The EMBO Journal vol.9 no.5 pp.1659 – 1664, 1990

The three-way DNA junction is a Y-shaped molecule in which there is no helix – helix stacking

Derek R.Duckett and David M.J.Lilley

Department of Biochemistry, The University, Dundee DD1 4HN, UK

Communicated by D.M.J.Lilley

We have studied the structure of a number of three-way DNA junctions that were closely related in sequence to four-way junctions studied previously. We observe that the electrophoretic mobility of the species derived by selective shortening of one arm of a junction are very similar whichever arm is shortened, and that this remains so whether or not magnesium is present in the buffer. This suggests that the angles subtended between the arms of the three-way junctions are similar. All thymine bases located immediately at the junction are reactive to osmium tetroxide, indicating that out-of-plane attack is not prevented by helix – helix stacking, and this is also independent of the presence or absence of metal cations. The results suggest that the three-way junction cannot undergo an ion-induced conformational folding involving helical stacking, but remains fixed in a Y-shaped extended conformation. Thus the three- and four-way junctions are quite different in character in the presence of cations.
Key words: DNA junctions/DNA structure/metal ion – nucleic acid interaction

Introduction

Junctions between DNA helices are important as intermediates in DNA rearrangements and as components in the secondary structure of single-stranded DNA molecules such as certain viral genomes. The most important of these is undoubtedly the four-way junction, the Holliday junction of genetic recombination. However, other junctions are possible, and in this work we have studied some examples of three-way helical junctions.

A major reason for studying the three-way junction was as a comparison with the four-way junction. We have proposed a general structure for the four-way junction—the stacked X-structure (Duckett *et al.*, 1988; Murchie *et al.*, 1989). In the presence of metal ions, helices stack pairwise to generate two coaxial, quasi-continuous helices, that are then rotated at about 60° to form an X-shaped structure. This is illustrated schematically in Figure 1. Two isomers of the structure are possible, depending on whether a given helix stacks with its neighbour to the left or to the right and the relative stability of the two isomers is determined by the local sequence at the junction. The structure is critically dependent on cation binding—in the absence of appropriate metal ions the helices can no longer undergo the mutual stacking process and the four arms of the junction remain fully extended in a square-planar configuration (Duckett *et al.*, 1988, 1990). This structure is completely supported by gel electrophoretic data (Duckett *et al.*, 1988) and fluorescence resonance energy transfer experiments (Murchie *et al.*, 1989) and is consistent with data from hydroxyl radical probing (Churchill *et al.*, 1988) and transient electric birefringence (Cooper and Hagerman, 1989). One question that arises from these studies concerns how general the folding principle in the stacked X-structure might be. For example, the stacking between neighbouring helices is quite similar in some respects to that between the acceptor and T stems in tRNA.

Can three-way junctions adopt some folded geometry analogous to the stacked X-structure of four-way junctions? Two structures appear possible for the three-way junction at first sight. If the three helices fail to undergo tertiary interaction, they should be extended approximately towards the corners of a triangle, forming a Y-shaped molecule. On the other hand, helix – helix stacking might be possible, analogous to that seen in the four-way junction. Since there are only three helices, just one such stack could form, leaving the third helix extended, and a T-shaped molecule would be the result. An experimental investigation of the structure adopted by the three-way junction might help to set limits on the generality of the folding principle of the stacked X-structure and we therefore set out to determine the structure of the three-way junction using techniques analogous to those that we have applied successfully to the four-way junction.

Results

Construction of three-way DNA junctions

We have previously described the construction of four-way helical junctions from synthetic 80 base oligonucleotides (Duckett *et al.*, 1988). The sequences of these were chosen so as to place unique restriction sites a short distance from the junction itself, by means of which any given arm of the junction could be shortened from 40 to 12 bp in length. Figure 2 illustrates the construction of three-way junctions from any two oligonucleotides used in the original four-way junctions, by the expedient of synthesizing an additional 80 base oligonucleotide that is complementary to the 5′ half of one original oligonucleotide and the 3′ half of another. By

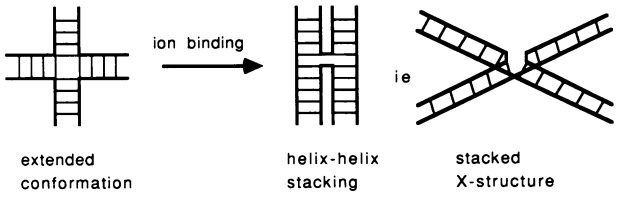

Fig. 1. Schematic to show the folding of a four-way helical junction into the stacked X-structure. In the absence of metal ions the helical arms of the four-way junction are unstacked and fully extended in a square planar configuration. On addition of metal ions (such as 100 μM Mg^{2+}) the arms become stacked in pairs, generating coaxial, quasi-continuous helices. These are rotated to generate an X-shaped species.

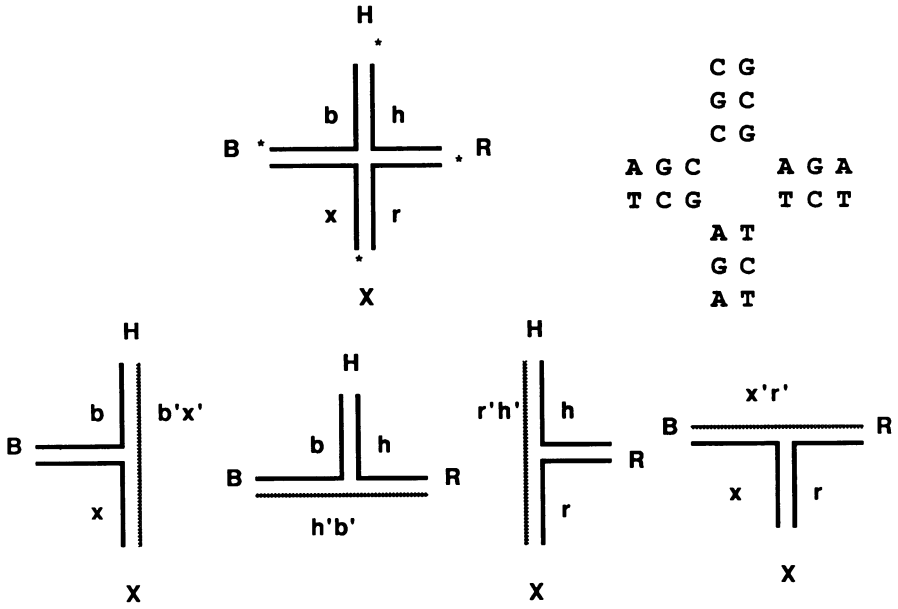

Fig. 2. Construction of three-way junctions and their relationship to a four-way junction. The three-way junctions were all based on the oligonucleotides used in the construction of junction 1 (Duckett *et al.*, 1988), the central sequence of which is shown. The arms are labelled with uppercase letters (B, H, R or X), while the strands are given the lowercase letters (b, h, r or x) derived from the arm in which the 5′-terminus is located (indicated by asterisks). The three-way junctions were generated by hybridization of a new oligonucleotide (stippled) to two existing oligonucleotides of junction 1. This could be accomplished to give the four different three-way junctions indicated.

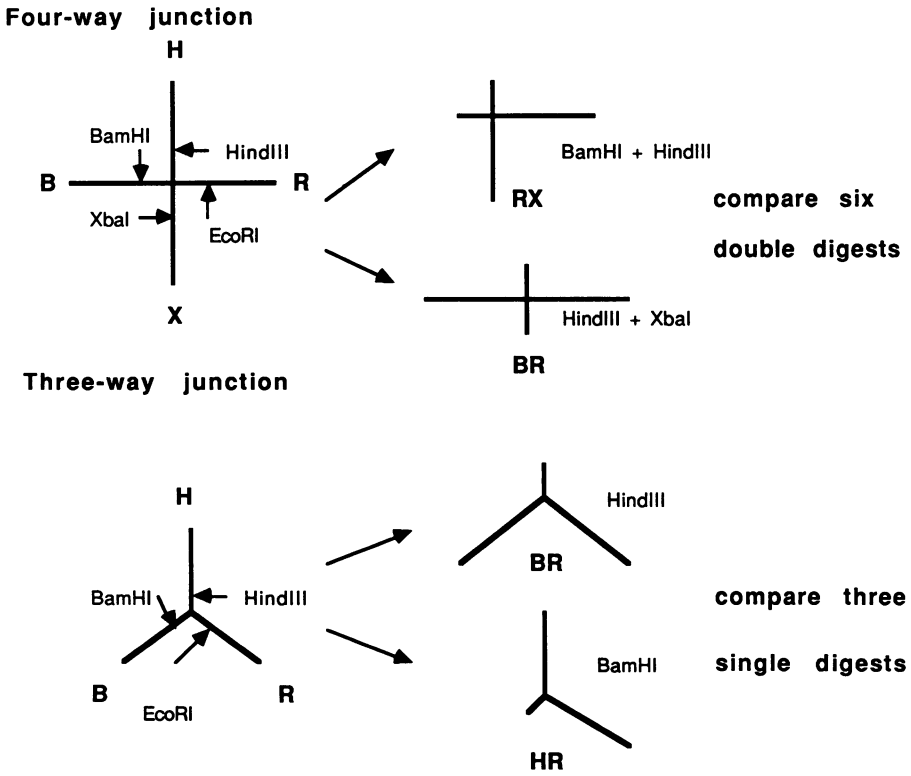

Fig. 3. Gel electrophoretic analysis of the three-way junctions. The method takes advantage of the ability to shorten any given arm by cleavage at a unique restriction site. Four-way junctions were analysed by comparing the gel electrophoretic mobility of the six possible species derived by restriction cleavage of two arms. By contrast, three-way junctions are analysed by comparing the three possible species that have been cleaved in a single arm. Note that the labelling of cleaved species in this and subsequent figures is derived by taking the label of the uncleaved (i.e. long) arms.

the synthesis of four such oligonucleotides, four three-way junctions were created from the oligonucleotides used for the construction of junction 1 (Duckett *et al.*, 1988). Each arm of the three-way junctions was 40 bp in length, and retained the sequence and restriction site of the equivalent arm of junction 1.

Gel electrophoretic analysis of a three-way junction

In our earlier analysis of four-way junctions, a series of isomeric species were generated by shortening two arms of the junction by restriction cleavage and comparing the relative gel electrophoretic mobility of the six possible species with two long and two short arms. An equivalent procedure was described by Cooper and Hagerman (1987), in which reporter arms were ligated to a central junction core. The underlying principle in such experiments is that the shape of the molecule, and hence its mobility in a polyacrylamide gel, will be related to the angle subtended between the longer arms. For the three way junctions the analysis is rather simpler. Digestion with a single restriction enzyme generates a species with two long and one short arm and hence three isomeric species may be compared by gel electrophoresis, differing only in which arm has been shortened. The basis of the analysis of four- and three-way junctions is summarized schematically in Figure 3.

A comparison is made between the relative mobilities in polyacrylamide of the six species resulting from junction 1 and the three from the three-way junction comprising B, H and X arms in Figure 4. The electrophoresis buffer contained 100 μM Mg^{2+}, a concentration known to fold four-way junctions into the stacked conformation (Duckett *et al.*, 1990). The four-way junction 1 gives the two fast, two intermediate and two slow species pattern (2:2:2 pattern) typical of the stacked X-structure (Duckett *et al.*, 1988). By comparison, the three-way junction gives three species of closely similar mobility—the relative differences in mobility of the three two-long-arm species are very much smaller than those between the different four-arm junction isomers. This suggests that the three angles between the arms of the three way junctions are more similar than those of the four-way junction.

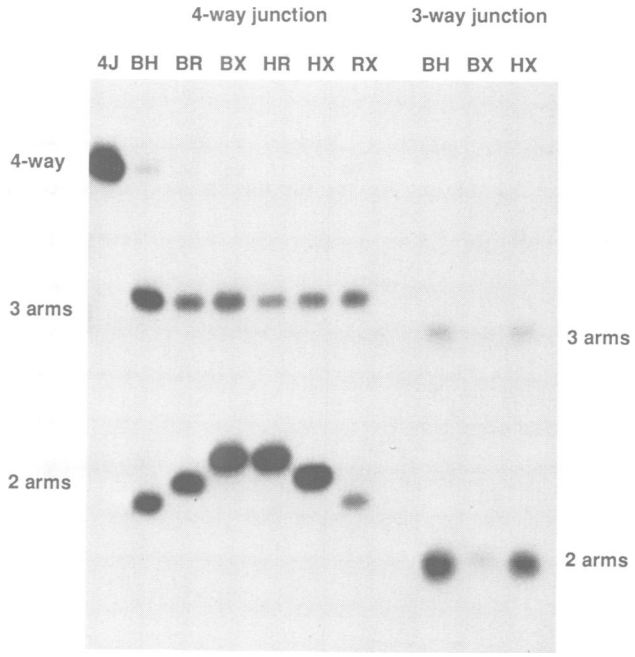

Fig. 4. Gel electrophoretic mobility of a three-way junction and comparison with a four-way junction. Autoradiograph of a 5% polyacrylamide gel containing the six double digests of junctions 1 (four-way) and the three single digests of the three-way junction with B, H and X arms. The first track (4J) contains uncleaved junction 1, followed by the six double digests, labelled as usual with the names of the long arms. The digests are always partial and the species generated have three long arms (i.e. are cleaved in one arm only) or two long arms, as indicated on the left. The pattern of fragments is the characteristic 2:2:2 motif. On the right of the gel are shown the three single digests of the three-way junction. Once again this is partial, and traces of uncleaved junction (three arms) are visible. The lower species (two arms) are the junctions that have been shortened in one arm. Note that the mobilities of these three species are virtually the same, by comparison with the two arm species generated from the four-way junction. The gel electrophoresis was performed in 90 mM Tris−borate (pH 8.3), 100 μM $MgCl_2$.

Analysis of four related three-way junctions

We have constructed four different three-way junctions (illustrated in Figure 2), and the electrophoretic mobility in polyacrylamide of the three single digests of each in the presence of 500 μM magnesium is shown in Figure 5. Just as for the BHX junction, the differences between the three isomers of each junction are rather small, and indeed the differences between the different junctions are also small. These results indicate that the structure of the three-way junction is quite different in character from the four-way junction, even in the presence of the relatively high cation concentration. The relative gel mobilities of the different species suggests that the angles between the arms of the junctions remain fairly constant. Clearly there are small differences in mobility between the three isomers of a given junction, indicating that the angles are not exactly identical, but the differences between them are nevertheless quite small.

Relative gel mobilities are not dependent on the presence of divalent cations

The conformation of four-way junctions is highly dependent on the presence of cations. In their absence the stacked X-conformation is not stable and an unstacked extended conformation is adopted, in which the four arms are directed towards the corners of a square (Duckett *et al.*, 1988). We therefore repeated the gel electrophoretic analysis of the three isomeric species of each three-way junction in a gel buffer in which magnesium was omitted and replaced by EDTA, conditions known to give the square planar configuration of the four-way junction. The resulting autoradiograph is presented in Figure 6. The results are closely similar to those in the presence of magnesium; once again there are only small differences between the different species. These patterns suggest that the structure of the three-way junction is little affected by the presence of metal ions and that the angles between the helical arms remain rather similar whether or not divalent cations are present.

Thymine bases at the junction are reactive to osmium tetroxide

The unstacked conformation of the four-way junction in the absence of ions is accompanied by a reactivity of thymine bases present immediately at the junction to osmium tetroxide, the reactivity being suppressed by the addition of sufficient cation to fold the junction into the stacked X-conformation (Duckett *et al.*, 1988, 1990). This reactivity is believed to result from the ability of the electrophile to attack the thymine 5,6 bond out-of-plane when it is unstacked on one side. We therefore probed the environment of junction thymines in a three-way junction. The BRX junction was chosen, in which the r strand bore a 5′-^{32}P radioactive

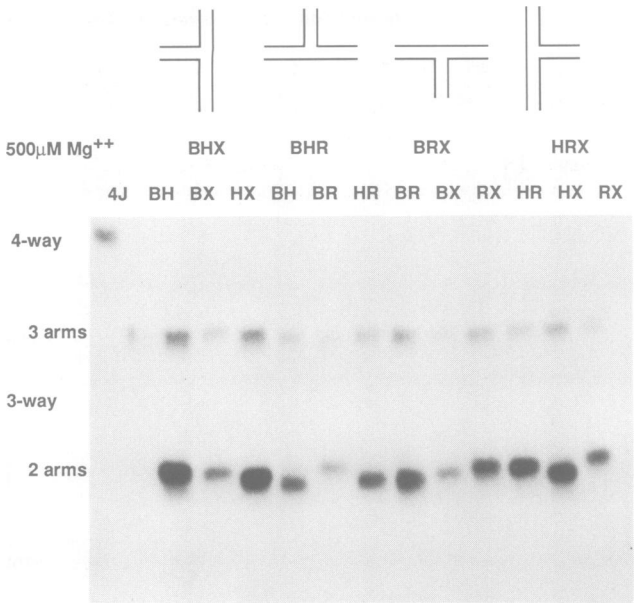

Fig. 5. Gel electrophoretic analysis of four three-way junctions. Comparison of the electrophoretic mobility in 5% polyacrylamide of the three species derived by single restriction digestion of the four three-way junctions. The leftmost track (4J) contains uncleaved junction 1 for comparison. The gel electrophoresis was performed in 90 mM Tris−borate (pH 8.3), 500 μM $MgCl_2$.

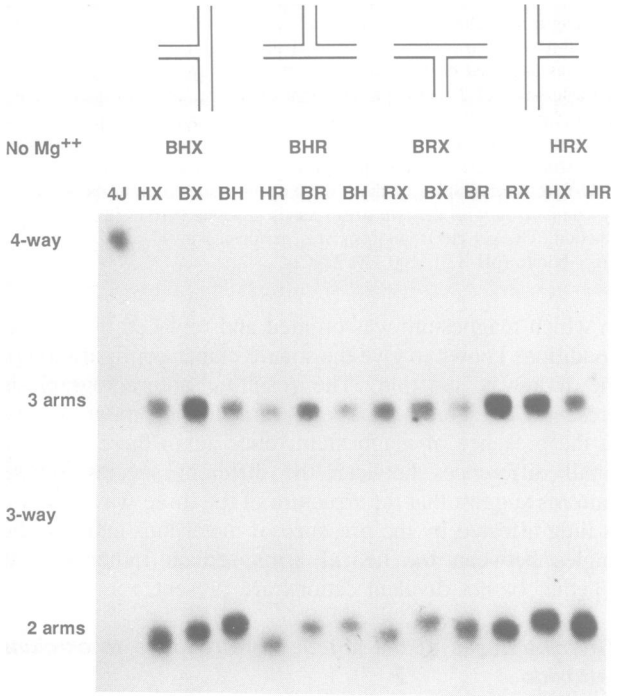

Fig. 6. Gel electrophoretic analysis of three-way junctions in the absence of cations. Comparison of the electrophoretic mobility in 5% polyacrylamide of the three species derived by single restriction digestion of the four three-way junctions. The leftmost track (4J) contains uncleaved junction 1 for comparison. The gel electrophoresis was performed in 90 mM Tris−borate (pH 8.3), 10 mM EDTA.

label, since this strand has two thymine bases present at the junction.

This junction was modified using 1 mM osmium tetroxide, 3% pyridine, in the presence of various concentrations of

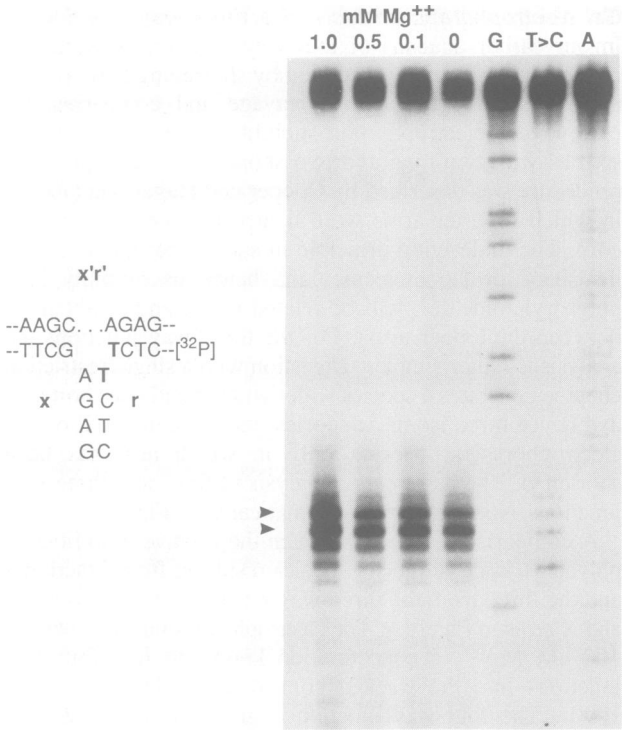

Fig. 7. Osmium tetroxide modification of thymine bases at the three-way junction with and without cations. The three-way junction with B, R and X arms was radioactively labelled at the 5′-terminus of the r strand. The central sequence is indicated on the left. The junction was reacted with osmium tetroxide, 3% pyridine in 90 mM Tris−borate (pH 8.3) containing the indicated concentration of magnesium. The DNA was then cleaved at thymine−osmium adducts by incubation with piperidine and electrophoresed in a sequencing gel, alongside sequence markers derived from the same radioactively labelled oligonucleotide. The autoradiograph is presented. Note that two thymine bases are particularly reactive (arrowed at left)—these are the thymine bases that are located immediately at the junction, shown in bold in the sequence. This reactivity is not suppressed by addition of magnesium ions.

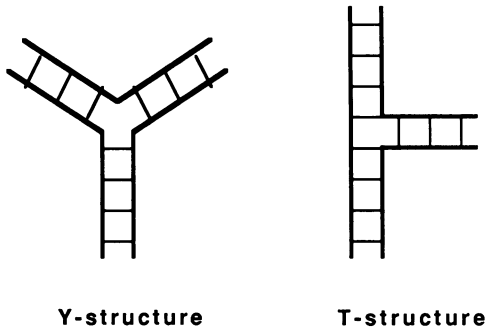

Fig. 8. Potential structures for a three-way helical junction. Two types of structure for the three-way junction can be envisaged. In the Y-shaped structure there is no helix−helix stacking, while in the T-shaped structure two of the helices stack (in the manner of the four-way junction) to form a coaxial, quasi-continuous helix.

magnesium ions, followed by piperidine cleavage of any osmium−pyridine adducts and electrophoresis on a sequencing gel. The autoradiograph is presented in Figure 7. It can be seen from this that there was strong modification at the centre of the r oligonucleotide, i.e. immediately at the junction. The strongest modification occurred at the two

thymine bases located immediately at the junction. Weaker modification was also seen at bases on either side, indicating that the formation of the three-way junction requires some distortion and/or weakening of the local DNA structure. The strong modification of the junction thymines suggests that neither the R nor the X helix is involved in helix−helix stacking. Moreover, the reactivity of these thymines is completely unaffected by addition of magnesium ions up to 1 mM, further indicating the lack of cation-dependent structure formation in the three-way junction.

Discussion

These studies of three-way DNA junctions have revealed two related aspects of their structure.

(i) Comparison of the relative electrophoretic mobility in polyacrylamide of junctions shortened in one arm suggests that the three angles between the arms are similar, much more so than the various angles of the four-way junction. These angles do not appear to vary significantly as magnesium ion is added or removed.
(ii) Thymine bases located at the junction are reactive to osmium tetroxide, irrespective of the presence or absence of magnesium ions.

These results indicate that the structure of the three-way junction is unaffected by cation binding and is held in a permanently unstacked, open conformation akin to the structure of the four-way junction in the absence of cations.

One might imagine that a three-way junction could adopt one of two kinds of structure, illustrated schematically in Figure 8. One would consist of three extended and unstacked helices, in an approximately Y-shaped geometry. The other would include a pair of colinear stacked helices as is seen in the ion-bound folded conformation of the four-way junction, in which case an approximately T-shaped geometry would result. The similarity in angles between the arms, together with the reactivity of junction thymines, suggests strongly that the Y-shaped structure is the closer approximation to the structure of the three-way junction.

A basic exercise in model building also leads to the same conclusion. We may attempt to construct a model of the three-way junction containing a stacked pair of helices by starting from a B-DNA helix, breaking a phosphodiester linkage and trying to add an additional helix. The distance between the phosphate groups of the third helix that must be joined into the fully stacked helix is 18 Å. Simple geometry suggests that it will be necessary to bend the first helix in order to introduce the third arm, generating a Y-shaped molecule with disrupted stacking.

Although the angles between the three arms appear to be closely similar, they need not necessarily be 120°, for the junction may not be planar. Although a planar Y shape is possible, we cannot exclude a pyramidal shape for the three-way junction. Moreover, while the inter-arm angles appear to be similar, the gel results suggest that there is some variability presumably due to local sequence-dependent effects on conformation. From ligation experiments it has previously been suggested that the angles of a three-way junction are variable in a flexible way (Ma *et al.*, 1986). This flexibility seems once again more consistent with an extended Y-shaped structure than a stacked T-shaped molecule.

In summary, we believe that the structure of the three-way junction is quite different from the stacked X-structure adopted by the four-way junction. There is no evidence for cation-dependent folding into a tertiary structure based on helix−helix stacking and instead, the junction remains permanently in an extended Y-shaped structure.

Materials and methods

Oligonucleotide synthesis and construction of junctions

Oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry (Beaucage and Caruthers, 1981; Sinha *et al.*, 1984) implemented on an Applied Biosystems 381A DNA synthesizer. Fully deprotected oligonucleotides were purified by gel electrophoresis in 12% polyacrylamide containing 7 M urea. The bands were excised and DNA electroeluted on to DEAE cellulose (Whatman DE52), eluted in 2 M NaCl and recovered by ethanol precipitation.

Junctions were constructed from 80 base oligonucleotides, based on those used to construct the four-way junction 1 of Duckett *et al.* (1988). For example, the b strand of junction 1 had the sequence 5′-CGCAAGC-GACAGGAACCTCGAG**GGATCC**GTCCTAGCAAGCCGCTGCTAC-CGG**AAGCTT**CTCGAGGTTCCTGTCGCTTGCG-3′ (*Bam*HI and *Hin*dIII sites shown in bold). In each strand, the sequence between the restriction sites was unique, but beyond this point the sequence of each arm was identical. The unique centre allowed correct assembly to occur, without detectable competition by hairpin forms. Annealing reactions were carried out by incubating stoichiometric amounts of the appropriate DNA fragments for 2 h at 65°C in 20 μl of 10 mM Tris−HCl (pH 8.0) and then allowing them to cool slowly. In each annealing reaction only one of the oligonucleotides was radioactively labelled, giving three different species for each junction. Junction DNA was purified by gel electrophoresis in 5% polyacrylamide and recovered by band excision and electroelution.

Enzymes and chemicals

Junction DNA was cleaved with restriction enzymes (Bethesda Research Laboratories and Boehringer) using conditions recommended by the manufacturers. Oligonucleotides were labelled at their 5′ termini using [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham). Osmium tetroxide was obtained from Sigma.

Osmium tetroxide reactions

Osmium tetroxide was dissolved in distilled water as an 8 mM stock solution. Purified junction DNA was incubated with 1 mM osmium tetroxide and 3% pyridine in 90 mM Tris−borate (pH 8.3), together with the concentrations of magnesium indicated in the text, in 50 μl final volume at 20°C for 15 min (Lilley and Palecek, 1984; Duckett *et al.*, 1988, 1990). Following osmium tetroxide reactions, the DNA was incubated in 1 M piperidine at 90°C for 30 min to cleave osmate adducts, followed by extensive lyophilization.

Sequencing reactions

Chemical sequencing reactions were performed on 5′-^{32}P-labelled oligonucleotides bound to Hybond M and G paper (Amersham), using dimethyl sulphate (G), formic acid (A + G), potassium permanganate (T > C) and hydroxylamine (C) reactions (Rosenthal *et al.*, 1985).

Gel electrophoresis

Purified junction DNA digested with the appropriate enzymes was loaded directly on to 5% polyacrylamide gels (29:1 monomer/bis ratio) and electrophoresed for 16 h at 90 V. Jacketed electrophoresis plates with circulated water ensured that the temperature of the gel remained at 20 ± 0.1°C throughout the electrophoresis experiment. The buffer system contained either 90 mM Tris−borate (pH 8.3), together with the indicated concentration of $MgCl_2$, or 90 mM Tris−borate (pH 8.0), 10 mM EDTA (TBE). Electrophoresis buffers were continuously re-circulated at 1 l/h. Junctions reacted with osmium tetroxide were analysed on 0.4 mm thick 10% polyacrylamide gels in TBE containing 7 M urea, which were run hot to the touch. Polyacrylamide gels containing radioactively labelled DNA were dried on to Whatman 3MM paper and autoradiographed at −70°C using Konica X-ray film with Ilford fast tungstate intensifier screens.

Acknowledgements

We thank Alastair Murchie for discussions and oligonucleotide synthesis, and the MRC, Wellcome Trust and Royal Society for financial support.

References

Beaucage,S.L. and Caruthers,M.H. (1981) *Tetrahedron Lett.*, **22**, 1859–1862.

Churchill,M.E.A., Tullius,T.D., Kallenbach,N.R. and Seeman,N.C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4653–4656.

Cooper,J.P. and Hagerman,P.J. (1987) *J. Mol. Biol.*, **198**, 711–719.

Cooper,J.P. and Hagerman,P.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7336–7340.

Duckett,D.R., Murchie,A.I.H., Diekmann,S., von Kitzing,E., Kemper,B. and Lilley,D.M.J. (1988) *Cell*, **55**, 79–89.

Duckett,D.R., Murchie,A.I.H. and Lilley,D.M.J. (1990) *EMBO J.*, **9**, 583–590.

Lilley,D.M.J. and Palecek,E. (1984) *EMBO J.*, **3**, 1187–1192.

Ma,R.-I., Kallenbach,N.R., Sheardy,R.D., Petrillo,M.L. and Seeman,N.C. (1986) *Nucleic Acids Res.*, **14**, 9745–9753.

Murchie,A.I.H.., Clegg,R.M., von Kitzing,E., Duckett,D.R., Diekmann,S. and Lilley,D.M.J. (1989) *Nature*, **341**, 763–766.

Rosenthal,A., Schwertner,S., Hahn,V. and Hunger,H.-D. (1985) *Nucleic Acids Res.*, **13**, 1173–1184.

Sinha,N.D., Biernat,J., McManus,J. and Koster,H. (1984) *Nucleic Acids Res.*, **12**, 4539–4557.

Received on January 26, 1990