

region has for elucidating subtle changes in molecular structure. The similarities in the immediate ligation environment, as indicated by the first-shell data for stellacyanin and the other proteins, lead us to favor a model in which the variation in the XANES is attributed to changes in the (EXAFS nondetectable) axial ligand. With this in mind, it is interesting to note that the increase in absorbance at 12317 eV (laccase < azurin, plastocyanin << stellacyanin) parallels the decrease in reduction potential for the native (Cu containing) forms of these proteins, although a temperature-dependent conformational change in laccase could confuse the trend.<sup>52</sup> To the extent that the Cu and Hg sites are similar, our data may therefore be consistent with the proposal<sup>5,53</sup> that axial ligation plays an important role in tuning the reduction potential of the type 1 site. Model compound XANES studies are in progress in an attempt to understand and exploit the dif-

ferences revealed in the spectra of the mercury-containing proteins.

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**Registry No.** Hg, 7439-97-6; L-histidine, 71-00-1; L-cysteine, 52-90-4.

**Supplementary Material Available:** Table of results pertaining to the wide-filter data and a listing of X-ray absorption vs energy for the spectra reported herein (25 pages). Ordering information is given on any current masthead page.

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## A Specific Quadrilateral Synthesized from DNA Branched Junctions

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**Abstract:** Four different three-arm branched DNA junctions have been synthesized and covalently linked together in a prescribed arrangement to form a macrocycle of previously specified sequence. One end of each individual junction is closed by a hairpin loop. Each open arm of the four junctions terminates in a unique single-stranded cohesive ("sticky") end; there are four pairs of complementary sticky ends among the eight arms, two pairs with 3' overhangs, and two pairs with 5' overhangs. The associations between junctions are directed by the sequences of these cohesive ends. The junctions that associate in this fashion are enzymatically joined together by the use of bacteriophage T4 DNA ligase. The final product is a "quadrilateral" the sides of which are each 16 nucleotide pairs long, approximately 1.5 turns of DNA. The quadrilateral is designed to be composed of two hextuply linked circles of DNA.

There is presently considerable interest in the potential of biological molecules to provide new reagents and structural units for synthetic chemistry. One goal of protein engineering, for example, is to design and synthesize binding sites for selected ligands, introducing functional groups with appropriate geometry, which may lead to new catalysts. On the macromolecular scale, one can imagine affixing larger functional molecules, such as protein domains, in particular arrangements. A requirement for achieving this type of structural control is the ability to construct a scaffolding to position and orient the functional units. We show here that the stable double helix of DNA can be used in conjunction with stable DNA branched junctions to create designated closed structures that could function in this manner. Assembly of this model scaffold is directed by the specificity of Watson-Crick<sup>1</sup> base pairing.

DNA branched junctions are analogues of structures found as intermediates in recombination and replication (e.g., ref 2). They consist of three or more strands of DNA which associate to form three or more double-helical arms that flank a central branch site or "junction". We and others have characterized the structure and stability of three- and four-armed junctions.<sup>3-10</sup> In principle,

these structures may be used as synthons for construction of N-connected polygonal and polyhedral stick figure devices and networks on the nanometer scale<sup>11,12</sup> rather than the Ångstrom scale more familiar to synthetic chemists (e.g., ref 13 and 14). This could be achieved by treating them as units to be linked together by the ligation techniques commonly employed in molecular biology.<sup>15,16</sup> From the standpoint of construction, line

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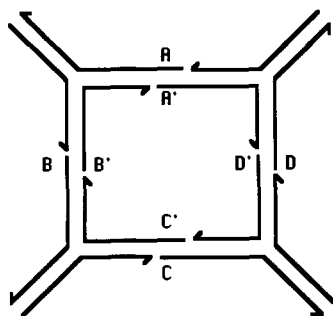
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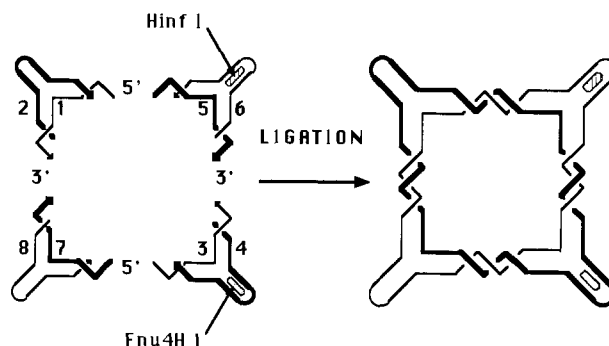


**Figure 1.** Tetramer designed from four components. Each of the individual components of this tetramer is a three-arm junction. The parallel lines represent regions of double-helical DNA. The half-arrowheads are at the 3' ends of the individual strands. The letters indicate the individual 5' overhangs which direct the cohesion of the different junction synthons: The sequence represented by A is complementary to the sequence represented by A'; similar relationships exist for B and B', C and C', and D and D'. Individual junctions thus have sticky ends A and B', B and C', C and D', and D and A'.

segments or "sticks" correspond to double-helical DNA, while the branch sites represent the vertices where they are connected. The details of incorporating the geometry of rigid junctions into the design such structures have been worked out,<sup>17</sup> and an interactive computer graphics program has been written to facilitate the design of structures formed from these components.<sup>18</sup> These structures offer the possibility of conveniently positioning and orienting molecules in space with high precision and stability. Recently, a possible application of this approach to biochip technology has been suggested by Robinson and Seeman.<sup>19</sup>

Several experimental milestones must be passed before junction-ligation procedures become part of a general design system. The first of these is the demonstration that it is possible to ligate junctions together. In 1986, we showed that one can readily oligomerize a three-arm junction, termed J<sub>3</sub>, by treatment with polynucleotide kinase and DNA ligase:<sup>20</sup> A ladder of linear junction oligomers is formed when the junctions are phosphorylated, ligated, and analyzed on denaturing gels. In addition, a separate ladder of macrocyclic species is seen, beginning with the trimer. We interpret this to mean that although the double-helical arms flanking the branch point are relatively stiff on the 5–7-nm scale,<sup>21</sup> the "valence angle" between them exhibits significant flexibility. More recently, we have shown that the same result may be obtained with four-arm junctions separated by 20 nucleotide pairs (approximately two helical turns of DNA);<sup>22</sup> macrocyclic trimers, as well as larger molecules, are obtained in addition to linear species when oligomerization takes place about any of the six angles formed by the arms of the junction. If the separation of four-arm junctions is changed to 16 nucleotide pairs (approximately 1.5 helical turns of DNA), the macrocycle ladder begins primarily with the tetramer.<sup>22</sup>

Having demonstrated that the same junction can be joined to itself to form oligomers, a logical next step is to bond different junctions together to form a nonrepetitive structure. This may be accomplished in a specific fashion by directing synthesis with different single-stranded complementary cohesive (sticky) ends. The sticky ends individually identify the particular arms that are to be ligated together, since the specificity of Watson–Crick base pairing will result in cohesion between complementary ends. When these sticky ends bind to each other in the presence of DNA ligase and its cofactors, separate arms are covalently bonded together with a connectivity that reflects this cohesion pattern. For ex-



**Figure 2.** Scheme of the synthesis of the DNA quadrilateral. The individual reactant junctions are shown on the left of the figure, and the target product is shown on the right. For clarity, the double helicity of the DNA has been represented merely as parallel lines in the vicinity of the branch sites and is confined to regions distal to the branch sites; nevertheless, all the twisting expected on the main cycle is shown on both sides of the figure. On the left, thick strands and the thin strands are associated in pairs to form three-arm junctions, in which one "exocyclic" arm is closed in a hairpin loop. Arrowheads represent the 3' ends of individual strands. Strand numbering is indicated on the left by the numbers from 1 to 8. The 5' and 3' symbols indicate the sense of the single-stranded overhangs. The overhangs are all on the short strands. The filled region in the exocyclic stem formed by strand 6 represents a *Hinf*I restriction site, while the filled region in the stem of strand 4 corresponds to a *Fnu*4H I site, as indicated. When the ligation is complete, the four junctions form two intersecting DNA circles which are linked six times.

ample, Figure 1 illustrates the design of a "square" to be made from four different three-arm junctions. The case in which  $A = B = C = D$  corresponds to the second macrocycle seen in the three-arm oligomerization study.<sup>20</sup> It is obviously much simpler and cheaper to oligomerize a given junction rather than to synthesize the N junctions corresponding to the desired junction oligomer. However, the synthesis of complicated structures requires a higher level of control over the products than is available from simple oligomerization. Therefore, we have chosen to use distinct cohesive ends to direct the association of four unique junction synthons. We demonstrate that the use of different cohesive ends to direct the synthesis results in formation of the selected DNA quadrilateral as the principal product and characterize the product.

### Design of Components

The individual components of the desired tetrameric macrocycle have been selected in order to demonstrate the efficacy of cohesive ends in directing association, while minimizing the synthetic effort required to complete the project. Therefore, we have chosen to separate junctions by 16 nucleotide pairs, approximately 1.5 turns of DNA.<sup>23–25</sup> This design contrasts markedly with that of the previous experiment involving a separation of 1.5 turns of DNA.<sup>22</sup> Helical twist was purposely ignored in that experiment, so that closing a cycle would stress the product. The results of that experiment suggest that at least two different junction structures formed at alternative corners of the tetramers which cyclized. Here, the helical twist is taken into account in the design, so as to promote closure of the tetramer. We have simplified both the synthesis and the analysis of product by adding loops of four thymidine residues (T<sub>4</sub>) at the ends of each of the exocyclic arms: Closed products will withstand digestion by the enzyme exonuclease III, which digests molecules with free 3' ends.<sup>26</sup> Each individual junction contains a different pair of the 16 possible cohesive ends which can be constructed from three G's or C's if the polarities of the overhangs (5' or 3') are taken into account.

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Table I. Sequences of Individual Component Strands

strand no.	sequence
1	5'-CGGGTGTCTACCAGATCCGC-3'
2	5'-GATCTGGACTATGGCTTTTGCATAGTTGAGCAC-3'
3	5'-CGCCCATAGTGGATTGCC-3'
4	5'-CAATCCTGAGCAGCTTTTGTCTCAACTATGG-3'
5	5'-CCGCATAGTGGATTGCCGG-3'
6	5'-GCAATCCCAGATCCTTTTGGATTCCGACTATG-3'
7	5'-GCGGGCTACGCGAAGCG-3'
8	5'-CTTCGCCGATTGCGTTTTCGCAATCGTGAGCC-3'

<sup>a</sup> Cohesive ends are indicated in boldface. Exocyclic loop regions are underlined.

Figure 2 illustrates the overall strategy for this synthesis. Each junction is composed of a long strand paired with a short strand. The long strand contains the T<sub>4</sub> loop in the middle and hence fulfills the role of two strands in a normal three-arm junction, such as those used in the three-arm oligomerization experiment.<sup>16</sup> Because the junction-to-junction separation is roughly 1.5 turns of average DNA, alternate junctions in Figure 2 have their upward and downward surfaces facing the reader. One strand of each junction ("outer") contains the loop, while the other strand ("inner") is shorter and contains both sticky ends. The helicity of B-DNA results in three reversals of the outer and inner strands in the space between branch points. Figure 2 shows that the desired product from ligation of the components should be two intersecting, single-stranded circles that make a tetrameric quadrilateral. In fact, the quadrilateral may be a rhombus or square, but there is no evidence that the molecule need be planar or have right angles. The two illustrated circles are designed to be hexuply linked in the absence of unlikely supercoiling; the exocyclic arms of the structure do not contribute to the linking number.<sup>27</sup>

Analysis of the ligation experiment requires the ability to excise each strand from the complex in a tractable form. This has been accomplished by including a special restriction site on one of the exocyclic arms of each of the cyclic single strands. In order to minimize sequence symmetry,<sup>11,12</sup> we have used five-base cutting sites for this purpose. A *Hin*I site (GANTC)<sup>28</sup> has been placed on one product circle, and a *Fnu*4HI site (GCNGC)<sup>28</sup> is situated on the other circle, as illustrated in Figure 2. Cleavage at either site by the appropriate restriction enzyme permits the dissociation of the complex and sequencing of the restricted strand. The sequences of the eight strands used are listed in Table I. Note that every double-helical arm of the unligated junctions contains at least six nucleotide pairs; a previous attempt at this synthesis which did not require six nucleotide pairs in each arm failed because a junction with an arm five pairs long did not form. The sequences employed here are variations on the four three-arm junctions defined by the truncation of a single arm from the four-arm junction J1.<sup>3,29</sup> Thus, they do not conform to the ideal minimization of sequence symmetry,<sup>11,12</sup> experimental precautions are taken to ensure that the additional symmetry causes no problems.

## Materials and Methods

**Synthesis and Purification of Oligonucleotides.** All oligonucleotides used in this study are synthesized on an Applied Biosystems 380B automatic DNA synthesizer, according to routine phosphoramidite procedures.<sup>30</sup> Protecting groups are removed by treatment for at least 12 h with 30% NH<sub>4</sub>OH at 55 °C. Strands are evaporated to dryness by a rotary evaporator, coevaporated twice with absolute ethanol, and dissolved in distilled water. The solution is heated to 50 °C and injected on a Varian 5000 HPLC equipped with a Macherey-Nagel 60-7 Nucleogen column heated to 50 °C. The A solution is 10 mM Tris, pH 7.1-4 M urea, and the B solution is 1.25 M NaCl in A solution. Strands are eluted with a linear gradient (1%/min) of B, precipitated with absolute ethanol, and dialyzed extensively against double-distilled water.

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A pure sample yields a single band on a denaturing gel. All oligodeoxynucleotide complexes are formed by dissolving individual strands in 66 mM Tris-HCl, pH 7.6-10 mM MgCl<sub>2</sub> (TMg), heating to 70-100 °C, and slowly cooling to anneal. Because the sequences are not minimally symmetric, junctions are annealed separately and kept cool when mixed together, in order to minimize strand invasion from like sequences.

**Enzymatic Reactions. (A) 5'-Phosphate Labeling.** A total of 1 μg of an individual strand of DNA is dissolved in 10 μL of TMg containing 1 mM spermidine, 15 mM dithiothreitol, and 0.2 mg/mL bovine serum albumin with 1 μL of [γ-<sup>32</sup>P]ATP (70 mCi/mL) and 1 unit of polynucleotide kinase (Boehringer Mannheim) for 45 min at 37 °C, when cold ATP is added to 1 mM. The reaction is stopped at 1 h by ethanol precipitation of DNA. Unlabeled ATP is substituted for radioactive ATP where appropriate. Complexes are formed as described above.

**(B) Ligations.** Ligations are performed in the same buffer as phosphorylations. A total of 400 units of T4 polynucleotide ligase (New England Biolabs) are added, and the ligation proceeds at 12 °C for 2 days.

**(C) Exonuclease III Digestion.** Exonuclease III digestions are performed by diluting an aliquot of the ligation mixture by 50% with the same buffer and adding 0.1 μL of exonuclease III (112 units/μL), at 37 °C for 30 min. Samples treated and untreated with exonuclease III are ethanol precipitated and analyzed on a denaturing polyacrylamide gel. Exonuclease III treated samples which will be analyzed by restriction analysis are extracted once with phenol/chloroform and then precipitated with ethanol.

**(D) Restriction Endonuclease Digestions.** The buffer for the enzyme *Fnu*4HI contains 10 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol. The buffer for *Hin*I contains the same components, in addition to 50 mM NaCl.<sup>28</sup> The digestions are performed with 2 units of restriction enzyme, at 37 °C for 4 h. The reaction is stopped by addition of 0.5 M EDTA (pH 7.5) to a final concentration of 10 mM. The products are then analyzed on a denaturing gel.

**(E) 3' Labeling for DNA Sequence Determination.** Purified product from a 10% denaturing gel is redissolved to 30 μL in a solution containing 5 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol and then digested with 2 units of *Hin*I at 37 °C for 2 h. The solution is heated to 60 °C for 5 min and allowed to cool to room temperature. To this solution are added 4 μL of [α-<sup>32</sup>P]dTTP (10 mCi/mL) and dATP to a final concentration of 1 mM. The solution is incubated with 5 units of Klenow fragment for 30 min at room temperature. The reaction is stopped by ethanol precipitation, and DNA fragments are gel purified.

**(F) DNA Sequence Determination.** The sequence of the product is determined by means of the chemically based rapid-sequencing protocol of Bencini et al.<sup>31</sup>

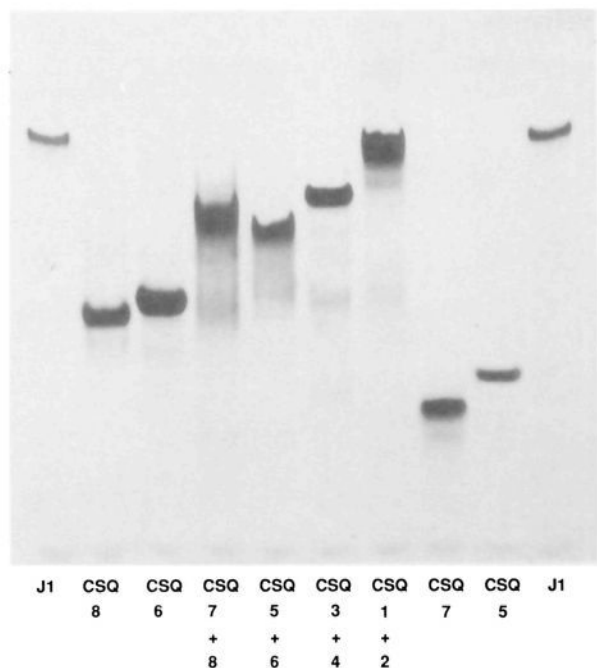
**Polyacrylamide Gel Electrophoresis. (A) Denaturing Gels.** Denaturing gels contain 7 M urea and are run at 55 °C. Unless otherwise noted, gels are 10% acrylamide [19:1 acrylamide:bis(acrylamide)]. The running buffer consists of 40 mM Tris-HCl, 20 mM acetic acid, and 2 mM Na<sub>2</sub>-EDTA, pH 8.1 (TAE). The sample buffer consists of equal volumes of TAE containing 40% formamide and glycerol, containing 0.02% each of bromophenol blue and xylene cyanol FF tracking dyes. Gels are run on Hoefer 600 electrophoresis units at about 20 V/cm, heated by a circulating bath, and are exposed to X-ray film for up to 15 h.

**(B) Native Gels.** Native gels are run at 4 °C. Unless otherwise noted, native gels are 20% acrylamide [19:1 acrylamide:bis(acrylamide)]. The running buffer consists of TAE plus 12.5 mM magnesium acetate (TAEMg). The sample buffer consists of equal volumes of TAEMg and glycerol, containing 0.02% each of bromophenol blue and xylene cyanol FF tracking dyes. Gels are stained in 1:1 formamide:water, containing 0.01% Stainsall dye. All native gels are run on Hoefer 600 electrophoresis units at about 10 V/cm, cooled by a circulating bath. Densitometric scanning of gels is done with a Hoefer GS 300 instrument in transmission mode.

## Results

Figure 3 illustrates a native gel showing the behavior of four of the individual long and short strands which comprise the junctions used in this experiment. In addition to the individual strands, this figure illustrates the electrophoretic behavior of the 1:1, complementary, pairwise combinations of these strands, i.e., the junctions to be used in the experiment. In comparison with J1, a previously characterized four-arm, four-strand junction with no sticky ends,<sup>3</sup> these junctions do not all migrate as discrete single bands. This is true even though the strands have been carefully

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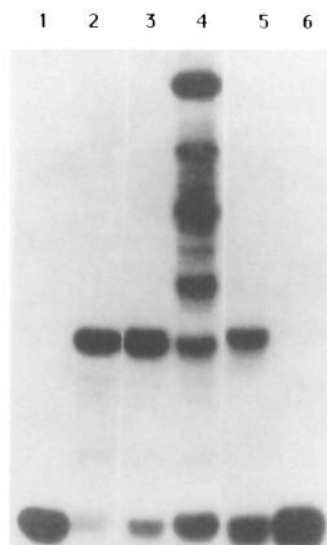


**Figure 3.** Native gel of the individual components. All gels are displayed so that the direction of migration is from the top to the bottom of the figure. The contents of each lane are indicated at the bottom of the gel. Strands from the quadrilateral are indicated by CSQ and their strand numbers. The well-characterized four-arm junction J1 is shown for comparison. Note that while the individual strands 7 and 8 are reasonably pure, their combination into a junction results in material that is much more heterogeneous than expected.

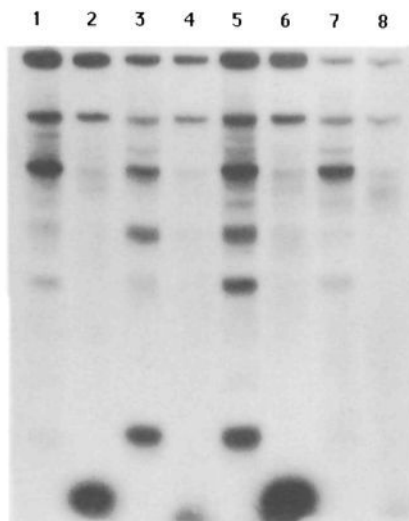
titrated together. The fact that some single strands are unavoidable in these mixtures suggests that the cautious strategy for ligating the junctions together is to do this in a sequential, stepwise fashion. This approach has been vindicated by experiment: mixing the four junctions together and ligating them gives the desired product with low yield; by contrast, proceeding in two stages results in a much higher yield of the target compound. The stages consist of first ligating the individual junctions together in pairs and then ligating the pairs together. Implementation of this strategy is equally successful when the 3' or 5' cohesive ends are ligated first, followed by the other set. No purification is done between ligation steps.

Figure 4 shows a denaturing gel with a sample set of reactants and products. Shown are two single strands, the results of three pairwise ligations, and the results of ligating two different pairwise products together in an attempt to form the target tetramer. The desired product is the band with the lowest mobility. This material is present as a significant fraction of the material in the reaction mix (roughly 15%, from a densitometer scan of this autoradiogram). As with most synthetic reaction mixtures, a "workup" is necessary to separate unreacted and incompletely reacted materials from the product. We eliminate these unwanted strands by treating the mixture with exonuclease III. Figure 5 shows a denaturing gel that illustrates four separate experiments in which crude ligated reaction mixtures are digested with exonuclease III. Only two components withstand exposure to this enzyme. These products are presumed to contain no 3'-terminal ends, or they, too, would be digested. Thus, two well-defined cyclic species result from the ligation reaction.

The smaller of the two products (about 5% of the total) corresponds to an unwanted product, in which only one of the two strands has cyclized. When not denatured away from its mated (unlabeled) strand, it is part of a nicked cyclic molecule. We show this in two ways in Figure 6. Figure 6A compares the size of the putative single-stranded circle with a known standard, a ligation ladder of Jy: Oligomerization of this junction by ligation results in the formation of both open and cyclic multiples of 20-mers.<sup>20</sup> Exonuclease III treatment of the Jy ligation mixture



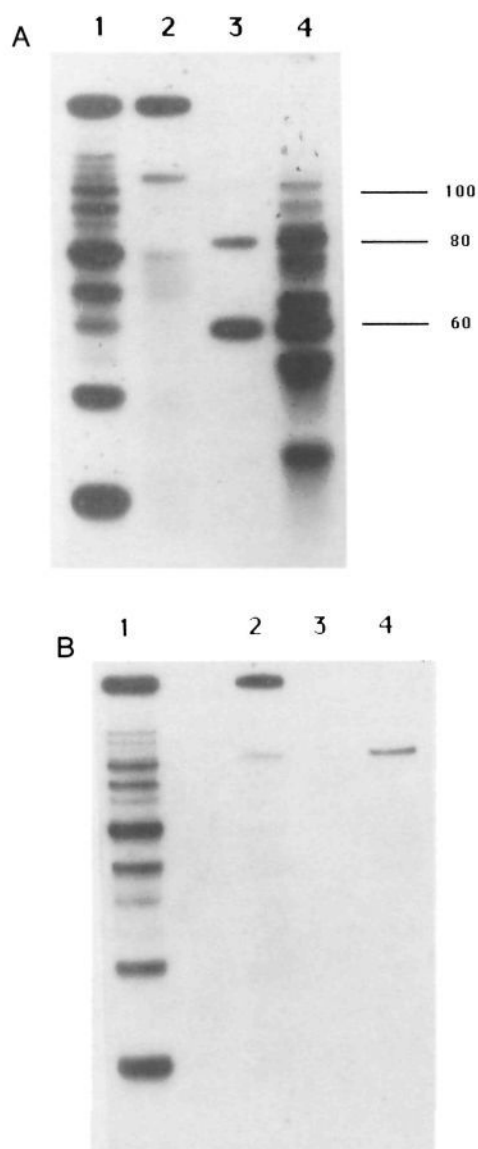
**Figure 4.** Ligation of components. The first and sixth lanes of this autoradiogram of a denaturing gel contain individual short strands 3 and 5, respectively. The second lane contains the results of ligating the junctions that contain the strands 3, 4, 7, and 8 (label on strand 3). The fifth lane is the result from ligating the other two junctions with 5' overlaps, containing strands 1, 2, 5, and 6 (label on strand 5). The third lane contains the results of mixing these two pair products, without ligation. The fourth lane contains the results of ligating the two pairs together. Note that the major product is the band with the slowest mobility.



**Figure 5.** Digestion of product with exonuclease III. This figure shows a denaturing gel of the products of the ligation with four different radioactive-labeling schemes. The lanes are grouped in pairs. The first lane of each pair is crude reaction product, and the second lane has been digested with exonuclease III. Starting from the left, the labeling for each pair has been respectively in strands 3, 2, 4, and 1. Note that only two products withstand digestion by exonuclease III.

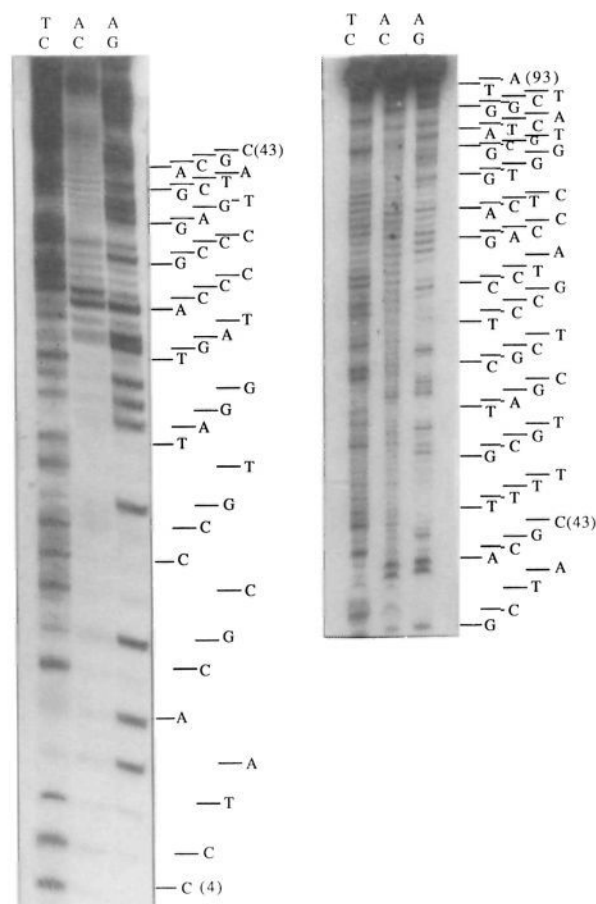
produces a ladder of length standards for cyclic single-stranded DNA molecules. The predicted length of the single-stranded circle from the components described above is 104 nucleotides. This compares well with the 100-mer standard next to it on the gel.

Figure 6B further defines the nature of the higher mobility component. In the first lane, we show the complete ligation mixture after reaction. Strand 6 is the only radioactively labeled strand in this mixture. The second lane shows the exonuclease III products. These products are treated with restriction endonuclease *HinfI* in the presence of exonuclease III. As seen in the third lane, both bands disappear, indicating that both of them contain the 1-6-3-8 cycle which is sensitive to this restriction



**Figure 6.** Characterization of exonuclease III resistant products. (A) Sizing the smaller circle. The autoradiogram shown is a denaturing gel containing the products of the ligation of the component junctions of the quadrilateral (lane 1) and the ligation of Jy to form oligomers (lane 4). The products of exonuclease III digestion of the quadrilateral reaction mixture are shown in lane 2, while similar treatment of Jy mixture is shown in lane 3. The bands in lane 3 form a series of molecular length standards for small single-stranded DNA circles: the lowest band is a 60-mer, and the two bands visible above it are an 80-mer and a 100-mer. The 100-mer migrates with only slightly higher mobility than the high-mobility product of the quadrilateral reaction, which is expected to be a circle of length 104. (B) Restriction analysis combined with exonuclease III digestion. Lane 1 of this denaturing gel contains the products of a ligation in which strand 6, which contains the *Hin*I site, is labeled. Lane 2 contains the same products after exonuclease III digestion. Lane 3 contains the products that result when *Hin*I restriction endonuclease is added to the exonuclease III digestion mixture: Everything visible in the autoradiogram is digested. Lane 4 contains the product of adding *Fnu*4HI to the exonuclease III digestion mixture: The unlabeled circle is restricted and digested, so the low-mobility band is chased into the high-mobility band, since the single-stranded circle containing the label remains intact.

enzyme. In the fourth lane, we show the results of treating the cyclic species with *Fnu*4HI, again in the presence of exonuclease III. This time the main product band disappears, but material accumulates in the single-stranded band. This is to be expected, for we have restricted the unlabeled strand while leaving the labeled strand intact.



**Figure 7.** Sequence of one strand of the quadrilateral. This gel shows the sequence of the strand derived when the product is cleaved by *Hin*I restriction endonuclease. The strand is 3' labeled, so the sequence should be read 5' → 3' from the top to the bottom. The numbers indicate the size of the fragment, rather than linear position in the sequence. There is an overlap region of eight bands between the two portions of the sequence. The procedure used involves reactions that are differentially sensitive to the presence of a pair of nucleotides, listed at the tops of the lanes, so that cleavage indicates the presence of one of those bases in the sequence at that point. The combination of two lanes resolves the ambiguity.

Proof that the junctions have assembled in the order directed by the sticky ends can only be established by determining the sequence of a strand of the product. We have done this by cleaving the product at the *Hin*I site and sequencing the linear single strand that contains all four ligation sites. The strand was labeled at the 3' end and the sequence determined by the method of Bencini et al.<sup>31</sup> on the basis of the chemical method of Maxam and Gilbert.<sup>32</sup> The sequence shown in the gel in Figure 7 demonstrates that the junctions have in fact assembled to form the quadrilateral in the designated order.

## Discussion

**Products of the Reaction.** The results obtained in this experiment indicate that it is possible to use the specificity of cohesive-ended ligation to make prespecified constructs from branched DNA junction components. It is particularly noteworthy that no larger products are observed in this study. In principle, flexible components with four unique sets of sticky ends are capable of forming a series including not only the observed tetramer but also the octamer, dodecamer, and higher products. One would also expect to see partially reacted intermediates on the way to those products, say molecules containing six ligated junctions. In the absence of any evidence for these species, it is likely that the

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

molecules capable of achieving closure do so rapidly enough to prevent the formation of longer molecules. We have demonstrated previously that three-arm junctions are sufficiently flexible over long periods of time that junction rigidity cannot be invoked to explain the single longest product.<sup>20</sup>

The lack of a ladder of molecules larger than the product suggests that the distribution of products seen on the gels may indeed represent an equilibrium mixture for the ligation reaction. This is in contrast to previous studies of junction ligation.<sup>20,22</sup> The presence of significant quantities of both the nicked circle and various linear molecules suggests that the DNA in those molecules is not capable of forming the final product, due either to incomplete phosphorylation, to damage, or to incomplete purification of the initial materials from slightly shorter failure sequences which result from the synthesis.

The low yield (2% by densitometric estimation) that results from ligating four different junctions in a single-pot reaction suggests that one should not rely too heavily on the fidelity of short cohesive ends under the nonstringent conditions optimal for the ligase. However, by separating the reactions into pairs, we also separate possibly competitive incorrect 3' or 5' ends at the first step of the ligation. The assembly of larger and more intricate molecules would require still more steps to achieve the desired product from starting materials this size. Fortunately, the continual improvement of DNA synthetic chemistry makes it possible to make much larger strands, and therefore much larger starting materials. These will be of value in the construction of three-connected figures, now in progress.

**General Procedures.** The demonstration that the component junctions have assembled in the sequence targeted for them is encouraging for the construction of other specific objects from nucleic acid junction synthons. Ligation directed by cohesive ends is widely used for specific assembly in molecular cloning techniques involving unbranched duplex DNA.<sup>26</sup> It appears to be effective as well in directing the products of ligation involving branched DNA junctions.

The purification scheme derived for this synthesis should be generally applicable to all closed syntheses. The nicked macrocycles are clearly separable from the target material by gel electrophoresis. In addition, it is always possible to remove linear failure molecules by exonuclease III digestion, which eliminates chains with a free 3' end. In this experiment, treatment with exonuclease III results in the desired product being 5-fold purified, increasing from 15% of the material to 75%. Exonucleolytic digestion may also be used conveniently, in order to purify intermediates: One can always make intermediates with closed-loop terminating arms; the arms may then be restricted to yield sticky ends on a pure molecule for the next step of synthesis, in the same way that we have restricted off closed loops for analytical purposes in this work.

**DNA Scaffolds.** The H-phosphonate chemistry described by Froehler<sup>33</sup> appears to be an effective means for the attachment of functional molecules or molecular fragments (such as protein domains) to a DNA scaffold: In this procedure, selected phosphate diesters are converted to phosphate triesters or phosphoramidates which can covalently link the functional molecules of interest. Use of linear duplex DNA as a scaffold is limited by the helical nature of the molecule. Many desirable distances between attachment sites are "masked", because they are on opposite sides of the double helix. More fundamentally, the helical symmetry of DNA makes it difficult to attach two large ligands to DNA via a single type of chemical bonding with kinematically stable fixture; this type of attachment requires three linkages to the scaffold. The nucleic acid junction appears to circumvent this difficulty: The arms which flank each junction in the final product are separated by 120° or less. As measured from a physical model,<sup>34</sup> the surface of a three-arm junction in the vicinity of the branch site contains pairs of phosphates separated by 13–50 Å, on either the top or bottom surface of the junction. A large range of unoccluded attachment site separations is available with the junction, regardless of the structure of the individual junction complex. Assuming only a single site per double-helical junction arm, 12 different molecules can be attached to the quadrilateral whose synthesis is described above. The branch-point to branch-point separation is in the 50–70-Å range. Additionally, the quadrilateral contains an inner surface, across which catalytic or binding groups can be opposed. This means that proteins or other large ligands can be rigidly affixed to a DNA scaffold by means of junctions, and not so readily otherwise. The scaffold provided here is not otherwise available with covalently closed double-helical DNA, where cyclization takes place at a larger size,<sup>35</sup> even in the most favorable circumstances.<sup>36</sup> As we have previously shown,<sup>20</sup> the components used here are flexible, so further constraints are probably necessary to make the scaffold rigid. The flexibility of quadrilaterals similar to the one formed here is currently under investigation. The use of junctions with four<sup>3</sup> or more (J. E. Mueller, N. R. Kallenbach, and N. C. Seeman, unpublished results) arms will extend control on the use of nucleic acid junctions as scaffolding devices by increasing the number of surfaces available.

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