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Interaction of Drugs with Branched DNA Structures

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I. INTRODUCTION

The idea that the structure of DNA corresponds to a homogeneous double helix under native conditions has gradually given way to the modern view that the local conformation of DNA is variable, reflecting the conformational preferences of the local sequence present. The sequence substructure present in a given molecule effectively produces what amounts to a "patchy" DNA duplex. The dynamic and structural properties, in principle, can differ drastically from one patch to another, depending also on external variables such as ionic conditions and temperature. In addition, cellular processes such as replication, transcription, and recombination can impose a second level of structural heterogeneity *in vivo*. The native double-helical structure is subject to transient deformation *in vivo* by enzymes or complexes of enzymes that unwind, unpair, repair, or form branches in normal duplexes. Any natural segment of DNA presents a highly variegated target for interaction with ligands, proteins, or small molecules. The focus of this article is on both the interaction between small molecule ligands with DNA and the binding properties of variant states of DNA in particular. Recent research has attempted to define the detailed binding properties of DNA with respect to several different types of ligands. A large number of carcinogens and mutagens have been found to be capable of reacting covalently with DNA, producing lesions that may escape the repair systems present in cells and have severe biological consequences. How such agents initially approach DNA and recognize target sites is likely to be determined by events that involve binding of the agent to a distinct "patch" or region of structural deformation. This article summarizes studies that reveal a strong selectivity of several drugs and ligands for binding variant structures in DNA, branches in particular but also base-pair "bulges" and other non-B DNA states, including triple helices and G-quartets. It is possible that preferential interactions as strong as those described here modulate the frequency of mutagenetic events that lead to carcinogenesis, for example.

II. PROPERTIES OF BRANCHED DNA MOLECULES

A. The Holliday Junction and Recombination

General recombination takes place between DNA molecules that contain extensive regions of sequence homology, resulting in the exchange of genetic markers along a chromosome (Figure 1). This process involves physical exchanges between strands of two or more duplexes, which are thought to proceed via a common four-stranded intermediate structure known as a Holliday junction.[1–6]

Holliday proposed a molecular model for strand exchange to explain the patterns of gene conversion, or nonreciprocal recombination

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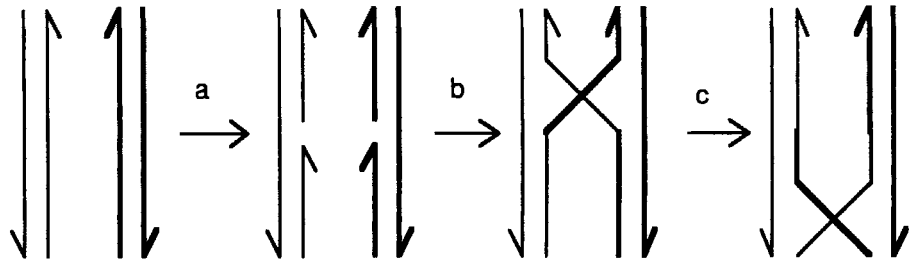

FIGURE 1. The Holliday model for general recombination. An outline of the model proposed by Holliday.[1] (a) Two homologous DNAs are aligned and the apposing strands nicked. (b) The Holliday recombination intermediate is formed by reciprocal strand exchange between two duplex DNA molecules. (c) Branch point migration generates a long heteroduplex region.

events in the smut fungus, *Ustilago maydis*.[1] The products of meiosis in crosses among certain mutants of *Ustilago* can be individually inspected as asci, making gene conversions easy to detect. Holliday postulated that two homologous DNA duplexes, each corresponding to one of the four chromatids present at meiosis, become nicked and exchange single strands, forming a segment of heteroduplex DNA symmetrically, as sketched in Figure 1. This recombinational intermediate can then be cleaved and rejoined in two different ways to yield distinct, genetically identifiable recombinant parental products.[1]

Holliday's original model describing the possible orientations of the branches about the junction and their functional consequences has been refined by subsequent studies.[4,5,7] At least three different kinds of models for Holliday junctions have been proposed on the basis of known structural features of DNA. In one version, postulated by Sigal and Alberts,[3] the branches of the four-stranded Holliday structure are base-stacked and coplanar so that the two recombining duplexes maintain relatively unperturbed helix axes. It was supposed that this structure could be formed from duplex DNA without unfavorable contacts, based on model building. A second representation views the junction as a planar, but not base-stacked, structure with the branches at right angles to each other, giving the structure an overall pseudo-4-fold symmetry.[7] An intermediate conformation in which the arms of the junction are arranged in a tetrahedron has also been suggested.[8] Some of the mechanical and dynamic properties of different models for junctions have been estimated.[9–12]

Since its proposal, Holliday's model has come to play an increasingly important role as a framework for current study of the molecular mechanisms in both general and site-specific recombination. The crucial property of the Holliday model is that it provides for covalent association between the two duplex DNA molecules that are to engage in recombination via formation of a heteroduplex joint (referred to here as the branch). This joint can translocate or "branch migrate" along the arms of the junction, generating long regions of heteroduplex DNA.[2,10]

Holliday junctions connecting two circular DNA molecules can produce a characteristic "figure-8" structure that have been visualized by electron microscopy in several phage and plasmid DNAs extracted from *E. coli*.[13] In several cases, these have been demonstrated to be joined at a region of homology.[14–16]

Visualizing recombining structures in the electron microscope allows definition of the overall configuration of junctions at low resolution. Information concerning finer details or the structural dynamics of Holliday junctions is sparse. Warner's group carried out a pioneering series of experiments measuring the kinetics of branch-point migration in topologically unconstrained branched structures.[10,11] The idea was to isolate a set of figure-8 intermediates at a given time, and monitor the time course of their resolution

into single circles at intervals by means of electron microscopy. The rate of branch migration per fork measured in this way turns out to be strongly dependent on temperature — a slow reaction at low T (ca. 60 base pairs/second at 10°C) and rapid at 30°C (10,000 base pairs/second at 30°C).[11] These workers also made the initial observation that ethidium bromide, a dye used to stain duplex DNA, could inhibit the rate of branch migration. The effect was observed at concentrations of dye that seemed to be lower than the normal dissociation constant for DNA binding, which is in the micromolar range. This prompts one to ask whether the effect involves a selective interaction between the dye and a branch or a more diffuse influence of the dye on the flexure of the arms that might retard movement of the branch. A difficulty encountered in these original experiments proved to be that the figure-8 structures studied were isolated using centrifugation in ethidium, and this proved hard to remove quantitatively to assess the intrinsic rate of migration.

A Holliday junction can undergo a second kind of structural transformation, referred to as a crossover isomerization,[3,4] whereby the two crossover strands can switch with the noncrossover strands. This kind of isomerization of Holliday junctions with stacked bases has been postulated to determine the relative incidence of genetic crossing over during recombination.[4,6,17] A question of interest in this research is whether or not the presence of a ligand can influence the crossover preference of a Holliday junction and, thereby, the yield of recombinants in a genetic exchange. One can also ask if transiently inhibited branches can serve as substrates for enzymes or proteins in the cell. In the discussion that follows, the interaction of drugs with branched and linear oligomeric DNA models is described. It has to be emphasized that the *in vivo* processes of recombination and resolution take place in a very different context. Eukaryotic DNA in meiosis, for example, is coated with protein molecules of several different kinds, and the entire complex can be seen to involve massive nucleoprotein structures referred to as nodules.[18] The models we consider offer a highly simplified analogue for the actual process.

B. Cruciforms from Circular DNAs

Under the influence of torsional stress, circular DNA molecules bearing sufficiently long palindromic sequences can be extruded into stable cruciforms capable of branch migration.[19] The extrusion of cruciform structures has been demonstrated at many inverted repeat sequences in the presence of negative supercoiling.[20–23]

The formation of cruciform structures *in vitro* has been established.[24–27] First, the cruciforms can be recognized by enzymes that cleave Holliday structures.[28,29] Two phage-encoded enzymes, T7 Endo I and T4 Endo VII, have been shown to recognize conformationally branched DNA, introducing a pair of nicks at or very near the base of a cruciform.[26,27] Second, cruciforms *in vitro* have been probed by single-strand specific enzymes that attack the loop, such as S1 nuclease,[23,25] Ba131 nuclease,[30] P1 nuclease,[31] and mung bean nuclease.[32] Third, reaction of cruciforms with single-strand selective chemicals, such as bromoacetaldehyde,[33] osmium tetroxide,[34] diethyl pyrocarbonate,[35,36] and bisulfite,[37] confirmed that a loop in the cruciform was formed, while the branch itself proved unreactive.

A cruciform structure is expected to be thermodynamically unstable relative to an unextruded linear or circular duplex because of the energetic cost of unpairing the bases of the loop together with the formation of the branch in the four-stranded junction. The unfavorable free energy of cruciform formation is overcome by the relaxation of supercoiling that accompanies extrusion in a negatively supercoiled molecule.[38] By measuring the change in helical densities accompanying extrusion, the free energy of cruciform formation can then be evaluated.[38] This has been repeated for cruciforms in pUC7 (18 kcal/mol),[39] ColE1 (18 kcal/mol),[30] and an $(A\text{-}T)_{34}$ sequence from *Xenopus laevis* (13 kcal/mol).[40] The stable oligonucleotide models described below also make it possible to measure the energetics of junction formation.[41]

C. Stable Oligonucleotide Models for Holliday Junctions

The structural and thermodynamic analysis of Holliday junctions has been facilitated by trap-

ping branched structures in synthetic oligodeoxynucleotide systems.[42] The idea is that oligonucleotides can be constructed that will preferentially associate to form junctions via Watson-Crick base pairing, while the sequences of these molecules do not have the symmetry necessary for the branch-point migration reaction to occur. A set of sequence rules was formulated by Seeman[42,43] that must be obeyed by oligonucleotide sequences, if they are to form immobile junction structures. These conditions must be supplemented by thermodynamic criteria to avoid opening up one or more of the arms and allowing disruption of the branch.[44]

Synthetic oligonucleotide junctions have made it possible to model properties of states of DNA in recombination that are normally unstable with respect to branch migration.[45] Structures with from three to six arms have been investigated.[46–59] These include branched systems in which the branch point is completely incapable of migrating along any of the duplex arms of the complex without breaking base pairs, which we refer to as migrationally immobile junctions.[42,43] Complexes in which the sequence surrounding the branch point possesses just enough symmetry to allow a predetermined small number of steps of the branch migration reaction to take place, migrationally restricted mobile junctions,[43,49] have also been studied.

The first synthetic immobile Holliday junction, termed J1 (Figure 2), was formed by annealing four 16-mer oligonucleotides of DNA that base pair to form a tetramer with a unique branch point and 8 bp arms.[46] Gel mobility assays showed that in the presence of Mg^{2+}, a unique structure is formed when all four strands are annealed, with a molar ratio of strands in the tetramer of 1:1:1:1 by gel analysis of unlabeled strands and by quantitation of radioactivity in each labeled single-strand band and the junction complex formed by labeled strands.[46,50]

J1 has been characterized physically using a variety of spectroscopic and other techniques. A ^1H NMR study of J1 shows that the base pairs flanking the branch remain paired at low temperature in the presence of Mg^{2+}.[47] Their lifetimes are lower than those of internal pairs in the arms, however. Thermal denaturation monitored by UV analysis of DNA hypochromism reveals that the junction molecule actually has a higher hypochromicity than the duplexes from which it was made, which also suggests that the junction is closed with the four arms completely base paired.[60] Circular dichroism spectroscopy and NMR indicate that most of the DNA in its arms retains an overall B helical conformation.[60,61]

Marky et al.[61] measured the stability of J1 relative to that of a set of octamers comprising its individual arms; the results indicate that the thermal stability of the junction is closer to that of an average of the four octameric arms rather than that of intact 16-mers. This implies considerable destabilization of the junction relative to two 16-mer DNA duplexes of the same sequence. Further analysis reveals that the effect is strongly temperature dependent.[41] At low T, the stability of a junction is actually very close to that of two component duplexes, while at high T the latter are more stable.[41] This is established using a set of eight strands that can form two junctions, J1 and $J1^c$, the latter consisting of the four strands, each complementary to those in J1, or four duplexes (see Figure 2).

Some overall structural features of immobile junctions in solution have emerged from four independent lines of experimental evidence. Cooper and Hagerman[51] studied a four-arm synthetic junction by gel electrophoresis under native conditions, using the fact that bent DNA molecules demonstrate reduced mobilities on polyacrylamide gels to an extent that varies with the apparent degree of the bend angle.[62] They then prepared a set of molecules with pairwise extended long duplex arms and observed different mobilities depending on the identity of the extended pairs in the presence of Mg^{2+}. They interpreted their results to indicate that the junction is not a completely flexible structure under these conditions; nor is it tetrahedral or planar-tetragonal. Instead, the four strands comprising the junction are structurally nonequivalent, with a geometry that depends on base sequence at the branch point.[51] These electrophoretic experiments suggest that the junction geometry is consistent with a stacked structure, roughly like a Sigal-Alberts model,[3] containing two helical strands and two crossover strands; the crossover strands have cis or antiparallel geometry.[51] Duckett and co-workers[53]

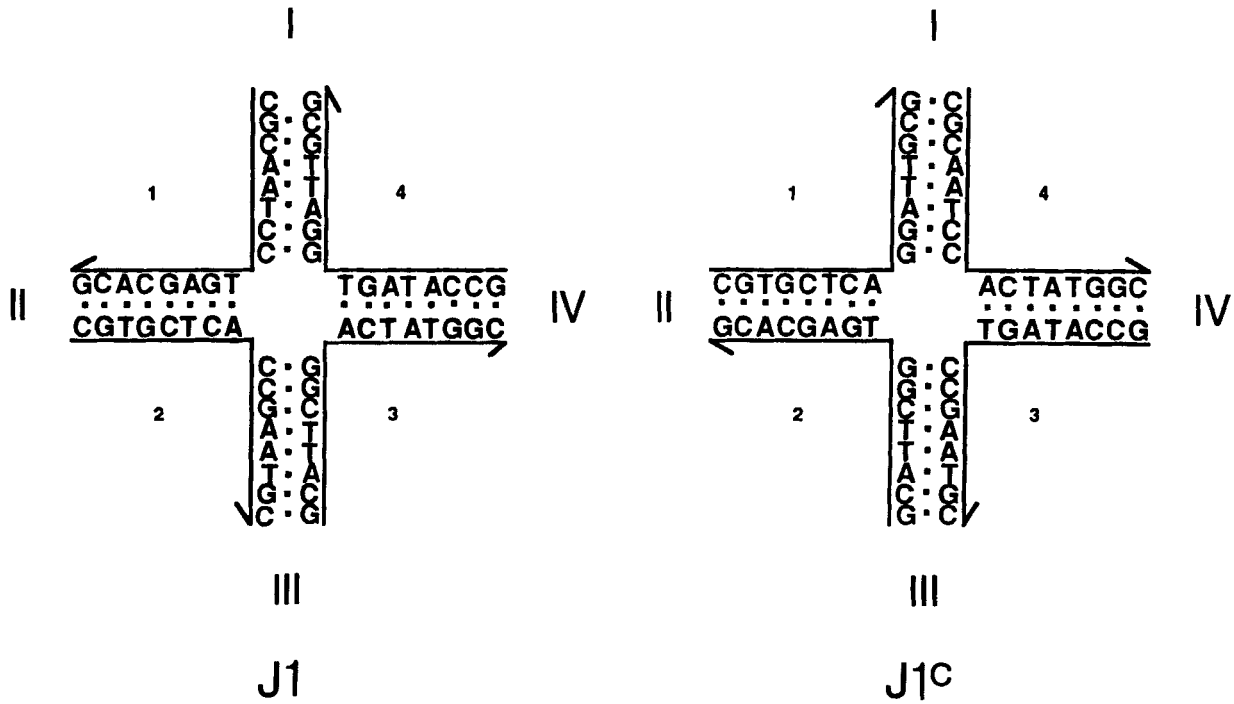

FIGURE 2. The sequences of two synthetic DNA four-arm junctions. The junctions, composed of the four 16-mer strands, are shown in schematic form with the half-arrowheads designating the 3′-OH terminus of each strand. The individual strands are numbered with Arabic numbers, and the arms are numbered with Roman numbers. The Watson-Crick base pairing is indicated by small dots between complementary pairs. The strand numbering indicated is used throughout the text. J1^c is formed from the four strands complementary to those in J1.

confirmed the results of gel electrophoretic experiments using other immobile junctions. They made the important new observation that changing the base pairs adjacent to the branch point results in changes in the relative gel mobilities of the junction with extended pairwise arms, while changes at positions one base-pair removed from the branch itself have no effect.

Churchill et al.[63] investigated the susceptibility of J1 to cleavage by hydroxyl radical probe. Analysis of the auto-footprint of J1 by Fe(II) · EDTA in the presence of ascorbate[64] revealed a twofold symmetric protection pattern in solution.[63] The residues flanking the branch in strands 2 and 4 were strongly protected from cleavage, while those same residues in strands 1 and 3 remained almost as susceptible as double-helical DNA. This led to the suggestion that J1 disposes its four arms into two roughly parallel helical domains, one containing arm I and arm II, while arm III and arm IV form the other domain (Figure 3), as in the Sigal-Alberts model[3] for the Holliday junction.[63] According to this proposal, strands 2 and 4 correspond to the crossover strands, while strands 1 and 3 are the noncrossover strands (see Figure 3). In a further study, Seeman and co-workers[50] compared Ferguson plots of the electrophoretic mobilities of "tethered" immobile junctions whose helical domains are constrained to be either parallel or antiparallel with that of J1; the mobility of the antiparallel tethered junction has the same dependence on acrylamide concentration as does the untethered J1. This covalent tethered-junction study as well as electrophoretic experiments[51,53] indicate that the preferred conformation of a junction has the two noncrossover strands antiparallel, as illustrated on the left of Figure 3.

Cooper and Hagerman[52] also used the method of transient electric birefringence to study the geometry of a synthetic four-arm DNA junction with extended pairwise arms by measuring the actual angles between junction arms. They find that, in the presence of Mg^{2+}, a four-arm DNA junction has a sequence-dependent geometry in solution in which pairs of arms are arranged in

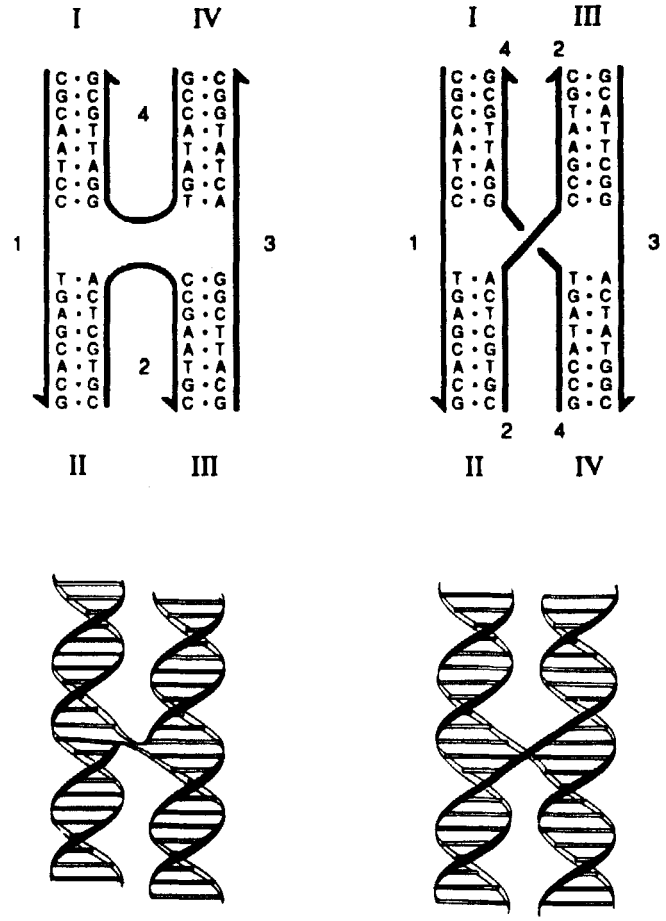

FIGURE 3. Structure of immobile four-arm DNA junction J1. The upper panels show the junction in schematic, while the lower panels show a model of the junction based on arms of 10.5-fold B-DNA. The structural conclusion based on the footprinting study[63] is indicated in this figure, by stacking arm I 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.

nearly colinear domains with the two helical strands antiparallel.[52] In the absence of Mg^{2+}, the structure is different.

Murchie and co-workers[54] carried out fluorescence energy transfer measurements to determine the relative distance between the ends of a four-arm DNA junction. Their results are consistent with the geometry of an "X" previously proposed by them from electrophoretic mobility experiments.[53] The interconnected helices are thought to be juxtaposed so that the continuous strands of each helix generate an antiparallel alignment, while the two interchanged strands do not cross at the center. The acute angle of the X structure is defined by a right-handed rotation of the helical axes about the axis perpendicular to the X plane (Figure 4).[54] One difficulty in accepting the detailed structural conclusions of this group[65] is that the differences in energy transfer rates between pairs of markers are smaller than the error level in the measurement.[65a] This model thus seems tentative at best, although likely to be correct in gross features.

The untethered synthetic junctions that have been studied in most of the above experiments have all been free to undergo isomerization be-

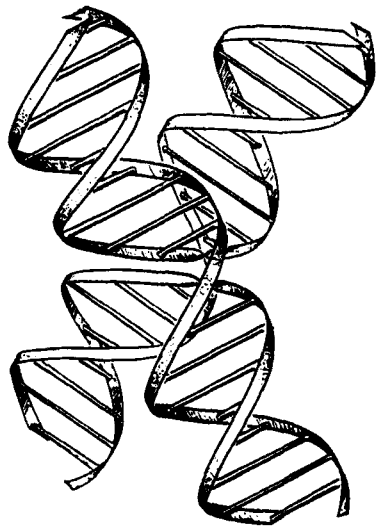

FIGURE 4. A structural model of four-arm DNA junction determined by fluorescence energy transfer measurement. Murchie et al.[54] proposed a right-handed cross of antiparallel junction structure by using fluorescence energy transfer between pairs of donor and acceptor fluorophores attached to the arms to determine the relative distances between the ends of a four-arm DNA junction.

tween parallel and antiparallel conformations.[50] Recently, Kimball et al.[66] studied a series of Holliday junction analogs that are designed to adopt either antiparallel or parallel conformations by covalently connecting two arms of a molecule with a short tether of thymidines. They find that applying a physical constraint at a distance from the branch point can determine which strands cross over. Use of tethered strands further allows one to estimate the free energy difference between the parallel and antiparallel isomers.[67] The result is that the free energy difference is small, with the antiparallel isomer more stable by only about 1 kcal/mol at 18°C. This difference suggests that the isomerization will not present a significant activation barrier to the rate of branch migration at this temperature.

Several experiments reveal that counterions play a crucial role in determining the structure of junctions. These include electrophoretic studies,[51,53,60,68] transient electric birefringence measurements,[53] as well as fluorescence energy transfer measurements.[54] In the absence of metal ions, junctions of any sequence assume a structure in which the four arms appear to be relatively extended.[51–54] NMR and chemical probing suggest that the base pairs at the branch in an immobile junction in the presence of Mg^{2+} are fully or partially paired at low temperatures.[47,53,68,69]

Chen et al.[49] characterized two junctions in which a single step of migrational freedom can occur, and they demonstrated that both migratory conformers exist for each junction in the presence of Mg^{2+} and low temperature. In agreement with the result of Duckett et al.,[53] Chen et al.[49] observe that two junctions, differing only in the base sequence immediately flanking the branch point, can manifest opposite hydroxyl radical protection patterns in the vicinity of the branch.[49] These results together with electrophoresis experiments indicate that the choice of double helical arms in the two helical domains is governed by the sequence flanking the branch.[49,53] This has been demonstrated clearly by Duckett et al.,[53] who find that the sequence at the penultimate position in four-arm junctions does not influence the structure.

Guo et al.[55] recently investigated the geometry of a three-arm DNA junction, JL, concluding that three-arm branched DNA molecules form an asymmetric structure in the presence of Mg^{2+} from electrophoretic mobility and chemical and enzymatic footprinting experiments. Further analysis[57] shows that the geometry of a three-arm junction is governed by the sequences at the branch and 1 bp removed from the branch, and the formation of a branch in a three-arm junction perturbs one turn of the helix from the branch in both the presence and absence of metal cations.[57] These results indicate that the structure of three-arm junctions is quite different in character from that of four-arm junctions.

III. ANALYSIS OF DRUG BINDING TO IMMOBILE FOUR-ARM JUNCTIONS

A. Site-Specific Interaction of J1 with MPE · Fe(II)

The interaction of drugs with DNA has been investigated using several approaches, including spectroscopic monitoring of bound ligands and inspection of isotherms. These traditional methods clarified a number of mechanisms of drug

binding by molecules that intercalate into duplexes or that associate externally with the grooves, for example. For the purposes of our present inquiry, it would be advantageous to be able to discriminate binding to different regions of the junction — the most obvious branch. This leads us to use footprinting techniques rather than classic binding analyses.

A variety of cleavage reagents have been used successfully as probes in footprinting experiments, including enzymatic nucleases such as DNase I[70–72] and UV irradiation,[73] including chemical species that modify DNA[74–76] or induce radicals in the presence of light or redox agents.[64,77–79] The "footprint" obtained by means of a particular reactive probe depends on both the nature of the active species and the mode and strength of interaction between the probe and DNA (Figure 5).

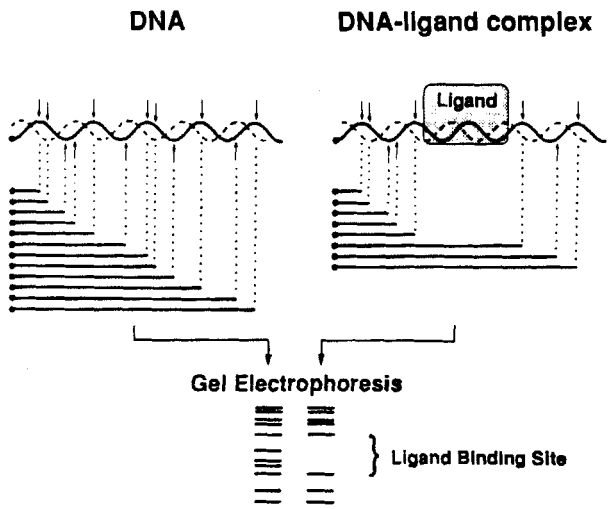

FIGURE 5. Schematic representation of the footprinting methods. The left side of the illustration shows a free DNA fragment and the right side the same DNA fragment containing ligand. Arrows show the cleavage sites by a chemical probe or endonuclease. The ligand protects the DNA backbone from the cleavage, while the free DNA is cleaved along its entire length. The resulting DNA fragments are analyzed by denaturing gel electrophoresis; the differences between the cleavage pattern of the free DNA and that of the ligand-DNA complex can be visualized by autoradiogram because only the radioactive labeled fragments are visible. The absence of bands in the autoradiogram reveals the region of DNA bound by ligand, as shown in the right-hand lane.

The strategy of the experiment for our application is straightforward: we compare the pattern of scission by a reactive probe molecule that induces chain scission in a strand of the junction with the pattern of scission of a duplex containing the same strand and its complement. In this approach, we are interested in the *difference* in patterns between the junction and duplex control. Four experiments are involved for a given four-arm junction, three for a three-arm one, with each strand of the complex labeled at a time. The reagents we have used are the following:

- *MPE·Fe(II).* The agent MPE·Fe(II) consists of an intercalating ring system (Figure 6, 7a) covalently tethered to an EDTA moiety that binds iron and can generate OH radicals in the presence of H_2O and peroxide or oxygen, presumably via a Fenton reaction.[80,81] Calibration of the reagent against DNase I indicates that it allows more precise location of tightly bound ligands on DNA than the enzyme
- *EDTA·Fe(II).* Tullius and Dombroski[82,83] found that EDTA·Fe(II) (Figure 7c) alone, which does not bind tightly to DNA, provides a sensitive and nonperturbative footprinting reagent for DNA and DNA-protein complexes. In selecting a negatively charged reagent, these authors sought to induce radical formation in the solvent surrounding the DNA. The short-lived OH radicals thus diffuse to the DNA from the surrounding solvent, a desirable feature in a footprinting reagent. The reagent was originally applied to DNA bound to calcium phosphate surfaces; it has since been found to respond differentially to a variety of structure changes in DNA.
- *$(OP)_2Cu(I)$.* The 2:1 complex of 1,10-phenanthroline with Cu(I) (Figure 7b), abbreviated here as $(OP)_2Cu(I)$,[79] has been found to have nucleolytic activity via oxidation of deoxyribose[84,85] in the presence of oxygen and a reducing agent such as mercaptopropionic acid. This reaction is apparently mediated by binding of the complex to duplex DNA,[86] since single-stranded species are not cleaved by this agent.[87] These reagents are used here together with DNase I and resol-

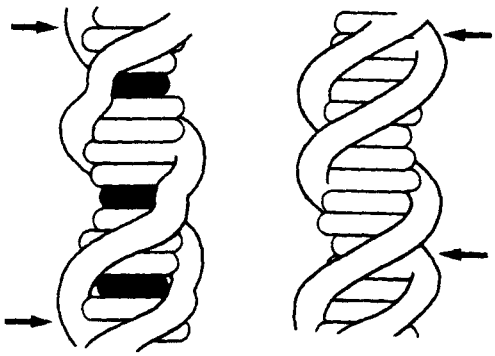

FIGURE 6. Schematic drawing showing intercalation of planar drug molecules into DNA double helix. Intercalation of planar drug molecules (black areas) into DNA double helix (left) distorts the regular sugar-phosphate backbone spacing (right).

vase enzymes that cleave the branch sites in junctions as probes of the binding of intercalative drugs to branched DNA.

Reaction of J1 with MPE·Fe(II) reveals a specific site of enhanced cutting near the branch by this agent, with several flanking sites showing lower reactivity relative to fully duplex controls.[88,89] The range of chain scission due to hydroxyl radicals emanating from the bound iron of MPE·Fe(II) extends several base pairs from the position of the ring.[78,90] The pattern of enhanced cuts is consistent with the presence of a preferred site of binding at or very near the branch point on one of the two stacks, but not the other (Figure 3). The MPE·Fe(II) scission experiments that initially revealed the presence of a site of enhanced cleavage by MPE·Fe(II) in the tetramer, absent in any corresponding duplexes were carried out at a ratio of 1 MPE·Fe(II) molecule per 16 tetramers,[88] with the strands in 16 μM concentration. In these conditions, both apparent enhancement and protection effects can be detected in junctions, relative to duplexes (Figure 8).[89] An experimental difficulty should be noted here. To compare two patterns on different lanes or gels, the two need to be "normalized" by looking for one or more sets of adjacent bands that appear closest in profile between the two and then adjusting the intensities of the two lanes accordingly. Despite loading approximately equal numbers of counts on each lane of a gel, there is variation both in the actual volumes delivered as well as in the extent of cutting between different experiments. Thus, for comparison of a double-strand pattern with that of a tetramer, for example, sets of bands with similar profiles tend to occur in the vicinity of positions 3–6 or 11–14 near the ends of the arms, which are likely to yield a common pattern of cuts. By using these sites as standards, it is often possible to ascertain the presence of both protection and enhancement on a given strand for the same comparison. However, when the standard positions are perturbed, for example, by presence of a sequence specific agent, a change in intensity cannot be distinguished from a scaling phenomenon.

As can be seen in Figure 8, the cleavage pattern produced by MPE·Fe(II) is consistent with enhanced probability for cutting at positions located on the left helical stack in J1, including strand 1:9–11, 4:10,11, and including residue 2:10. Protection at several sites distal to the branch point is observed, too, including 1:6,7,13–15, 3:6,7 (weak), and 4:5–8. Increasing the ratio of probe to DNA from this level up to 30 MPE·Fe(II) molecules per tetramer — nearly 1 MPE per base pair — results in a similar pattern of enhancement. This indicates that the site of higher affinity is occupied first and is maintained, even in the presence of drug levels corresponding to saturation of normal intercalative binding modes.[91] However, the enhancement of 2:10 disappears at high ratios of drug to J1. Interestingly, the pattern of protection in strand 1 breaks down with increasing drug, while that at 4:5–8 persists. No evidence is seen that a large relative excess of MPE·Fe(II) perturbs the junction configuration, since the strong drug-binding site is maintained.[89] In the MPE·Fe(II) experiment, the preferred site could be eliminated by competition with a second intercalator, propidium iodide (PI, Figure 7d). This suggests to us that the binding site or sites involved might be favorable for intercalative binding agents in general.[88,89]

B. Propidium Iodide and Actinomycin D Interaction with J1

Other footprinting agents have been used in an effort to define the enhanced binding site in

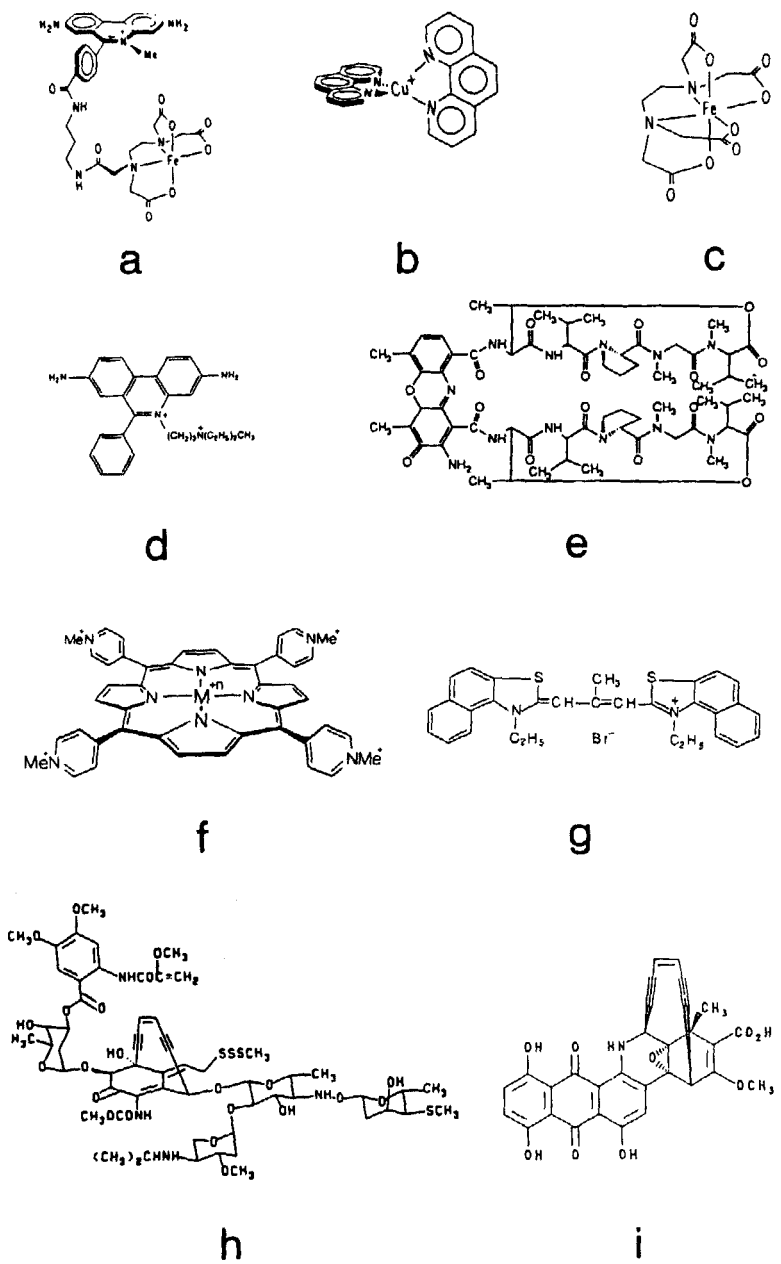

FIGURE 7. Chemical structures of ligands. (a) methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)]; (b) bis (*o*-phenanthroline)copper(I) [$(OP)_2Cu(I)$]; (c) Fe(II)·EDTA; (d) propidium iodide; (e) actinomycin D; (f) metalloporphyrins; (g) Stains-All; (h) esperamicin A_1; (i) dynemicin.

the four-arm junction, J1, more precisely. An outline of the experimental plan is shown in Figure 9, for clarity.[89] The probes used are $(OP)_2Cu(I)$, EDTA·Fe(II) at different ratios of probe to DNA, in the presence and absence of excess PI or actinomycin D. Sigman and his group have investigated the nucleolytic properties of Cu complexes with o-phenanthroline.[79,84–87] The activity of this agent appears to be more localized in range than the radicals resulting from γ-irradiation,[79] although Williams and Goldberg[92] argue that a diffusible intermediate is nevertheless involved. The precise fashion in which this probe interacts with duplex DNA is not known: $(OP)_2Cu(I)$ appears to bind DNA via the minor groove.[79,93] It is not a traditional intercalator, *since*

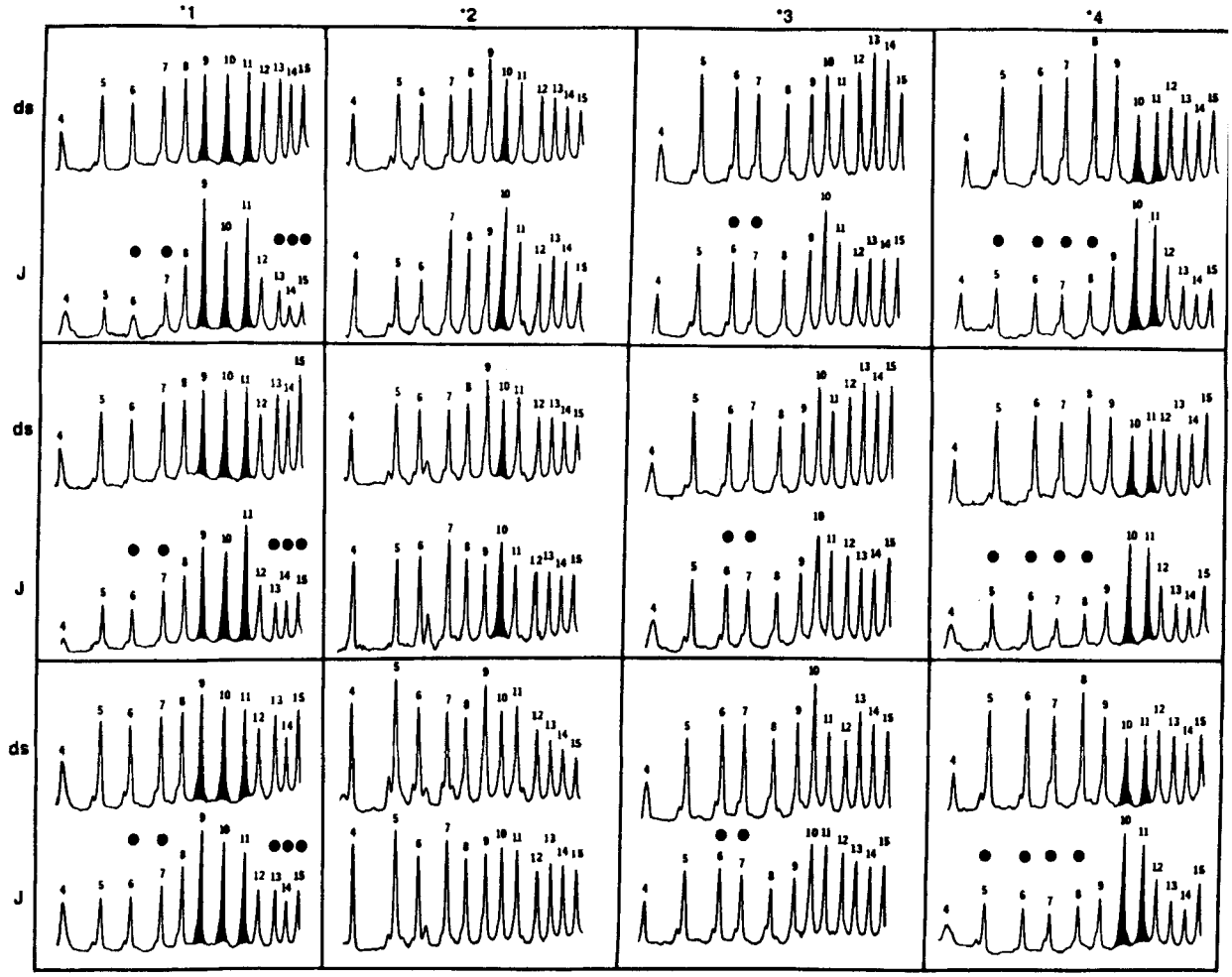

FIGURE 8. Densitometric scans of the cleavage pattern of J1 due to different concentrations of MPE·Fe(II).[89] Each column of this figure corresponds to labeling of the indicated strand of J1 and contains three panels, from top to bottom. The top panel corresponds to cleavage by MPE·Fe(II):J1 tetramer at a ratio of $^1/_{16}$, the middle panel to a MPE·Fe(II):J1 ratio of 3:1, and the bottom to a MPE·Fe(II):J1 ratio of 30:1. Each panel contains two scans, one with the labeled strand in the tetramer complex (J) and one with the labeled strand in the linear duplex formed by pairing 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. Thus, positions 9–11 on strand 1 and positions 10 and 11 on strand 4 show enhanced cleavage in the junction relative to the duplex.

a geometrically equivalent but nonscissile analog of $(OP)_2Cu(I)$, *bis* 2,9-dimethyl-1,10-phenanthroline-Cu(I), does not increase the viscosity of DNA on binding to the extent ethidium does.[94]

The $(OP)_2Cu(I)$ cleaving patterns of the four strands of J1 corresponding to the duplexes alone evidently vary more in intensity than those produced by EDTA·Fe(II) or MPE·Fe(II).[89] This variation has been discussed by Drew and Travers[93] as well as by Veal and Rill.[95,96] The latter propose that partial intercalation of $(OP)_2Cu(I)$ results in a strong sequence preference for A·T rich units (trimers or tetramers), with inhibition due to the 2-amino group of G, at selected positions in these units. This probe appears to reflect local structural features in the minor groove of DNA, since cuts on opposite strands are staggered by three bands, the number that spans the two strands across the minor groove.[93] Inspection of the corresponding J1 profiles shows that $(OP)_2Cu(I)$ is also more sensitive to differences between linear duplexes and branched molecules than the other probes that have been tested.[89]

To further characterize the drug-J1 complex,

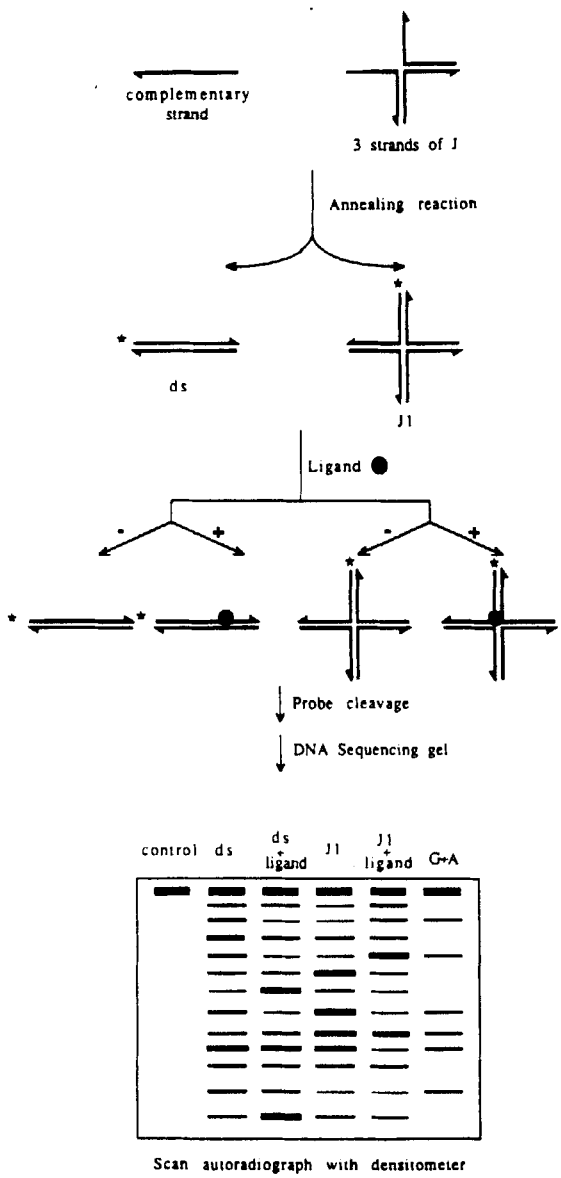

FIGURE 9. Strategy of chemical footprinting analysis of intercalation into a DNA junction. Each strand of the tetramer junction is labeled in turn and paired with roughly stoichiometric concentrations of (a) its complete complementing strand and (b) the three remaining strands of the tetramer. Each sample is split into two aliquots, one for ligand binding and one as a control. This gives four separate lanes for each labeled strand, as indicated in the diagram. In addition, control uncut strands and a sequence are run.

we have used the $(OP)_2Cu(I)$ to footprint it. The effect of PI at a ratio of 2 molecules to 1 tetramer on the $(OP)_2Cu(I)$ cutting profile of J1 can be seen.[89] The striking enhancement at 4:10 is lost in the presence of PI. Enhancement at the opposite residue in the same arm, 1:8, is also lost, as is that at 1:9, across the branch point, and at 3:8,9. However, in contrast, the enhancements seen at 2:7,9 and 3:10,11 are not affected by PI binding.[89] Interestingly, site 4:9 is protected in the presence of PI. If this probe binds as a partial intercalator,[95,96] this might reflect exclusion of binding adjacent to the intercalated PI molecule.

In an attempt to determine the stoichiometry of PI binding, a series of complexes of J1 containing different ratios of PI were exposed to the

$(OP)_2Cu(I)$ probe.[89] The scission is monitored on strands 3 and 4. The profiles shown reveal different dependences on the relative PI concentration at lower PI concentration, suggesting (but not proving) that the two sites reflect the effect of ligands with different affinity.

To find where PI binds, complexes of PI with J1 were exposed to OH radicals generated by EDTA·Fe(II) in the presence of hydrogen peroxide.[63,64,89] A "footprint" of PI bound to J1 emerges at sites 1:9 and 2:6, but only at relatively high ratios of drug to DNA. In contrast to MPE·Fe(II), PI at high concentrations may alter the conformational equilibrium of the junction, since the characteristic crossover isomer pattern of protection at 2:8,9 and 4:8,9 in the tetramer relative to duplexes[63] can be seen to reverse, giving protection at 1:8,9 and 3:8,9.[89] Since the effect is seen only at high PI:J1 ratios, it is probably not due to a single drug molecule having a preference for the reversed crossover isomer; the reversal of crossover pattern is likely to be a function of the combined interactions of several molecules with J1.[89]

It is interesting to contrast these findings concerning the site of enhanced affinity for intercalators in a branched molecule with the observations of sites of enhanced affinity in duplexes containing base "bulges."[92,97,98] The scission pattern resulting from the presence of an excess or bulged base at a specific position in a duplex is similar in some respects to that for a branch site. The MPE·Fe(II) pattern of enhancement extends three residues 3′ from the actual position of the excess base.[98] However, no protected sites have been reported. Similarly, three residues 3′ to the extra base are enhanced with respect to $(OP)_2Cu(I)$ cutting, with the second base showing the strongest effect.[92] The $(OP)_2Cu(I)$ patterns associated with the particular branch that has been studied show enhancement at two positions 3′ to the branch site on the two noncrossover strands, 1 and 3, but positions 5′ to the branch site between positions 8 and 9 also clearly show enhancement.[89]

Actinomycin D (Figure 7e) consists of a planar ring system capable of intercalation into DNA, with two pentapeptide side chains.[99] Preferential interaction of AD with GpC and related sequences in DNA has been demonstrated clearly by MPE footprinting[100,101] as well as by spectroscopic and thermodynamic methods.[102] The efficacy of MPE in delineating tight sites of AD binding in normal DNA implies that AD is not displaced by the probe at the concentrations used; similar behavior is found in the case of branched DNA.[89] This footprint strongly suggests an interaction of AD in the vicinity of the site for preferential interaction of MPE and PI, probably centering on the G·Cs flanking the branch site in arms I and III (see Figure 3). Inhibition of cutting at 1:8–10, 2:9, 3:8,9 and 4:5–10 in J1 relative to the duplexes can be seen, together with elimination of the interaction at 2:10.[89] In addition, there is an indication of a switch in intensities between sites 3:12 and 13 in the tetramer compared with duplex.[89] This is an A·T sequence adjacent to three G·Cs near the end of arm IV, and the effect may arise from occlusion by binding by the adjacent duplex. The number of sites affected suggests that more than one AD may be involved.

C. Selective Interaction of Porphyrins with J1

Among the most remarkable and perhaps unlikely systems that have been asserted to exhibit intercalative binding modes are a group of water-soluble cationic porphyrins (see Figure 7f); porphyrins are large fused systems of heterocycles that play essential roles as prosthetic groups in many proteins and enzymes.[103] While no X-ray crystal structures of intercalated porphyrin-DNA complexes have yet been reported, the water-soluble meso-substituted porphyrin, tetrakis (4-*N*-methylpyridyl)porphine (H_2TMpyP-4), was first proposed to exhibit intercalative modes of binding by Fiel and his co-workers,[104] based on viscosity measurements, increase in Tm, and unwinding studies of circular DNA. The interaction of H_2TMpyP-4 with covalently closed DNA circles is accompanied by a degree of unwinding that is almost identical to that of ethidium.[105] Intercalative modes appear to be strongly favored only for alternating CpG sequences.[105–107] NMR measurements using ^1H and ^{31}P detect the presence of at least one type of complex in which the porphine ring appears to insert fully and symmetrically between adjacent CpG base

pairs.[108] However, only in a pure CG sequence could this represent the exclusive or dominant mode of binding. Intercalation has been shown to take place in mixed sequences containing some GC base pairs.[109] In addition, some mode or modes of external binding take place with A·T or mixed sequences.[106,107,110,111] Similar intercalative modes are observed for the tetrakis (3-*N*-methylpyridyl)porphine,[112] but the tetrakis (2-*N*-methylpyridyl)porphine appears to be incapable of intercalation,[106] presumably because of steric hindrance by the *meso* ring systems.

The interaction of these molecules with DNA is modulated strongly by the presence and nature of metal ligands on the porphine.[109,113] The basic observation is that metal derivatives lacking axial ligands, Cu(II) and Ni(II), for example, behave like H_2TMpyP-4 in exhibiting intercalative binding modes with preference for CG sites.[106] Metal derivatives that possess axial ligands, such as Zn(II), Co(III), Fe(III), or Mn(III), appear to be incapable of traditional intercalation and interact instead via external modes[106] with a preference for the minor groove of A·T sequences, as can be demonstrated most directly by porphyrin-mediated chemical cleavage of DNA.[74,114]

The binding of H_2TMpyP-4, two axially unliganded metal complexes, CuTMpyP-4 and NiTMpyP-4, and three axially liganded porphyrins, ZnTMpyP-4, CoTMpyP-4, and MnTMpyP-4, to a branched DNA molecule has been investigated.[115] The results show that H_2TMpyP-4 and its axially unliganded complexes of Cu(II) and Ni(II) interact preferentially at the crossover site, with the latter two inducing a much stronger response to $(OP)_2$Cu(I) cleavage than does propidium. The axially liganded Zn(II), Co(III), and Mn(III) complexes also respond in the vicinity of this site, but with quite different characteristics that may be associated with partial intercalative modes of interaction.[115]

Each of the porphyrins or metalloporphyrins examined shows some evidence for differential interaction near the branch point of the junction.[115] The interaction differs qualitatively and quantitatively from that seen in the case of the axially unliganded H_2, Ni, or Cu species. Returning to the question of whether the latter porphyrins actually interact with the junction via intercalation, a competition experiment similar to that carried out on MPE alone[88] was performed to answer this: propidium is an intercalative drug frequently used in DNA binding studies. It has the ethidium ring system, with a side chain having two positive charges rather than the single one in ethidium (Figure 7d). The results shows that PI can compete for NiTMpyP-4 and ZnTMpyP-4 in binding J1.[115]

These data provide strong evidence that the region in J1 near the branch point provides a high-affinity binding site for the extended porphyrin ring system as it does for more traditional intercalators.[88,89,115] These results are of interest in connection with the nature of porphyrin-DNA interactions as well as the special properties of branched DNA that make it a target for intercalative binding agents. Each of the porphyrin derivatives that has been tested interacts differentially with the junction in the vicinity of the branch point.[115] In each case, differential effects with respect to linear duplexes are detected in a "belt" of sites surrounding the branch point. The enhancements in $(OP)_2$Cu(I) scission, which is observed here with NiTMpyP-4 and CuTMpyP-4 in particular, are much larger than what have been seen for any other agents.[115] However, the competition experiment with PI shows that the mechanism of this hyperreactivity does not involve tight binding by the porphyrins. Rather, some conformation in the adjacent DNA may favor $(OP)_2$Cu(I) binding and/or scission.[115] The Ni and Cu porphyrin derivatives have been found capable of intercalation into normal duplex DNA with a preference for GC sequences, based on several of the stated criteria for intercalation.[106] The fact that PI competition eliminates the porphyrin enhancement in $(OP)_2$Cu(I)-induced scission is consistent with a model in which these molecules intercalate at or very near the branch point.[115]

The behavior of the axially liganded species is more complicated.[115] The signature of ZnTMpyP-4 interaction with J1 is elimination of the enhancements characteristic of $(OP)_2$Cu(I)-induced cleavage of free J1. Since interaction of ZnTMpyP-4 with J1 is also competitive with PI, can we conclude that the Zn derivative also intercalates near the branch point of J1? The answer is that this question cannot be decided from the information available. The effects of the axially

liganded porphyrin derivatives on MPE·Fe(II) scission are modest compared with the effects seen on $(OP)_2Cu(I)$ scission.[115] Unfortunately, the mode of interaction of $(OP)_2Cu(I)$ with DNA has not been precisely defined. One hypothesis is that $(OP)_2Cu(I)$ is a partial intercalator itself,[95,96] so that this process of binding may also be favored by the local structural deformation at the branch point. If the five-coordinate ZnTMpyP-4 can also partially intercalate at this site (both MnTMpyP-4 and CoTMpyP-4 are six-coordinate), the strong competition in this experiment for $(OP)_2Cu(I)$ cutting there might reflect the common mode of interaction it shares with the probe. The axially unliganded porphyrins, presumably more fully inserted, may deform the local structure in order to enhance scission by $(OP)_2Cu(I)$ at adjacent positions on the junction arms. However, it should be pointed out that Thederahn et al.[116] propose a minor groove binding model for the interaction between $(OP)_2Cu(I)$ and duplex DNA, with no obvious insertion of either ring into the helix.

D. Selective Interaction of the Dye Stains-All with J1

The thiacarbocyanin dye, Stains-All (4,5,4,5′-dibenzo-3,3′-diethyl-9-methylthia-carbocyanine bromide, Figure 7g) is one of a large number of cyanin dyes introduced as photosensitizers in the photographic industry.[117] This dye is used in histology as a differential stain for DNA, RNA, polysaccharides, and membrane phospholipids.[118] It has the eponymous property of staining each of these components a distinct color. In early experiments with J1, it was observed that the dye Stains-All colors dilute solutions of J1 differently from any of the duplex arms alone. The further study shows 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.[119]

Figure 10 shows the result of an absorption experiment[119] in which dilute solutions of the dye in 10-cm cuvettes are titrated against the junction and one control duplex. The absorption spectrum of the dye alone is extremely sensitive to both dye concentration and the presence of different ions in the solvent.[120,121] 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.[120] 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.[120] This spectrum is altered in the presence of 10 m*M* $MgCl_2$, as seen in panel A of Figure 10, to one with three bands at 470, 530, and 570 nm.[119] Progressive addition of tetramer or duplex produces the changes shown in Figure 10, 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 association 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.[119] Thus, the spectral changes are distinct, indicating a differential mode of association of dye molecules with the branched species.

Figure 11 summarizes the patterns obtained in the footprinting experiments using probes MPE·Fe(II), $(OP)_2Cu(I)$, and diethyl pyrocarbonate (DEPC) on J1 with and without an equimolar concentration of Stains-All.[119] By eliminating 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.[80,81,84] The DEPC patterns suggest that binding dye induces a local change in conformation of the major grooves of each strand at the branch point (Figure 11C).[119] The differential patterns, in fact, are opposite for the two crossover strands in J1.

To assess the binding affinity of the dye for J1, a dilution series is performed 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.[119] The inhibition of these characteristic enhancement of positions is observed to disappear between 3×10^{-8} *M* and 6×10^{-8} *M* dye, indicating an apparent binding constant of about 45 n*M* for the interaction. This value is

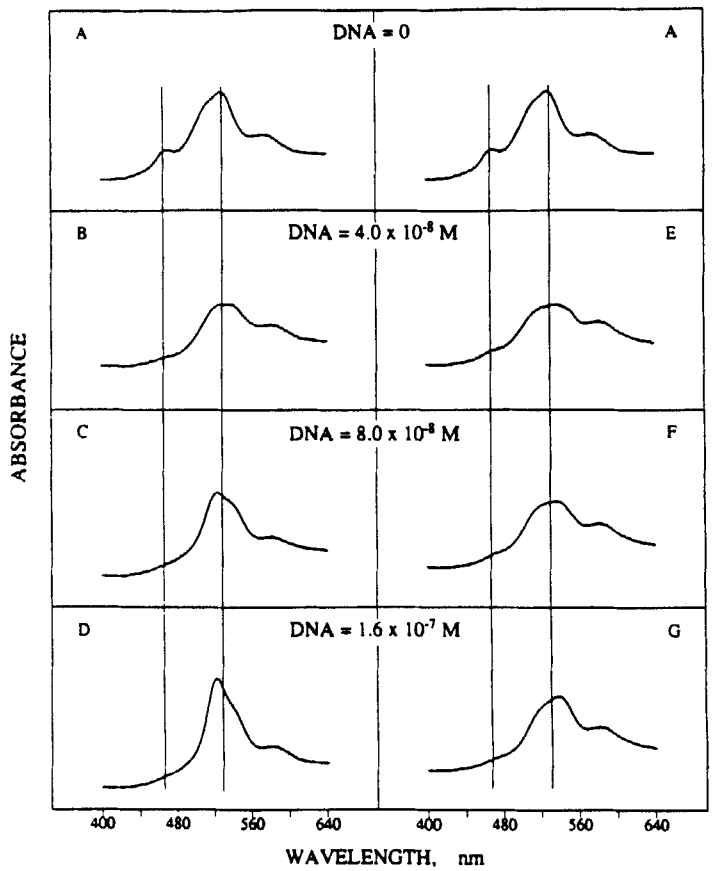

FIGURE 10. Absorption spectra of 1.0×10^{-6} *M* Stains-All solution with different amounts of DNA added to 10 m*M* sodium cacodylate solution (pH 8.0).[119] Curve B–D: absorption of DNA junction titration; curve E–G: absorption of control duplex titration (absorption scale in relative units).

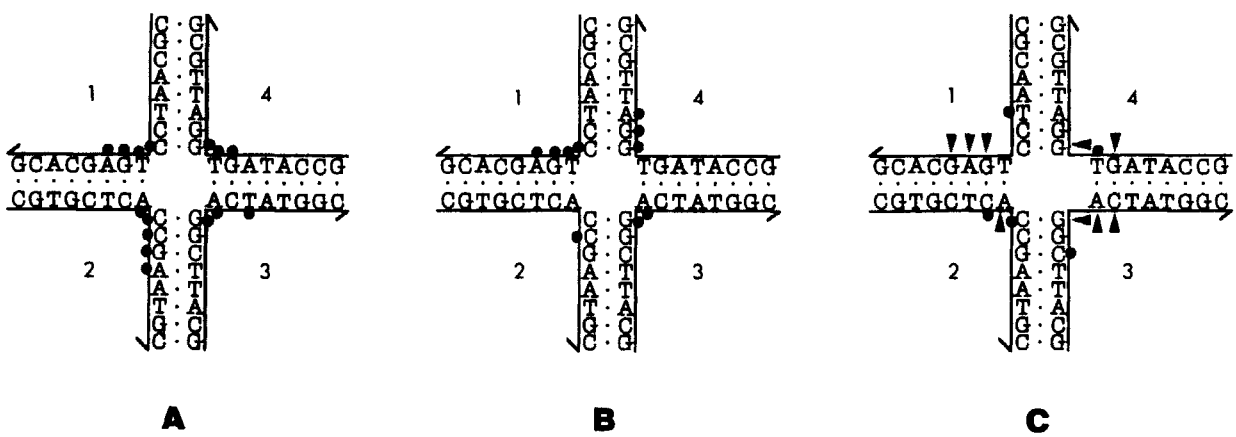

FIGURE 11. Sites of protection from chemical cleavage of J1 with Stains-All. Results of the scission experiments[119] are summarized in these panels. Sites of differential protection due to the cleavage of J1 with MPE·Fe(II) competing with Stains-All are indicated by filled circles in panel A. Panel B shows those sites due to the scission 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.

roughly two 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.[119] In this case, the competitive effect of the MPE·Fe(II) probe can be neglected, since it is estimated that its affinity is about 100 times weaker from a similar titration experiment. 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. 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.[89]

From the spectral data and footprinting experiments,[119] 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. Based on the dissociation constant that is measured,[119] 45 n*M*, the former model is favored. 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.

E. Interaction of Dynemicin and Esperamicins with Immobile Junctions

There is considerable interest in characterizing the activity of reactive antitumor antibiotics that contain an enediyne moiety that can generate radical species capable of cleaving DNA. These are among the most potent known antibiotics, active at concentrations well below those of the adriamycins, for example. This class of agents has the capacity to bind DNA and cleave the phosphodiester backbone on formation of a phenyl diradical from the enediyne core in the presence of thiols of NADPH.[122–127] How the branch sites in junctions behave with respect to these molecules is of interest to this inquiry.

The structure of esperamicin A_1 (esp A_1) consists of a diacetylenic ring system attached to four sugar residues and an aromatic ring (see Figure 7h). *In vitro*, esp A_1 and its simpler variants have a preference for cleaving pyrimidines in short oligopyrimidine tracts in duplex DNA.[127] The staggered double-strand cut pattern observed suggests scission across the minor groove of DNA, consistent with the reported inhibition of the double-strand DNA cleaving activity of esp A_1 by netropsin,[127] a classic minor-groove binding drug. Comparison of the sequence preferences of esperamicins A_1, C, and D suggests that the enediyne moiety itself is responsible for the sequence preference, while the sugars and anthranilate groups nonspecifically promote DNA binding.[127] In terms of relative activity in producing DNA breakage with intact eukaryotic cells, esp A_1 is estimated to be 200-fold more effective than esp C and 3000-fold more than esp D.[122] Removal of an isopropylamino sugar from esp D to produce the esp E structure retains the sequence preference of the other esperamicins, lending further support to the hypothesis that the ring itself is responsible for the sequence selectivity of DNA damage by these agents.[128]

The branch of J1 contains a TCCT sequence at the branch site of one of the strands assigned as noncrossing,[63] offering an opportunity to test whether esperamicins preserve their normal sequence preference in branched structures and what factors are involved in determining this activity. Scission at this normally highly reactive sequence is greatly diminished in the branched structure.[128] Comparison of the relative activities of esperamicins A_1 through E indicates that the protection is exerted by the ring itself, and not by the sugars or the anthranilate ring. Presumably, scission is reduced by alteration in the groove structure of the DNA at the branch rather than by a steric effect.

As shown in Figure 12, the main effect near the branch is a reduction in activity of all the esperamicins.[128] Since the strong DNA binding

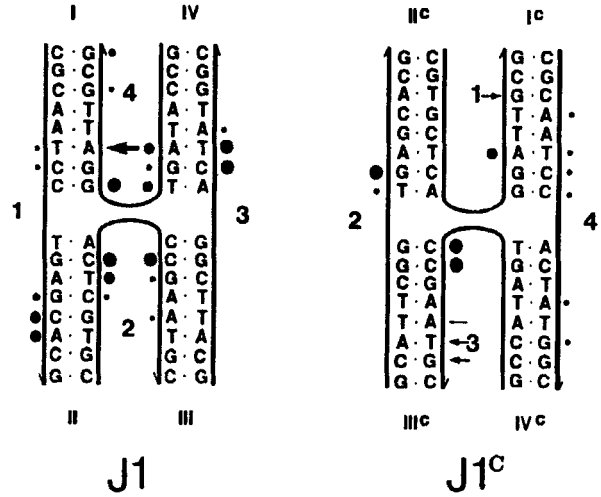

FIGURE 12. Sites of differential reactivity of esperamicin A_1 in branched DNA junctions J1 and J1^c relative to duplexes.[128] Sites of protection relative to duplexes are indicated by filled circles; sites of enhanced cutting relative to duplexes are indicated by arrows. The size of the filled circle or the arrow indicates the quantitative intensity at each responsive site.

activity comes from the sugars and aromatic group, it might be supposed that the effect is due to inhibition of this binding by changes in the local conformation near the branch. However, the fact that esperamicins A_1, C, D, and E all show parallel effects argues that it is the core of the drug that is responsible.[128] Note that sites of apparent enhancement arise also; these are always two or more base pairs away from the branch. Such an effect could arise from redistribution of drug following exclusion at the branch. A local structural basis for these sites cannot be ruled out, however.

Dynemicin (Figure 7i) contains an anthracycline moiety linked to a cyclic enediyne system that occurs also in esperamicins[122,123] and calicheamicins.[124] Dynemicin has been shown to cleave normal duplex DNA with relatively low sequence specificity compared with esperamicin A_1 or calicheamicin.[125,126] The response of the branch site in J1 and J1^c to dynemicin then is informative. If the sequence specificity of the cleavage of linear duplexes by esperamicins is dominated by the enediyne core itself.[122,128] The fact that bases close to the branch in a junction are less reactive to cleavage by esperamicins[128] suggests that the enediyne core itself is inhibited from access to a branch. On the other hand, if the selective interaction of the anthracycline moiety dominates the sequence specificity, enhanced cutting at or near the branch might still result.

Figure 13 contrasts the patterns of cleavage at 18°C of the four strands of J1 by dynemicin with the control patterns from the same strands in fully double-helical duplexes.[129] Differences in the cleavage patterns can be detected: certain bands increase in amplitude in the junctions relative to the duplexes. The enhanced cutting defines a site or sites of preferential interaction of dynemicin with these junctions, since the effect is not sensitive to the relative concentrations of dynemicin and DNA.[129] In J1, residues 1:9 and 2:8 increase significantly in amplitude in the tetramer as opposed to duplexes. At 18°C, cleavage at strand positions demarcating the site of enhanced affinity in both junctions is observed to be 70 to 100% more efficient than at the corresponding sequence positions in the control duplex DNA molecules.[129]

If the different selectivity of dynemicin involves intercalation of the anthraquinone core into the DNA, with subsequent radical attack emanating from the enediyne core, propidium should compete with dynemicin for the preferential binding to the branch site of the junctions. To test this, we repeated the cleavage experiments with dynemicin in the presence of a two-fold excess of PI.[129] The patterns of preferential cleavage at these sites were significantly altered in the presence of excess PI.

The behavior of dynemicin is thus distinct from that of esperamicins in its interaction at the branch of a junction.[128,129] Nevertheless, the reactivity of dynemicin for DNA is much lower than that of esperamicins A_1, B, and C. While it may be extremely potent as a drug, dynemicin cuts DNA far less efficiently than even the core moiety of the esperamicin system. This might suggest that interaction of the anthracycline ring system of dynemicin with DNA inhibits the mode of association of the enediyne moiety that is operative in the esperamicins. This would account for both the weaker interaction of dynemicin relative to esperamicins and the altered sequence specificity. The second feature that distinguishes these molecules is that the esperamicins show lower reactivity at positions close to the branch,[128]

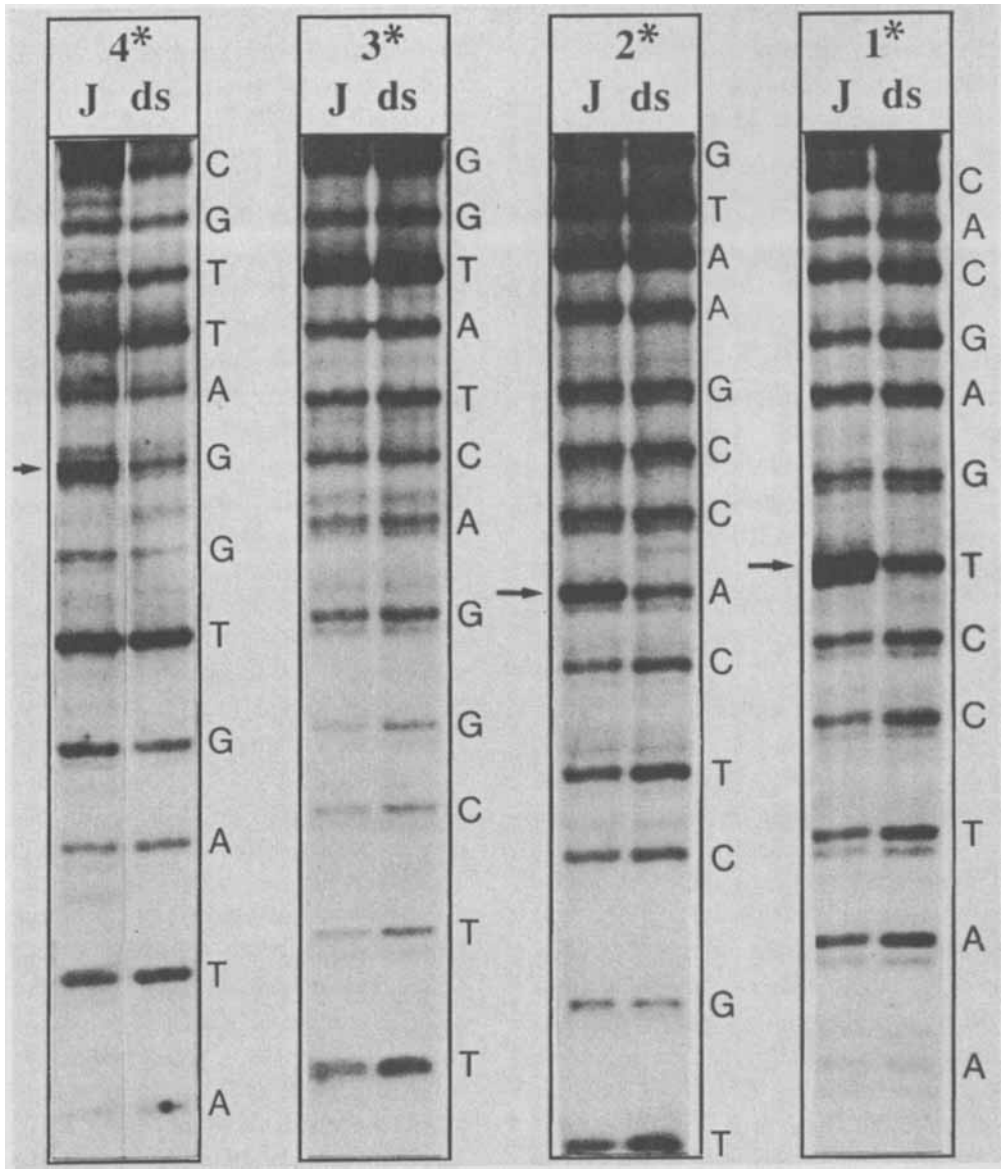

FIGURE 13. Dynemicin scission of J1 and control duplexes.[129] An autoradiogram is shown. Junction 5′-labeled with ^{32}P in one strand only and the duplex with same radioactive strand were cleaved by dynemicin and electrophoresed on a 20% polyacrylamide sequencing gel. The radioactive labeled strand is indicated by the number of the strand in the 5′ half of the figure. The arrows indicate the enhanced cutting sites of junction relative to the control duplex.

whereas dynemicin proves to be hyperreactive.[129] The fact that the pattern is sensitive to the presence of PI is consistent with the idea that the anthracycline ring in dynemicin is involved. Thus, while the enediyne moiety in esperamicins controls the sequence preference, in dynemicin this is dominated by the anthracycline ring system, so that its patterns of scission are distinct from esperamycin A_1 or any of its hydrolysis products. The crystal structure of a daunomycin-DNA complex indicates that the anthracycline ring system can intercalate into duplex DNA.[130] The action of PI on dynemicin-induced scission implies some kind of interplay in the action of the two mole-

cules. However, the scission pattern observed in the presence of excess PI is complex. One possibility is that this might be due to a structural change at the branch imposed by PI binding. A conformational change resulting from the interaction of the PI seems more consistent with our observation.[131]

F. Drug Inhibition of Endo I Resolution of J1

Several enzyme activities have been identified that can catalyze resolution of Holliday junctions into unbranched duplexes from phage,[17,19,26,27,53,132–137] bacteria,[138–140] and eukaryotes.[141,142] Of the resolvases that function in general as opposed to specialized recombination processes, the enzymes Endo VII from bacteriophage T4[19,27,134,137,143] and Endo I from bacteriophage T7[132,133] have been characterized most completely.

The two enzymes share a number of common properties. Both are pleiotropic, functioning in packaging phage DNA and degradation of host DNA as well as in recombination per se. Mutants in the gene for T7 Endo I, gene 3 of T7, and that for T4 Endo VII, gene 49 of T4, are deficient in phage recombination.[144,145] Branched DNA intermediates accumulate during infection of cells with phage defective in either gene.[146] T7 Endo I can, in fact, complement T4 phage that are defective in gene 49.[139] They cleave three- as well as four-arm branched DNA duplex substrates at or near the branch,[17,19,26,53,134,135] including structures containing single strands at the branch.[135] Four-arm branched substrates that have been tested include migrationally immobile and mobile structures.[19,26,53,134,135] The enzymes differ in that Endo I is asserted to cleave 5′ to the branch, while Endo VII cleaves 3′ to the branch. The T4 enzyme has been found to be specific for the crossover strands in migrationally immobile DNA junctions.[17,53]

We have investigated the substrate requirements and inhibition by drugs of Endo I, using purified enzyme to cleave three different four-arm junctions.[143] The enzyme cleaves branches with arms containing fewer than five base pairs, in contrast to Endo VII. The rate of cleavage is strongly dependent on temperature: at 4°C, the enzyme is effectively inactive, while at 9°C, cleavage is detectable. In contrast to Endo VII, Endo I shows a preference for the branch sites of the two strands in the immobile species studied that retain their duplex conformation, as judged by their lack of protection from Fe(II)·EDTA-induced cleavage at the branch.[49,56,63] In the case of a junction that has two sites for branch migration, Endo I cleaves all four strands with no preference.[147]

Since the branch site in junctions interacts tightly with a number of DNA binding drugs,[56,88,89,115,119,147] we can ask whether the bound ligands can influence resolution of junction substrates into duplexes by Endo I. Since inhibition might also result from direct interaction of ligands with the active site of the enzyme, these possibilities must be distinguished.

Figure 14 illustrates the results of an inhibition experiment.[143] Cleavage reactions were monitored in the absence and presence of ligands by labeling strand 1 of J1. As can be seen from Figure 14, propidium, CuTMpyP-4, and Stains-All inhibit the cleavage reactions at micromolar concentrations: propidium is the strongest apparent inhibitor; the dye Stains-All is weakest. The effect is relieved by addition of dsDNA to the inhibited reaction.[143] If inhibition occurs by interaction of ligands with enzyme, a parallel loss of the strong single-strand nuclease activity of Endo I should be seen. However, the experimental results show that there is no such loss following 5 h of exposure to PI at a concentration sufficient to inhibit resolution of J1.[143] However, there is a detectable loss of single-strand activity following the longer exposure times used to demonstrate drug inhibition. We cannot conclude that the inhibition is thus purely a substrate effect, and this point will need examination by kinetic studies.

An interesting result of this study[143] is that ligands that associate strongly with branched DNA at the branch site can inhibit resolution of the junction into the duplexes. One possible mechanism for this effect is a substrate inhibition in which the substrate complexed by drug is not available for cleavage. One test of the substrate inhibition hypothesis would be to determine the apparent kinetics in the presence of the ligands;

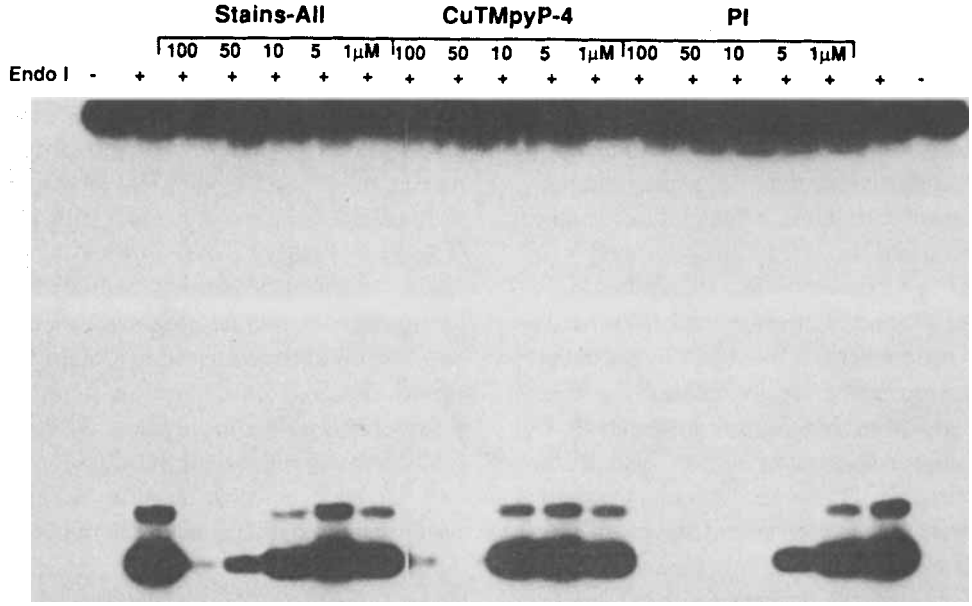

FIGURE 14. Ligand inhibition of Endo I resolution.[143] An autoradiogram of the gel is shown. J1 5′-labeled with ^{32}P in strand 1 only was cleaved with Endo I in the presence and absence of different concentrations of ligands and electrophoresed on a sequencing gel. All samples were incubated with 0.10 unit of Endo I at 18°C for 72 h.

an agent that removes active substrate via a reversible interaction should act kinetically as a competitive inhibitor. Excess calf thymus DNA added to the reaction solution relieves the inhibition,[143] but does not show that inhibition is reversible or distinguish the inhibition of enzyme from that of substrate. In any case, inhibition of resolvase activity by ligands raises the possibility that ligands might directly influence the yield of products of recombination. Design of agents that do this with increased effectiveness might provide a new avenue to influence genetic recombination by inhibiting the resolution process.

G. Drug Binding Properties Are Determined By Junction Geometry

What is the relationship between the structure of a junction and its ligand-binding properties? To address this issue, we have investigated the conformation of a four-arm DNA junction, J1^c as well as its interaction with drugs.[56]

The structure of J1^c (see Figure 2), the corresponding junction formed from the four strands complementary to each of those in J1, was explored by using hydroxyl radicals protection assay.[63] The results[56] suggest that J1^c forms stacked helical structures, just as the complementary junction J1 does, but has the opposite pattern, with strands 1 and 3 crossing over, while strands 2 and 4 are helical. In another experiment,[56] the digestion products of J1^c with T7 Endo I has been examined and the result shows that Endo I cleaves preferentially in strands 2 and 4. This is in contrast to J1 in which strands 1 and 3 are cleaved by Endo I.[143] Both hydroxyl radical protection and T7 resolvase-cutting experiments indicate that the conformational preference between the two crossover isomers in J1^c is not as strong as in J1. In light of the information from junctions of several different sequences that have been characterized to date,[53,63] the results in this study[56] are interpreted to mean that the alternative structures coexist in a dynamic equilibrium determined by the relative strength of the stacking interactions between the pairs flanking the branch together with the ease of folding the pairs that crossover at the branch.

The analogous binding properties of J1^c were investigated,[56] and the footprinting data show that J1^c has a site for enhanced binding of MPE·Fe(II) and $(OP)_2Cu(I)$ as does J1.[88,89] Comparison of the scission patterns of J1^c and J1 by the probes

MPE·Fe(II), $(OP)_2Cu(I)$, and DEPC indicates that the junction geometry is the major influence in determining ligand-binding properties.[56] Detailed comparison of the cleavage profiles of J1[89] and J1^c induced by $(OP)_2Cu(I)$ indicates a relationship between the junction structure per se and the pattern of scission.[56] Positions 2:10,11 in J1^c show enhanced reactivity in $(OP)_2Cu(I)$-induced cutting, whereas the corresponding positions in J1 occur at 1:9,10. Since the segment 1:9–16 in J1 has the same sequence as 2:9–16 in J1^c, sequence alone is not responsible for the differential reactivity. The positions of enhanced reactivity in MPE·Fe(II)-induced scission in J1^c also differ from those found in J1, where the enhanced cutting sites cluster on the left helical stack.[56] These data are also consistent with the idea that high-affinity MPE binding to a junction is essentially structure specific, with MPE scission producing excess damage on one helical arm of a junction, resulting in an asymmetric pattern of differential scission with respect to the control duplex. In the case of J1, it is hypothesized that one crossover isomer predominates,[50,63] while J1^c is a mixture of two more nearly equal crossover isomers.[56] This can explain why the MPE·Fe(II) cleavage pattern of J1^c is more nearly fourfold symmetric than that of J1.[56,88] Similarly, in the DEPC-induced cutting experiment, positions 2:8 in J1 and 1:8 in J1^c correspond to the same A residue in the two junctions, but no difference in reactivity at position 3:9 in J1 (which corresponds to 4:9 in J1^c) is detected.[56,119] If the major site of protection from DEPC reaction on strand 1 of J1^c represents the fractional presence of the strand 1-strand 3 crossover isomer, while the weaker protection on strand 4 comes from the other crossover isomer, we have a consistent explanation for the results of each of the probes that have been used in these experiments.

H. Drug Binding to Parallel and Antiparallel Holliday Model Junctions

Synthetic DNA junctions can be constrained to adopt parallel or antiparallel conformations[66] as models for two of the structural isomers accessible by a free junction. Two Holliday junction analogs, JA and JP, with identical base-paired sequences but different arms connected with a short tether of thymidines, have been constructed.[67] Each of these molecules is constrained conformationally to assume either an antiparallel or a parallel structure.[66] Availability of conformationally constrained model junctions allows us to investigate how the branch sites in JA and JP (Figure 15) might differ in their substrate activities, structural properties, and in their response to ligands.[67] It is of interest to know if these conformationally constrained analogues of J1 respond similarly to ligands or if the response of a tethered analog to ligands is perturbed from that of the unrestricted junctions.

We want to know how JA and JP differ from each other or with respect to J1 in structural terms. There are several kinds of experiments we have done to characterize the structure of JA and JP.[67] First, both JA and JP migrate as distinct bands on a nondenaturing electrophoresis gel with a lower mobility than any of the other combinations,[46] consistent with the formation of two structurally well-defined complexes.[67] Second, hydroxyl radical cleavage has been successfully used to identify the crossover strands of junction molecules. The cleavage patterns of junctions JA and JP are compared with the cleavage patterns for the same sequences in normal duplexes.[67] Differences reveal structural features of the junction. Protection from cleavage at the branch point, the signature of the crossover strands,[63,66] is seen on strands 2 and 4 of JA and strands 2 and 4-3 located in 5′ end of JP, demonstrating that both JA and JP can adopt the crossover state that predominates for free junctions. Third, one source of structural information about JA and JP comes from comparing the substrate properties of these different junctions for the resolvase Endo I.[53,135,143] The cleavage pattern of JA indicates attack preferentially in the positions that correspond to those in J1.[67] The pattern in JP is quite different: cleavage at each of the four strands at the branch is observed, with little preference.[67] Endo I thus distinguishes the two junctions, with no indication of cleavage in the tether itself. This implies a structural difference between the branch sites in JA and JP, and also provides further evidence that J1 favors antiparallel conformation in solution a resemblance of JA. Fourth, JA and JP have differential reactivity with diethyl pyrocarbon-

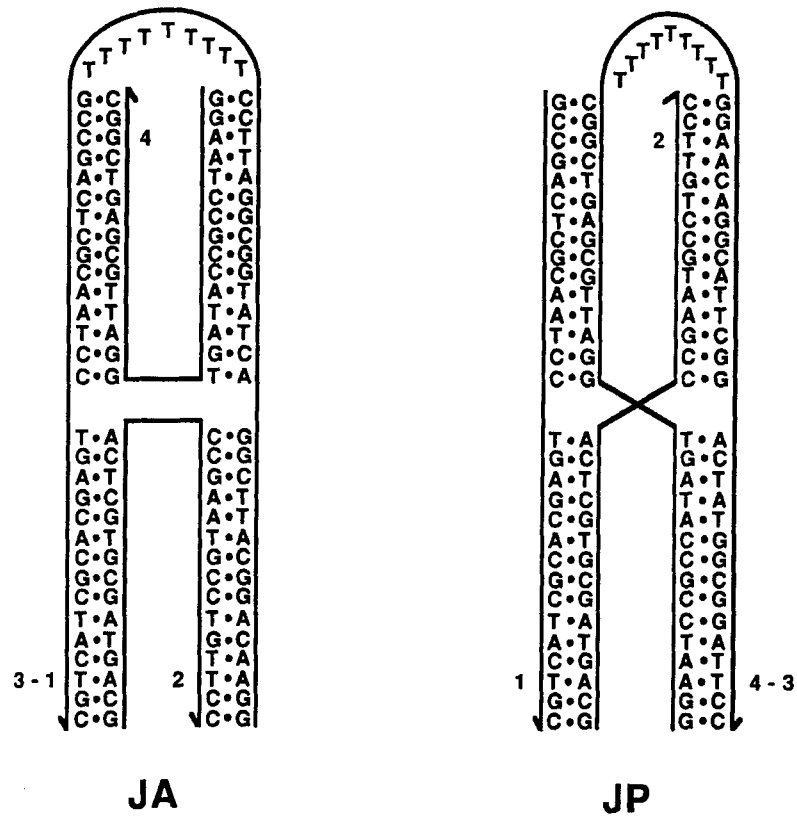

FIGURE 15. Sequence and schematic representations of two conformationally constrained junctions JA and JP.[67] They contain identical base-paired arms in which the core sequence is the same as J1. The junction on the left, JA, is tethered so that the stacking domains are antiparallel, while the junction on the right, JP, is tethered so that the stacking domains are parallel. The numbering system corresponds to the positions of strands in the untethered junction J1 (see Figure 3). The loops consist of $d(T)_9$.

ate.[67] Both JA and JP show minor protection at position 2:8 from DEPC-induced scission relative to that seen in J1 as a control. In addition, JP shows enhanced reactivity at 1:10,12 and 4:9,10. Enhanced reactivity might indicate distortion of the major groove in the vicinity of these positions relative to fully duplex control or local opening of the helical structure. This result again points to a difference in the folded geometry of JP in the presence of Mg^{2+} relative to JA or J1.

We have seen that a number of intercalators and other DNA-binding ligands interact strongly with the branch site in both three- and four-arm DNA junctions. In J1, these agents preferentially cut at several sites flanking the branch in each of the four strands of the junction. No strong difference in the cleavage patterns by these reagents can be detected between JA and JP or between these and J1.[67,88,89] These results indicate that in both cases, the branch point is a site of enhanced binding for drugs, as it is in the untethered four-arm junction containing the same sequence at the branch.[67]

The tethered models are not identical to an untethered junction at the branch, however.[67] Despite the small difference in free energy between the two (tethered models), the DEPC experiment indicates that there is a local structural difference between JA and JP at the branch site. At present we cannot specify the basis for this difference. The fact that the difference in free energy between JA and JP is sensitive to the magnesium ion concentration[67] might suggest that the two phosphates located at the branch on the crossing over strands differ in proximity, geometry, or both. This would account for the reactivity dif-

ference detected by the enzymes. A difference in conformation among the thymines in the tethering loops of JA and JP cannot be excluded from the results presented here. However, the fact that Endo I and DEPC differ in their activity on the constrained junctions argues that there is a difference at the branch, whether or not the tethering sequences are identically structured. The fact that the free and tethered models do not differ in their ability to interact preferentially at the branch site with the ligands tested implies that this interaction does not require a large-scale conformational change in the junction.

IV. DRUG BINDING TO A THREE-ARM DNA JUNCTION

Since three-arm branched DNAs can also arise as intermediates in general recombination in the absence of replication,[134,148,149] there is reason to determine the structural and ligand binding properties of these structures in particular.

Three oligonucleotides, each of 16-mer nucleotides with the sequences shown in Figure 16, have been synthesized and hybridized under stringent conditions. The complete junction migrates as a narrow band with a much lower mobility than any of the other combinations consistent with the formation of a structural well-defined species.[55]

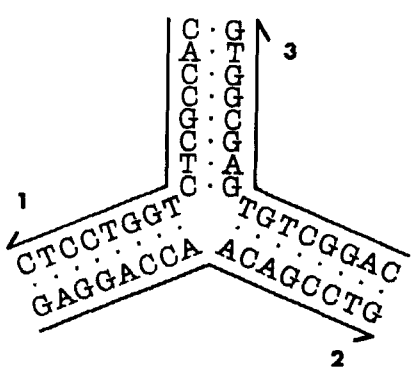

FIGURE 16. Sequence of the three-arm DNA junction JL.[55] The junction is shown schematically, and consists of three 16-mer strands of DNA. The 3′-ends of the strands are indicated by half-arrowheads.

First, we need to know something about the structure of this complex. The electrophoretic mobilities of three species of a three-arm junction in which pairs of arms are elongated are found to differ in the presence of Mg^{2+}: one combination of elongated arms migrates significantly faster than the other two.[55] This effect is eliminated in the absence of Mg^{2+}, indicating that counterions are crucial to the conformation of a three-arm junction, as in the case of four-arm structures. These results[55] also suggest that the three-arm DNA junction forms an asymmetric structure due to preferential stacking of two of the arms at the junction in the presence of Mg^{2+}. Furthermore, the electrophoretic mobility experiment shows that the geometry of a three-arm junction is governed by the base sequence at the branch and 1 bp removed from the branch.[57]

Chemical and enzymatic probing also suggest that JL lacks threefold symmetry.[55] The patterns of self-protection of each 16-mer strand of the core complex exposed to Fe(II)·EDTA scission is unique, consistent with an asymmetric structure. A similar correspondence is seen in the profile of scission induced by DNase I.[55] Strands 1 and 3 show protection of several residues 3′ to the branch site; also, strand 1 shows enhanced reactivity at position 7,8 relative to the duplex. Strand 2 appears fully protected, both in the vicinity of the branch point and at all sites 3′ to it. The structure of the active site of this enzyme has been determined by X-ray crystallography, and it appears to require access to the minor groove for scission and to bind to both strands of a duplex substrate.[150] The presence of an additional arm in a substrate DNA blocks the latter interaction resulting in protection of several residues flanking the branch.[151] These footprinting results also confirm that the three arms of JL are not structurally equivalent in solution in the presence of Mg^{2+}.

The interaction of three-arm DNA junctions with resolving enzymes has been investigated previously.[134,135] Jensch and Kemper[134] find that all three strands in a three-arm junction are susceptible to T4 Endo VII, and Dickie et al.[135] reach the same conclusion with respect to T7 Endo I. We also performed the T7 Endo I experiment on JL.[55] T7 Endo I cleaves this junction with great specificity, cleaving one or two phosphodiester

linkages per strand, and JL is cleaved predominantly at positions 1:8, 2:7, and 3:7.

Purine bases at the branch of JL have been shown to be reactive to diethyl pyrocarbonate.[55] This reagent is purine specific, with a preference for N-7 of A over that of G,[152] and sensitive to local conformation in DNA.[153] Purine bases in the vicinity of the branch of four-arm junctions show enhanced reactivity to DEPC at low salt concentrations relative to duplexes of the same sequence, and this reactivity is suppressed by addition of magnesium to the reaction buffer to fold the junction into a twofold stacked conformation.[147] We have also probed the branch purine bases of a three-arm junction.[55] Strands 1 and 3 show minor enhancement in DEPC reactivity at 1:10 and 3:9,10. Strand 2, in contrast, shows clear enhancement at 2:8,9, indicating that the major groove of this strand is also distorted from that of its duplex control near the branch. Enhanced reactivity might indicate partial opening of base pairs at these positions. Thus, while the structure of the three-arm junction appears to reflect preferential stacking of particular arms in the presence of Mg^{2+}, it differs in fundamental respects at the branch from that observed in four-arm structures.

Since reaction of four-arm junctions with the reagents MPE·Fe(II) and $(OP)_2Cu(I)$ reveals a specific region of enhanced reactivity in the junction relative to fully duplex controls,[88,89] what is the response of the branch site of a three-arm junction JL to cleavage by the two chemical probes? Enhanced reactivity to MPE·Fe(II) is seen at the branch relative to the duplexes.[55] Similarly, $(OP)_2Cu(I)$ become hyperreactive in the junction relative to the duplexes. The pattern of enhanced cuts is consistent with the presence of preferred site of binding positively charged ligands at or very near the branch point.[55] The results from the MPE·Fe(II) and $(OP)_2Cu(I)$ cleavage experiments underline the role of branched DNA molecules as targets for drug molecules. The cleavage patterns differ from those we have examined in four-arm junctions[88,89] but indicate preferential interactions in the vicinity of the branch point as is found in four-arm species. Does the ligand selectively binding the branch of JL influence the conformation in solution in the presence of Mg^{2+}? To address this issue, the electrophoretic mobility experiment was repeated in the presence of propidium.[55] In the presence of a 2:1 ratio of PI to trimer, the results are very similar to those in the absence of PI, suggesting that ligand binding to the branch does not affect the junction conformation.[55]

V. DRUG BINDING TO A PARTIALLY MOBILE DNA JUNCTION

While the physical properties of branched DNA molecules in which branch migration is fully inhibited have been discussed above,[47,61,63] the corresponding properties of branches with a limited number of accessible steps of branch migration are not so well characterized. Chen et al.[49] studied two junctions in which a single step of migrational freedom can occur. In each case, a Fe(II)·EDTA footprinting experiment reveals a protection pattern spanning three residues at the branch site, showing that both migrational isomers are present.[49] One dominant preference for the crossover strand is observed, which depends on the sequence flanking the branch.[49,53,54] In these cases, it was pointed out that the clear crossover preference implies both positional isomers share the dominant crossover strand choice.

In the case of a bimobile four-arm junction, K24, there are three different positional isomers, leading to a large number of potential conformers for this system. The structure of K24, formed from the four 50-mer oligonucleotides with the sequence shown in Figure 17, has been investigated. The susceptibility of strands of K24 to hydroxyl radicals[64] has been explored.[147] The hydroxyl radical protection pattern of K24 is roughly fourfold symmetric. The fact that the patterns in strands 1 and 3 of K24 do not match those in strands 2 and 4 suggests that K24 is not an ensemble of fourfold symmetric "X" structures.[147] It follows that, if protection from Fe(II)·EDTA-induced cleavage at the branch point relative to duplex controls is the signature of the crossover strands in these stacked structures, each strand in K24 acts as a crossover in the ensemble of these structures. Since three or four nucleotides, rather than two, are protected in K24, the bimobile junction K24 has a different pattern from that of an immobile junction such as J1 and the

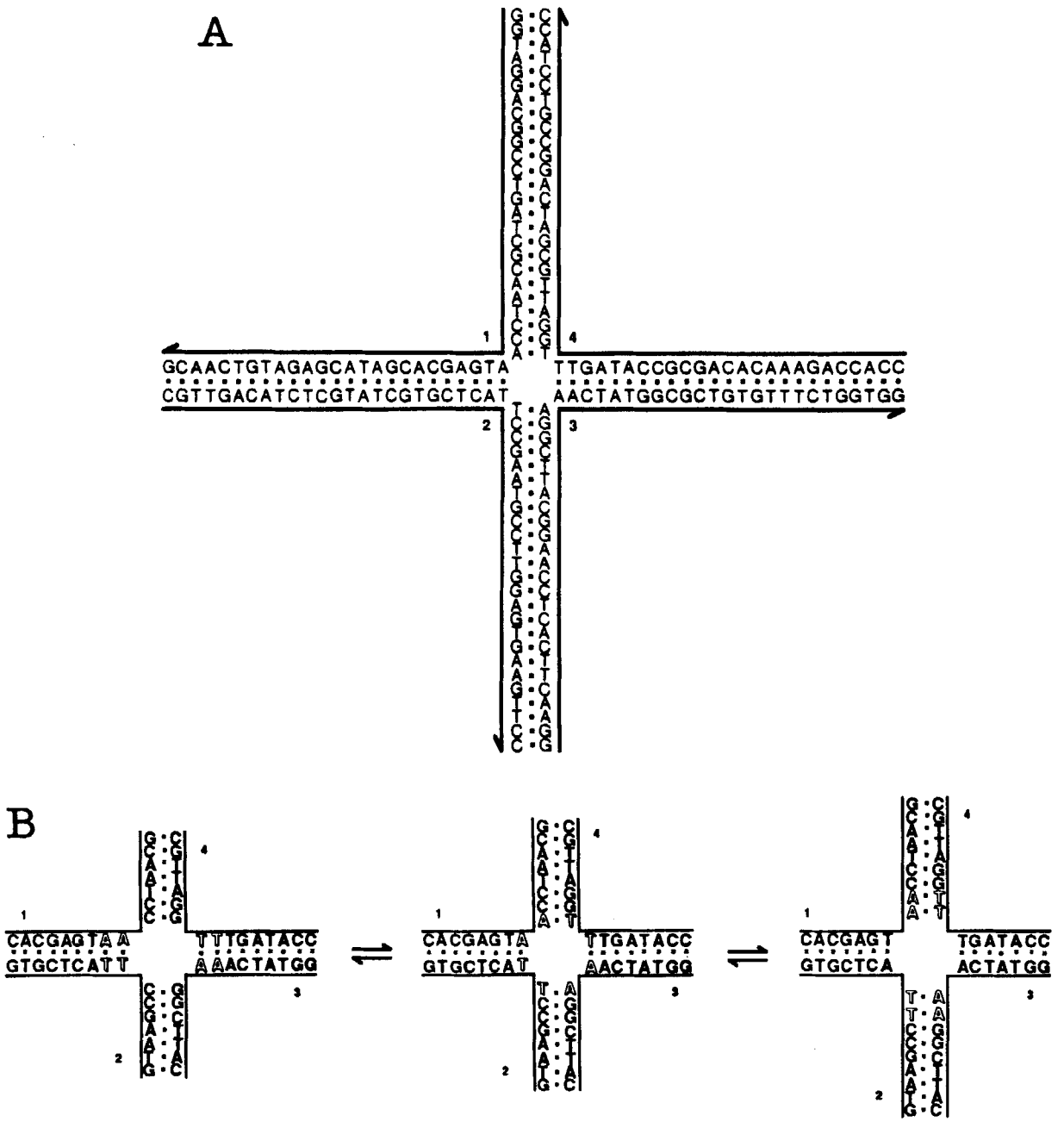

FIGURE 17. Analysis of a bimobile four-arm junction.[147] A bimobile four-arm junction was assembled by the hydridization of four 50-mer synthetic oligonucleotides. (A) The sequence of bimobile junction K24. The 3′ ends of the strands are indicated by half-arrowheads. (B) Diagram showing the allowed branch positions in K24, illustrated only the core sequence. The twofold sequence symmetry at the center of junction K24 permits the two branch migration steps indicated. The equilibrium illustrated shows how the pairing of the central bases can switch among "symmetric" pairs on the middle, "vertical" pairs on the left, and "horizontal" pairs on the right. No preferred stereochemical arrangements are implied here.

protections are significantly weaker in each case. This suggests that all three migrational isomers of K24 are present in solution. If each retains a twofold stacking pattern, with preferential choice determined by the identity of the flanking bases, these isomers do not all share a common conformational preference as has been seen in the study of two junctions with a single step of branch migrational freedom.[49]

A digestion experiment by resolvase enzymes has also been used to characterize K24 structure.[147] Holliday resolvases such as T4 Endo VII and T7 Endo I were found to cleave junctions at or near the branch. It was demonstrated that T4 Endo VII selectively cleaves the crossover strands in four-arm junctions.[17,52] On the other hand, T7 Endo I prefers to cleave the structurally continuous strands of model junctions.[143] If the bimobile junction K24 is an ensemble of stacked twofold symmetric structures with different conformational preference, one would expect to find that all four strands are cut near position 25 by both resolvases. The results show that both resolvases T4 Endo VII and T7 Endo I cleave each of the four strands of K24 with almost equal intensity.[147] This is consistent with the conclusion derived from hydroxyl radical protection experiments. However, Endo I digestion of K24 shows a slightly different pattern[147]: one single cutting site is seen at position 24 of strands 1 and 3, whereas three sites occur in strand 2 and two in strand 4. Thus, the three migratory isomers may not be distributed equally, or the conformational preferences among them may differ in strength. These results are interpreted to mean that K24 represents a mixture of stacked helical structures with different isomers.

Since the branch point of the immobile four-arm DNA junctions is a preferred site for ligand binding, then an interesting question is whether the branch site of a bimobile junction K24, which migrates at the center, preferentially interacts with drugs. Chemical footprinting properties of K24[147] with both MPE·Fe(II) and $(OP)_2Cu(I)$ in the presence and absence of the dye Stains-All show that the branch in K24 serves as a site for enhanced binding of these probes as does that in an immobile four-arm junction. Thus, the branch sites in immobile and bimobile junctions have similar ligand-binding properties despite the two-step migratory difference at the center of junction.

It has been noted previously that formation of J1 requires counterions — either divalent ions or high concentrations of monovalent — presumably to screen the repulsion of phosphates in close proximity at the branch point.[45] Cooper and Hagerman[51,52] used electrophoresis and transient electric birefringence experiments to show that in the absence of metal ions, a four-arm junction adopts a structure that is different from that in the presence of metal cations. Other studies confirmed these experiments on other junctions using both electrophoresis[53] and fluorescence energy transfer measurements.[54] It is therefore interesting to ask if only the stacked two-helical domain structure of a junction is required for the drug binding.

Before investigating the drug-binding experiments in the absence of Mg^{2+}, it is necessary to establish that the equimolar mixtures of the four strands of K24 associate under the experimental conditions used. The 1:1:1:1 mixture of strands runs as a single species on native polyacrylamide gel, with lower mobility than any of the other combinations.[147] Then the diagnostic experiments with MPE·FE(II) and $(OP)_2Cu(I)$, omitting magnesium from annealing buffer, are repeated. In contrast to the experiment in the presence of Mg^{2+}, no enhanced cleavage is seen by either of these probes at the branch site in K24 that is relative to the duplex patterns.[147] This indicates that junction geometry is the major influence in determining ligand-binding properties. If base pairs flanking the branch site are partially open in the absence of metal ions, these positions should become more reactive to attack by diethyl pyrocarbonate. The reactivity of purine bases in K24 at low salt concentrations to diethyl pyrocarbonate was therefore examined.[147] Several purines in the vicinity of the branch in K24 show enhanced reactivity to DEPC relative to duplexes of the same sequence, and this reactivity is suppressed by addition of magnesium to the reaction buffer. The hypersensitivity of these purine bases at or near the branch suggests the junction exhibits a markedly different structure in the absence of Mg^{2+}, consistent with unpairing of bases in this region.

The interaction of the branch site in K24 with ligands produces results that suggest a different signature for each of the strands.[147] Inspection of the cleavage patterns shows that the MPE·Fe(II) and $(OP)_2Cu(I)$ scission profiles distinguish each strand, and that inhibition of their cutting by Stains-All retains this feature. In principle, binding of either reagent in a symmetric site can induce an asymmetric cutting pattern in an immobile junction.[88,89] Binding any of the ligands that have been studied in principle could influence the positional or crossover isomerization equilibria or both. The positions of differential response in K24 include additional sites near the branch not seen in the comparable case of immobile junction J1.[88,89] In light of the report by Warner's group that ethidium inhibits branch migration,[10] this might mean that the presence of drugs freezes the equilibrium rather than favoring one of the competing migrational isomers over the others.

In terms of its arm length and ability to branch migrate, K24 is arguably a more realistic model for natural Holliday intermediates than most junctions that have been characterized so far.[17,49,51–54,63] The finding that the crossover-strand bias randomizes as more steps of migrational freedom become accessible is, perhaps, not surprising. However, the dynamics of the process or how the manifold of present structures arises are not known yet. The underlying importance of the stacking interactions at the branch may still exert a major influence on the products of recombination. Appending duplex arms of about 100 bp to an immobile junction does not perturb the (equilibrium) process of selecting helical partners for stacking, as can be seen by comparing the results of Cooper and Hagerman[51,52] with those of J1 with arms of 8 bp.[71,63] Sufficiently long helical arms should influence the rate of interconversion between alternative pairs. These questions have obvious implications for the *in vivo* reaction and, thus, need to be investigated. By analogy with calculations showing that the spontaneous rates of unwinding DNA are too slow to allow replication at *in vivo* rates, it might be anticipated that cofactors designed to overcome this kind of rate limitation might exist. The *in vivo* process also occurs in a different context: bacterial DNA, for example, is coated with recA protein and probably other molecules that influence the rate of branch migration.

VI. CONCLUSIONS AND FURTHER STUDIES

The above series of experiments reveal a strong and selective interaction between a variety of DNA binding drugs and ligands and the site of branching in immobile junctions with three or four arms as well as partially mobile junctions with four arms. The interactions occur preferentially at or near the branch itself. The questions we consider now are the following. What is the nature of the complexes formed between ligands and branched molecules? What are the relevant thermodynamics? Does the interaction have a biological role?

Despite a wealth of footprinting and binding experiments, we presently lack any information concerning the detailed structure of any of the complexes involved. The strength of footprinting analysis is that it can define the site and strength of an interaction with precision and convenience. Issues such as the stoichiometry of binding are not so directly addressed by these experiments. It is also a mistake to attempt to infer geometrical properties from such data. For this, we need data from NMR or crystallography, which as yet are not available. Attempts to determine the structures of any of these complexes to atomic resolution are presently under way,[154] but up to now, details are lacking.

One fundamental question that arises concerns how ligand binding affects the conformational equilibrium in a junction. As we have pointed out, a four-arm junction such as J1 does not have a unique single conformation in solution, even in the presence of Mg^{2+}. Instead, at least two kinds of structural isomerization occur in which the choice of stacking partners and the relative orientation of the helices varies.[155] Does ligand binding restrict these equilibria? One way to test this is to compare the interaction of a ligand with model junctions in which this conformational repertory is restricted, for example, by introducing covalent oligo dT tethers.[66,67] The conformationally constrained model junctions, such as JA and JP, can be used to investigate how the

junction geometry in JA and JP might correlate with their substrate activities and ligand-binding properties.[67] Preliminary footprinting data indicate that tethering does not alter the protection patterns of MPE·Fe(II) on JA and JP. Hence, no gross conformational change in the four-arm junction seems to accompany drug binding.

With respect to the question of the potential biological role of the interactions we are considering, a standard and general objection can be raised at the outset. The argument is that even if a branch binds a ligand 100 times more tightly than normal duplex DNA since the number of crossovers present in a cell is typically small — say less than 10 — it is unlikely that a ligand will locate any branch and thus interact with it. In a prokaryotic organism such as *E. coli*, for example, with roughly 10^6 bp of DNA, there would be about a 10^5-fold excess of duplex sites available, effectively swamping all the ligand.

To evaluate the potential of a drug to locate a branch within a buffer of duplex sites, a key issue is the rate of drug binding and dissociation relative to the rate of branch migration. So far, we have little information about these values, and this argues that measurements of the on and off rates of branch binding relative to duplex binding need to be made. The argument presented above is a purely equilibrium one; depending on how rapidly a branch migrates and a drug dissociates from an occupied site, the yield of recombinants could be severely affected. We have performed two kinds of experiments directed at determining some of the *in vivo* features of ligand-branch interactions. We have asked whether or not a process that requires a site-specific recombination event, such as bacteriophage lambda integration or excision, is sensitive to the presence of these ligands. Low concentrations of propidium inhibit the lysogenic pathway in this phage, effectively producing a clear plaque phenotype. This experiment does not establish the mechanism of the inhibition; for example, direct action of PI on the integrase itself could equally well account for the observed result.

Additional information was obtained in an experiment conducted with the collaboration of Howard Nash, whose laboratory has developed an *in vitro* recombination system with components involved in integration of phage λ.[156] This is a site-specific recombination event between two sites on the phage DNA (attP) and two on the bacterial DNA. The *in vitro* reaction requires the phage attP to be on a negatively supercoiled DNA molecule, a DNA containing the BOB′ attachment sites, and the int and IHF proteins. To monitor effects of drugs on the integration, a synthetic 57 bp DNA fragment containing the BOB′ sequence[157] was labeled at the 5′ end and allowed to interact with a circular pUC DNA molecule carrying the attP sites in the presence of the proteins both with and without drug. The products of the reaction were examined on 1% agarose gels. The Holliday junction can be detected as a labeled α structure in this experiment, while the completed product migrates as linear DNA. Concentrations of Stains-All in the range of 10 to 100 μM can be seen to lead to increased levels of the a structures, thus implying a stabilizing effect on the Holliday intermediate in this reaction.[158]

These preliminary results point to the ability of ligands to influence recombinational events via stabilizing branched intermediates. The consequences merit further exploration in connection with viral integration processes as well as general recombination in bacteria and eukaryotes.

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