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Cleavage of a four-way DNA junction by a restriction enzyme spanning the point of strand exchange

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The four-way DNA junction is believed to fold in the presence of metal ions into an X-shaped structure, in which there is pairwise coaxial stacking of helical arms. A restriction enzyme *Mbo*II has been used to probe this structure. A junction was constructed containing a recognition site for *Mbo*II in one helical arm, positioned such that stacking of arms would result in cleavage in a neighbouring arm. Strong cleavage was observed, at the sites expected on the basis of coaxial stacking. An additional cleavage was seen corresponding to the formation of an alternative stacking isomer, suggesting that the two isomeric forms are in dynamic equilibrium in solution.

Key words: DNA structure/Holliday junction/recombination/restriction enzymes

Introduction

The four-way DNA junction has been postulated to be an important intermediate in homologous genetic recombination (Holliday, 1964; Broker and Lehman, 1971; Sobell, 1972, 1974; Sigal and Alberts, 1972; Meselson and Radding, 1975; Potter and Dressler, 1976, 1978; Orr-Weaver *et al.*, 1981) and there is evidence for its involvement in the integrase class of site-specific recombination (Hoess *et al.*, 1987; Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987; Jayaram, *et al.*, 1988). There has been significant progress in understanding the structure of the four-way junction based on gel electrophoresis (Gough and Lilley, 1985; Cooper and Hagerman, 1987; Duckett *et al.*, 1988), spectroscopy (Murchie *et al.*, 1989), probing experiments (Churchill *et al.*, 1988; Duckett *et al.*, 1988; Lu *et al.*, 1989; Murchie *et al.*, 1990) and molecular modelling (von Kitzing *et al.*, 1990), and a reasonable consensus is emerging. We have proposed a geometry for the junction, termed the stacked X-structure, based on pairwise stacking of helical arms to generate two antiparallel quasi-continuous helices, that are rotated with a right-handed sense to present an X-shape with a small angle of $\sim 60°$. We noted that two isomeric forms of the structure are possible, depending on which pairing of stacked arms is made. Resolving enzymes appear to interact selectively with one side of the junction (von Kitzing *et al.*, 1990) and thus the alternative isomeric forms are cleaved differently (Duckett *et al.*, 1988; Mueller *et al.*, 1988).

While the X-shape is the simplest interpretation of gel electrophoretic and fluorescence energy transfer experiments, these data do not demand that the arms are fully colinear. A lower symmetry would be consistent with these data, and this has been argued on the basis of electric birefringence measurements (Cooper and Hagerman, 1989). Nucleic acids exhibit a propensity to undergo base stacking, exemplified by the T and acceptor arms of tRNA where a coaxial helical stack is formed (Rich, 1977), and this is consistent with results of osmium tetroxide probing of the DNA junction as a function of metal ions (Duckett *et al.*, 1988, 1990). We sought confirmation of this stacking using the restriction enzyme *Mbo*II (Gelinas *et al.*, 1977). This enzyme has a recognition site that lies 7 bp from the site of cleavage. The basic principle of the experiment was to construct a junction in which the recognition target was located in one arm, such that cleavage would presumably require helix–helix stacking to locate the cleavage site in a coaxial helix. If colinear helices were generated in the folding of the four-way junction, then it might be anticipated that cleavage would proceed just as for a normal duplex of the same sequence. Furthermore, if stacking isomers were present in solution, then incubation with the enzyme would result in different strands becoming cleaved, depending on which arm was stacked with that containing the recognition target. Thus *Mbo*II cleavage might shed light both on the colinearity of helical arms in the folded conformation of the four-way junction, and the existence of isomeric forms of the junction in solution.

Results

A four-way junction containing a site for MboII

*Mbo*II is one of the class of type II restriction enzymes in which the cleaved phosphodiester bonds lie outside the recognition sequence. As shown in Figure 1, cleavages are introduced into any sequence that is 8 bases (top strand) and seven bases (bottom strand) from the GAAGA binding site. We constructed a four-way junction with a central sequence identical to that of junction 1 previously extensively studied in this laboratory (Duckett *et al.*, 1988, Murchie *et al.*, 1989), in which one of the four helical arms contained an *Mbo*II recognition target, shown in Figure 1. Introduction of this sequence required no alteration of the original junction 1 sequence that was closer to the point of strand exchange than 5 bases. The junction was constructed from four synthetic oligonucleotides, each of 30 nucleotides, thus generating arms of 15 bp. In the stacked X-structure proposed for the four-way junction (Duckett *et al.*, 1988), there is pairwise, coaxial stacking of the arms, generating quasi-continuous helices of 30 bp. Gel electrophoretic and fluorescence energy transfer experiments have shown that formation of the stacked X-structure by junction 1 involves stacking of X and R arms; thus if the stacked pair of arms resembles a continuous DNA helix, cleavage of the R arm (i.e. the h and r strands) by *Mbo*II might be anticipated (Figure 1).

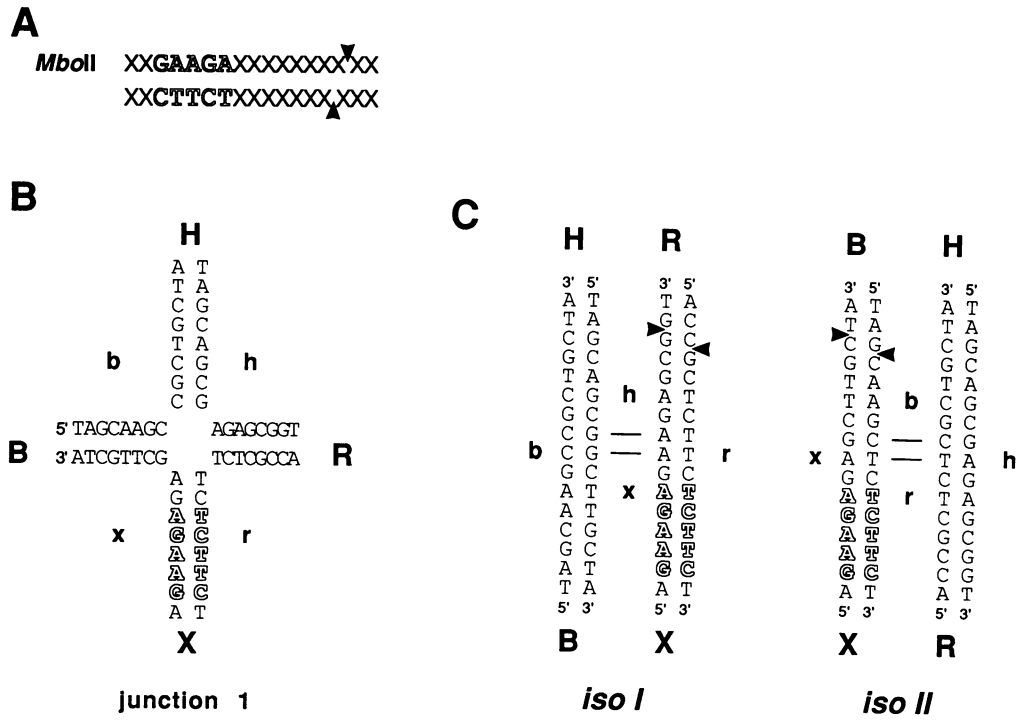

Fig. 1. The sequence of junction 1, in which an *Mbo*II site has been introduced. **A.** The sequence requirements for *Mbo*II cleavage of DNA. The enzyme recognizes the sequence 5'GAAGA3', and cleaves eight bases further in the 3' direction as indicated. **B.** The central sequence of junction 1 modified to include an *Mbo*II site in the X arm (open characters). Uppercase letters denote the four arms of the junction, while strands are given lowercase letters corresponding to the arm in which the 5' end is located. In the junction that was used experimentally the arms were each 15 bp in length; only the central 8 bp of each arm are shown here, but the full sequences are given in the Materials and methods section. **C.** Two possible ways of pairwise stacking of the arms. *IsoI* is generated by stacking H and B arms, while *isoII* is formed by B on X stacking. Gel electrophoretic and fluorescent spectroscopic evidence indicate that junction 1 is well described by *IsoI*. The coaxial stacking of X and R arms in *isoI* might be expected to lead to cleavages in the h and r strands, indicated by the arrows. Alternatively, *isoII* might be cleaved in the x and b strands as indicated.

Cleavage of four-way junction 1 by MboII

Four preparations of the junction were made, each 5'-^{32}P-labelled in one of the four strands, in order to deduce the cleavage sites in each strand unambiguously. After incubation of each radioactively labelled junction with *Mbo*II, the digested DNA was analysed by electrophoresis on a sequencing gel, and the resulting autoradiograph is shown in Figure 2.

Several points can be noted from this gel. It is clear that the junction is cleaved by *Mbo*II. Strong cleavages are seen in the h and r strands, as predicted from the model in which there is R on X arm stacking to form a continuous helix. Comparison with sequence tracks derived by chemical cleavage of the radioactive single strands shows that the cleavages are at the bonds expected for a continuous X−R stack, and indicates that there is no gross structural distortion at the exchange point (such as a large change in twist).

Examination of the sequence of junction 1 in the *isoI* conformation (Figure 1) shows that stacking of the R and X arms generates a second *Mbo*II recognition site flanking the point of strand exchange. However, no cleavage is seen corresponding to this site.

MboII sites are cleaved at unequal rates

Closer inspection of Figure 2 reveals that the intensity of the cleavages introduced by *Mbo*II into the h and r strands are not equal, indicating a possible difference in accessibility between the two sites. In order to quantify this difference we compared the rates of cleavage on both strands with those on equivalent duplex molecules. Radioactively labelled r and h strands were incorporated into either four-way junctions or duplex molecules, and digested with *Mbo*II at 23°C for different times. Time courses are shown in Figure 3. The extent of cleavage as a function of time was estimated by gel electrophoresis and phosphorimaging, from which relative rates of cleavage for the chosen experimental conditions could be measured. The rates of cleavage of junction 1 are significantly lower than those on the duplex molecules, despite the fact that the sequences cleaved are identical. The h strand is cleaved approximately five times more slowly when assembled into a four-way junction, compared to a duplex. Thus the structure of the junction hinders cleavage by *Mbo*II. Nevertheless, given sufficient time, 100% of the h strand is cleaved by the restriction enzyme, albeit at a reduced rate. Rate constants calculated from the kinetic measurements (Figure 3) showed that there is an approximately four-fold difference in the rates of cleavage of the h and r strands.

Evidence for a second stacking isomer

In addition to the cleavages that are seen in the h and r strands, closer inspection of Figure 2 also reveals a weak cleavage that was introduced into the x strand. In order to obtain cleavage in the x strand it is necessary for the junction to isomerise, generating a new stacked X-structure in which the X and B arms form a stacked, coaxial pair. The intensity of this cleavage (50 times weaker than that of the h strand) suggests that the population of this isomer is smaller than

Fig. 2. Cleavage of a four-way DNA junction by *Mbo*II. Junction 1, 5′-^{32}P-labelled in the indicated strand, was cleaved with *Mbo*II, and the DNA electrophoresed on a sequencing gel and autoradiographed. The central lane (G) was derived by DMS modification of the h strand. The strong bands at the top are the full length 30 base oligonucleotides, and bands further down arise from cleavage by the restriction enzyme.

the B on H isomer. Our original gel electrophoresis experiments also require that any distribution of isomers should favour the *isoI* form (Duckett *et al.*, 1988). Nevertheless, these results show that the alternative isomeric form exists. This implies that there is an equilibrium in solution between the two isomers, that is biased towards the B on H isomer.

Cleavage of bulge-kinked DNA molecules

The cleavage by *Mbo*II in junction 1 suggests that the X arm (containing the enzyme binding site) and the R arm (where it cleaves) are probably close to being colinear. In order to examine this in further detail we constructed DNA molecules known to deviate from linearity. We and others have shown previously that bulges (unopposed bases in a duplex) generate a precise kinking of the helix axis (Bhattacharyya and Lilley, 1989; Hsieh and Griffith, 1989; Rice and Crothers, 1989), the magnitude of which depends on the number and type of unopposed bases. We therefore constructed duplex molecules of the same sequence as an X on R stack, but containing variable sized oligoadenine bulges at the position

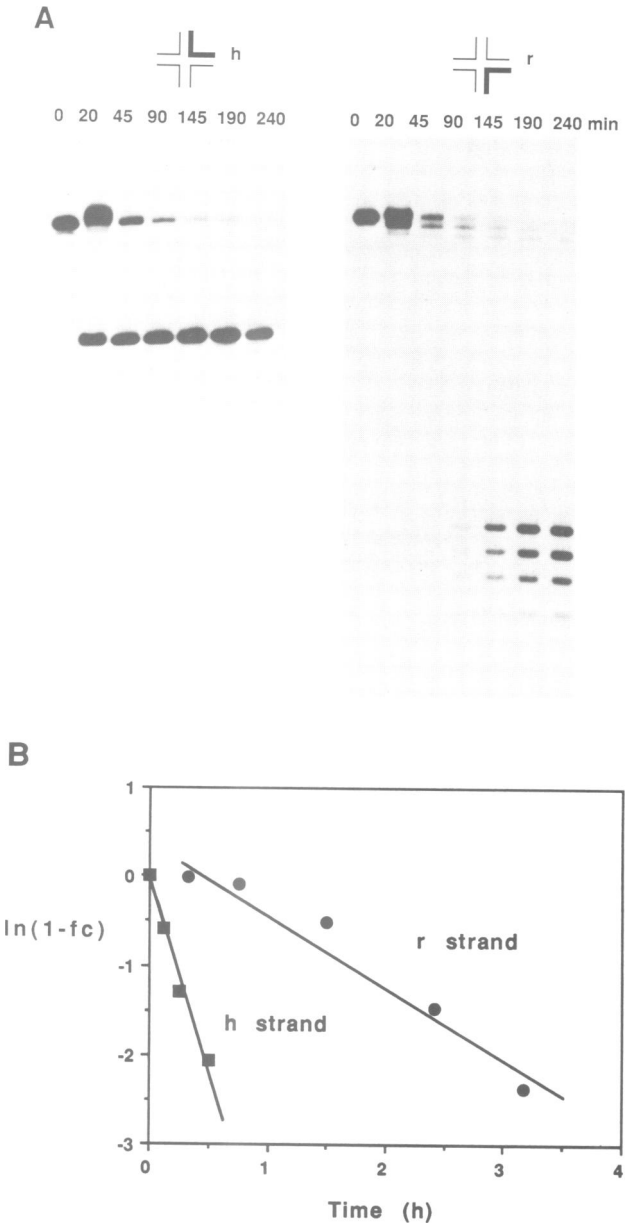

Fig. 3. *Mbo*II cleaves the strands of junction 1 at different rates. **A.** Junction 1, 5′-^{32}P-labelled in either the h or the r strands, was incubated with *Mbo*II at 23°C for the times indicated, followed by electrophoresis and phosphorimaging and autoradiography. The autoradiographs are presented. Note the complete digestion of the h strand after four hours. The r strand is subject to some exonucleolysis; the extent of this varies between preparations of the restriction enzyme. **B.** Comparison of the rates of cleavage of h and r strands. Plot of the logarithm of the uncleaved fraction (f_c is the fraction of DNA cleaved by *Mbo*II) of DNA as a function of time. Rate constants were calculated from the gradients.

opposite to the point where strand exchange occurs in the junction (Figure 4). These species were cleaved under identical conditions, and the products examined by gel electrophoresis as before. The resulting autoradiograph is shown in Figure 4. While a single adenine perturbs the cleavage pattern only in minor detail, inclusion of an A_3 or A_5 bulge into the duplex considerably alters the pattern of cleavages. On the non-bulged strand (equivalent to h strand cleavage in the junction), there is marked cleavage one base nearer the recognition sequence, and for the A_5 bulge the

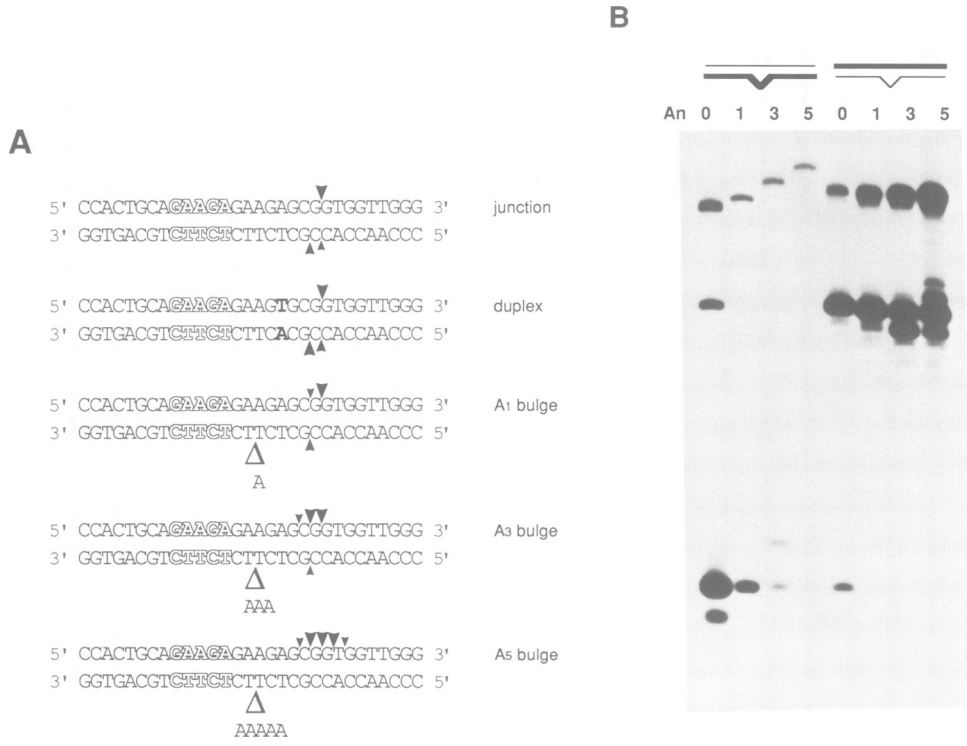

Fig. 4. Cleavage of bulged DNA duplexes by *Mbo*II. **A.** Sequences of the bulged duplexes. These were based on the sequence of the stacked X and R arms of junction 1. Adenine bulges were introduced into the position opposite to the point of strand exchange in the junction. In the case of the unbulged duplex, the 3′ *Mbo*II site was mutated (basepair shown bold) to prevent corresponding cleavage. **B.** *Mbo*II cleavage of the bulged duplex molecules. Duplexes constructed from one 5′-^{32}P-labelled and one unlabelled strand were cleaved with *Mbo*II, and the products separated by electrophoresis on a sequencing gel. In the case of the unbulged duplex there is some cross contamination of radioactive strands, due to an incompletely inactivated kinase. Relative extents of cleavage were estimated by phosphorimaging, and the results indicated by the size and locations of arrows in the sequences shown in A.

strand becomes strongly cut at several positions. Cleavage on the bulged strand is severely, and ultimately completely, inhibited by the presence of the bulge. These results suggest that deviation from colinearity of binding and cleavage sites changes the interaction between the catalytic site of the restriction enzyme and the DNA. The precise cleavages introduced into the R arm of the four-way junction are therefore consistent with coaxial stacking of the R and X arms in the junction, although small deviations from colinearity cannot be excluded.

Discussion

*Mbo*II is a restriction enzyme for which the recognition and cleavage sites are separated by virtually a complete turn of B-form DNA. We have found that when these sites were placed in different helical arms of a four-way junction, there was extensive cleavage at the target sites expected on the basis of formation of coaxially stacked pairs of arms. It is especially interesting that strong cleavage was observed in the h strand of the R arm, despite the fact that this strand is not covalently continuous with the recognition site for the enzyme (see Figure 1).

Stacking of helical arms in the four-way junction

Previous studies of the four-way junction have suggested pair-wise stacking of helical arms, and this was a feature of early models for the junction (Sigal and Alberts, 1972). Our gel electrophoretic experiments (Duckett *et al.*, 1988) indicated that in the presence of magnesium ions, the junction adopts an X-shape, and important aspects of the structure were confirmed by fluorescence energy transfer experiments (Murchie *et al.*, 1989). Incorporation of the well known tendency of basepairs to undergo stacking into the model suggests that the stacked X-structure is constructed by coaxial stacking of arms, yet the symmetry suggested by either electrophoresis or fluorescence experiments does not demand this. However, the pattern of cleavage in junction 1 by *Mbo*II, and comparison with model duplex molecules kinked by base bulges, is fully consistent with coaxial stacking of the R and X arms. Since the cleavages introduced into the junction are not translated in either direction, there is no evidence for significant over- or underwinding at the junction. This interpretation is subject to the caveat that the enzyme may itself influence the structure of the junction, although this probability does not seem high to us. Moreover, we cannot interpret our data to say that the arms must be exactly colinear; we cannot exclude the possibility that the formation of the junction results in some kinking to which the *Mbo*II is not overly sensitive. Good stacking at the exchange point is consistent with the results of chemical probing by osmium tetroxide (Duckett *et al.*, 1988, 1990). Osmium addition occurs at thymine bases present immediately at the point of strand exchange in the ion-free unfolded conformation, but not in the folded conformation, indicating that good stacking between arms prevents the required out-of-plane attack by the electrophile in the stacked-X structure. Continuity of structure through the junction is also consistent with results of hydroxyl radical probing (Churchill *et al.*, 1988).

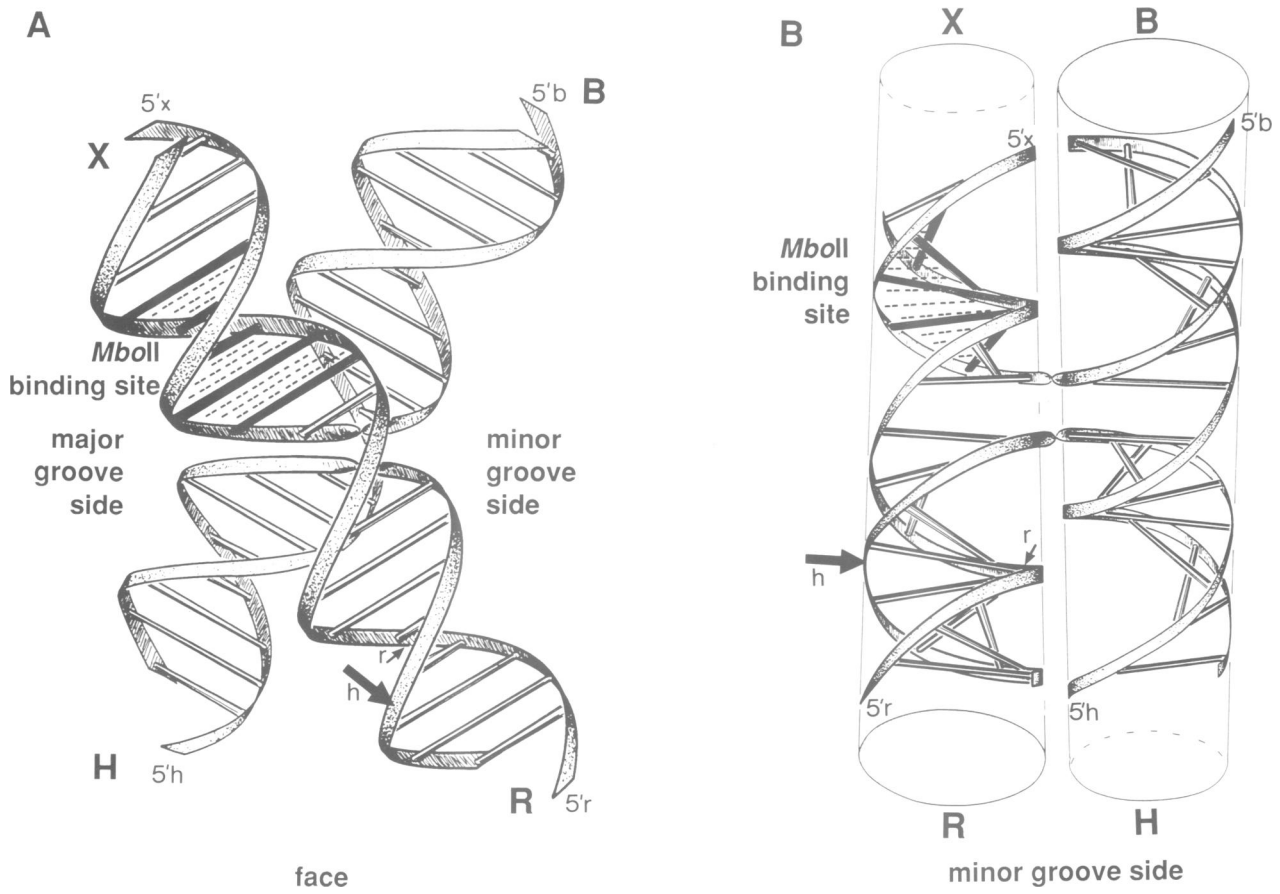

Fig. 5. Location of the *Mbo*II binding and cleavage sites shown on the proposed stacked X-structure of junction 1. Two views of the structure are presented in ribbon form; the face view (**A**) and the minor groove side (**B**). The recognition site for *Mbo*II in the X arm is shown stippled—the major groove should be accessible to the enzyme without steric hindrance from the stacked BH pair of arms. The cleavage sites in the h and r strands are indicated by arrows. The phosphodiester bond cleaved in the h strand is very accessible from the side of the junction, while that in the r strand is in close proximity to the H arm which will therefore hinder attack by the enzyme at this site. This explains the experimental observation that the h strand is cleaved about four times faster than the r strand in the junction.

Accessibility of binding and cleavage sites in the four-way junction

Cleavage of the four-way junction by *Mbo*II shows that the binding site for the enzyme is accessible to the restriction enzyme. Figure 5 shows the position of the *Mbo*II recognition site on a ribbon representation of the proposed antiparallel, right-handed structure of the junction. The major groove is quite accessible from the face and minor groove side of the junction, and hindrance by the other stacked pair of arms should be minor. By contrast, the major groove at the point of strand exchange is less accessible, explaining the failure of some restriction enzymes with recognition sites that span this region (including the 3′ *Mbo*II site of junction 1) to cleave these substrates (data not shown). The positions of the cleavages introduced by *Mbo*II are indicated on the model. While the cleavage site in the h strand is on the outside of the molecule, that on the r strand is on the opposite side of the R helix, where access will be hindered by the H arm. Thus the four-fold lower rate of cleavage on the r strand is readily explained in terms of the tertiary structure of the junction.

The mechanism by which *Mbo*II cleaves at a fixed and significant distance from the recognition target is unknown. The lower rates of cleavage found in the junction compared with the duplex implies that the proximity of the second helix may interfere with this to some degree. However, the fact that accurate site-specific cleavage occurs indicates that this interference is relatively minor, and suggests that the restriction enzyme is located along one face of the molecule, on the outer face of the junction.

An equilibrium between stacking isomers

The cleavage of the x strand in junction 1 by *Mbo*II is significant. This corresponds to formation of the alternative stacking isomer, in which the X arm is stacked with the B arm. The weak cleavage at this site suggests that the proportion of this isomer is low, in agreement with our earlier gel electrophoretic results (Duckett *et al.*, 1988). However, this cannot be quantified from these data since the extent of cleavage will not be exactly proportional to the relative amount of this isomer that is present in solution, for two reasons. First, the sequence at the cleavage site is not identical for the two isomers, and second, the presentation of the recognition and cleavage sites changes significantly on isomerization.

The second isomer is in equilibrium with the major isomer, since extended digestion with *Mbo*II results in 100% cleavage of the h strand. It is not possible for 100% of one isomer to coexist with a small percentage of the other, since they are mutually exclusive conformations, unless continual interconversion occurs. Thus the results suggest a dynamic equilibrium between the two isomers. In gel electrophoretic experiments, only a single set of bands is observed (Cooper and Hagerman, 1987; Duckett *et al.*, 1988), indicating that

the interconversion between isomers is fast compared to the rate of equilibrium in the gel pores. It is quite possible that the rate limiting process for the isomerization process will be helical unstacking. NMR studies of imino proton exchange processes in duplex DNA (Guéron *et al.*, 1987) indicate that helix opening is relatively slow and infrequent. However, opening on the millisecond timescale may nevertheless be fast relative to that of an electrophoresis experiment. The equilibrium between isomers is particularly significant, as helical unstacking is probably an integral part of, and perhaps a prerequisite for, branch migration processes in junctions with appropriate sequence symmetry.

Conclusions

The cleavage of a four-way DNA junction by *Mbo*II permits the following conclusions to be drawn: (i) The accuracy with which the enzyme cleaves the junction is consistent with coaxial helix−helix stacking. (ii) The accessibility of the recognition sequence, and the relative cleavage rates of the two strands, is in agreement with the right-handed, antiparallel structure of the junction. (iii) The observation of cleavages in both the R and B arms suggests that both stacking isomers are present in solution, and that they are interconverted in a dynamic equilibrium. These observations may be taken as further evidence for the proposed stacked X-structure. Furthermore, the structure should now be regarded as potentially dynamic structure in which there is a conformational equilibrium between isomers.

Materials and methods

Oligonucleotide synthesis

Oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry (Beaucage and Caruthers, 1981; Sinha *et al.*, 1984) implemented on Applied Biosystems 381A and 394 synthesizers. Deprotected oligonucleotides were purified by electrophoresis in 20% polyacrylamide containing 7 M urea, and electroelution. Oligonucleotides were 5′-^{32}P-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The sequences of the oligonucleotides were the same as the 30-mers used in Murchie *et al* (1990), except for the modifications necessary to introduce the *Mbo*II restriction site indicated in the text. Thus the four oligonucleotides were:

b strand 5′ CCCGTCCTAGCAAGCCGCTGCTACCGGAGG 3′

h strand 5′ CCTCCGGTAGCAGCGAGAGCGGTGGTTGGG 3′

r strand 5′ CCCAACCACCGCTCTTCTCTTCTGCAGTGG 3′

x strand 5′ CCACTGCAGAAGAGAGCTTGCTAGGACGGG 3′

Construction of four-way junctions and bulged duplexes

Stoichiometric quantities of each oligonucleotide were hybridized by slow cooling from 65°C in 450 mM NaCl, 45 mM Na citrate, 1 mM $MgCl_2$, and purified by electrophoresis in 8% polyacrylamide and electroelution.

Restriction enzyme digestion

*Mbo*II was obtained from IBI. DNA was incubated with 5 units of *Mbo*II in 5 μl volumes containing 25 mM Tris−HCl, pH 7.8, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 100 μg/ml BSA. We noted that the quality of the enzyme varied with source, and even with batch. Some preparations of *Mbo*II appeared to contain some exonuclease activity (see Figure 3 for example).

Kinetics of MboII cleavage

1 pmol of DNA was cleaved with 15 U of *Mbo*II at 23°C. 2 μl aliquots were removed at various times and the reaction stopped by addition of formamide and freezing in liquid nitrogen. At the end of the time course, all DNA aliquots were denatured at 90°C for 90 s and loaded onto a 20% polyacrylamide gel. After electrophoresis, gels were exposed to storage phosphor screens (Kodak) and quantification of radioactivity performed using a 400S PhosphorImager (Molecular Dynamics). Extent of cleavage was assessed as the ratio of the radioactivity of cleaved to total DNA (f_c). Restriction cleavage rate constants were calculated by linear regression from the gradient of $\ln(1-f_c)$ versus time.

DNA sequencing

Guanine-specific sequencing lanes were generated from 5′-^{32}P-labelled oligonucleotides using a modification of the DMS reaction (Maxam and Gilbert, 1980; Williamson and Calander, 1990). DMS modification was performed in 10 μl volumes containing 0.05% aqueous DMS (v/v) for 10 min at 20°C. Adducts were cleaved by 1.2 M pyrrolidine at 90°C for 15 min, evaporated to dryness and repeated coevaporation with water, before dissolving in formamide and gel electrophoresis.

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