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Site-Specific Interaction of Intercalating Drugs with a Branched DNA Molecule†

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ABSTRACT: The interaction of a stable branched DNA molecule with an intercalative drug is probed by hydroxyl radical scission. Methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)], consisting of an intercalating ring system tethered to EDTA·Fe(II), produces the hydroxyl radicals by means of a Fenton reaction. The cleavage patterns of each labeled strand in a branched tetramer of four 16-mers are compared with those of the same strands in unbranched duplex controls. Strong differences between the profiles corresponding to scission of branched and duplex DNA molecules are seen in each of the strands at low MPE/DNA ratios. A specific site in the branched structure interacts preferentially with the drug, while other regions of the molecule are protected from cleavage. At 4 °C, cutting at strand positions demarcating the site of enhanced affinity is observed to be 60–100% more efficient than at the corresponding sequence positions in the control duplex DNA molecules; the degree of protection is comparable. Cleavage in the vicinity of the preferred site occurs at residues flanking the branch point. The reactive Fe(II) group appears to be centered within two residues of the branch point, and the site of preferential intercalation may be between the two base pairs abutting the branch point in one of the two helical domains. The pattern of preferential cutting at this site is eliminated in the presence of excess propidium diiodide, another intercalative drug.

Chemical probes of the structure and conformation of nucleic acids make it possible to investigate details of DNA or RNA structure to a resolution of a single base pair (Gilbert et al., 1976; Singer, 1975). In combination with electrophoresis, a variety of reactive probes that are relatively insensitive to base sequence enable one to detect the presence of unusual structural features in nucleic acids by "footprinting" (Dervan, 1986; Sigman, 1986; Tullius & Dombrowski, 1985, 1986; Tullius, 1987). We have been interested for some time in the physical properties of a system of stable, branched DNA molecules formed from oligonucleotide strands (Seeman, 1982; Seeman & Kallenbach, 1983). These molecules enable one to model properties of potentially unstable states of DNA, such as those that occur in recombination or cruciform extrusion. Structures with three, four, and up to six arms have been investigated, including two kinds of branched systems: those in which the branch point is completely incapable of migrating along any of the duplex arms of the complex, which we refer to as immobile junctions (Seeman, 1982; Kallenbach et al., 1983; Figure 1), and those in which the sequence surrounding the branch point possesses precisely enough symmetry to allow a predetermined small number of steps of the branch migration reaction to take place—partially mobile junctions (Seeman, 1982; Chen et al., 1988). The physical properties of one four-arm junction, J1 (with the sequence shown in Figure 1), have been determined in greatest detail so far, including measurements of the electrophoretic mobility on polyacrylamide gels (Kallenbach et al., 1983; Chen et al., 1988), UV (Marky et al., 1987), CD (Seeman et al., 1985; Marky et al., 1987), and NMR (Wemmer et al., 1985) spectra, thermodynamic parameters (Marky et al., 1987), and the distribution of macrocyclic rings of different size resulting from covalent joining of single three-arm or four-arm junctions to form chains (Ma et al., 1986; Petrillo et al., 1988).

A crucial piece of structural information about immobile and partially mobile junctions in solution has emerged from investigating the sensitivity of different sites in the component strands to hydroxyl radical cleavage (Churchill et al., 1988; Chen et al., 1988). Analysis of the autofootprints of the junctions by Fe(II) EDTA in the presence of ascorbate reveals a 2-fold symmetric protection pattern of each molecule in these branched complexes relative to their patterns in control linear

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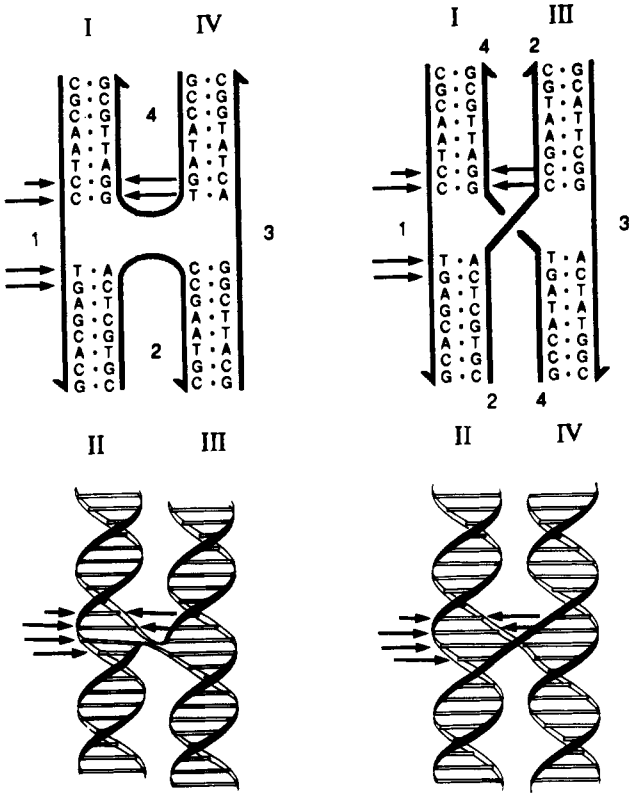

FIGURE 1: Sequence and structure of the branched junction J1 and its cleavage by MPE·Fe(II). The upper panels show the junction in schematic, while the lower panels show what the junction would look like if it were formed from 10.5-fold B-DNA. The junction is composed of four strands of DNA, indicated in the upper panels by Arabic numerals. Each strand participates in forming two double helical arms, indicated by Roman numerals. The structural conclusion of previous work (Churchill et al., 1988) is indicated in this figure, by stacking arm I on arm II and arm III on arm IV to form two helical domains. Both of the possible coplanar arrangements of these helical domains are shown, with the antiparallel arrangement on the left and the parallel arrangement on the right. The sites of enhanced hydroxyl radical cleavage due to the interaction of J1 with MPE·Fe(II) are indicated by arrows. The length of the arrow indicates the qualitative intensity of the cleavage site.

duplexes. This pattern implies that both types of junction exist predominantly as two sets of stacked helices, in which the structures of two strands are unperturbed relative to the same strands in duplex models, while two others form the crossover strands (see Figure 1). A similar conclusion has been reached by Cooper and Hagerman (1987) using an electrophoretic analysis. Which arms participate in particular helical domains is determined by the base sequence flanking the branch point (Chen et al., 1988; Duckett et al., 1988). The precise relative orientation of the helical domains is not yet known, however.

An important issue with respect to characterizing DNA junctions concerns how they respond to different ligands capable of interacting with double-stranded DNA. Reports in the literature have suggested that certain drugs interact specifically with branched DNA structures. Experiments by Fishel and Warner (Fishel, 1981) indicate that the intercalative drugs, ethidium and propidium, can act as potent inhibitors of branch migration in DNA at concentrations in the micromolar range. This result does not distinguish a specific conformational effect due to the drug acting in the vicinity of the branch point from a general effect of drug on altering the flexure of the arms. Inhibition of branch migration is detected at relatively low occupancy of ethidium sites, suggesting that the former mechanism might be operative. An effect of ethidium on reducing the electrophoretic mobility of DNA cruciforms in native gels has also been reported (Dieckmann & Lilley, 1987), but again this cannot be attributed to a site-specific interaction of the drug in the vicinity of the branch point. On the other hand, a highly selective interaction of a $Rh(DIP)_3^{3+}$ ligand in the vicinity of the extruded site of cruciforms in phage and plasmid DNAs has recently been reported (Kirshenbaum et al., 1988).

We present here experimental evidence that the reagent developed by Dervan's laboratory (Dervan, 1986), methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)], which binds to duplex DNA and cleaves strands in the presence of peroxide or ascorbate (Hertzberg & Dervan, 1982, 1984; Van Dyke & Dervan, 1983a,b), selects one preferential site at or close to the branch point in the immobile four-arm junction J1 at low ratios of drug to DNA. MPE consists of a DNA intercalator similar to the ring system in ethidium linked via a short hydrocarbon tether to an EDTA group (Hertzberg & Dervan, 1982). MPE·Fe(II) has been shown to bind normal duplex DNA with relatively low sequence specificity, cleaving the sugars of the backbone proximal to the binding site via hydroxyl radicals generated by a Fenton reaction in the presence of oxidants (Hertzberg & Dervan, 1984).

Materials and Methods

Nucleic Acids. All strands in these experiments are synthesized on an ABI 380B automated synthesizer, by use of standard phosphoramidite chemistry (Caruthers, 1987). Strands are purified after deprotection and removal from the synthetic columns by preparative HPLC on a Du Pont Zorbax Bio Series oligonucleotide column, using a gradient of NaCl in a solvent system containing 20% acetonitrile and 80% 0.02 M sodium phosphate. Fractions from the major peak are collected, concentrated, desalted, and lyophilized.

Kinase Reaction and Strand Purification. A total of 20 μg of a specific DNA strand is dissolved in 10 μL of a solution containing 66 mM Tris·HCl, 1 mM spermidine, 100 mM $MgCl_2$, 15 mM dithiothreitol (DTT), and 0.2 mg/mL nuclease-free bovine serum albumin (BSA) from BRL and mixed with 5 μL of 1.25 mM [γ-^{32}P]ATP (10 mCi/mL) and 2 units of T4 polynucleotide kinase (Boehringer) for 15 min at 37 °C. This reaction is quenched by adding 1 mM cold ATP and 1 unit of T4 polynucleotide kinase and stopped by freezing in dry ice. The mixture is rapidly heated for 5 min at 70 °C, cooled to room temperature, and loaded on a 20% denaturing polyacrylamide gel. This gel is run at 1000 V for 3 h at room temperature and exposed briefly to X-ray film (Kodak X-Omat AR or Amersham Hyperfilm βmax); the band corresponding to the 16-mer is cut out and soaked overnight at 37 °C in 0.5 mL of buffer (0.5 M ammonium acetate, 1 mM EDTA). This material is centrifuged for 5 min at room temperature in a microfuge, and the gel is reextracted with 0.1 mL of the same buffer, precipitated twice with ethanol, and lyophilized.

MPE Cutting. Our procedure follows that of Van Dyke and Dervan (1983a), with minor changes due to the short strands involved and the requirement for Mg^{2+} to stabilize junctions (Seeman et al., 1985). Junctions are formed by annealing a stoichiometric mix of strands at 16 μM concentration in 50 mM Tris·HCl, pH 7.5, with 1 mM $MgCl_2$. An Eppendorf tube containing the solution is immersed in boiling water for 2 min, cooled slowly to room temperature, and finally chilled to 4 °C. Double-stranded controls are formed similarly by use of a cold strand complementary to the labeled junction strand. For cutting branched and linear molecules, freshly prepared solutions of MPE·Fe(II) are made up in a buffer of 10 mM Tris·HCl, pH 7.4, with 50 mM NaCl and 1 mM $MgCl_2$,

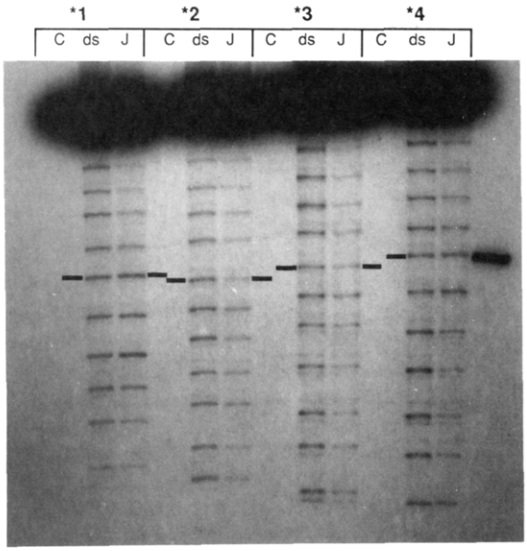

FIGURE 2: Autoradiogram showing the cleavage pattern of J1 by MPE·Fe(II). Each strand is run three times on this autoradiogram, to make four sets of three lanes each. The leftmost member of each set contains the cleavage pattern of the strand when it is complexed into the junction. The middle member contains the cleavage pattern of the strand when it is complexed with its Watson–Crick complement to form a linear duplex. The rightmost member is a control that has been untreated. The position of the tenth residue for each set is indicated by a black line.

containing 2 μM Fe(II) and 1 μM MPE (Van Dyke & Dervan, 1983b). The DNA is exposed to the reagent for 15 min at 4 or 37 °C, followed by addition of 4 mM DTT for 30 min, and the reaction is stopped by freezing on dry ice. After drying, the sample is taken up in formamide loading buffer, heated briefly to 90 °C, cooled, and then run on a denaturing polyacrylamide gel for 3 h at 2000 V and 40 °C. No dyes are added in these runs. The gel is dried immediately on a vacuum drying apparatus (Hoefer) and exposed at room temperature to film without an intensifier screen.

Results

Figure 2 contrasts the patterns of cleavage at 4 °C of the four strands of J1 by MPE·Fe(II) with the control patterns from the same strands in fully double helical duplexes. The duplexes are formed by annealing each labeled strand with its complementary 16-mer as described. Densitometer scans of the autoradiogram in Figure 2 are shown in Figure 3. It is useful to keep in mind that residues 8 and 9 of each strand in J1 flank the branch point. Two kinds of differences in the cleavage patterns can be seen: certain bands increase in amplitude in the junction relative to the duplex, while others decrease. The former define a site of preferential interaction of the drug with the junction. The latter define other sites of reduced drug interaction with the junction, lying on all arms of the junction. In particular, bands corresponding to chains of 8, 9, and 10 residues in length in strand 1 increase significantly in amplitude in the tetramer as opposed to the duplex. Residues 9 and 10 in strand 4 also show an increase in intensity compared to the corresponding duplex. By contrast, strand 3 shows no such effect. At 4 °C, increased cleavage at residue 9 on strand 2 can be seen, but is not seen at higher MPE/J1 ratios. On the other hand, residues at positions 4–8 in strand 4, 12 and 13 in strand 1, 12 and 13 in strand 4, and 4–6 and 11 and 12 in strand 3 are all protected from cleavage in the tetramer relative to the duplexes.

The cleavage pattern of J1 induced by MPE·Fe(II) is different from that noted for J1 in the presence of Fe(II) EDTA

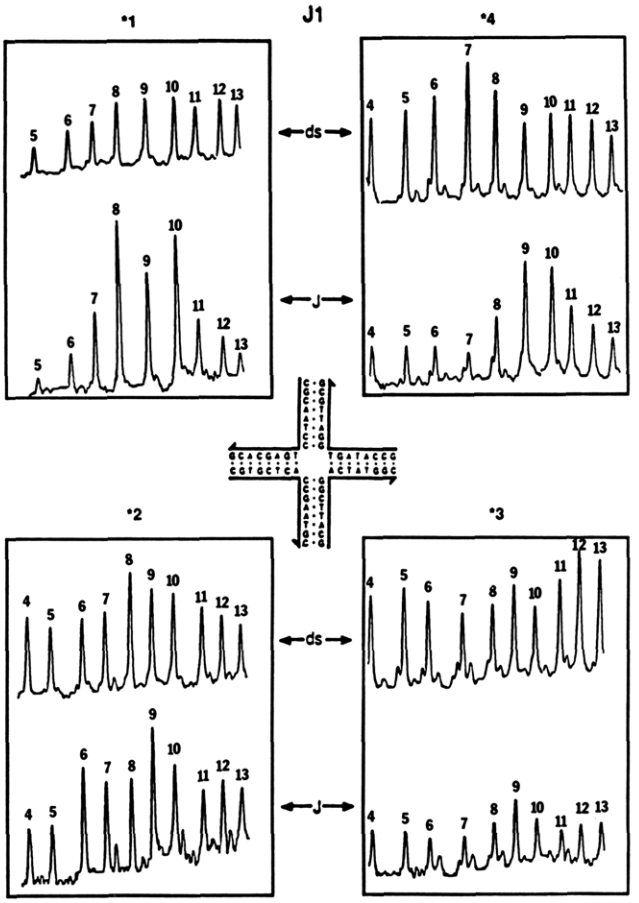

FIGURE 3: Densitometric scans of the cleavage pattern of J1 due to MPE·Fe(II). Each quadrant of this figure corresponds to a given strand of J1, whose sequence is indicated in the center of the figure. Each panel of scans contains two scans, that with the strand labeled in the junction (J) and that with the strand labeled in the linear duplex (ds). The branch point falls between residues 8 and 9 on each strand. Note the enhanced cleavage at residues 7, 8, and 9 on strand 1 and at residues 8, 9, and 10 on strand 4, as well as at residue 8 on strand 2.

(Churchill et al., 1988); this is true for all strands, whether they are complexed in junction or in a linear duplex. A corresponding pattern of enhanced cut sites is revealed at 37 °C, with the exception that the effect observed at residue 9 on strand 2 is weaker. Some dissociation of the tetramer might occur at 37 °C and at the concentrations we use (Marky et al., 1988), which can account for the difference.

The experiment illustrated in Figures 2 and 3 is carried out with a ratio of MPE·Fe(II) to junction of 1:16, to favor populating the tightest binding site(s) in the junction. Inspection of a simple model (Figure 1) suggests that the most efficiently cut positions demarcate a specific location of the reactive Fe(II) group in the tetramer. The range of chain scissions by diffusible oxidizing species emanating from a hypothetical Fe(II) group in the minor groove of normal B-DNA has been estimated by Dervan's group (Dervan, 1986). In MPE-Fe(II), the iron atom is thought to lie over one of the base pairs adjacent to the intercalation site of the ring (P. B. Dervan, personal communication), yielding a slightly asymmetric cleavage pattern. Strongest cut sites are expected at two residues to the 3′ side down each strand from the location of the Fe(II), the positions of the closest sugars in the duplex. Weaker cutting is also predicted at other sites

(5') n n n n n N N N N N n n (3')
•
(3') n n n N N N N n n n n n (5')

where the dot indicates the position of Fe(II) in the minor groove, capital N's denote nucleotide residues that are cleaved, and sites of greatest cutting intensity are indicated by boldface letters.

Not unexpectedly, the pattern of intensity of scissions in arm I of the junction is not identical with the predicted pattern for a single site located in a segment of the B-DNA duplex. The pattern we observe is consistent with preferential insertion of the methidium ring within one base pair from the branch point, positioning the Fe(II) over the CpT/AG sequence (the two base pairs abutting the branch site in arm I; see Figure 1). The local helix environment here might differ considerably from B-DNA, although CD spectra reveal no overall departure in the junction from B-DNA (Seeman et al., 1985; Marky et al., 1987). In B-DNA, and in the duplex controls we have investigated, ethidium binding shows minor sequence preferences, in comparison to A-RNA, for example, in which strong sequence preferences exist (Nelson & Tinoco, 1984; Kean et al., 1985). A second feature of the patterns in Figure 3 is the presence of bands that clearly exhibit protection from MPE-induced cleavage in the tetramer relative to duplex controls. This effect is most evident at bands corresponding to chains of 4–8 residues in length in strand 4; it can be discerned also at positions 12 and 13 in strand 1 and in strand 4.

Are these altered sites in the tetramer susceptible to competition from other intercalators? We added a 5-fold excess of propidium diiodide (PI) over MPE·Fe(II) to the reaction mix without iron, equilibrated the mix for 15 min, and repeated the MPE·Fe(II) cutting experiment to check this. The resulting densitometric traces are shown in Figure 4. Several features of these profiles are of interest. First, the tetramer cutting profiles of strands 1 and 4 lack some of the distinctive features noted in these strands in Figure 3. Comparisons of the duplex and junction patterns with strand 1 labeled now show no convincing differences. Interestingly, however, protection at positions 7 and 8 in strand 4 persists, although lacking the result of the MPE experiment, this might have been taken to be a "footprint" of PI (Van Dyke & Dervan, 1983a,b). A difference at position 12 in strand 3 is still present, indicating that the site or region of inhibition of drug binding in arm IV affects both drugs.

At present, we do not know the extent to which either drug affects the nature of the equilibrium conformation of the junction. It can be seen that the patterns of scission of the duplexes alone differ quite strikingly between Figures 3 and 4. Hence the presence of PI changes the probabilities of a cut in duplex DNA by the methidium reagent. Presumably, the interplay between Mg^{2+} and MPE·Fe(II) for sites on the DNA is perturbed by the addition of PI, a divalent species capable of intercalative binding also.

Discussion

These experiments provide direct evidence that drug binding to a branched DNA junction differs from that to duplex DNA in two ways. First, a specific and relatively tight drug binding site is present on arm I of J1, resulting in an asymmetric binding mode that breaks the 2-fold overall symmetry we (Churchill et al., 1988; Chen et al., 1988) and others have found (Cooper & Hagerman, 1987) and reconfirmed (Duckett et al., 1988) in unliganded junctions. Second, other sites are seen at which drug binding is weakened to a comparable extent. The simplest explanation for these other sites is that neighboring arms of the two-domain structure (Churchill et al., 1988) of the DNA molecule (Figure 1) occlude the site of drug binding.

Although the 2-fold symmetry of the structure is broken in terms of the preferential site revealed by differential MPE intercalation, these results nevertheless reinforce the results previously obtained by hydroxyl radicals generated from Fe(II)·EDTA unbonded to MPE. The site of enhanced drug binding found lies squarely in the leftmost of the two helical domains shown in Figure 1, and cleavage affects both arms in this domain. While some cleavage is seen in the portion of strand 4 located in the rightmost domain abutting the junction, no preferential cleavage due to the intercalating drug is apparent on strand 3, which should be remote from the intercalation site as determined from molecular models (Seeman, 1988). This pattern is in striking contrast to cleavage by endonuclease VII resolvase, which cuts the crossover strands in an approximately 2-fold symmetric fashion (Duckett et al., 1988; Mueller et al., 1988).

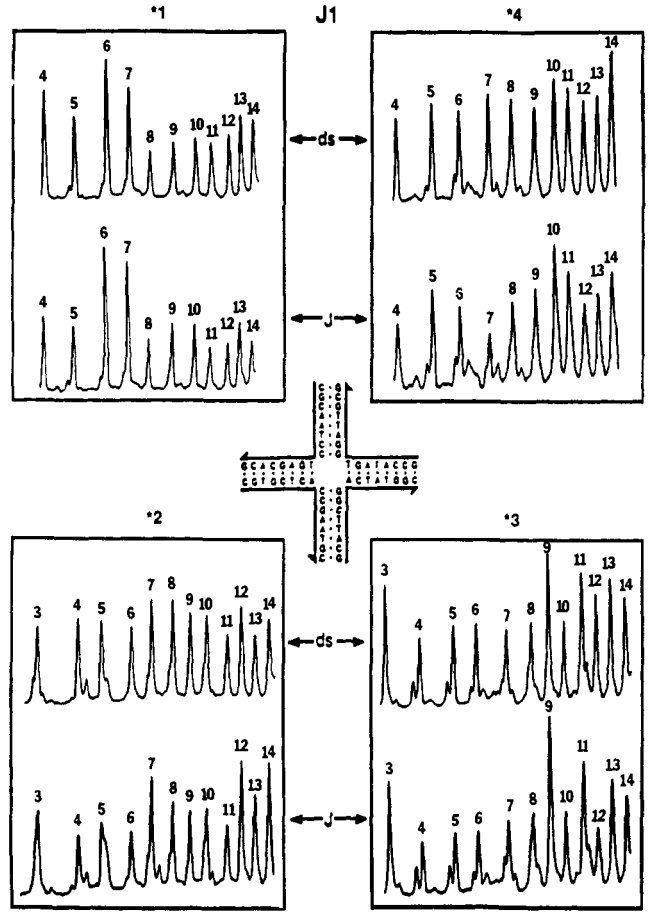

FIGURE 4: Densitometric scans of the cleavage pattern of J1 due to MPE·Fe(II) competing with propidium. The same conventions apply to this figure as to Figure 3. Note, in particular, the disappearance of enhanced cleavage on strand 1.

Protection of sites in the tetramer with respect to duplex DNA could be due to steric occlusion of the drug, or to a local structural change in the DNA imposed by the presence of the branch site, or both. Inspection of models of J1 (Seeman, 1988) reveals that occlusion of the minor groove of one domain by the other can account for several of the protected sites. Figure 4 shows that the protection effect is also sensitive to the presence of added propidium.

Previous autofootprinting experiments of junctions by hydroxyl radicals (Churchill et al., 1988; Chen et al., 1988) have been interpreted to reflect accessibility of the free Fe(II) EDTA probe to the scissile group. It is worth noting that a base line is established for the additional strain of intercalating MPE into DNA by comparison of junction cleavage with double helical cleavage. The propidium iodide experiment

controls for the possibility that enhanced cleavage results from junction strain in the presence of an unintercalated probe that generates hydroxyl radicals: The observation that the cleavage intensity in the vicinity of the branch site in arm I decreases in the presence of this competitive intercalator suggests that the methidium is bound in a similar mode and not just closely associated with a fragile site in an outside-binding mode.

It is interesting to contrast our observation of a site of enhanced drug interaction with the selective cleavage in the vicinity of an extruded cruciform in pBR322 following UV irradiation of a bound organometallic ligand, $Rh(DIP)_3^{3+}$ (Kirshenbaum et al., 1988). In that study, two specific cut sites on complementary strands (of the same plasmid arm) located 4 and 16 base pairs from the branch point were detected. While this effect is also asymmetric with respect to the branch point of the cruciform, it appears unlikely to involve a site of binding close to or at the branch point unless some additional conformational change, such as bending of the duplex, is involved (the two cut sites would be about 41 Å apart in B-DNA).

The tetramer of four 16-mer strands that we investigate here requires Mg^{2+} for stabilization (Seeman et al., 1985), indicating an anomaly in the charge distribution in this complex, compared to the dimer controls. This probably occurs at the branch point where two phosphate groups are constrained to lie in close proximity (Churchill et al., 1988; Chen et al., 1988). The bases flanking the branch point in J1 have been shown by NMR spectroscopy to remain paired (Wemmer et al., 1985), while chemical probes suggest similar behavior in the case of extruded cruciforms (Dieckmann & Lilley, 1987; Scholten & Nordheim, 1986). Binding of positively charged ligands in the vicinity of the branch point can thus be rationalized, although no structural details are available to define the precise mode of attachment. It should be mentioned that preferential ethidium binding at or near other distinctive structural features in nucleic acids has been found, including sites of base mismatching or "bulges" (Kean et al., 1985). It seems possible that branch points in nucleic acid junctions may prove differentially responsive to a variety of ligands, natural or synthetic.

Acknowledgments

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