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Drug Binding by Branched DNA: Selective Interaction of the Dye Stains-All with an Immobile Junction†

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ABSTRACT: The thiacarbocyanine dye Stains-All (4,5:4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine bromide) is one of a large number of cyanine dyes introduced as photosensitizers in the photographic industry. Stains-All is used in histology as a stain for nucleic acids, proteins, polysaccharides, and lipids. We report here that the dye colors branched DNA molecules differently from linear duplexes and use footprinting experiments with methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] and bis(*o*-phenanthroline)copper(I) [$(O\text{-}P)_2Cu(I)$] to show that Stains-All interacts preferentially at the branch point of a four-arm DNA structure. A titration experiment allows us to estimate that the interaction of the dye with the branch has a dissociation constant below 45 nM, tighter than that of ethidium or methidium by over 2 orders of magnitude. Probing the interaction with the purine-specific reagent diethyl pyrocarbonate (DEPC) implies that the dye induces an asymmetric distortion near the branch in the major grooves of double helix in the junction.

The properties of unstable Holliday recombination intermediates have been modeled by stable synthetic branched DNAs formed from oligonucleotides in which the sequence symmetry needed for branch migration is eliminated (Seeman, 1982; Kallenbach et al., 1983; Kallenbach & Seeman, 1986). Experiments in solution (Churchill et al., 1988; Chen et al., 1988) show that the dominant stable conformation of the tetramer consists of a pair of adjacent duplexes, each consisting of two neighboring arms stacked (or nearly so) over each other. This structure is determined by the sequence of bases flanking the branch (Chen et al., 1988), as well as by the nature and concentration of cations in the solution (Seeman et al., 1985). In the presence of Mg^{2+}, the immobile junction formed from four 16-mers, referred to as J1, favors the conformation in which the two noncrossover strands lie antiparallel, as illustrated in Figure 2 (Cooper & Hagerman, 1987, 1989; Churchill et al., 1988; Seeman et al., 1989), and this appears to hold for immobile junctions of other sequences as well (Duckett et al., 1988). Base pairs flanking the branch site in J1 appear to remain hydrogen bonded (Wemmer et al., 1985).

In early experiments with J1, we observed that the dye Stains-All (see Figure 1 for structure) (Hamer, 1975) colors dilute solutions of J1 differently from any of the duplex arms alone. This dye is used as a differential stain for DNA, RNA, polysaccharides, and membrane phospholipids (Green, 1975). It has the eponymous property of staining each of these components a distinct color. We report here that J1 is stained differently by the dye from 16-mer DNA duplexes with the same sequences as the arms in J1 and use different footprinting agents for precise characterization of the Stains-All binding site in the four-arm junction, J1. We estimate the dissociation constant for the dye–J1 interaction to be about 45 nM.

Materials and Methods

Nucleic Acids. All DNA strands used in these experiments are synthesized on an ABI 380B automated synthesizer, using standard phosphoramidite chemistry (Caruthers, 1982). Strands are purified following deprotection and removal from

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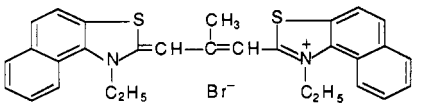

FIGURE 1: Structure of Strains-All (4,5:4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine bromide).

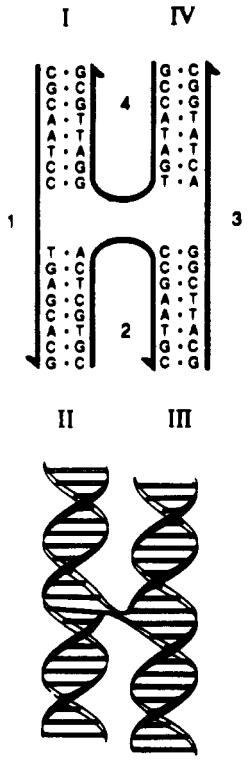

FIGURE 2: Sequence and structure of the immobile branched junction J1. The upper panel shows the junction in schematic, while the lower panel is a representation of what the junction would look like if it were formed from 10.5-fold B-DNA. The junction is composed of four strands of DNA, indicated in 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. The antiparallel conformer of J1 shown is that which we believe to be the predominant form in solution (Seeman et al., 1989).

the synthetic columns by preparative HPLC on a Du Pont Zorbax Bio Series oligonucleotide column, using a NaCl gradient 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.

Phosphorylation and Strand Purification. Twenty micrograms of a specific DNA strand is dissolved in 10 μL of a solution containing 66 mM Tris-HCl, 1 mM spermidine, 10 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. The gel is run at 2000 V (ca. 50 V/cm) for 3 h at room temperature and exposed briefly to X-ray film (Kodak XOMAT AR); the band corresponding to 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, precipitated twice with ethanol, and lyophilized.

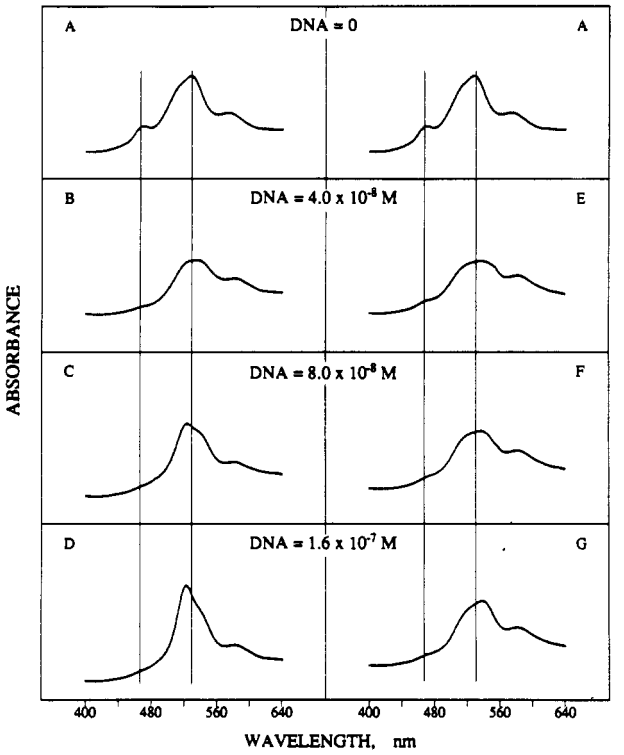

FIGURE 3: Absorption spectra of 1.0×10^{-6} M Stains-All solution with different amounts of DNA added in 10 mM sodium cacodylate solution (pH 8.0) (absorption scale in relative units). Curves B–D: Absorption of DNA junction titration. Curves E–G: Absorption of control duplex titration.

MPE·Fe(II) Cutting. Our procedure follows that of Van Dyke and Dervan (1983a,b), with minor changes due to the short strands involved and the addition of 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 10 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 using a stoichiometric amount of cold strand complementary to the labeled junction strand. For cutting both 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, containing 1 mM Fe(II) and 2 mM MPE (Van Dyke & Dervan, 1983a,b). DNA (16 μM) is exposed to the reagent for 15 min at 4 °C, followed by addition of 4 mM DTT for 30 min, and the reaction is stopped by freezing on dry ice. After extracting with 1-butanol to remove drug and precipitating with ethanol, the dried 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 (ca. 50 V/cm) 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.

$(OP)_2Cu(I)$ Cutting. For cutting branched and linear molecules, freshly prepared solutions of 200 mM *o*-phenanthroline (Kodak), 45 mM $CuSO_4$, and 1 mM ascorbate (replacing 3-mercaptopropionic acid; Kuwabara et al., 1986) are made up in a buffer of 50 mM Tris-HCl, pH 7.5, with 10 mM $MgCl_2$. The DNA is exposed to the reagent for 45 min at 4 °C, followed by extraction with 1-butanol and precipitation with ethanol. The dried sample is taken up in formamide loading buffer, heated briefly to 90 °C, cooled, and run on a denaturing polyacrylamide gel for 3 h at 2000 V (ca. 50

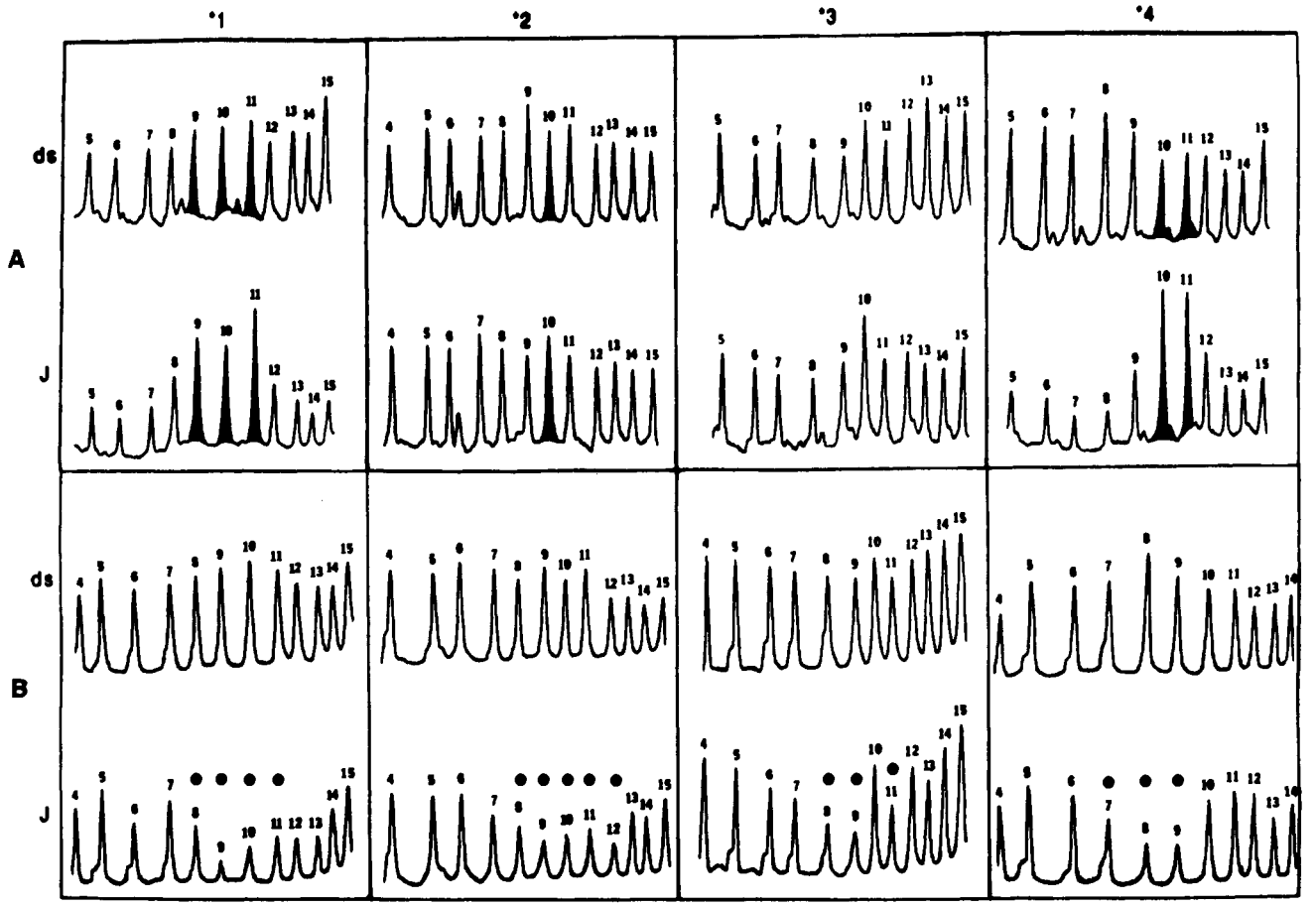

FIGURE 4: Densitometric scans of the cleavage pattern of J1 due to MPE·Fe(II) and MPE·Fe(II) competing with Stains-All. Each column of this figure corresponds to a given strand of J1 and contains two panels. Panel A shows densitometric scans of the cleavage pattern of J1 by MPE·Fe(II); panel B shows densitometric scans of the cleavage pattern of J1 due to MPE·Fe(II) competing with Stains-All. Each panel of scans contains two scans, one with the strand labeled in the tetrametric junction (J) and one with the strand labeled in linear duplex formed with its complementary strand (ds). The branch point lies between position 8 and 9 on each strand. Sites of differential enhancement in this experiment are indicated as black bands in ds and J. Sites of differential protection are indicated by filled circles over the corresponding bands in the J profiles. The sites of differential activity are summarized in Figure 7.

V/cm) 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 X-ray film without an intensifier screen.

Diethyl Pyrocarbonate Reaction. Linear DNA duplexes and the tetramer are modified by diethyl pyrocarbonate following the procedure of Herr (1985). DNAs (16 μM) are suspended in a buffer of 50 mM Tris-HCl, pH 7.4, with 10 mM $MgCl_2$. The Eppendorf tubes are placed on ice, and 1 μL of diethyl pyrocarbate is added to each sample. The samples are incubated for 60 min at 4 °C. Because diethyl pyrocarbonate is relatively insoluble in water, the samples are thoroughly mixed at the beginning as well as halfway through the 60-min incubation. The reactions are terminated by two sequential rapid ethanol precipitations and then lyophilized. The DNAs are cleaved at the sites of diethyl pyrocarbonate modification by treatment with 80 μL of 1 M piperidine at 90 °C for 30 min.

Densitometry and Comparison of Profiles. Autoradiograms are scanned on a Hoefer GS300 densitometer. No base-line corrections are applied. The data from the experiments we report consist of pairs of profiles corresponding to the relative probability of chain scission at a series of positions with respect to a labeled 5′-phosphate for each strand in a DNA 16-mer duplex and in the junction tetramer. These profiles can differ in the overall intensity of scission, as well as the relative intensity at individual bands. In many cases, it is possible to select a set of three or more bands with comparable relative intensities and use these to normalize by eye two profiles. These bands often lie near the chain termini, where effects from the branch site might be expected to be less evident. We identify positions of enhancement or protection relative to these benchmarks.

Results

Figure 3 shows the result of an absorption experiment, in which a dilute solution of the dye in a 10-cm cuvette is titrated with the junction and one control duplex, consisting of strand 1 of the junction (Figure 2) paired with its Watson–Crick complementary 16-mer. The absorption spectrum of the dye alone is extremely sensitive to both dye concentration and the presence of different ions in the solvent (Kay et al., 1964a,b). Many of these differences are attributed to new bands (J bands) arising from self-association of dye molecules and effects of various agents on this process (Kay et al., 1964a; Lehmann, 1988). In aqueous solutions below about 10^{-6} M in dye, buffered with cacodylate, the spectrum of the dye simplifies to two bands in the visible, at 530 and 570 nm (Kay et al., 1964a). This spectrum is altered in the presence of 10 mM $MgCl_2$ as seen in panel A of Figure 3 to one with three bands, at 470, 530, and 570 nm. Progressive addition of tetramer or duplex produces the changes shown in Figure 3, indicating interaction of Stains-All with both DNAs, but a difference between the duplex and J1. Under the conditions used, 10^{-6} M dye with lower DNA concentrations, the spectral shifts seen might still correspond to J bands from dye asso-

ciation nucleated by the DNA. In the case of the junction, a band near 520 nm increases with increasing J1 concentration, while a band at 540 nm increases with the duplex. Thus the spectral changes are distinct, indicating a differential mode of association of dye molecules with the branched species.

A number of drugs or ligands, including propidium and porphyrins, have been found to interact preferentially at the branch site in J1 (Guo et al., 1989, 1990; Lu et al., 1990). This can be demonstrated most directly by "footprinting" experiments (Galas & Schmitz, 1978; Schmitz & Galas, 1979), using reactive probes, such as MPE·Fe(II) (Hertzberg & Dervan, 1982, 1984) or $(OP)_2Cu(I)$ (Kuwabara et al., 1986), to monitor the probability of cleavage along the DNA backbone in the presence and absence of ligand.

Figure 4 shows the results of such an experiment on J1 with and without an equimolar concentration of Stains-All. Panel A shows the densitometric trace of an autoradiogram of the products resulting from exposure to MPE·Fe(II) of the free junction and four control duplexes formed from pairing each strand in J1 with its fully complementary 16-mer strand. The pattern of enhanced scission at residues **1**:9–11, **2**:10, and **4**:10,11 with protection at positions **1**:6,7, **1**:13–15, and **4**:6–8 identifies an interaction between the probe and the junction in the vicinity of the branch site that differs from comparable interactions in duplexes of the same sequence (Guo et al., 1989). Panel B shows the effect on the duplex and tetramer profiles of adding equimolar Stains-All to the DNA in the same reaction mixture. While there are only modest differences between the duplex profiles in the presence and absence of dye (compare panel A ds with panel B ds), the pattern of enhanced reactivity at the positions noted above is sharply altered. In the presence of Stains-All, residues **1**:8–11 are protected from scission relative to the duplex (panel A J vs panel B J), while **2**:8–12, **3**:8,9, **3**:11, and **4**:7–9 also show protection and the enhancement at **4**:10,11 is reduced or eliminated.

A comparable picture emerges when $(OP)_2Cu(I)$, which exhibits more selective sequence and structure effects on DNA junctions than MPE·Fe(II), is used as a probe (Guo et al., 1990). Without dye, enhanced reactivity of $(OP)_2Cu(I)$ in the junction relative to duplexes normally occurs at **1**:9,10, **2**:7,9, **3**:8–11, and **4**:10 (data not shown). In the presence of the dye, **1**:8–11, **2**:10, **3**:8,9, and **4**:9–11 are protected relative to these positions in duplexes. The dye thus protects sites close to the branch point (between residues 8 and 9 in each strand in J1) from scission by both probes, while it has relatively little effect on the corresponding duplex profiles. Taken with the observation in Figure 3 of a new band at 520 nm associated with interaction of dye with the junction, this indicates a unique interaction of Stains-All at the branch point of J1. Relative to other drug ligands we have tested, Stains-All appears more selective for the branched species, since its effect on the duplex profiles is only moderate, while it eliminates the characteristic enhancement patterns of MPE·Fe(II) and $(OP)_2Cu(I)$ completely. It is possible that the effect is due to a bifunctional interaction of the ring systems of the dye with the two adjacent duplexes in J1.

To assess the binding affinity of the dye for J1, we perform a dilution series, monitoring the scission by MPE·Fe(II) at the sensitive sites at **1**:9–11 and **4**:10,11 as a function of the dye concentration. Panel A in Figure 5 shows the control status of these two strands in the absence of Stains-All; the positions indicated are hyperreactive to MPE·Fe(II) (Guo et al., 1989). In the presence of 60 nM dye (panel D), these positions are protected, and the protection is eliminated at 30 nM dye (panel C) or 10 nM dye (panel B). Assuming single occupancy of a high-affinity site is responsible for the effect, the apparent dissociation constant is about 45 nM, indicative of very tight binding. In this case, the competitive effect of the MPE·Fe(II) probe can be neglected, since we estimate that its affinity is about 100 times weaker from a similar titration experiment.

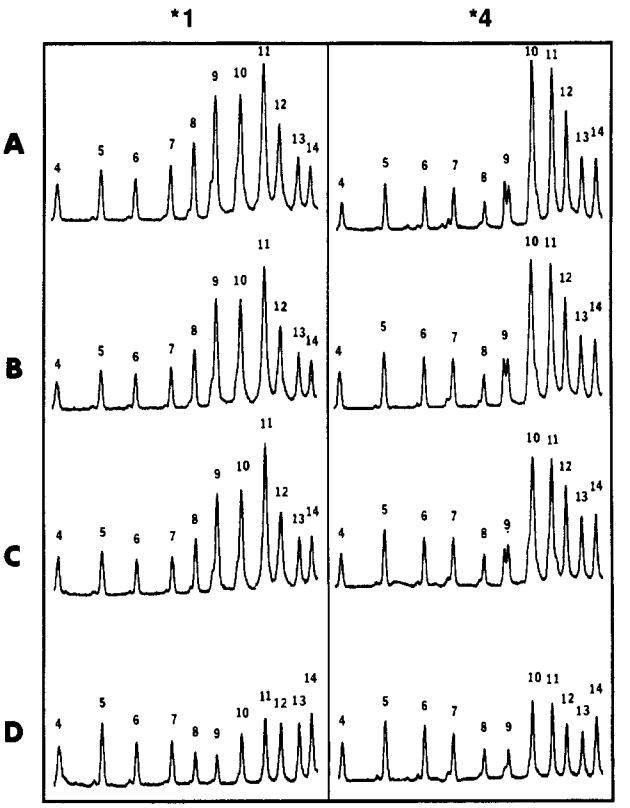

FIGURE 5: Densitometric scans of MPE·Fe(II)-induced cleavage of J1 in the presence of different concentrations of Stains-All. Two strands in J1—strand 1 (left column) and strand 4 (right column)—are monitored, which show enhanced cleavage by MPE·Fe(II) relative to duplex controls in the absence of dye (Guo et al., 1989). Panel A: Control in the absence of dye. Panel B: 10 nM Stains-All. Panel C: 30 nM dye. Panel D: 60 nM dye.

Additional information about the complex with Stains-All is obtained from a footprinting experiment with diethyl pyrocarbonate, a reagent that carbethoxylates purines in DNA at N7 (Leonard et al., 1971), with a preference for A over G. Figure 6 shows the profiles of DEPC reaction of the J1 tetramer and four duplex controls. An interaction of the dye with two of the duplexes can be seen by comparing the ds lanes in panel A with panel B. Cleavage at positions **1**:5–10, **1**:12, and **2**:11 are enhanced in the presence of dye, as are **3**:7,8 and **4**:7,9,10. Comparison of the ds with J profiles in panel A, without dye, shows only minor differences between the tetramer junction and any of the four duplex controls. However, in the presence of dye an interesting pattern of protection and enhancement is seen. Position **1**:6 is *protected* relative to the duplex control in the ds lane of panel B (J vs ds), while positions **1**:10–12 are *enhanced*. Similarly, **3**:6 is protected and **3**:8–10 enhanced relative to the duplex of strand 3 and its complement (J vs ds). The two strands we have previously identified as crossover strands in the unperturbed junction (Churchill et al., 1988) show two opposite patterns: positions **2**:7 and **2**:9 are protected, while **2**:8 is enhanced; positions **4**:7 and **4**:9 are enhanced and **4**:8 is protected.

Discussion

Branched DNA molecules represent novel structures for characterization by physical and chemical methods. The

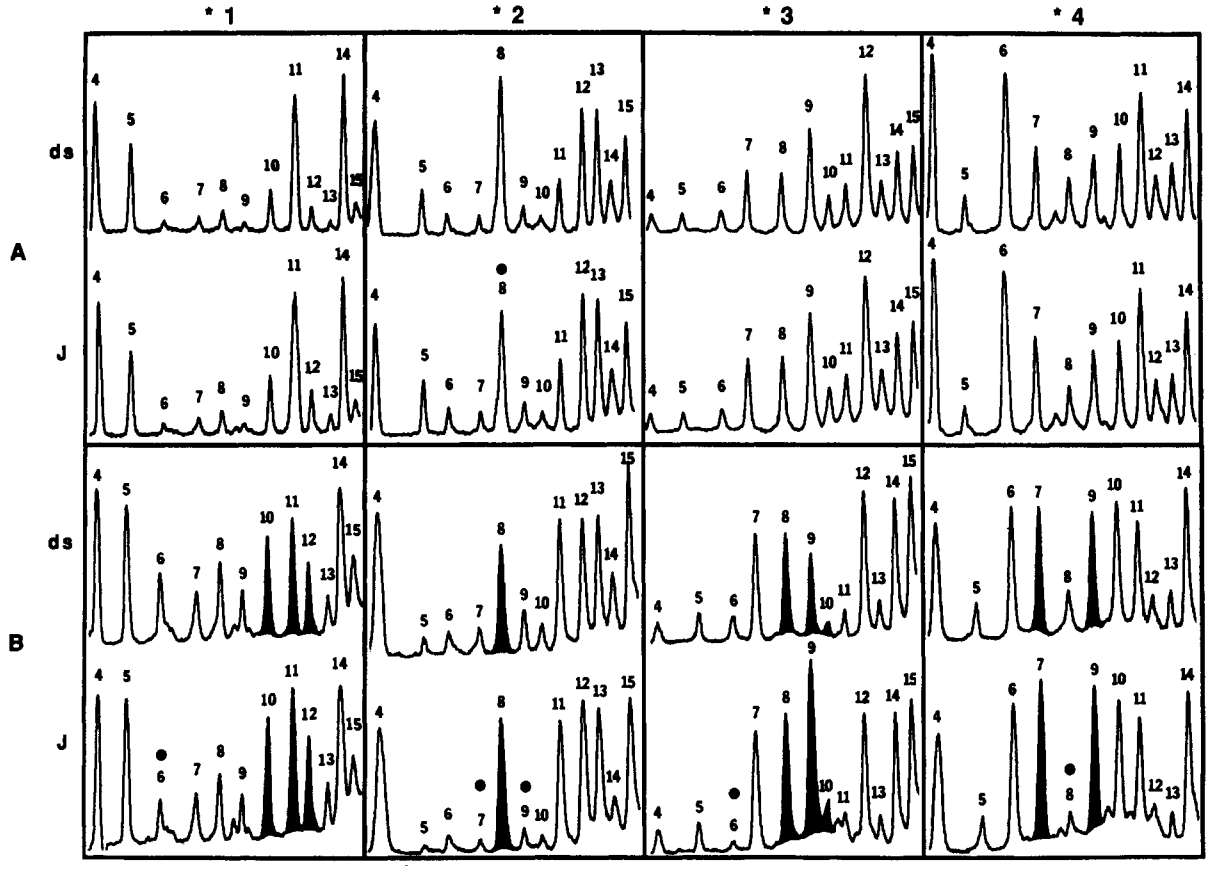

FIGURE 6: Densitometric scans of the cleavage pattern of J1 due to DEPC in the presence and absence of Stains-All. The same conventions apply to this figure as to Figure 4. Panel A shows densitometric scans of the clevage pattern of J1 by DEPC; panel B shows densitometric scans of the clevage pattern of J1 by DEPC competing with Stains-All. The filled circles identify bands that show differential protection in the junction relative to the duplex, while sites of enhanced reactivity are indicated by black bands. The sites of differential activity are summarized in Figure 7.

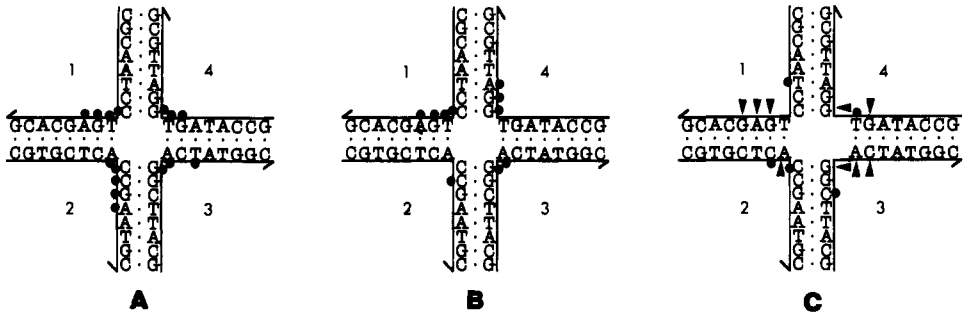

FIGURE 7: Sites of protection from chemical cleavage of J1 with Stains-All. Results of the scission experiments reported in this study are summarized in these panels. Sites of differential protection due to the cleavage of J1 with MPE·Fe(II) competing with Stains-All are indicated by filled circles in panel A. Panel B shows those sites due to the scision of J1 with $(OP)_2Cu(I)$ competing with Stains-All. Panel C shows those sites as well as the sites of enhancement by triangles due to the cleavage of J1 with DEPC competing with Stains-All.

four-arm immobile junction J1 has been extensively studied spectroscopically (Wemmer et al., 1985; Seeman et al., 1985) and thermodynamically (Marky et al., 1987), as well as by electrophoresis (Seeman et al., 1989) and chemical footprinting (Churchill et al., 1988; Chen et al., 1988). The dominant conformation of this tetramer in solution consists of two helical domains, each containing a pair of stacked arms as sketched in Figure 2. Other conformers are possible and indeed can be accessed by suitably constraining the arms (Kimball et al., 1989). We have found that the branch point in J1 provides a site of strong interaction for a number of ligands, including methidium, ethidium, and propidium (Guo et al., 1989, 1990), porphyrins (Lu et al., 1990), and ditercalinium (Lu et al., unpublished results) as well as Stains-All. This seems reasonable in view of the fact that the branch point of an immobile junction represents an anomalous site with respect to both its structure and its charge distribution. We (Seeman et al., 1985) and others (Cooper & Hagerman, 1987, 1989) have pointed out that the structure of immobile junctions depends on the counterion concentration, implying that the close proximity of two phosphates at the branch provides a site of unusual charge density. Both the charge and the local deformation at the branch appear to favor interaction with positively charged ligands. The unusual proximity of adjacent duplexes permits in principle association of more than one ligand near the branch (Guo et al., 1990). So far, we have not been able to define the stoichiometry of binding any ligands precisely. A titration of J1 with propidium suggests a differential concentration response in two strands (Guo et al., 1990), consistent with more than one strong binding site. The MPE·Fe(II) cleavage pattern of J1 is invariant to the ratio of MPE:junction from 1/30 to 30/1, a range of nearly 100-fold, indicating that

the branch site interacts very tightly relative to the excess of weaker sites available. To assess the binding constant for Stains-All at the junction, we exposed solutions containing a fixed concentration of J1 and different dye concentrations to scission by MPE·Fe(II), with the result shown in Figure 5. The inhibition of the characteristic enhancement of positions **1**:9–11 and **4**:10,11 shown by MPE·Fe(II) in the absence of dye is observed to disappear between 3×10^{-8} and 6×10^{-8} M dye, indicating an apparent dissociation constant of about 45 nM for the interaction. This value is roughly 2 orders of magnitude tighter than the apparent MPE interaction with J1 and suggests that the dye might actually be inserting bifunctionally rather than into a single site. Since the interaction takes place in the presence of 10 mM Mg^{2+}, it can be argued also that electrostatic interactions are responsible only for part of the binding energy. However, the titration described sees the interaction only from the perspective of the junction, and binding could be still tighter than the dissociation constant estimated from Figure 5.

Figure 7 summarizes the patterns we have obtained concerning the mode of interaction of Stains-All with the immobile junction J1. By elimination of the positions of enhancement of MPE·Fe(II) and $(OP)_2Cu(I)$ cleavage near the branch point, it can be argued that the dye inserts at or near the branch itself. Both these agents probe the conformation at the minor groove in a DNA duplex (Hertzberg & Dervan, 1982, 1984; Kuwabara et al., 1986). The DEPC patterns suggest that binding dye induces a local change in conformation of the major grooves of each strand at the branch point. The differential patterns in fact are opposite for the two crossover strands in J1. From the spectral data and footprinting experiments, it is not possible to arrive at a structural model for the complex between J1 and Stains-All. At least two possibilities can be envisioned: one in which a single dye molecule serves as a bifunctional intercalator, inserting partially into both of the adjacent helical domains of J1, and a second in which only one ring of the two ring systems of the dye inserts into the junction, while the second provides a strong nucleus for association of additional dye molecules, favoring dye–dye excitonic shifts in the spectrum [see Lehmann (1988)]. On the basis of the dissociation constant we measure, 45 nM, we favor the former model. In this case, the spectral change reflects electronic interactions of both rings with DNA, a situation not likely to occur in linear duplexes. This model raises the interesting possibility that bifunctional ligands might be identified that are active at very low concentration on junctions such as J1 and only at much higher concentrations in normal DNA. Hence one might be able to identify relatively selective inhibitors of recombination processes, perhaps, which can function in a background of much weaker binding to the vast excess of nonspecific duplex DNA sites.

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