwise elongated complexes; if not, of course, the mobility differences will persist. We therefore constructed the junction JL 4, which is closely related to JL 1, except that the sequences flanking the branch on all three arms are uniformly CA/GT (Figure 4A). The results of the gel electrophoretic analysis are shown in Figure 4. The three pairwise elongated complexes of this junction now have equal mobility within the limits of the data, in the presence and absence of Mg^{2+}. These results indicate that the sequences at the branch and 1 bp removed from the branch—including the stacking interactions between them—dominate the geometry of a three-arm junction.

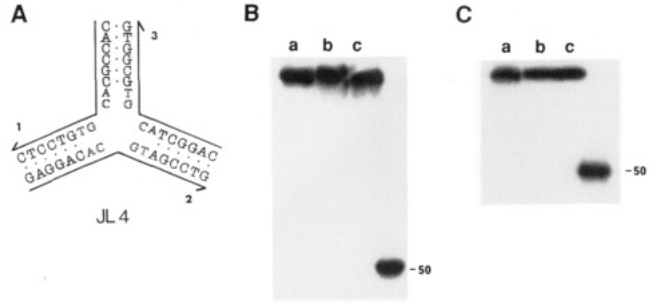

FIGURE 4: Analysis of a fourth three-arm junction (JL 4), with same sequences at and 1 bp removed from the branch on all three arms. (A) Sequence of the three-arm junction JL 4. (B) Autoradiograph of a 20% polyacrylamide gel of three pairwise extended complexes in the absence of Mg^{2+}. The numbering convention refers to that of Figure 1. The gel marker is 50-bp linear duplex DNA. (C) Autoradiograph of a 20% polyacrylamide gel of three pairwise extended complexes in the presence of Mg^{2+}.

Formation of a Branch in a Three-Arm Junction Perturbs One Turn of the Helix from the Branch with or without Mg^{2+}. Several experiments have revealed that counterions play a crucial role in the folding of four-arm DNA junctions (Seeman, et al., 1985; Cooper & Hagerman, 1987, 1989; Duckett et al., 1989, 1990; Lu et al., 1990a). In the absence of divalent metal ions, four-arm junctions of any sequence adopt a more extended structure (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988, 1990; Murchie et al., 1989). The conformation of a three-arm junction is also highly dependent on the presence of Mg^{2+} (Guo et al., 1990a). The bases at the branch are reactive to diethyl pyrocarbonate and osmium tetraoxide, independent of the presence or absence of Mg^{2+} (Duckett & Lilley, 1990; Guo et al., 1990a). This suggests that formation of a three-arm branch might entail opening or local unpairing of bases in the arms, and hence that some instability exists in these molecules. To investigate this, we have used both diethyl pyrocarbonate and osmium tetraoxide to probe each of the three-arm junctions with extended arms. In the absence of Mg^{2+}, purine bases at the branch and 2 bp removed from the branch are reactive to diethyl pyrocarbonate relative to their rates of reaction in duplex DNA (Figure 5); this reactivity is not suppressed by addition of Mg^{2+} (data not shown). By contrast, the thymine residues along one turn of the helix from the branch are reactive to osmium tetraoxide (Figure 5). Interestingly, this hyperreactivity does not depend on the distance of the thymine from the branch. The effect is not influenced by addition of Mg^{2+} either (data not shown). We find similar results in the cases of JL 2 and JL 3 (data not shown). One explanation for this is that the hyperreactivity reflects loosening or distortion of base pairing and that the formation of the branch in a three-arm junction induces strain, which is released gradually within the first helical turn of the DNA in the arms. How might metal ions such as Mg^{2+} induce the conformational folding of a three-arm junction and yet not eliminate the hyperreactivity at the branch by these chemical probes? Perhaps magnesium ions bind strongly to the branch and "freeze" the conformation, instead of imposing new structure.

Increasing Temperature Eliminates Differences in Junction Mobility. It has been shown that the electrophoretic anomaly associated with DNA bending is sensitive to temperature (Koo & Crothers, 1987). By 50 °C the difference in mobility associated with phased AT tracts disappears, for example. The mobility differences among the pairwise elongated complexes of three-arm junctions prove to be also strongly dependent on the temperature. If the samples were incubated at 25 °C for

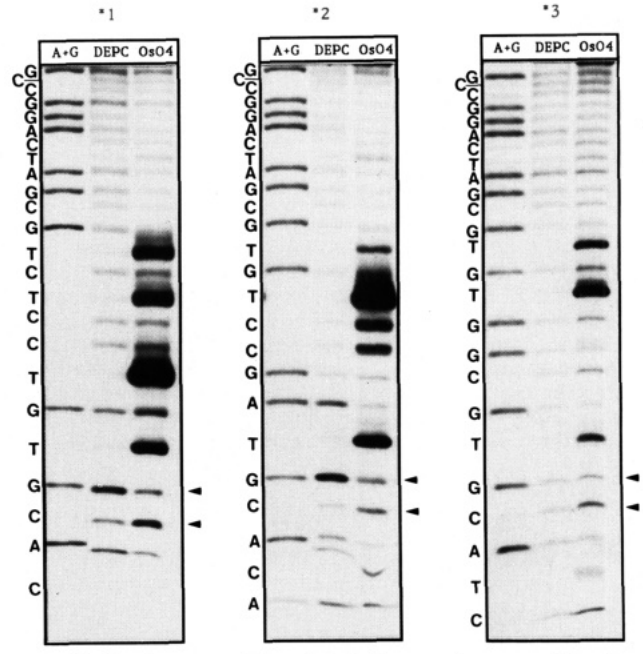

FIGURE 5: Osmium tetraoxide and diethyl pyrocarbonate modification reactions in JL 4. An autoradiograph of the modification experiments in the absence of Mg^{2+} is shown. The complexes a, b, and c were radioactively labeled at the 5′-terminus of strand 3, 1, and 2, respectively. The chemical modification reactions were carried out, as described under Materials and Methods. Samples were electrophoresed on a sequencing gel alongside A + G sequence markers derived from the same radioactive strand. Radioactively labeled strands are indicated by the numbers of the strand in the 5′ half. The triangles indicate the positions of the branch. Note that the thymine bases in one turn of the helix from the branch are hyperreactive. This reactivity is not suppressed by addition of Mg^{2+} (data not shown).

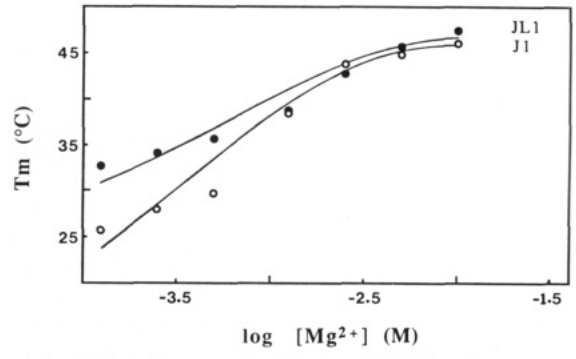

FIGURE 6: Effect of magnesium ion on stabilizing four-arm and three-arm junctions. The melting temperatures were measured, as described under Materials and Methods. The four-arm junction J1 consists of four hexadecadeoxynucleotides (Kallenbach et al., 1983).

1 h before being loaded into a native gel (the temperature of the gel remained 25 ± 0.1 °C), the electrophoretic difference between three pairwise elongated complexes of each of the junctions we have studied disappears (data not shown). The electrophoretic retardation associated with three-arm junctions appears to be more labile to temperature than the oligo(A) bending phenomenon.

Magnesium Has a Smaller Effect on Stabilizing Three-Arm Than Four-Arm Junctions. To further characterize the difference between three- and four-arm junctions, we measured the extent of stabilization of the two junctions by Mg^{2+}, using the relative increase in T_m produced by Mg^{2+} ($dT_m/d \log [Mg^{2+}]$) as a basis for comparison. As shown in Figure 6, the T_m of JL 1 changes by roughly 10.7 °C per decade change in Mg^{2+} concentration, while that of J1, a four-arm junction, changes by 15.0 °C. Thus Mg^{2+} has a smaller effect on

stabilizing JL 1 than it does on a four-arm structure with the same size arms, an effect that probably reflects the lower strandedness of the former complex, and thus the lower electrostatic repulsion among the strands. This is not inconsistent with the idea that some strain arises in three-arm junctions on binding Mg^{2+}.

Discussion

These data bring out a number of interesting differences between the structure and stabilization of three- and four-arm DNA junctions. In the latter case, the bases flanking the branch remain paired, although they exchange more rapidly than pairs within a duplex, while the sequence of bases at the branch dictates the choice of preferred stacking partners (Chen et al., 1988; Duckett et al., 1988; Guo et al., 1991). Isomerization between antiparallel and parallel configurations of the two stacked arms occurs in four-arm junctions, with the antiparallel isomer favored slightly in stability over the parallel (Lu et al., 1991a).

The behavior of three-arm junctions is different in the following important respects:

(1) The asymmetric structure in three-arm junctions varies depending on the identity of the bases flanking the branch and 1 bp removed from the branch, as can be seen by the changing order of mobilities in Figures 1–3. This implies that there is no single stable configuration of the arms in three-arm junctions, as there is in the four-arm case.

(2) Formation of three-arm junctions entails a conformational alteration in the vicinal arms, since thymine residues up to 10 residues from the branch are hyperreactive to osmium tetraoxide in three-arm structures. No similar effect occurs in four-arm junctions that have been studied to date (Duckett et al., 1988, 1990). Interestingly, this effect is not detected at any sites other than those near the branch itself by using the purine reagent DEPC.

(3) Three-arm junctions interact differently with Mg^{2+} ions, and probably divalent ions generally, from four-arm junctions, as seen by the smaller effect of Mg^{2+} on T_m of the former.

(4) Three- and four-arm junctions differ also in their roles as substrates for resolvase enzymes. T7 endo I and T4 endo VII do not selectively cleave double strands at the branch sites in three-arm junctions (Jensch & Kemper, 1986; Guo et al., 1990a), another difference from four-arm ones (Mueller et al., 1988; Lu et al., 1991b).

It appears then that the three-arm branch is structurally different from the four-arm version, in agreement with the observations of Duckett and Lilley (1990), although the conformation of both is influenced by Mg^{2+} and they share the ability to interact selectively with a variety of DNA binding ligands (Guo et al., 1989, 1990a,b). It is difficult to be more specific about the conformation of three-arm branches with the available data. One may wonder if the geometry of the junction is perturbed by appending the arms required for mobility analysis (Cooper & Hagerman, 1987). The patterns of reactivity to DEPC and osmium tetraoxide of JL 1 are identical with those in its three pairwise elongated complexes, implying that no major perturbation is involved (unpublished data). Since these two reagents are more reactive at the branch in three-arm but not four-arm junctions, the three-arm branch appears to be a more open structure than that in four-arm models. However, this structure is evidently a more complex one than the simple Y-shaped molecule proposed by Duckett and Lilley (1990).

One can only speculate about the nature of the structure in these junctions at this point. Two quite different classes of models can be considered. In one, the different mobilities are assumed to correspond to particular equilibrium geometries of the system. The alternative is that each junction consists of a mixture of conformational isomers and that the mobilities reflect the average of this mixture. These two possibilities are discussed separately below.

In the first case, we can imagine two different geometrical models for a junction. In one, preferred stacking of two arms occurs, with exclusion of the third, producing a "T"-shaped molecule (Guo et al., 1990a). However, this model is inconsistent with the relative mobility patterns in Figures 2 and 3 unless the effective angle of the T is variable. JL 1 and JL 3 have identical base-stacking interactions across the branch yet have different mobilities. If the next-neighbor stacking interactions at the branch determine the preferential geometry, JL 1 and JL 3 might be expected to have the same structure and mobility. One might also expect the mobilities of the three pairwise elongated complexes of JL 2 to be the same on this basis, and they are not. It therefore seems more likely that some kind of preferential kinking or local unfolding occurs at the branch, which leads to structures having variable effective angles between the arms. Whether these structures are planar or pyramidal is hard to judge, although the fact that the mobilities with and without Mg^{2+} differ suggests that the structure in the presence of Mg^{2+} might be more "puckered", favoring a pyramidal geometry.

The second hypothesis, in which the junction consists of several interconverting isomers, can account for the mobility behavior but raises the question of how rapidly these isomers interconvert and whether the observed effects are static or dynamic in nature. The observed loss of mobility differences with increasing temperature unfortunately does not provide a clue, because this is a property of the presumed static bending in duplex DNA (Koo et al., 1986). It might be expected also for a set of rapidly interconverting isomers. That there is plasticity in the structures of both three- and four-arm junctions is indicated by the ease with which ligation of sticky-ended junctions yields cyclic trimers, tetramers, etc. (Ma et al., 1986; Petrillo et al., 1988; Chen et al., 1989). Jensch and Kemper (1986) have made the interesting observation that the enzyme *EcoRI*, which requires a double-stranded restriction site for cleavage, is able to cut each of the three GAATTC sequences spanning the branch of a three-arm junction they constructed in which this sequence occurs at the branch on each of the component strands. This implies that (in the presence of enzyme) each arm is transiently duplex at the branch and hence that isomerization of the junctions may occur. However, one does not know the time scales involved in these isomerization events, and further experimentation will be needed to address these questions.

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