Wilson, W. D., Krishnamoorthy, C. R., Wang, Y.-H., & Smith, J. C. (1985) *Biopolymers 24*, 1941–1961.
Wilson, W. D., Dotrong, M. H., Zuo, E. T., & Zon, G. (1988) *Nucleic Acids Res. 16*, 5137–5155.
Wilson, W. D., Tanious, F. A., Buczak, H., Venkatramanan, M. K., Das, B. P., & Boykin, D. W. (1990) in *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions, The 23rd Jerusalem Symposium on Quantum Chemistry and Biochemistry* (Jortner, J., & Pullman, B., Eds.) Reidel, Dordrecht, Holland (in press).
Wright, R. G. M., Wakelin, L. P. G., Fieldes, A., Waring, M. J., & Acheson, M. (1980) *Biochemistry 19*, 5825–5836.
Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.
Young, P. R., & Kallenbach, N. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 11*, 6453–6457.
Zimmerman, S. C., Lamberson, C. R., Cory, M., & Fairley, T. A. (1989) *J. Am. Chem. Soc. 111*, 6805–6809.

Asymmetric Structure of a Three-Arm DNA Junction†

Qiu Guo,‡ Min Lu,‡ M. E. A. Churchill,§ T. D. Tullius,§ and N. R. Kallenbach*,‡
Department of Chemistry, New York University, New York, New York 10003, and Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218
Received July 18, 1990; Revised Manuscript Received September 5, 1990

ABSTRACT: We present here experimental evidence that three-arm branched DNA molecules form an asymmetric structure in the presence of Mg^{2+}. Electrophoretic mobility and chemical and enzymatic footprinting experiments on a three-arm branched DNA molecule formed from three 16-mer strands are described. The electrophoretic mobilities of three species of a three-arm junction in which pairs of arms are extended are found to differ in the presence of Mg^{2+}: one combination of elongated arms migrates significantly faster than the other two. This effect is eliminated in the absence of Mg^{2+}, leading us to 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+}. The pattern of self-protection of each 16-mer strand of the core complex exposed to Fe(II)·EDTA and DNase I scission is unique, consistent with formation of an asymmetric structure in the presence of Mg^{2+}. We conclude that three-arm junctions resemble four-arm junctions in showing preferential stacking effects at the branch site. Comparison of the scission patterns of linear duplexes and the branched trimer by the reactive probes methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] and Cu(I)–[*o*-phenanthroline]$_2$ [$(OP)_2Cu^I$] further indicates that the branch point represents a site of enhanced binding for drugs, as it does in the four-arm case. Reaction with diethyl pyrocarbonate (DEPC), a purine-specific probe sensitive to conformation, is enhanced at the branch site, consistent with loosening of base pairing or unpairing at this point. The resolving enzyme, bacteriophage T7 endonuclease I, cleaves each of the three stands of the three-arm junction one nucleotide away from the branch with roughly equal efficiency. 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.

There is growing interest in the biological and physical properties of variant states and structures of DNA that are functionally relevant, including sites of base mismatches (Hunter et al., 1986), bulges (Williams & Goldberg, 1988a,b), bends of the helix axis (Hagerman, 1986; Koo et al., 1986), cruciforms (Mizuuchi et al., 1982), and other branched species (Seeman et al., 1989), as well as H-DNA (Htun & Dahlberg, 1988). Certain states of DNA, such as Holliday branched recombination junctions (Holliday, 1964), are stable only transiently; it is necessary to trap these intermediates in order to avoid resolution of the system into two duplexes by the process of branch migration (Kim et al., 1972; Meselson & Radding, 1975; Warner et al., 1978). By selecting symmetry-breaking sequences that are thermodynamically stable, the branch point in a four-arm model Holliday intermediate can be fully or partially immobilized, providing a general system of stable oligonucleotide models for recombination intermediates (Seeman et al., 1989). Much work on this system has focused on the four-arm junction, which allows one to investigate properties of Holliday-type intermediates (Kallenbach & Seeman, 1986; Cooper & Hagerman, 1987, 1989; Duckett et al., 1988). Since three-arm intermediates can also arise as intermediates in general recombination in the absence of replication (Broker & Doermann, 1975; Minagawa et al., 1983; Jensch & Kemper, 1986), there is reason to investigate the structural and substrate properties (Jensch & Kemper, 1986; Dickie et al., 1987) of these species as well.

Gel electrophoresis has been widely used to investigate the properties of stable DNA junctions (Cooper & Hagerman, 1987; Duckett et al., 1988; Seeman et al., 1989). Cooper and Hagerman (1987) studied a four-arm synthetic junction by gel electrophoresis under native conditions, utilizing the fact that bent DNA molecules have reduced mobilities on polyacrylamide gels to an extent that varies with the apparent degree of the bend angle (Koo et al., 1986). Cooper and Hagerman (1987) formed a set of molecules in which long duplex arms were appended pairwise to a four-arm complex

† This research was supported by Grants CA-24101, GM-40894, and CA-01208 from the National Institutes of Health; T.D.T. has received an NIH Research Career Development Award, an Alfred P. Sloan Fellowship, and a Camille and Henry Dreyfus Teacher–Scholar Award.
* Author to whom correspondence should be addressed.
‡ New York University.
§ The Johns Hopkins University.

0006-2960/90/0429-10927$02.50/0

and, in the presence of Mg^{2+}, observed different mobilities depending on which pairs of arms were extended. These electrophoretic experiments suggest that the junction geometry is consistent with a stacked structure, roughly like a Sigal–Alberts model (Sigal & Alberts, 1972). Duckett et al. (1988) repeated the gel electrophoretic experiments using other immobile junctions, and Duckett and Lilley (1990) have recently extended the analysis to three-arm junctions with results, however, at variance with our findings here (see below).

In addition to measurements of electrophoretic mobility on the native structures, the properties of variant states of DNA can be studied by high-resolution footprinting methods. Both nucleases and chemical reagents have been used successfully as probes in footprinting experiments (Galas & Schmitz, 1978; Brenowitz et al., 1986; Ward et al., 1987). Tullius and coworkers have found that EDTA·Fe(II), which does not bind tightly to DNA, affords a sensitive and relatively nonperturbing footprinting reagent for both DNA structure per se (Burkoff & Tullius, 1987) and DNA–protein complexes (Tullius & Dombroski, 1985, 1986). Diethyl pyrocarbonate (DEPC) is a conformation-sensitive reagent that carbethoxylates purines at the N-7 position (Leonard et al., 1971; Herr, 1985). The 2:1 complex of 1,10-phenanthroline with Cu(I), abbreviated here as $(OP)_2Cu^I$ (Sigman, 1986), has nucleolytic activity via oxidation of deoxyribose (Kuwabara et al., 1986; Goyne & Sigman, 1987) in the presence of oxygen and a reducing agent such as mercaptoproionic acid. The probe MPE·Fe(II) consists of an intercalating ring system covalently tethered to an EDTA moiety that binds iron and can generate OH radicals in the presence of H_2O and peroxide or oxygen (Hertzberg & Dervan, 1982, 1984).

In this study, we have investigated the geometry and ligand binding properties of a three-arm DNA junction, JL, the sequence of which is shown in Figure 1A. We present two kinds of experimental evidence that the three-arm junction has an asymmetric structure in the presence of Mg^{2+}. First, we have measured the electrophoretic mobility of three species of the junction (Figure 1C), each with a different pair of duplex arms extended (Cooper & Hagerman, 1987). We find that one combination migrates faster than the other two and that this effect is eliminated in the absence of Mg^{2+}. Next, we apply a variety of chemical and enzymatic probes which confirm that the three-arm structure in Mg^{2+} is intrinsically asymmetric and demonstrate also that the three-arm junction resembles the four-arm junction in providing a tight ligand binding site at the branch. DEPC is found to become reactive to purines at the branch in the presence of Mg^{2+}.

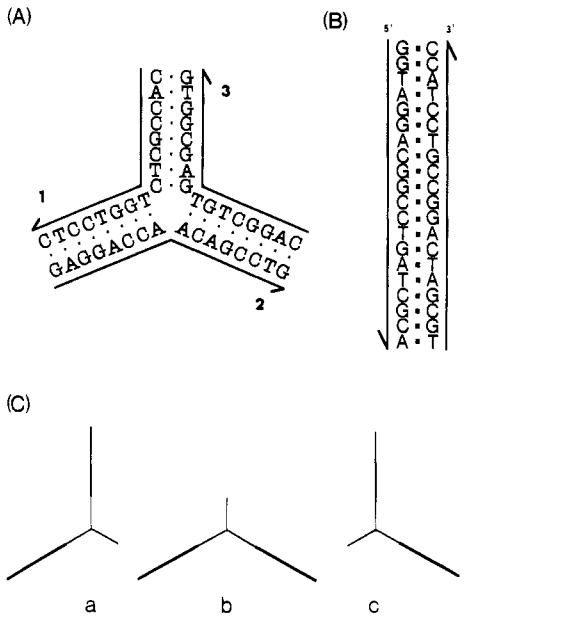

FIGURE 1: Analysis of a three-arm DNA junction JL. (A) Sequence of the three-arm junction JL. The junction is shown schematically and consists of three 16-mer strands of DNA. The 3′-ends of the strands are indicated by half-arrowheads. The strand numbering indicated is used throughout the text. (B) Sequence of the 20-bp molecule used for elongation of two arms of JL in turn in electrophoretic mobility experiment. The termini indicated by 5′ and 3′ are free ends in the arm-extended junctions. (C) Outline of the construction of three possible species for analysis by polyacrylamide gel electrophoresis. Each species was assembled by the hybridization of one 56-mer and two 36-mer oligonucleotides in which two of three arms are each elongated by 20 bp.

Materials and Methods

Synthesis and Purification of Oligonucleotides. Oligonucleotides used in these experiments were synthesized on a ABI 380B automated synthesizer and deprotected by routine phosphoramidite procedures (Caruthers, 1982). All strands 30 nucleotides long or less were purified by preparative HPLC on a Du Pont Zorbax Bio Series oligonucleotide column, following the manufacturer's recommended elution protocol. Longer strands were purified by polyacrylamide gel electrophoresis. Oligonucleotides were labeled at their 5′-termini by using T4 polynucleotide kinase (Boehringer); the labeled strands were purified by polyacrylamide gel electrophoresis.

Annealing Reactions. Junctions were formed by annealing a roughly stoichiometric mix of strands at 15 μM concentration in 50 mM Tris-HCl, pH 7.5, with 10 mM $MgCl_2$. An Eppendorf tube containing the solution was immersed in boiling water for 2 min, cooled slowly to room temperature, and finally chilled to 4 °C. Double-stranded controls were formed similarly, adding a stoichiometric concentration of cold strand complementary to the labeled junction strand.

Gel Electrophoresis. Native gels (20%, 19:1 monomer/bis ratio) were run for 70 h at 80 V. The electrophoresis plates were jacketed and cooled with circulating water to provide a running temperature of 4 ± 0.1 °C in the gel throughout the electrophoresis. The buffer system contained 40 mM Tris, 20 mM acetic acid, pH 8.1, 1 mM EDTA (TAE), or the same buffer with 10 mM $MgCl_2$ (TAE–Mg) or TAE–Mg in the presence of 1 μg/mL propidium. The gels were exposed for 1 h without an intensifying screen. For denaturing gels, the products of cleavage reactions were taken up in formamide loading buffer, heated briefly to 90 °C, cooled, and then run on a denaturing polyacrylamide gel (20%, 19:1 monomer/bis ratio) for 3 h at 2000 V (ca 50 V/cm) and 40 °C. No dyes were added in these runs. The gel was dried immediately on a vacuum drying apparatus (Hoefer) and exposed at room temperature to film without an intensifier screen.

Hydroxyl Radical Protection Experiments. The procedure described by Churchill et al. (1988) for EDTA–Fe(II) cutting of DNA junctions was used, except that the ratio of labeled to unlabeled strands is kept nearly stoichiometric. Samples are loaded onto a 20% sequencing gel after ethanol precipitation.

Cleavage by DNase I. A DNase I (Sigma, bovine pancreatic) stock solution was made by dissolving the dry enzyme in 50% glycerol–50 mM Tris-HCl, pH 7.0, and stored at 4 °C. This solution was diluted with DNase I buffer (50 mM Tris-HCl, pH 7.0, 32 mM $MgCl_2$) to provide activated enzyme. For cutting branched and linear DNA molecules, 10-μL samples of DNA (15 μM) were exposed to 1 unit of the activated DNase I complex for 2 min at 4 °C, precipitated twice with frozen ethanol, and dissolved in loading buffer after drying.

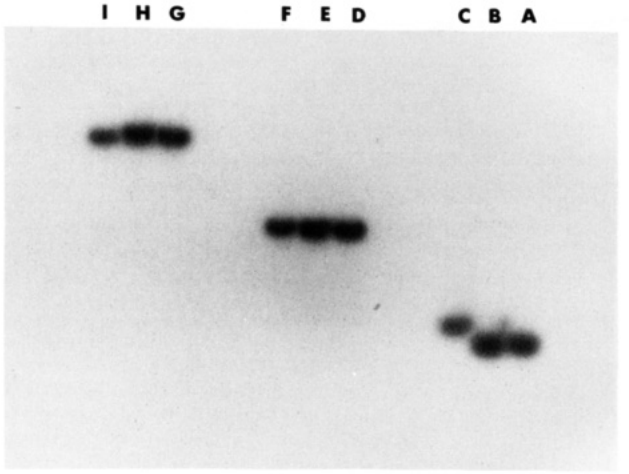

FIGURE 2: PAGE of oligodeoxynucleotide strands and mixtures. An autoradiogram of a native gel is shown, in which each of the three strands of JL is labeled in turn and electrophoresed alone or in combination with one or two other cold strands in excess. Lanes A–C contain the individual strands 1–3, respectively. Lanes D–F contain the adjacent pairs 12, 23, 31, respectively. Lanes G–I contain the trimer junction complex. Note that the trimeric junction migrates as a single well-defined band of lower mobility than any other monomer or dimeric species.

Diethyl Pyrocarbonate Modification. Linear duplexes and trimer DNA were modified by diethyl pyrocarbonate essentially as described by Herr (1985) and Lu et al. (1990c).

Cleavage by MPE·Fe(II). Our procedure followed that of Van Dyke and Dervan (1983), with minor changes due to the short strands involved and the addition of Mg^{2+} to stabilize junctions (Seeman et al., 1985). Both branched and linear molecules were cleaved by MPE·Fe(II) as described (Hertzberg & Dervan, 1982; Guo et al., 1989).

Cleavage by $(OP)_2Cu^I$. The procedure of Kuwabara et al. (1986) was followed, except for substitution of ascorbate for mercaptopropionate. Both junction and duplex controls were reacted with $(OP)_2Cu^I$ as described (Guo et al., 1990).

Cleavage by T7 Endonuclease I. Digestions of a three-arm junction JL with T7 endonuclease I (LKB Pharmacia) were performed in 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/mL bovine serum albumin. Reactions were stopped by chloroform and isoamyl alcohol (24:1 V/V) extraction and DNA was precipitated by ethanol.

Sequencing Reaction. Purine-specific (A + G) sequencing ladders were generated from each 5′ ^{32}P-labeled oligonucleotide by using the piperidine–formate reaction (Maxam & Gilbert, 1977).

Densitometry. Autoradiograms were scanned on a Hoefer GS300 densitometer, without baseline corrections. We use a number to designate the strands, separated by a colon from the number of the residue(s) in a given strand (Guo et al., 1990).

RESULTS

Construction of the Three-Arm Junction, JL. Three oligonucleotides each of 16 nucleotides with the sequences shown in Figure 1A have been synthesized and hybridized under stringent conditions. Mixtures of the various components of JL labeled with ^{32}P in one strand only are subjected to electrophoresis in polyacrylamide gels, as shown in Figure 2. Lanes A–C of the gel shown contain the individual 16-mer strands, lanes D–F contain the three possible duplex mixtures of strands, and lanes G–I contain the trimer junction complex. The complete junction migrates as a narrow band with a much lower mobility than any of the binary combinations, consistent with the formation of a structurally well-defined three-stranded complex.

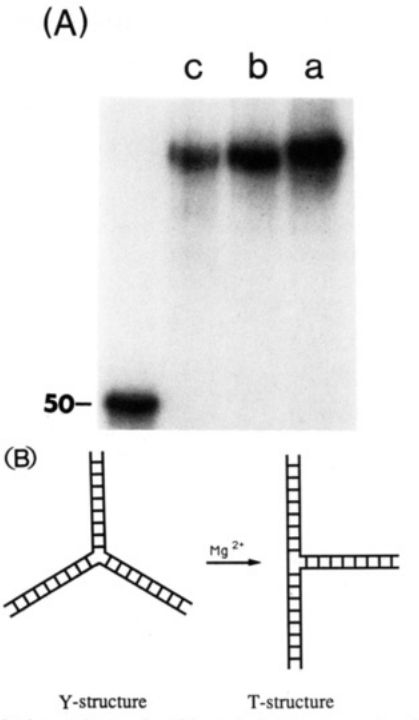

FIGURE 3: Native polyacrylamide gel electrophoresis of three-arm DNA junction JL with pairwise combinations of elongated junction arms. (A) Autoradiograph of a 20% polyacrylamide gel of three pairwise elongated species in the presence of Mg^{2+}. The numbering convention refers to that of Figure 1. Gel marker is 50-bp linear duplex DNA. (B) The structure of three-arm junction JL. Shown is a schematic of the T-shaped structure. (C) Ferguson plots for the three pairwise elongated species. The mobilities in these plots were determined from densitometer traces of gels, measured with respect to the distances traveled by the 50-mer linear duplex.

Electrophoresis of Native Complexes with Pairs of Arms Extended Reveals That JL Lacks 3-Fold Symmetry. In the experimental scheme of Cooper and Hagerman (1987), the electrophoretic mobility of the junction is measured in a series of complexes in which each pair of arms is extended by a "reporter sequence". Figure 1B illustrates the sequence of the reporter duplex used in our experiments and the three different combinations that have been studied (Figure 1C). The mobility results are shown in Figure 3A. One combination of extended arms yields a complex that migrates faster than the other two. The electrophoresis buffer contained 10 mM Mg^{2+}, which has been found to stabilize folding of four-arm junctions into a stacked conformation (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988, 1990). We conclude from these data that JL does not have a predominantly symmetric trigonal structure in solution, in the presence of Mg^{2+}. Instead, the complex appears to be asymmetrical, with strands 2 and 3 forming a bend, while the conformation of strand 1 forms a less acute angle than the other two. The resulting structure might resemble a roughly T-shaped structure, as sketched in Figure 3B; however, we have no information about the angle(s) involved at this time. In order to demonstrate that the mobility difference reflects a shape change, we carried out a Ferguson analysis (Rodbard & Chrambach, 1970) of the mobilities of the three complexes, with the result shown in Figure 3C. One extended complex (a) exhibits a different retardation coefficient from the other two, which are quite similar, consistent with the above interpretation.

Relative Electrophoretic Mobilities Are Dependent on the Presence of Mg^{2+}. We have previously noted that formation

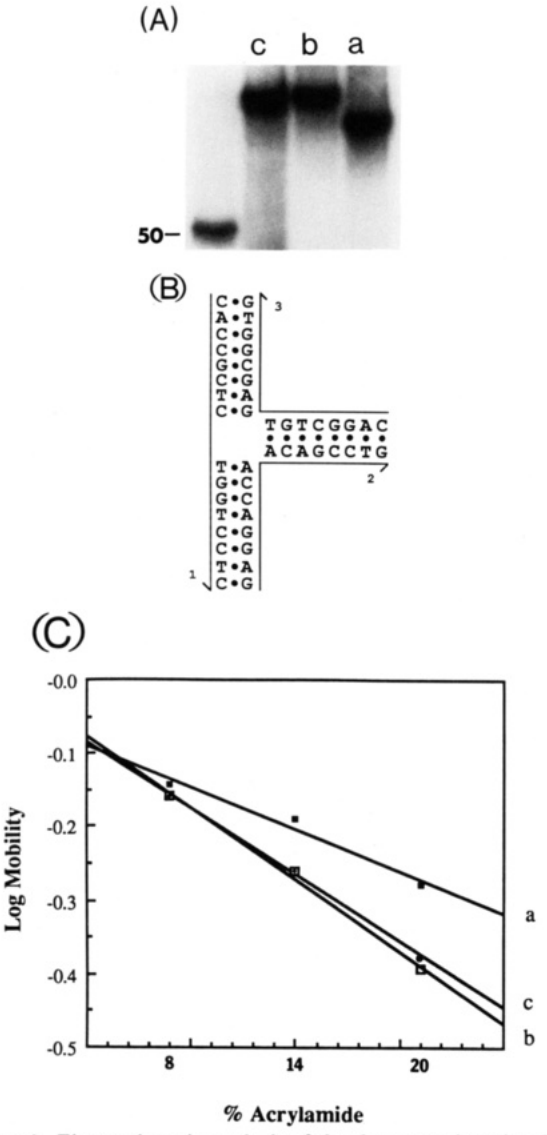

FIGURE 4: Electrophoretic analysis of the three-arm junction JL in the absence of Mg^{2+}. (A) Autoradiogrphy of a 20% polyacrylamide gel of three pairwise elongated species in the absence of Mg^{2+}. The same conventions apply to this figure as to Figure 3. (B) Schematic of junction folding.

of four-arm junctions requires counterions—either ions or high concentrations of monovalents—presumably to screen the repulsion of phosphates in close proximity at the branch point (Kallenbach & Seeman, 1986). Cooper and Hagerman (1987, 1989) have used electrophoresis and transient electric birefringence experiments to show that, in the absence of metal ions, a four-arm junction adopts a structure in which the four arms are unstacked and maximally extended. Lilley and coworkers have confirmed these experiments on other junctions by using both electrophoresis (Duckett et al., 1988, 1990) and fluorescence energy transfer measurements (Murchie et al., 1989). We therefore asked if the conformation of three-arm junctions is dependent on the presence of cations.

We repeated the electrophoretic mobility experiment, omitting magnesium from both annealing buffer and gel buffer. The results are presented in Figure 4A. In contrast to the experiment in the presence of Mg^{2+}, there are only small differences between the different species. This indicates that counterions are crucial to the conformation of a three-arm junction, as in the case of four-arm structures. These results also suggest that three-arm DNA junctions adopt some folded geometry as do four-arm junctions as shown in Figure 4B.

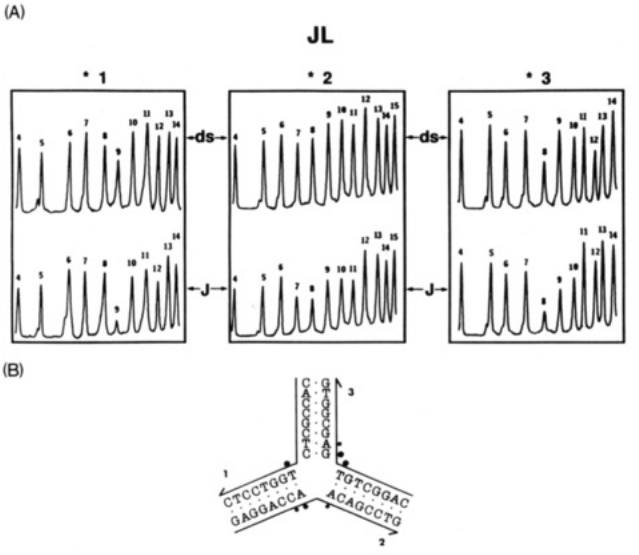

FIGURE 5: Hydroxyl radical cleavage of JL and control duplexes. (A) Densitometric scans of the cleavage reactions. In this experiment, each strand of JL is labeled in turn, mixed with its complementary 16-mer or with the two strands needed to form JL, and exposed to Fe(II)-EDTA. Each panel shows two scans, one of a gel of the reaction products with the labeled strand in the trimer complex (J) and the control with the labeled strand in the linear duplex formed by pairing the same strand with its complementary strand (ds). The branch point lies between position 8 and 9 on each strand. (B) Sites of differential activity of the probe in JL relative to duplexes. Sites of protection relative to duplexes are indicated by filled circles. The size of the filled circle is a measure of the quantitative intensity at each responsive site.

Chemical and Enzymatic Probing Suggests That JL Lacks 3-Fold Symmetry. Figure 5A shows the profiles of scission by Fe(II)·EDTA of each strand of JL in the trimer and in a control duplex formed from an equimolar mix of the strand with its complementary 16-mer, to form an uninterrupted linear helix. The control duplex profiles (labeled ds) control for any sequence dependence of strand scission that occurs in normal DNA (Tullius & Dombroski, 1985). Differences between the profiles of cleaving the junction (J) and duplexes (ds) identify sites of protection or enhancement of scission in the three-arm junction relative to the duplex (Churchill et al., 1988). Positions 1:9, 2:7–9, and 3:8–10 show a reduction of cleavage, relative to the corresponding duplexes. The cleavage pattern of each strand in JL is unique (Figure 5B), consistent with an asymmetric structure.

A similar correspondence is seen in the profile of scission induced by DNase I (Figure 6). Strands 1 and 3 show protection of several residues 3′ to the branch site, 1:9,10,12,13 and 3:7,9–11; 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 (Suck et al., 1988). The presence of an additional arm in a substrate DNA blocks the latter interaction, resulting in protection of several residues flanking the branch (Lu et al., 1989). These footprinting results also confirm that the three arms of JL are not structurally equivalent in solution, in the presence of Mg^{2+}.

Purine Bases at the Branch Are Reactive to Diethyl Pyrocarbonate. This reagent is purine specific, with a preference for N-7 of A over that of G (Herr, 1985), and is sensitive to local conformation in DNA (Johnston & Rich, 1985). Purine bases in the vicinity of the branch of four-arm junctions show

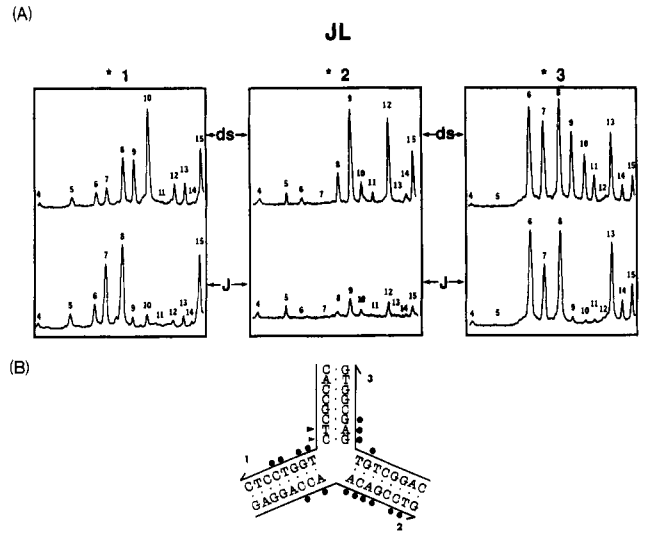

FIGURE 6: DNase I scission of JL and control duplexes. (A) Densitometric scans of the scission reactions. The same conventions apply to this figure as to Figure 5A. In this case, DNase I is used as a reactive probe of the junction (J) relative to duplex controls (ds). Protection of residues 3′ to the branch in all three strands is evident. (B) The sites of differential activity. Sites of enhanced cutting relative to duplexes are indicated by arrows.

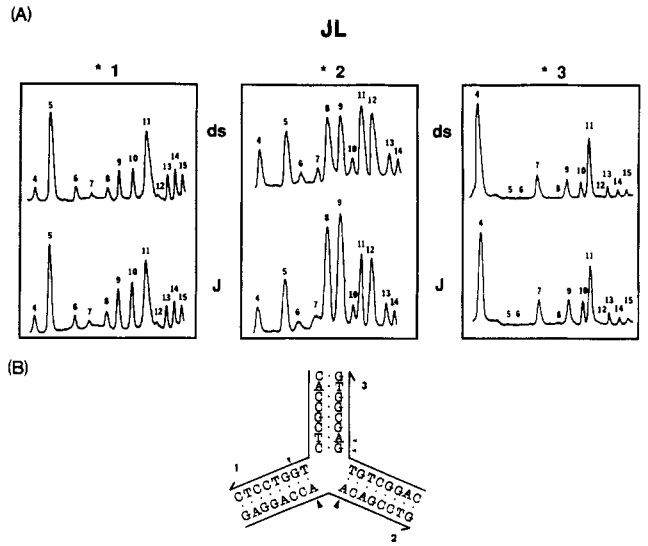

FIGURE 7: Diethyl pyrocarbonate modification of purine bases at JL. (A) Densitometric scans of the modification experiment. The same conventions apply to this figure as to Figure 5A. This reagent preferentially reacts with N-7 of adenines and hence probes the major groove environment of the junction. (B) Sites that react differentially in the junction.

enhanced reactivity to diethyl pyrocarbonate 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 2-fold stacked conformation (Lu et al., 1990a). We therefore probed the branch purine bases of a three-arm junction. The reactivity of diethyl pyrocarbonate with JL and control duplexes in the presence of Mg^{2+} is shown in Figure 7. 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. The hypersensitivity of these purine bases at or near the branch of JL in the presence of Mg^{2+} suggests that the folded geometry of a three-arm junction in the presence of Mg^{2+} is different

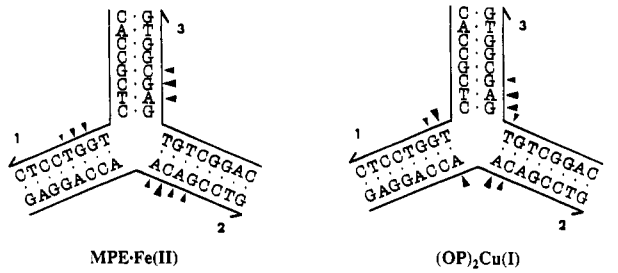

FIGURE 8: Sites of enhanced cleavage on JL by MPE·Fe((II) and $(OP)_2Cu^I$. The same conventions apply to this figure as to Figure 6B. These reagents have been found to interact preferentially with four-arm junctions near or at the branch site (Guo et al., 1989, 1990). A similar effect is seen here, with the sites affected shown.

from that of four-arm junctions.

The Branch Site in JL Binds Ligands Preferentially. We have found that a number of drugs or ligands interact preferentially at the branch site in four-arm junctions (Guo et al., 1988, 1990; Lu et al., 1990a–c). We have previously reported that 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 (Guo et al., 1989, 1990; Lu et al., 1990a). The pattern of enhanced cuts is consistent with the presence of a preferred site of binding positively charged ligands at or very near the branch point. It is then of interest to explore the response of the branch site of a three-arm junction JL to cleavage by MPE·Fe(II) and $(OP)_2Cu^I$. In Figure 8, we summarize the effects of exposing the junction and control duplexes to the two chemical probes. Enhanced reactivity to MPE·Fe(II) is seen at 1:10–12, 2:9–12, and 3:10–12 in the J profiles relative to the duplexes (data not shown). Similarly, $(OP)_2Cu^I$ become hyperreactive in the junction relative to the duplexes, as evidence by induced scission at 1:9,10, 2:8,10, and 3:8,11 (data not shown).

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 in four-arm junctions we have examined (Guo et al, 1989, 1990) but indicate preferential interactions in the vicinity of the branch point as is found in four-arm species. Does the ligand selectively binding to the branch of JL influence the conformation in solution in the presence of Mg^{2+}? In order to address this issue, we repeated the electrophoretic mobility experiment in the presence of propidium. In the presence of a 2:1 molar ratio of PI to trimer, the results are very similar to those in the absence of PI (data not shown), suggesting that ligand binding to the branch does not affect the junction conformation.

Cleavage by a Resolvase Enzyme. The interaction of three-arm junctions with resolving enzymes has been investigated previously (Jensch & Kemper, 1986; Dickie et al., 1987). Jensch and Kemper find that all three strands in a three-arm junction are susceptible to T4 endonuclease VII (Jensch & Kemper, 1986), and Dickie et al. (1987) reach the same conclusion with respect to T7 endonuclease I. We have performed the T7 endonuclease I experiment on JL. As seen in Figure 9A, T7 endonuclease I cleaves this junction with great specificity, cleaving one or two phosphodiester linkages per strand. The deduced positions of cleavage are summarized in Figure 9B. JL is cleaved predominantly at positions 1:8, 2:7, and 3:7.

Discussion

These results provide evidence that the three arms in JL are not conformationally equivalent, provided Mg^{2+} is present.

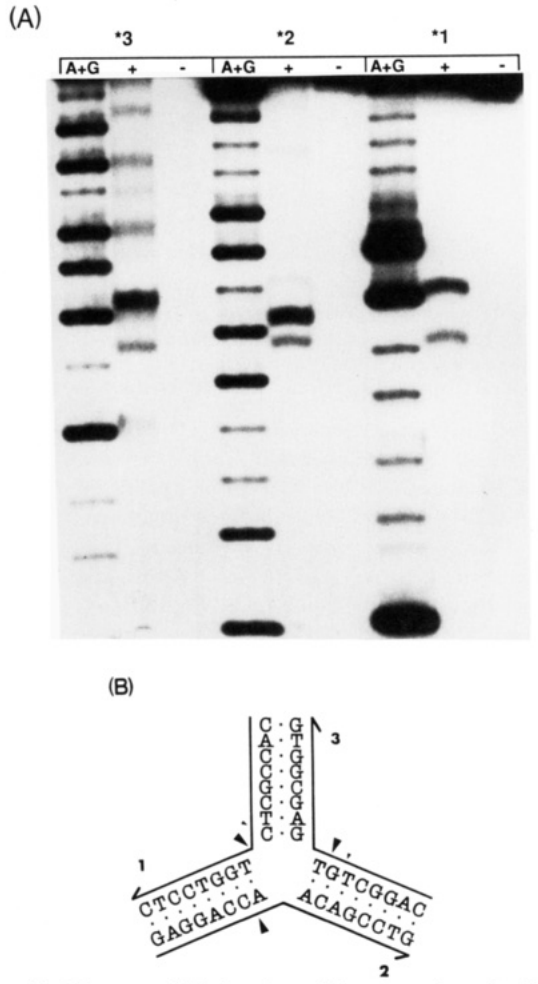

FIGURE 9: Cleavage of JL by phage T7 endonuclease I. (A) An autoradiograph of the gel is shown. Junction 5′-labeled with ^{32}P in one strand only is cleaved with T7 endonuclease I and electrophoresed on a sequencing gel alongside the A + G sequence marker derived from the same radioactive oligonucleotide. The radioactively labeled strand is indicated by the number of the strand in the 5′-half of the figure. Note that definition of the cleavage position at the nucleotide level is accomplished by comparing the mobilities of the product fragments to those of fragment ladders generated by sequence analysis and DNase I digestions. (B) Cleavage sites in T7 endonuclease I digestion.

The profiles of cutting by each of the reactive probes we have applied show that the three strands of JL have distinctive patterns of scission, hence are not equivalent structurally. While footprinting data do not provide direct information about stereochemistry, the mobility experiment with pairs of extended arms reveals that complex a behaves as a more linear overall structure than complexes b and c (Figure 3). The fact that this mobility difference is eliminated in the absence of Mg^{2+} suggests an analogy between the folded conformations of the three- and four-arm immobile junctions. In the case of four-arm junctions, the structure in the presence of Mg^{2+} is 2-fold, instead of 4-fold, symmetric and consists of two duplex domains linked by a pair of crossover strands that are relatively deformed compared to the two helical strands (Churchill et al., 1988). This 2-fold symmetry is eliminated in the absence of Mg^{2+} or other suitable counterions (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988, 1990; Murchie et al., 1989). Our observations here on JL parallel this behavior; the three strands are not equivalent except which Mg^{2+} is removed. Protection from Fe(II)·EDTA scission has been found to arise from both physical occlusion by neighboring duplex and local charge effects due to repulsion of the reagent from the branch (Lu et al., 1990d). The DNase I profiles reflect the larger spatial requirements of the active site of this nuclease (Suck et al., 1988). The enzyme has been shown to bind across the minor groove of DNA (Drew, 1984; Suck et al., 1988), with interactions extending over one turn of helix, and we have found that it cannot cleave five residues 3′ to the branch in four-arm junctions (Lu et al., 1989). However, in the three-arm case, strand 2 is actually protected over five residues, in both 3′- and 5′-directions from the branch site (Figure 6), a pattern we have not seen before.

While it is premature to deduce any precise structures from the data at hand, it seems reasonable to conjecture from the mobility data that strands 2 and 3 might comprise the two folded strands in an overall T-like structure, with 1 remaining relatively unperturbed as sketched in Figure 3B. This kind of model is consistent with several features of four-arm branched DNAs that have been established experimentally so far:

(i) In the presence of Mg^{2+}, a 2-fold symmetric conformation is observed (Churchill et al., 1988). In low salt, it appears that charge repulsions at the branch prevail, and a more open structure results (Cooper and Hagerman, 1987, 1989; Duckett et al., 1988, 1990; Murchie et al., 1989).

(ii) The bases flanking the branch determine which pairs of stacked arms are favored (Chen et al., 1988; Duckett et al., 1988). Recent results from this laboratory suggest that the quantitative extent of preference varies with sequence also.

(iii) The effective "valence angle" at the branch is flexible, even in the presence of Mg^{2+}, as determined by macrocyclization experiments with blunt- or sticky-ended three-arm (Ma et al., 1986) and four-arm (Petrillo et al., 1988) immobile junctions. Both kinds of junctions readily cyclize to form closed rings of three, four, or more units. This implies that the angle subtended by the arms can vary over a significant range, on the time scale of the joining reaction.

A roughly T-form model for JL is compatible with these features. Allowing one pair of arms to stack preferentially gains a duplex stacking interaction in the presence of Mg^{2+} or other divalent counterions (Seeman et al., 1985) as opposed to in their absence. The choice of arms 2 and 3 in Figure 3 as the favored stacking pair presumably reflects optimal stacking and/or flexural interactions at the branch, as also occurs in the four-arm case (Cooper & Hagerman, 1987; Chen et al., 1988; Duckett et al., 1988).

The existence of preferential stacking interactions at the branch of a three-arm junction does not imply that three- and four-arm junctions have identical structural features at this point. The DEPC result indicates that there is a real difference. DEPC does not reveal any sites of enhanced reactivity near the branch of four-arm junctions in the presence of Mg^{2+} (Lu et al., 1990a,c). Here we observe that each strand shows a pattern of such reactivity for DEPC near the branch. In the presence of Mg^{2+}, the pattern for each strand is distinct, supporting an asymmetric model. The increased reactivity is consistent with some degree of base opening at the branch, although it does not prove it. However, Duckett and Lilley (1990) report a similar enhancement of reactivity for osmium tetroxide, a probe for T in the unpaired state.

It remains to explain why the results we obtain completely contradict those obtained by Duckett and Lilley (1990) who performed a similar Cooper–Hagerman analysis on a related system of three-arm junctions. The title of their article in fact is "The three-way DNA junction is a Y-shaped molecule in which there is not helix-helix stacking". One important parameter in this analysis is the ratio of long to short arms in

the complexes containing pairs of extended arms. In their original experiment, Cooper and Hagerman (1987) used a reporter arm of 106 bp to analyze a junction with 20-bp arms, giving a ratio of over 6:1 in length. Subsequently, Lilley's group repeated these assays using a significantly lower ratio of arm lengths, 3.3 (Duckett et al., 1988). This significantly reduces mobility differences among the pairwise-extended-arm species in their investigation relative to Cooper and Hagerman's results, although the resolution is nevertheless sufficient to discriminate among different four-arm structures. However, Duckett and Lilley (1990) appear to assume that comparable resolution is available to analyze the corresponding behavior of three-arm structures in gels *with the same percent acrylamide*. The Ferguson plot shown in Figure 3C shows that this is not the case. The ratios we use in our experiment are 3.5, very close to the conditions of Duckett and Lilley (1990). Figure 3C indicates that we would anticipate little or no mobility difference among the three complexes we have analyzed if we confined our measurements to 5% polyacrylamide gels, as Duckett and Lilley (1990) have done. In addition, the degree of cross-linking of the gels plays a major role (Chrambach & Rodbard, 1971). Our gels are more highly cross-linked than theirs, in principle giving us higher resolving ability. The issue is readily capable of being decided by their rerunning their complexes under our gel conditions. The difference in interpretation between the results presented here and theirs is not subtle. They conclude, on the basis of what we believe to be a misleading mobility measurement using 5% gels (29:1 monomer/bis ratio), that there is no asymmetry in three-arm junctions. Our mobility data and footprinting experiments indicate that, in the presence of Mg^{2+}, the structure of JL is asymmetric. Our experiment is internally controlled, because the difference in mobility is eliminated in the absence of Mg^{2+}. We will present an analysis of the sequence determinants of this symmetry elsewhere.

Registry No. JL, 130246-71-8; d(GGTAGGACGGCCTGATCGCA)·d(TGCGATCAGGCCGTCCTACC), 130246-72-9; Mg, 7439-95-4.

References

Brenowitz, M., Senear, D. F., Shea, M. A., & Ackers, G. K. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8462–8466.

Broker, T. R., & Doermann, A. H. (1975) *Annu. Rev. Genet. 9*, 213–244.

Burkhoff, A. M., & Tullius, T. D. (1987) *Cell 48*, 935–943.

Caruthers, M. H. (1982) in *Chemical and Enzymatic Synthesis of Gene Fragments* (Gassen, H. G., & Lang, A., Eds.) pp 71–79, Verlag Chemie, Weinheim.

Chen, J.-H., Churchill, M. E. A., Tullius, T. D., Kallenbach, N. R., & Seeman, N. C. (1988) *Biochemistry 27*, 6032–6038.

Chrambach, A., & Rodbard, D. (1971) *Science 172*, 440–451.

Churchill, M. E. A., Tullius, T. D., Kallenbach, N. R., & Seeman, N. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4653–4656.

Cooper, J. P., & Hagerman, P. J. (1987) *J. Mol. Biol. 198*, 711–719.

Cooper, J. P., & Hagerman, P. J. (1989) *Proc. Natl. Acal. Sci. U.S.A. 86*, 7336–7340.

Dickie, P., McFadden, G., & Morgan, A. R. (1987) *J. Biol. Chem. 262*, 14826–14836.

Drew, H. R. (1984) *J. Mol. Biol. 176*, 535–557.

Duckett, D. R., & Lilley, D. M. J. (1990) *EMBO J. 9*, 1659–1664.

Duckett, D. R., Murchie, A. I. H., Diekmann, S., von Kitzing, E., Kemper, B., & Lilley, D. M. J. (1988) *Cell 55*, 79–89.

Duckett, D. R., Murchie, A. I. H., & Lilley, D. M. J. (1990) *EMBO J. 9*, 583–590.

Galas, D. J., & Schmitz, A. (1978) *Nucleic Acids Res. 3*, 3157–3170.

Goyne, T., & Sigman, D. S. (1987) *J. Am. Chem. Soc. 95*, 7850–7857.

Guo, Q., Seeman, N. C., & Kallenbach, N. R. (1989) *Biochemistry 28*, 2355–2359.

Guo, Q., Lu, M., Seeman, N. C., & Kallenbach, N. R. (1990) *Biochemistry 29*, 570–578.

Hagerman, P. J. (1986) *Nature 321*, 449–450.

Herr, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8009–8013.

Hertzberg, R. P., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 313–315.

Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.

Holliday, R. (1964) *Genet. Res. 5*, 282–304.

Htun, H., & Dahlberg, J. E. (1988) *Science 241*, 1791–1796.

Hunter, W. N., Kneale, G., Brown, T., Rabinovich, D., & Kennard, O. (1986) *J. Mol. Biol. 190*, 605–618.

Jensch, F., & Kemper, B. (1986) *EMBO J. 5*, 181–189.

Johnston, B. H., & Rich, A. (1985) *Cell 42*, 713–724.

Kallenbach, N. R., & Seeman, N. C. (1986) *Comments Mol. Cell Biophys. 4*, 1–16.

Kim, J. S., Sharp, P., & Davidson, N. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1948–1952.

Koo, H.-S., Wu, H.-M., & Crothers, D. M. (1986) *Nature 320*, 501–506.

Kuwabara, M., Yoon, C., Goyne, T., Thederahn, T., & Sigman, D. (1986) *Bicohemistry 25*, 7401–7408.

Leonard, N. J., McDonald, J. J., Henderson, R. E. L., & Reichmann, M. E. (1971) *Biochemistry 10*, 3335–3342.

Lu, M., Guo, Q., Seeman, N. C., & Kallenbach, N. R. (1989) *J. Biol. Chem. 264*, 20851–20854.

Lu, M., Guo, Q., Mueller, J. E., Kemper, B., Studier, W. F., Seeman, N. C., & Kallenbach, N. R. (1990a) *J. Biol. Chem. 265*, 16778–16785.

Lu, M., Guo, Q., Pasternack, R. F., Wink, D. J., Seeman, N. C., & Kallenbach, N. R. (1990b) *Biochemistry 29*, 1614–1624.

Lu, M., Guo, Q., Seeman, N. C., & Kallenbach, N. R. (1990c) *Biochemistry 29*, 3407–3412.

Lu, M., Guo, Q., Wink, D. J., Seeman, N. C., & Kallenbach, N. R. (1990d) *Nucleic Acids Res. 10*, 3333–3337.

Ma, R.-I., Kallenbach, N. R., Sheardy, R. D., Petrillo, M. L., & Seeman, N. C. (1986) *Nucleic Acids Res. 14*, 9745–9753.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

Meselson, M. S., & Radding, C. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 358–361.

Minagaw, T., Murakami, A., Ryo, Y., & Yamagishi, H. (1983) *Virology 126*, 183–193.

Muzuuchi, K., Mizuuchi, M., & Gellert, M. (1982) *J. Mol. Biol. 156*, 229–243.

Murchie, A. I. H., Clegg, R. M., von Kitzing, E., Duckett, D. R., Diekmann, S., & Lilley, D. M. J. (1989) *Nature 341*, 763–766.

Petrillo, M. L., Newton, C. J., Cunningham, R. P., Ma, R.-I., Kallenbach, N. R., & Seeman, N. C. (1988) *Biopolymers 27*, 1337–1352.

Rodbard, D., & Chrambach, A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 970–977.

Seeman, N. C., Maestre, M. F., Ma, R.-I., & Kallenbach, N. R. (1985) in *The Molecular Basis of Cancer* (Rein, R., Ed.) pp 99–108, Alan Liss, New York.

Seeman, N. C., Chen, J. H., & Kallenbach, N. R. (1989) *Electrophoresis 10*, 345-354.
Sigal, N., & Alberts, B. (1972) *J. Mol. Biol. 71*, 795-798.
Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180-186.
Suck, D., Lahm, A., & Oefner, C. (1988) *Nature 332*, 465-468.
Tullius, T. D., & Dombroski, B. A. (1985) *Science 230*, 679-681.
Tullius, T. D., & Dombroski, B. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5469-5473.
Van Dyke, M. W., & Dervan, P. B. (1983) *Nucleic Acids Res. 11*, 5555-5567.
Ward, B., Rehfuss, R., & Dabrowiak, J. C. (1987) *J. Biomol. Struct. Dyn. 4*, 685-695.
Warner, R. C., Fishel, R. A., & Wheeler, F. C. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 957-968.
Williams, L. D., & Goldberg, I. H. (1988a) *Biochemistry 27*, 3004-3011.
Williams, L. D., & Goldberg, I. H. (1988b) *Nucleic Acids Res. 16*, 11607-11615.

Patterns of Strongly Protein-Associated Simian Virus 40 DNA Replication Intermediates Resulting from Exposures to Specific Topoisomerase Poisons†

Cha-Gyun Shin and Robert M. Snapka*

Department of Radiology and Department of Medical Microbiology and Immunology, The Ohio State University, Columbus, Ohio 43210-1214

Received April 12, 1990; Revised Manuscript Received August 8, 1990

ABSTRACT: Exposure of infected CV-1 cells to specific type I and type II topoisomerase poisons caused strong protein association with distinct subsets of simian virus 40 (SV40) DNA replication intermediates. On the basis of the known specificity and mechanisms of action of these drugs, the proteins involved are assumed to be the respective topoisomerases. Camptothecin, a topoisomerase I poison, caused strong protein association with form II (relaxed circular) and form III (linear) viral genomes and replication intermediates having broken DNA replication forks but not with form I (superhelical) viral DNA or normal late replication intermediates which were present. In contrast, type II topoisomerase poisons caused completely replicated forms and late viral replication forms to be tightly bound to protein—some to a greater extent than others. Different type II topoisomerase inhibitors caused distinctive patterns of protein association with the replication intermediates present. Both intercalating and nonintercalating type II topoisomerase poisons caused a small amount of form I (superhelical) SV40 DNA to be protein-associated in vivo. The protein complex with form I viral DNA was entirely drug-dependent and strong, but apparently noncovalent. The protein associated with form I DNA may represent a drug-stabilized "topological complex" between type II topoisomerase and SV40 DNA.

Topoisomerase poisons stabilize a reaction intermediate in which the enzyme is covalently bound to DNA at the site of a DNA strand break (Liu, 1989). In the case of eukaryotic type II topoisomerase, the protein subunits are bound to the 5′ side of each DNA strand break, and the breaks may be either single stranded or double stranded. Eukaryotic topoisomerase I makes single-strand breaks exclusively, with the protein covalently attached on the 3′ side. Type II topoisomerase poisons such as etopside, teniposide, and adriamycin are standards in the arsenal of anticancer drugs (Liu, 1989). More recently, type I topoisomerase poisons have shown promise for the treatment of human colon cancer (Giovanella et al., 1989).

The antineoplastic action of topoisomerase poisons may be related to their ability to interfere with cellular processes which require topoisomerase action. The movement of DNA replication forks requires a topoisomerase to reduce the linkage of the parental DNA strands and remove positive superhelical stress ahead of the forks. This is known as the swivel function, and both theory (Champoux & Been, 1980) and a large body of experimental evidence (Brill et al., 1987; Goto & Wang, 1985; Uemura & Yanagida, 1984; Yang et al., 1987) indicate that either type I or a type II topoisomerase can serve as the "swivel". It is also clear that type II topoisomerase is required for the separation of newly replicated daughter DNA strands both in bacteria (Steck & Drlica, 1984) and in eukaryotes (Sundin & Varshavsky, 1981; Snapka, 1986; Snapka et al., 1988; DiNardo et al., 1984; Uemura & Yanagida, 1986; Holm et al., 1985).

The DNA tumor virus SV40 has been very useful for understanding the roles of topoisomerase in eukaryotic DNA replication. SV40 is considered a model for the mammalian replicon because of its extensive use of cellular DNA replication machinery and chromosomal proteins (DePamphilis et al., 1979). This system has been used to show that the type I topoisomerase poison camptothecin breaks replication forks at all stages of DNA replication (Snapka, 1986; Avemann et al., 1988), while type II topoisomerase poisons slow or block the replication of the last few hundred base pairs of the viral genome and the separation of daughter chromosomes (Snapka

†This work was supported by U.S. Public Health Service Grant CA45208, by The Ohio State University Comprehensive Cancer Center, and by National Cancer Institute, DHHS, Grant P30 CA-16058 15.
*Address correspondence to this author at the Department of Radiology.

0006-2960/90/0429-10934$02.50/0