The tertiary structure of the four-way DNA junction affords protection against DNase I cleavage

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ABSTRACT

The accessibility of phosphodiester bonds in the DNA of four-way helical junctions has been probed with the nuclease DNase I. Regions of protection were observed on all four strands of the junctions, that tended to be longer on the strands that are exchanged between the coaxially stacked pairs of helices. The protected regions on the continuous strands of the stacked helices were not located exactly at the junction, but were displaced towards the 3' side of the strand. This is the region of backbone that becomes located in the major groove of the opposed helix in the non-crossed, right-handed structure for the junction, and might therefore be predicted to be protected against cleavage by an enzyme. However, the major grooves of the structure remain accessible to the much smaller probe dimethyl sulphate.

INTRODUCTION

Homologous genetic recombination between homologous DNA molecules creates genome diversity, and is important in the course of evolution. The four-way DNA (Holliday) junction is believed to be the central intermediate in homologous recombination [1–6], as well as certain kinds of site-specific recombination events [7–10]. An important stage of recombination is the resolution process, that regenerates unconnected duplexes. This is achieved by Holliday resolvase enzymes, that recognise the structure of the junction and cleave it. Cleavage can generate two different recombination products, depending on the strands of the junction that are cleaved, and thus the nature of the interaction between the enzyme and the DNA may determine the genetic outcome of the recombination process.

Recent progress in understanding the structure of the four-way DNA junction has come from the study of small junctions assembled *in vitro* [11–14]. We demonstrated [14] that the inclusion of a four-way junction into a DNA fragment was equivalent to forming a sharp bend or kink at that position, to which gel electrophoresis was very sensitive, and we showed that the binding of metal ions altered the gel mobility of these species [15]. Cooper and Hagerman [16] studied the relative electrophoretic mobilities of small synthetic junction to which

reporter arms had been ligated in various combinations, concluding that the shape could not be tetrahedral, and deduced an asymmetric configuration of the four helical arms.

Using a related technique, we studied the configuration of a series of junctions constructed from eighty base oligonucleotides, and as a result we proposed a general model for the helical junction [17], termed the stacked X-structure. The essential details of the model were subsequently confirmed and extended using fluorescence energy transfer spectroscopy [18]. The main features of the proposed structure are:

1. In the presence of metal ions, the arms of the junction associate in pairs by coaxial helix-helix stacking. The choice of stacking partners depends on local sequence, and two isomers are possible. However, in general we observe predominantly a single isomer for a given sequence.

2. A major factor in the structure adopted by DNA junctions is electrostatic repulsion. This is minimised by rotation of the coaxially paired helices, rather in the manner of opening a pair of scissors. This does not affect helix-helix stacking, but serves to increase the majority of phosphate-phosphate distances.

3. The two coaxial helical stacks lie across each other with a right-handed sense.

4. The two-fold symmetry of the structure generates two sets of inequivalent strands in the structure. One set (the continuous strands) retain quasi-helical symmetry though the junction, and are related by the same helix axis throughout. The other (the exchanging strands) pass between the two coaxial stacks at the point of strand exchange.

5. The exchanging strands are disposed about the smaller angle of the X-structure, and thus do not cross. This generates an approximately antiparallel alignment of DNA helices, in contrast to the parallel structure most frequently depicted.

The features of the stacked X-structure are summarised schematically in Figure 1. The structure is consistent with all available experimental evidence. The non-crossed structure was proposed originally on the basis of gel electrophoretic experiments [17], and confirmed by fluorescence resonance energy transfer experiments in which the six relative end-to-end distances were compared within small junctions [18]. The two fold symmetry of the structure is consistent with hydroxyl radical probing experiments [19]. Cooper and Hagerman [20] have recently studied the structure of a junction by transient electric

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Figure 1. The stacked X-structure for the four-way helical junction. In the presence of appropriate cations, four-way DNA junctions undergo a folding process in which the four helical stems stack in pairs, to generate two connected coaxial quasi-continuous helices. These are rotated in a right-handed sense to generate the stacked X-structure in which the exchanged strands do not cross.

birefringence, and observed two small and four larger interarm angles, and the small angles are just those that are consistent with a non-crossed interpretation of their gel electrophoretic results.

The right-handed cross of the quasi-continuous helices was anticipated by both stereochemical reasoning [18] and molecular mechanical energy minimisation calculations [21], and was confirmed by fluorescence energy transfer experiments [18]. For the non-crossed, right-handed structure, phosphate-phosphate repulsion is minimised by placing the continuous strands emerging from the region of the exchange point in the major groove of the other helix. Such tertiary interactions might be expected to result in differential accessibility to probes of DNA structure. Hydroxyl radicals have been employed as sequence-neutral probes [23] of strand accessibility in synthetic junctions [19, 22], and some protection was observed near the exchange point. The hydroxyl radical is very small, and would not be expected to be readily thwarted by steric effects. We have therefore chosen the much larger probe DNase I, that has been extensively used for the study of DNA-protein interactions [24]. It is less sequence-neutral than hydroxyl radicals, but more likely to be sensitive to larger scale tertiary structure in the molecule. We have found that the structure of the junction results in significant protection against DNase I cleavage, and that the results are in good agreement with those expected on the basis of the non-crossed, right-handed stacked X-structure. Lu *et al* have very recently also reported the observation of protection against DNase I cleavage in a different DNA junction [25].

MATERIALS AND METHODS

Construction of junctions and duplexes

Oligonucleotides were synthesised by β -cyanoethyl-phosphoramidite chemistry [26,27], implemented on an Applied Biosystems 381 DNA synthesiser. Fully deprotected oligonucleotides were purified by electrophoresis in polyacrylamide gels containing 7 M urea. When required to be radioactive, oligonucleotides were labelled at 5'-OH using [γ - 32 P] ATP and T4 polynucleotide kinase (Amersham).

For each junction, four 80 nt or 30 nt oligonucleotides were synthesised, having the sequence of either junction 1 or 2 [17]. For the complete sequences of the oligonucleotides used to generate the 30 mer junctions 2 and 1, the interested reader should refer ahead to Figures 5 and 6. Strands complementary to each strand of the junctions were required for the construction of duplexes. For example, the strand complementary to the b strand of the 30 mer junction 2 (strand b') was CCTCCGGTAGCA-GCCGCTTGCTAGGACGGG. Junctions and duplexes were

Figure 2. The principle of the DNase I probing experiment. Junctions are constructed in which just one of the four strands is radioactively labelled with [$5'$ - 32 P]. The same strand is also hybridised to its perfect complement to generate a duplex, and the DNase I digestion of the labelled strand is compared between junction and duplex. This is repeated for the four strands in turn. The arms of the junction are labelled B, H, R and X as in [17], and the strands take the lower case label derived from the arm in which the 5' end is located, ie b, h, r and x.

constructed by annealing stoichiometric quantities of the appropriate oligonucleotides (one of which carried a [$5'$ - 32 P] radioactive label) for 2 h at 65°C in 450 mM NaCl, 45 mM Na citrate, 1 mM MgCl₂ followed by slow cooling. Junctions and duplexes were purified by gel electrophoresis in 5 or 10% polyacrylamide, excision of bands, electroelution and ethanol precipitation.

DNase I cleavage of DNA

DNase I (Sigma) was prepared as a 7200 unit/ml stock solution in 150 mM NaCl, 1 mM MgCl₂, and diluted to a working concentration in a buffer containing 10 mM Tris (pH 7.4), 20 mM NaCl, 20 mM MgCl₂, 4 mM MnCl₂ immediately before use. 1.8 pMol of junctions or duplexes were dissolved in 5 μ l of 10 mM Tris (pH 7.4), 20 mM NaCl, 100 μ M MgCl₂, and an equal volume of DNase I solution added to a final concentration of 0.3 unit/ml. These were incubated for 3 to 5 min at 16°C, and stopped by addition of 1 μ g of calf thymus DNA and EDTA to a final 25 μ M, followed by ethanol precipitation. The precipitates were dissolved in deionised formamide, containing 0.1% each of xylene cyanol and bromophenol blue.

Dimethyl sulphate modification of DNA

Dimethyl sulphate (BDH) was dissolved 1 in 200 (v/v) in either 90 mM Tris.borate (pH 8.3), 10 mM EDTA, or 90 mM Tris.borate (pH 8.3), 1 mM MgCl₂. To 200 μ l of 0.5% dimethyl sulphate were added 3.6 pMol of junctions, together with 0.1 μ g calf thymus DNA, and incubated for 6 min at 10°C. The reactions were stopped by addition of 1.5 M Na acetate (pH 7.0), 1 M β -mercaptoethanol and 100 μ g/ml tRNA, and ethanol precipitation. The DNA was cleaved at methylated guanine bases by reaction in 1 M piperidine at 90°C for 30 m, followed by extensive lyophilisation, and redissolved in deionised formamide.

Figure 3. DNase I cleavage of junction 2 80 mers. Junction 2 was constructed from oligonucleotides comprising 80 bases—this is the same DNA that was used in the gel electrophoretic experiments of Duckett *et al* [17]. For each strand, the junction and duplex were digested with DNase I, and electrophoresed on a 10% sequencing gel, followed by autoradiography. The position of the point of strand exchange (ie the centre of the oligonucleotide) is indicated by an arrow for each strand. Protected regions are clearly visible in each strand, and are indicated by vertical bars.

Analysis of cleavage products

Samples were heated to 90°C for 2 min prior to electrophoresis on 10% (80 mers) or 20% (30 mers) polyacrylamide sequencing gels containing 7 M urea. Gels were autoradiographed at -70°C using Konica X-ray film with an intensifier screen.

Sequencing of DNA

Radioactively labelled oligonucleotides were sequenced by chemical degradation [28]. DNA was reacted with dimethyl sulphate (G specific) or hydrazine (C + T specific), and cleaved with 1M piperidine.

RESULTS

The nuclease DNase I has been used extensively for the study of DNA-protein interactions. In general the pattern of cleavage

of phosphodiester bonds along a piece of DNA is compared with and without bound protein, and any protection conferred due to protein binding—the footprint—is taken as indicating the physical presence of the protein at that site. The aim of these experiments was to see if the three-dimensional structure adopted by the four-way DNA junction would itself afford protection against cleavage by DNase I.

The principle of the experiment is indicated in Figure 2. Four-way junctions were assembled from synthetic oligonucleotides, such that the central sequences were identical to those of Junctions 1 and 2 previously studied by us by means of gel electrophoresis and fluorescence resonance energy transfer [17, 18]. Each strand of the junctions were studied independently (ie in four separate experiments), by means of selectively labelling the 5'-terminus of a single oligonucleotide with [³²P], and junction assembly with three unlabelled oligonucleotides. In order to reveal effects due to the structure of the junction on the cleavage by DNase

Figure 4. DNase I cleavage of junction 2 30 mers. Junction 2 was constructed from oligonucleotides comprising 30 bases, such that the sequence (except for the two terminal nucleotides) was identical to that made from 80 mers. For each strand, the junction and duplex were digested with DNase I, and electrophoresed on a 20% sequencing gel, followed by autoradiography. For each strand there are three tracks; the sequence marker (G or CT), the digest of duplex and the digest of the junction. Dimethyl sulphate guanine reactions were used as sequence markers for b, h and x strands. A hydrazine cytosine plus thymine reaction was used for the r strand, because of the small number of guanines in this oligonucleotide.

I, the cleavage of a given strand within a junction was compared to the cleavage of that same strand hybridised to its perfect complement (termed the duplex). Any differences between the

Figure 5. Regions of junction 2 protected against DNase I cleavage, drawn on the stacked structure. The regions of DNA protected against DNase I cleavage in the junction relative to duplex DNA were derived from the analysis of many experiments. The results are summarised as the stippled regions.

DNase I cleavage of the strand in the junction and the duplex must arise from modulation of cleavage frequencies by the structure of the junction. It should be noted that as divalent cations were present in the incubation buffers, the junctions should remain in the folded stacked X-conformation during the DNase I cleavage. An exactly analogous procedure was used [19, 22] for the study of cleavage of DNA junctions by hydroxyl radicals.

The structure of a junction confers protection to cleavage by DNase I

The results of a DNase I probing experiment on a junction based on the sequence of junction 2 [17] are presented in Figure 3. The four 80 mer junctions containing a single radioactively labelled strand were digested with DNase I, and electrophoresed on a sequence gel that was subjected to autoradiography. For

Figure 6. Regions of junction 1 protected against DNase I cleavage, drawn on the stacked structure. The stippled boxes indicate regions of DNA protected against DNase I cleavage in the junction relative to duplex DNA. Junction 1 has four base changes relative to junction 2, the result of which is an exchange of stacking partners [17, 18].

each strand, the data are presented as pairs of tracks, where the strand was incorporated into a duplex or a junction, and comparison between tracks immediately reveals any protection in the latter.

Since the cleavage rates of DNA by DNase I is subject to considerable modulation by local sequence, even ladders along the length of the duplexes are not found. However, comparison with the junction-containing tracks shows that the pattern is altered in a significant way due to junction formation—for all strands a region near the centre of the strand is protected against DNase I cleavage. This suggests that the three-dimensional structure of the junction creates steric problems for the nuclease, such that the strands near the point of exchange are inaccessible to an enzyme of this size and shape. We note that the observed

Figure 7. Dimethyl sulphate modification of junction 1. Junction 1 constructed from 30 mers was reacted with dimethyl sulphate in the absence or presence of 1 mM Mg^{++} . Reactions were carried out separately on four singly-labelled junctions as before. Modified DNA was reacted with 1 M piperidine to cleave the backbone at methylated guanines, and electrophoresed on a 20% sequencing gel followed by autoradiography. Note that all guanines may be modified by dimethyl sulphate, in both folded (+ Mg^{++}) and unfolded (– Mg^{++}) conformations.

protected regions are much larger than those seen by hydroxyl radicals [19], presumably due to the greater dimensions of the enzyme probe.

In order to delineate the protected regions around the junction, a smaller version of junction 2 was created from 30 nt oligonucleotides. These were radioactively labelled on individual strands as junctions and duplexes, and digested with DNase I just like the larger junctions, and representative results are presented in Figure 4. By comparison with sequence markers, the extent of the protected regions can be placed on the sequences of the four strands. By analysis of a number of such gels, the borders of the protected regions can be deduced with a precision of a single base in most cases; a few are less accurate. These results are summarised in Figure 5, drawn on to the stacked structure of junction 2 deduced from gel electrophoresis [17] and

Figure 8. Regions protected against DNase I cleavage in the four-way junction. The regions protected against DNase I cleavage are shown coloured on the ribbon model of the non-crossed, right-handed structure of the four-way junction. **a.** View of the side at which the major grooves point towards the viewer. **b.** Front view, showing one quasi-continuous helix passing over the other. The major and minor groove sides of the structure are indicated. **c.** View of the side at which the minor grooves point towards the viewer. Both side views show clearly that the protected regions of the continuous strands on the 3' side of the point of strand exchange are partially obscured in the major groove of the opposite helix. The minor groove side view shows the widening of the minor groove close to the point of strand exchange, resulting in protection against DNase I cleavage.

fluorescence energy transfer [18] experiments. Several points can be made from this. First, all four strands are protected. Second, the extents of the protected regions are significantly greater on the exchanging strands (ie those that pass between the two pairs of stacked helices—in the case of junction 2 these are strands b and r) than those of the continuous strands (strands h and x for junction 2). Third, the protected regions on the continuous strands are not disposed symmetrically about their centres, but are displaced towards the 3' end of the strands.

Analogous protection against DNase I cleavage is found for a junction of opposite isomeric structure

We have found that two isomeric forms are possible for four-way DNA junctions, that differ in the helical stacking partners chosen [17]. The observed stable isomeric form for junction 2 has B and X arms stacked, as are H and R arms. However, we noted that by making two changes at the junction itself, we could construct a junction (junction 1), in which B and H arms were stacked. Clearly it was of interest to study the DNase I cleavage of junction 1, in order to see if analogous protection was conferred by the folding of this junction.

DNase I cleavage experiments were performed in an entirely analogous manner to those above. The protected regions were analysed from a number of experiments, and placed on the sequence of junction 2 in Figure 6. As with the other junction, the protection on the continuous strand is displaced from the centre in the 3' direction.

The structure of the junction does not hinder modification of guanine bases by dimethyl sulphate

Dimethyl sulphate (DMS) methylates N7 positions of guanine bases in DNA. Since purine N7 is located in the major groove, DMS provides a probe of the accessibility of this groove in a given structure. We can therefore ask whether the stacked X-structure adopted by the four-way junction prevents access by DMS into the major groove at any point.

The experiments were performed on junction 1, and we took advantage of the ability to use this probe in the absence of metal ions (in contrast to DNase I) to compare the folded and unfolded conformations. In place of the approach of comparing the attack of the probe on junction and duplex, we studied the modification of the singly-labelled junction with or without added magnesium,

ie in the folded and extended conformations respectively. The chemical modification was revealed by subsequent reaction with 1 M piperidine, in order to achieve strand scission. An example of the resulting data is presented in Figure 7. In contrast to the results with DNase I probing, there are almost no differences apparent between the modification of guanine bases due to folding of the junction. Some guanines exhibit a small variation in modification in the junction (such as the guanine on the x strand at the point of strand exchange, that becomes protected on addition of magnesium), but this modulation is weak. We conclude that the major groove is sufficiently unprotected by the structure of the junction that it is freely accessible to DMS molecules.

DISCUSSION

We have clearly demonstrated that the folding of the four-way DNA junction into the stacked X-conformation confers protection to cleavage of the strands in the region of the junction itself. The patterns of protection are characterised by the following observations:

1. All four strands of the junction are protected in discrete regions at the point of strand exchange. The extent of the protection tends to be greater on the exchanged strands.

2. The centre of the region protected on the continuous strands is located to the 3' side of the point of strand exchange.

From stereochemical principles, we proposed that the four-way junction should be most stable in a right-handed, non-crossed conformation, as this achieves greatest reduction of interaction between phosphate groups on different helices near the junction. A related interaction between double-stranded DNA helices has been observed in a crystal structure of a dodecanucleotide [29]. The handedness of the junction was confirmed experimentally using fluorescence resonance energy transfer measurements of dye-conjugated junctions [18]. We must ask how the above results on DNase I cleavage fit in with this model.

Three views of the stacked X-structure are presented in Figure 8, drawn in a simplified ribbon representation for clarity. The regions of backbone that are protected against DNase I cleavage are indicated. The exchanging strands are well protected in the region of the exchange, as would be expected in most models. Part of this protection may arise from interaction of these strands with the metal ions that are tightly bound in the formation of the folded structure [17, 30]. Perhaps more interesting is the protection afforded to the continuous strands. This is located 3' to the point of strand exchange, which is just the region where this strand turns through the major groove of the opposing helix. This is seen most clearly in the side views of the structure. DNase I is known to bind in the minor groove of DNA [31, 32] and the cleavage rate is highest when the groove width is optimal. Energy minimisation studies of the junction structure [21] suggest that it will be necessary to widen the minor groove locally in order to permit the required strand exchange, and this can be seen clearly in the view of the junction from the minor groove side (Figure 8c). The region of groove widening at the junction corresponds well with the regions of backbone protected against DNase I cleavage. The protection is thus likely to result from a combination of structural factors, including occlusion of the continuous strands in the major groove, and variation in width of the minor groove. The right-handed, non-crossed structure for the junction can therefore explain all the features of the DNase I protection pattern. Other models (such as crossed, or parallel,

structures) are less consistent with the data, although a detailed analysis is not possible in the absence of specific structures. Small differences between the protection patterns of junctions 1 and 2 may indicate some structural variability, such as the angle of the X, with sequence.

Despite the protection against DNase I cleavage that junction formation affords, the major groove remains largely accessible to a probe of the size of DMS. Thus, although the right-handed, non-crossed structure locates the continuous strands of each helix in the major groove of the opposing helix, the fit is clearly loose enough to allow free access to a small molecule the size of DMS. Similarly, only very weak protection was observed against hydroxyl radicals [19] in the equivalent 3' positions.

In summary, the patterns of protection seen in these small four-way DNA junctions are fully consistent with the previously derived model for the structure of the junction, and may therefore be seen as further evidence supporting it.

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