Antiparallel DNA Double Crossover Molecules As Components for Nanoconstruction

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Abstract: Double crossover molecules are DNA structures containing two Holliday junctions connected by two double helical arms. There are several types of double crossover molecules, differentiated by the relative orientations of their helix axes, parallel or antiparallel, and by the number of double helical half-turns (even or odd) between the two crossovers. We have examined these molecules from the viewpoint of their potential utility in nanoconstruction. Whereas the parallel double helical molecules are usually not well behaved, we have focused on the antiparallel molecules; antiparallel molecules with an even number of half turns between crossovers (termed DAE molecules) produce a reporter strand when ligated, so these have been characterized in a ligation cyclization assay. In contrast to other molecules that contain branched junctions, we find that these molecules cyclize rarely or not at all. The double crossover molecules cyclize no more readily than the linear molecule containing the same sequence as the ligation domain. We have tested both a conventional DAE molecule and one containing a bulged three-arm branched junction between the crossovers. The conventional DAE molecule appears to be slightly stiffer, but so few cyclic products are obtained in either case that quantitative comparisons are not possible. Thus, it appears that these molecules may be able to serve as the rigid components that are needed to assemble symmetric molecular structures, such as periodic lattices. We suggest that they be combined with DNA triangles and deltahedra in order to accomplish this goal.

Introduction

A key aim of biotechnology and nanotechnology[1,2] is a rational approach to the construction of new biomaterials, such as individual geometrical objects and nanomechanical devices, but including extended constructions, particularly periodic matter.[3−5] The informational macromolecules of biological systems, proteins and nucleic acids, are thought to have the potential to serve as building blocks for this endeavor, because they are used for similar purposes in the cell. For the past several years, our laboratory has been engaged in the nanoscale construction of stick figures, using branched DNA molecules as building blocks. The edges of these figures consist of double helical DNA, and the vertices correspond to the branch points of stable DNA branched junctions.[6,7] We have reported the assembly in solution or on solid supports of molecules whose helix axes have the connectivities of a quadrilateral,[8] of a Platonic polyhedron, a cube,[9] and of an Archimedean polyhedron, a truncated octahedron.[10] If the edges of DNA polyhedra are designed to contain an integral number of double helical turns, every face corresponds to a cyclic strand of DNA, which is linked to each of its neighbors. Thus, the cube is a hexacatenane, and the truncated octahedron is a 14-catenane of DNA; the extent of linking between faces is equal to the number of turns in the edge where they meet. Both the polyhedra contain two turns per edge, so each of their cyclic strands is doubly linked to each of its neighbors.

The construction of the truncated octahedron by means of a solid support based methodology[11] suggests that there is no apparent obstacle to constructing any Platonic, Archimedean, or Catalan polyhedron from DNA. Logically, the construction of discrete closed structural entities, such as polyhedra, can be controlled readily, because the symmetrical features of the molecules can be limited by the molecular design or by the synthetic protocol. Thus, the ligation of identical DNA sticky end pairs (to yield an edge) can be separated from each other in time, by protection techniques;[11] the use of sticky ends with unique sequences also provides control over the assembly of finite objects.[8] The methodology we have developed enables implementation of this logic.

By contrast, this situation does not apply to the construction of periodic matter (crystals), where translational symmetry is an inherent characteristic of the system, because the contacts between all unit cells are identical. It is possible to envision deprotection schemes to unmask, successively, individual polyhedra or polyhedral clusters containing the same sticky ends by means of different restriction enzymes.[12] Likewise, one can imagine the construction of "pseudocrystals", having the same backbone structure and topology in each unit cell, but differing in sequence at key sites. Such schemes may be applicable to DNA computing,[13] but they are both cumbersome and expensive. They do not offer a practical route to the assembly of large repetitive constructs, even if one pictures hierarchical assembly of subsections of the target crystal. Here we seek to develop new components that will facilitate the extension of

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DNA nanoconstruction beyond individual objects, to the synthesis of periodic matter.

There are at least three key elements necessary for the control of three-dimensional structure in molecular construction that involves the high symmetry associated with crystals: [1] the predictable specificity of intermolecular interactions between components; [2] the structural predictability of intermolecular products; and [3] the structural rigidity of the components.[14] DNA branched junctions are excellent building blocks from the standpoint of the first two requirements, which are also needed for the construction of individual objects: [1] Ligation directed by Watson−Crick base pairing between sticky ended molecules has been used successfully to direct intermolecular specificity since the early 1970s,[15] and [2] the ligated product is double helical B-DNA, whose local structural parameters are well-known.[16]

The key problem in working with branched DNA as a construction medium is that branched junctions have been shown to be extremely flexible molecules.[17,18] The ligation of three-arm and four-arm DNA branched junctions leads to many different cyclic products, suggesting that the angles between the arms of the junctions vary on the ligation time scale; these angles are analogous to valence angles around individual atoms. Likewise, a five-arm DNA branched junction has been shown to have no well-defined structure, and a six-arm DNA branched junction has only a single preferred stacking domain.[19] Leontis and his colleagues have shown that a three-arm branched junction containing a loop of two deoxythymidine nucleotides has a preferred stacking direction.[20] Ligation along this direction shows a lower propensity to cyclization (21.3%) than other directions,[14] but it is not possible to treat the stacking domain in the Leontisian junction as a rigid component.[21]

Consequently, we have continued to seek DNA structures that fail to cyclize significantly in the course of ligation reactions. Here, we report the study of DNA double crossover molecules in ligation assays. DNA double crossover molecules are model systems for structures proposed to be involved in genetic recombination initiated by double strand breaks[22,23] as well as meiotic recombination.[24] We have explored the structural features of these molecules recently, and we have shown that there are five different isomers of double crossover molecules.[25] We have used double crossover molecules to establish the sign of the crossover node in the Holliday junction,[26] to construct symmetric immobile branched junctions,[27] to establish the crossover isomer preferences of branched junctions,[28] and to examine the effects of domain orientation on cleavage by the Holliday junction resolvase, endonuclease VII.[29] The helical domains are parallel in three of the five isomers, and they are antiparallel in the other two. Those with parallel domains are not as well-behaved as those with antiparallel domains,[25] so we have concentrated on antiparallel double crossover molecules here. There are two isomers of antiparallel double crossover molecules. The two isomers differ by containing an even (DAE) or odd (DAO) number of helical half turns between crossover points.

In order to characterize the suitability of double crossover molecules for constructing periodic matter, we have used the same assay that we used previously in this system, the ligation-closure experiment.[14,18,19] Our criterion for suitability is lack of cyclization. This is a practical assay, because cyclization poisons the growth of periodic systems. In addition, cyclization at a series of different short lengths suggests high molecular flexibility.[14,18,19] Of course, no molecular system is completely rigid, and at some point the formation of periodic matter is dependent on the phenomena that lead to lattice formation. Nevertheless, molecules that are highly flexible appear much less likely to form homogeneous periodic nuclei than those that are more rigid. This point is particularly pertinent to the construction of periodic matter from branched DNA molecules, because this system is the inverse of the conventional macromolecular crystal-forming system: Normally the structure is well-defined (if unknown), but the details of the intermolecular contacts are uncontrolled; here, the intermolecular contacts can be controlled by specifying sticky ends, but a rigid motif must be sought.

DAE molecules are much easier to analyze than DAO molecules. The upper portion of Figure 1a illustrates a DAE molecule and a DAO molecule in a schematic representation that illustrates their strand structures. The lower portion of Figure 1a shows that the ligation experiment involving an antiparallel molecule with an even number of half turns (DAE) is amenable to simple analysis, because one strand can be isolated as a molecule that reports both linear and cyclic products. The products of ligating the molecule with an odd number of half-turns between crossover points (DAO) are linear or cyclic unbranched catenanes of single stranded closed cyclic DNA molecules; a simple reporter molecule is not available. Therefore, we have analyzed only DAE molecules and have not analyzed DAO molecules. Much to our surprise, we find that DAE double crossover molecules can be ligated extensively without showing a large propensity to cyclize. This is true for two subclasses of DAE molecules: [1] molecules in which the cyclic strand between junctions is a simple unclosed loop and [2] molecules in which the cyclic strand between junctions participates in a three-arm bulged junction. Both classes of molecules are less likely to cyclize than branched junctions with the same sequence; the simple double crossover appears to be at least as stiff as linear duplex DNA of the same sequence. These results suggest that antiparallel DNA double crossover molecules are likely to be useful components in nanoconstruction.

Materials and Methods

Strand and Sequence Design. We have used DAE molecules closely related to those characterized previously.[25] There is little question about the preferred crossover isomers in the DAE (or DAO) system, but we have chosen sequences to flank the junctions that correspond to those in the well-characterized branched junction, J1.[30] We have closed the second domain with dT_4 loops that terminate arms containing 5-nucleotide pairs. This procedure permits us to minimize the number of strands that must be titrated together, and it also ensures

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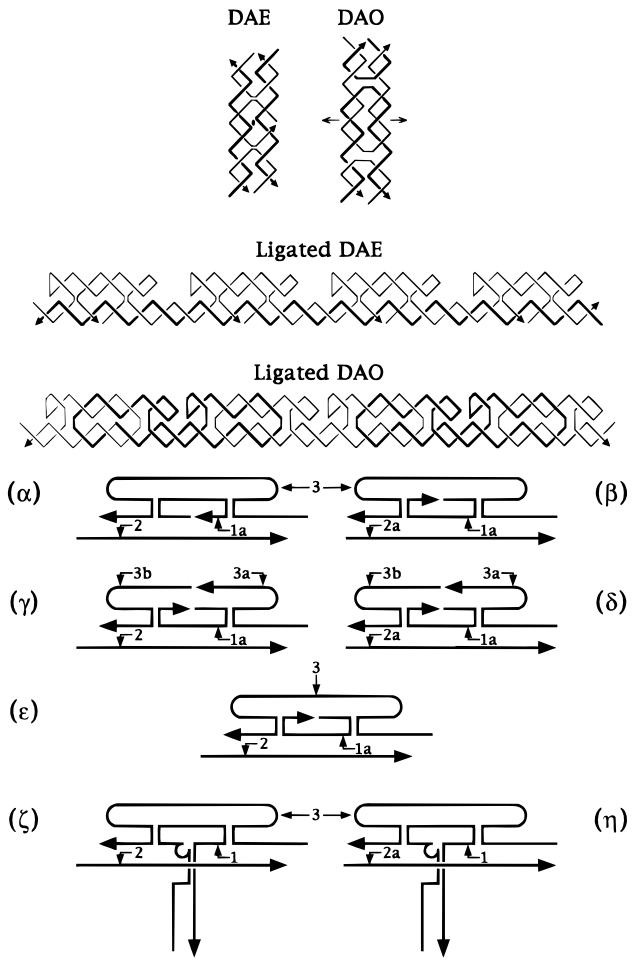

Figure 1. (a) Schematic representations of antiparallel double crossover molecules. Shown at the top of the diagram are the two types of antiparallel double crossover molecules, DAE, with an even number of double helical half-turns between the crossover, and DAO, with an odd number of half-turns between the crossovers. The DAE molecule contains five strands, two of which are continuous, or helical strands, drawn with thick lines, and three of which are crossover strands, drawn with thin lines, including the cyclic strand in the middle. The 3′ ends of each strand are indicated by an arrowhead. The DAO molecule is drawn to the right of the DAE molecule, and it contains only four strands. Two of these are drawn with thick lines and two with thin lines. The twofold symmetry element is indicated perpendicular to the page for the DAE molecule, and it is horizontal within the page for the DAO molecule. Thick lines are symmetrically related to thick lines and thin to thin lines in the DAE molecule, whereas thick lines are related to thin lines by symmetry in the DAO molecule. Sealing the cyclic strand in the middle of the DAE molecule would be necessary for the symmetry to be exact for that molecule. The drawing below these diagrams represents DAE and DAO molecules in which one helical domain has been sealed by hairpin loops, and then the molecules have been ligated together. The ligated DAE molecule contains a reporter strand, drawn with a thick line. By contrast, the ligated DAO molecule is a series of catenated molecules, which are drawn here with alternating thick and thin lines. Note that the ligated DAE molecule would be a catenane of two strands if it were to cyclize. (b) Molecular species used in these studies. The molecular species used here are shown in schematic form. The 3′ end of each strand is indicated by an arrowhead. The strand numbers are also indicated. Each species is assigned a Greek label. The molecules indicated as α, γ, and ζ have been used in the ligation experiments described below. These molecules contain sticky ends, because they contain reporter strand 2. By contrast, the molecules indicated as β, δ, and η all have blunt ends, and contain strand 2a. These molecules were used for nondenaturing gel electrophoresis, because the sticky ends cohere in the gel. Molecule ϵ has been used to illustrate this fact on the nondenaturing gel. Molecules ζ and η contain strand 1, rather than strand 1a, so they have an extra three-arm branched junction with an indicated bulge, between the crossovers. Molecules γ and δ contain a nick in strand 3 opposite the nick in strand 1a, so they are double junction molecules, but not double crossover molecules.

Table 1. DNA Sequences Used

Sequences Used to Produce the Non-Denaturing Gel and Double Junction Control

Name	Length	Sequence
Strand 1	42	5'-GGAGCGGACGTTGCACACCTATTTCCACCCT GCGTCCGCTCC-3'
Strand 1a	20	5'-TTCCACCCTGGCACACCTAT-3'
Strand 2	30	5'-CGGCTGATCTCCTGTGCCAGGGACAACTTG-3'
Strand 2a	30	5'-TGATCTCCTGTGCCAGGGACAACTTGCGGC-3'
Strand 3	58	5'-GCCGCAAGTTGTGGAGCTTTTGCTCCTGGAA ATAGGACATTTTTTAATGTGGAGATCA-3'
Strand 3a	31	5'-GCCGCAAGTTGTGGAGCTTTTGCTCCTGGAA-3'
Strand 3b	27	5'-ATAGGACATTTTTTAATGTGGAGATCA-3'

The combination of strands 1a, 2a and 3, above, forms species β.
The combination of strands 1a, 2, 3a and 3b, above, forms species γ.
The combination of strands 1a, 2a, 3a and 3b, above, forms species δ.
The combination of strands 1a, 2, and 3, above, forms species ε.
The combination of strands 1, 2a, and 3, above, forms species η.

Sequences Used in Hydroxyl Radical and Ligation Experiments

Strand 1	42	5'-GGAGCGGACGTTGCACACCTATGCGCACCCT GCGTCCGCTCC-3'
Strand 1a	20	5'-GCACACCTATGCGCACCCTG-3'
Strand 2	30	5'-CGGCTGATCTCCTGTGCCAGGGACAACTAC-3'
Strand 2c	30	5'-GCCGGTAGTTGTCCCTGGCACAGGAGATCA-3'
Strand 3	58	5'-GCCGGTAGTTGTGGAGCTTTTGCTCCTGCGC ATAGGACATTTTTTAATGTGGAGATAC-3'

The combination of strands 1a, 2, and 3, above, forms species α.
The combination of strands 1, 2, and 3, above, forms species ζ.
The combination of strands 2 and 2c, above forms the duplex control.

the integrity of the product. Slight modifications have been made to the initially designed sequence in the course of the work. The sequences of the strands that we have made are listed in Table 1. We have combined these strands in various ways throughout the course of this work in order to generate the species that we need. These species are illustrated in Figure 1b. The species used in ligation experiments are α, γ, and ζ, and the species used in the nondenaturing gel are β, δ, ϵ, and η. In contrast to the detailed strand structures shown in Figure 1a, the representation used in Figure 1b (as well as Figures 3 and 6) uses parallel lines to represent regions of linear duplex; this simpler form of drawing DNA is valid topologically in branched systems, so long as there are an integral number of double helical turns between the branch points of the molecule.

Synthesis and Purification of DNA. All DNA molecules used in this study are synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected using routine phosphoramidite procedures.[31] All strands are purified by polyacrylamide gel electrophoresis.

Formation of Hydrogen-Bonded Complexes. Complexes are formed by mixing a stoichiometric quantity of each strand, as estimated by OD_{260}. This mixture is then heated to 90 °C for 5 min and cooled to the desired temperature by the following protocol: 20 min at 65 °C, 20 min at 45 °C, 30 min at 37 °C, 30 min at room temperature, and (if desired) 30 min at 4 °C. Exact stoichiometry is determined, if necessary, by titrating pairs of strands designed to hydrogen bond together and visualizing them by native gel electrophoresis; absence of monomer is taken to indicate the endpoint.

Hydroxyl Radical Analysis. Individual strands of the DAE complexes are radioactively labeled and are additionally gel purified from a 10−20% denaturing polyacrylamide gel. Each of the labeled strands [approximately 1 pmol in 50 mM Tris·HCl (pH 7.5) containing 10 mM $MgCl_2$] is annealed to a tenfold excess of the unlabeled complementary strands, or it is annealed to a tenfold excess of a mixture of the other strands forming the complex, or it is left untreated as a control, or it is treated with sequencing reagents[32] for a sizing ladder. The samples are annealed by heating to 90 °C for 3 min and then cooled slowly to 4 °C. Hydroxyl radical cleavage of the double-strand and double-crossover-complex samples for all strands takes place at 4 °C for 2 min and 40 s,[33] with modifications noted by Churchill *et al.*[30] The reaction is stopped by addition of thiourea. The sample is dried,

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

dissolved in a formamide/dye mixture, and loaded directly onto a 10−20% polyacrylamide/8.3 M urea sequencing gel. Autoradiograms are analyzed on a BioRad GS-250 Molecular Imager.

Polyacrylamide Gel Electrophoresis. Denaturing Gels. These gels contain 8.3 M urea and are run at 55 °C. Gels contain 10% acrylamide (19:1, acrylamide:bisacrylamide). The running buffer consists of 89 mM Tris·HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA (TBE). The sample buffer consists of 10 mM NaOH, 1 mM EDTA, containing 0.1% xylene cyanol FF tracking dye. Gels are run on an IBI Model STS 45 electrophoresis unit at 70 W (50 V/cm, constant power), or on a Hoefer SE 600 electrophoresis unit at 60 °C (31 V/cm, constant voltage). They are then dried onto Whatman 3MM paper and exposed to X-ray film for up to 15 h.

Nondenaturing Gels. Gels contain 8% acrylamide (19:1, acrylamide:bisacrylamide). DNA is suspended in 10−25 μL of a solution containing 40 mM Tris·HCl, pH 8.0, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate (TAEMg); the quantities loaded vary as noted below. The solution is boiled and allowed to cool slowly to 16 °C. Samples are then brought to a final volume of 10 μL with a solution containing TAEMg, 50% glycerol, and 0.02% each of bromophenol blue and xylene cyanol FF tracking dyes. Gels are run on a Hoefer SE-600 gel electrophoresis unit at 11 V/cm at 16 °C, and exposed to X-ray film for up to 15 h or stained with Stainsall dye.

Enzymatic Reactions. A. Kinase Labeling. An individual strand of DNA (10 pmols) is dissolved in 20 μL of a solution containing 66 mM Tris·HCl, pH 7.6, 6.6 mM $MgCl_2$, and 10 mM dithiothreitol (DTT) and mixed with 2 μL of 2.2 μM γ-^{32}P-ATP (10 mCi/mL) and 6 units of polynucleotide kinase (U.S. Biochemical) for 2 h at 37 °C. Radioactive labeling is followed by addition of 1 μL unlabeled of 10 mM ATP, and incubation proceeds for another 10 min. The reaction is stopped by heat inactivation, followed by gel purification.

B. Ligations. T4 polynucleotide ligase (10 units) (U.S. Biochemical) in 30 μL of a buffer supplied by the manufacturer are added to 10 pmol of each strand, and the reaction is allowed to proceed at 16 °C for 10−16 h. Thus, the concentration of DNA in each ligation reaction is about 333 nM. The reaction is stopped by phenol/chloroform extraction. Samples are then ethanol precipitated.

C. Restriction Endonuclease Digestions. Restriction enzymes are purchased from New England Biolabs and used in buffers suggested by the supplier. Digestion is performed at 37 °C for 2 h with 20 units of Hha I or 10 units of Rsa I. The reaction is stopped by two or three phenol extractions, followed by ethanol precipitation.

D. Exonuclease III Treatment. Exonuclease III (exo III) (100 units), purchased from U.S. Biochemical, is added directly to the ligation mixture, and the reaction is allowed to proceed for 0.5−2 h at 37 °C. The reaction is stopped by heat inactivation.

E. Exonuclease I Treatment. Exonuclease I (exo I) (10 units), purchased from U.S. Biochemical, is added directly to the ligation mixture, and the reaction is allowed to proceed for 0.5−2 h at 37 °C. The reaction is stopped by heat inactivation.

Results

Assembly of the Molecules. Previously, we have shown that it is possible to assemble DAE-type double crossover molecules by hybridizing their constituent strands.[25] Nevertheless, this is the first time that we have attempted to assemble DAE molecules containing two closed ends. Furthermore, it is important to have a clear picture of the molecules being studied, in order to be certain that the properties noted actually reflect the species being examined. Figure 2 illustrates a nondenaturing gel containing the constituents of the DAE molecule being studied here. As indicated in Table 1, there have been modifications to the sequence used here, relative to those used for hydroxyl radical analysis (see below) and for the ligation experiments: [1] The d(AC) sequence at the 3′ end of strand 2 is here a d(TG), and residues 5 and 6 of strand 3 differ accordingly; and [2] the sequence of strand 2 has (for most lanes) been permuted cyclically (called strand 2a), so that the sequence d(CGGC) is at the 3′ end of the strand, thereby

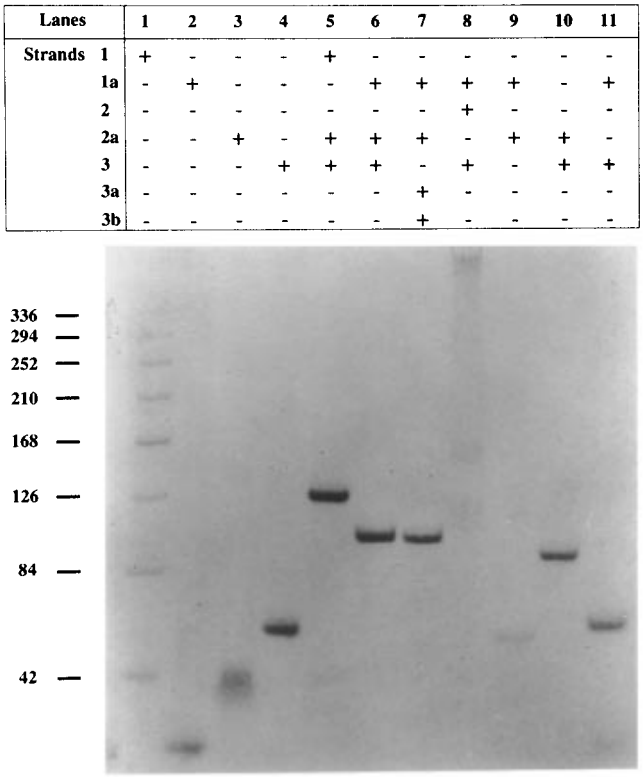

Figure 2. A Nondenaturing Gel of the components used here. Lanes 1−4 contain single strands 1 (with the bulged junction), 1a (without the bulged junction), 2a (cyclically permuted to eliminate the sticky end), and 3. Strand 1 fortuitously forms a ladder of 42-mers, that help to calibrate the gel. Lane 5 contains the trimer of strands 1, 2a, and 3, similar to the DAE molecule with a junction, but with the sticky ends rephased, so that the molecule is well-behaved on this gel (molecule η, Figure 1b). Lane 6 contains the DAE molecule without an extra junction (molecule β, Figure 1b), and lane 7 contains the doubly nicked version of that molecule (molecule δ, Figure 1b); the nick on the central strand faces the nick in strand 3 (generated by using two shorter strands, 3a and 3b) here. Both lanes 6 and 7 contain the rephased version of strand 2 used in lane 5. Lane 8 contains the unrephased version of strand 2 (molecule ϵ, Figure 1b), and its cohesion is seen to make characterization of the molecule difficult. Lanes 9−11 contain mixtures of the strands in which one strand has been omitted.

eliminating the sticky end. Figure 1b illustrates that for complete molecules, this modification generates molecules β, δ, and η from molecules α, γ, and ζ, respectively, although strand 1a is rephased by 10 nucleotides for the $\alpha \rightarrow \beta$ conversion. The sequence was modified from the sequence used for this gel, because ligation experiments revealed that ligase sometimes inserted strand 1a (Figure 3a) at the 5′ end of the product in place of strand 2. The cyclic permutation of strand 2 was done to eliminate association of the cohesive ends on the gel that obscured the information sought. The version used here contains the nick in the central strand permuted to be across from strand 3.

Lanes 1−4 contain the single strands that make up the various components of the complexes used. The multimerization of strand 1 (the 42-mer that forms the junction from strand 1) in lane 1 provides convenient molecular weight markers; the multimerization seen here is fortuitous, probably due to the complementarity of the 5′ and 3′ ends of the molecule. Strand 1a, in lane 2, is designed just to produce the DAE molecule, without the extra junction. Lane 5 contains the complete double crossover with the extra junction, formed using strand 1 (molecule η, Figure 1b). Lane 6 contains the complete double crossover formed using strand 1a (molecule β, Figure 1b). Lane

(33) Tullius, T. D.; Dombroski, B. *Science* **1985**, *230*, 679−681.

7 contains the same molecule, but strand 3 (now composed of strands 3a and 3b) has been nicked across from the nick in permuted strand 1a, to create two bonded junctions, rather than a double crossover molecule (molecule δ, Figure 1b). Lanes 9−11 contain the paired complexes with strand 1. Lane 8 illustrates the nature of the complex when the unpermuted version of strand 2 is used (molecule ϵ, Figure 1b): Very little of the material has penetrated the gel, and that which has is smeared. Note that, in contrast, the material in lanes 5−7 moves as a single discrete band, indicative of closed complexes, rather than open complexes.[34] Relative to the markers in lane 1, these complexes migrate with mobilities similar to those expected for their molecular weights. Thus, they appear to be well-formed discrete complexes.

Hydroxyl Radical Autofootprinting Analysis. We have used hydroxyl radical autofootprinting in the past to characterize a number of unusual DNA molecules, such as branched junctions,[19,30,35,36] antijunctions, and mesojunctions,[34,37] in addition to previous work with double crossovers,[25,27,28] These experiments are performed by labeling a component strand of the complex and exposing it to hydroxyl radicals. The key feature noted in these experiments is decreased susceptibility to attack in the comparison between the pattern seen when the strand is part of the complex, relative to the pattern derived from duplex DNA. Decreased susceptibility is interpreted to suggest that access to the hydroxyl radical may be limited by steric factors at the sites where it is detected. Likewise, similarity to the duplex pattern at points of potential flexure is assumed to indicate that the strand has adopted a helical structure in the complex, even though this is not required by the secondary structure. In previous studies of double crossovers, protection has been seen both at the crossover sites and at noncrossover sites where strands from the two domains appear to occlude each other's surfaces from access by hydroxyl radicals.[25,27,28]

The results of these experiments are shown in Figure 3. Each panel contains one-dimensional traces of the gel containing the products of hydroxyl radical treatment, in which linear duplex (DS) is compared with the double crossover molecule (DC). The results are summarized below in schematic form: Filled triangles of large and small sizes indicate nucleotides in the double crossover molecule that show large and small protections (respectively) relative to the linear duplex baseline. In the simple DAE molecule (Figure 3a), extensive protection is visible at the two nucleotides flanking the branch points and the one 5′ to them, as noted previously.[25,27,28,30] A weak protection site is also visible 3′ to the junction to the right on strand 3. In addition, strong protection is seen on the noncrossover strands in the central part of the molecule, four nucleotides 3′ to the crossover sites. These sites have also been noted previously and appear to be due to occlusion of the two strands by each other. Analogous protection is also visible on strand 3 at a site four nucleotides 3′ to the left junction. Weak protection is also noted on the third nucleotide of the two hairpins. This protection pattern is similar to patterns noted previously for DAE double crossover molecules,[25] suggesting that the molecule has adopted the conformation expected for it.

The pattern in the molecule containing the bulged junction is shown in Figure 3b. Protection is again visible in the nucleotides flanking the junctions on strands 1 and 3, but the protection is somewhat weaker here, in comparison with the pattern seen for molecule without the bulged junction. In addition, protection is seen 5′ and 3′ to the bulged junction on strand 1 as well as 5′ to it on strand 2. We are unaware of previous hydroxyl radical analysis of a bulged junction generated by free Fe(II)EDTA^{2-}. However, Zhong *et al.*[38] have analyzed an analogous molecule with other chemical probes, including hydroxyl radicals that were generated by MPE-Fe(II)EDTA^{2-}. The expectation is that the two domains of this junction containing strand 2 should stack upon each other, with the third domain being somewhat perpendicular to it.[20,39] The protections seen on the two sections of strand 1 that are nominally bent are in agreement with this model and with previous hydroxyl radical experiments on conventional three-arm junctions,[37,40] in which one strand is virtually unprotected, and the other two are more

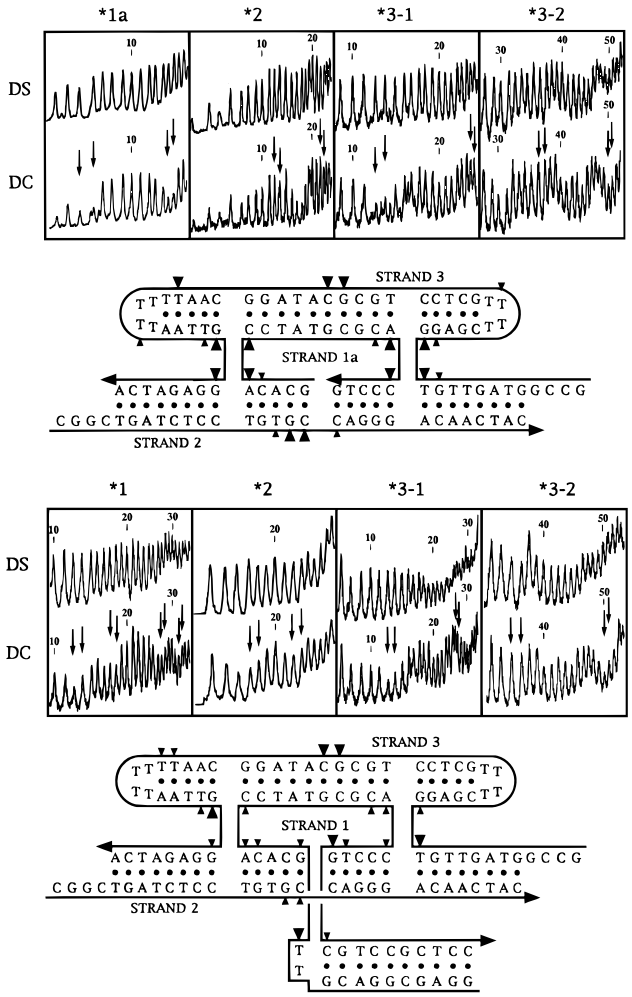

Figure 3. Hydroxyl radical cleavage patterns of the DAE molecules. (a) The conventional DAE molecule. The densitometer traces for each strand are shown above a summary drawn on the sequence of the molecule. Each panel shows the strand that has been labeled at the top. The double strand (DS) is compared with the double crossover (DC). The arrows indicate the sites of the crossovers. Residues are numbered at every tenth position. In the lower section of the panel, sites of greater protection are indicated by large triangles, and sites of lesser protection are marked by small triangles. The protections noted are in agreement with previous studies of DAE molecules.[25] (b) The DAE molecule containing a bulged three-arm branched junction. The same conventions apply as in (a). A protection pattern similar to that in (a) is seen in the corresponding residues, although it is of somewhat lower intensity. In addition, the site near the extra junction also shows protected nucleotides.

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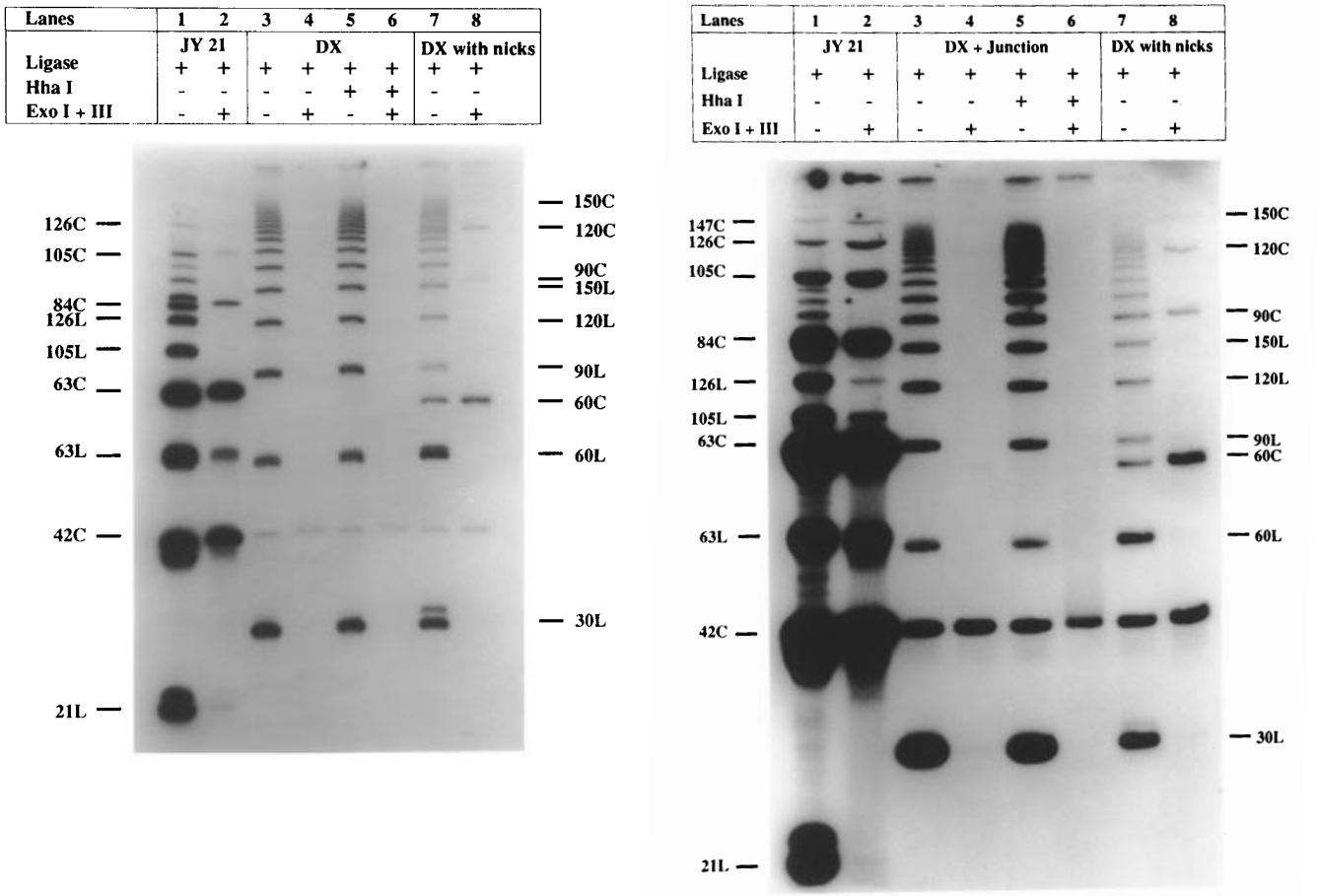

Figure 4. Ligation of DAE Molecules. (a) The Conventional DAE Molecule. This is an autoradiogram of a denaturing gel. Lanes 1 and 2 contain a standard three-arm branched junction,[41] "JY21", that generates linear and circular markers at a separation of 21 nucleotides; lane 2 has been treated with exonucleases, to yield only a 21-mer single-stranded circular DNA ladder. Linear markers are indicated by the suffix "L", and single-stranded cyclic markers are indicated by the suffix "C". Lanes 3−8 contain the DAE molecule (3−6) or its doubly nicked version. The column heading "DX" refers to double crossover molecules. A fixed amount of a cyclic 42-mer has been added to each of lanes 3−8, so that the intensity of each lane can be compared directly, regardless of the total radioactivity of the lane. Lanes 3 and 5 contain ligation ladders of labeled strand 2 of the DAE molecule (molecule α, Figure 1b), lanes 4 and 6 have been treated with exonucleases I and III, and lane 6 has also been treated with Hha I restriction endonuclease. No cyclic material is visible in these lanes. Any that appeared in lane 4 would correspond to single strand ligations, rather than double strand ligations; those can only be revealed if the molecule has been restricted to cleave strand 3. The molecule with two nicks, constituting two linked junctions (molecule γ, Figure 1b), but not a double crossover, shows cyclization as the dimer, with a visible ladder extending to at least the 180-mer near the top of the gel. (b) The DAE molecule containing an extra bulged three-arm junction (molecule ζ, Figure 1b). The same conventions apply here as in (a). In contrast to the conventional DAE molecule, a small amount of a single-stranded circular ladder is seen in lane 4, corresponding to cyclic dimers, trimers, etc. Faint quantities of these bands are also visible in lane 6. Thus, a small amount of cyclization is present with this molecule that is absent from the conventional DAE molecule. Nevertheless, the amount of cyclization is much less than with conventional branched junctions, as seen in lane 8.

strongly protected. We ascribe the protection on strand 2 to its occlusion by strand 3, as in the molecule lacking the bulged junction. These data are consistent with a model in which one strand of the bulged junction is not very different from helical, and two strands contain bends. Thus, the hydroxyl radical patterns of both molecules are consistent with a reporter strand (strand 2) that does not differ significantly from a helical conformation. The partial structural characterization by hydroxyl radical analysis contains no indications that the molecules are behaving in an unexpected fashion.

Ligation Experiments. We have ligated the two DAE molecules, one without a branched junction in the central section, and a second containing a branched junction in that region. Figure 4a shows a denaturing gel that contains the results of the ligation experiments in the molecules lacking the extra junction. Lane 1 contains the products of ligating a standard marker that produces a ladder of both linear and cyclic single strands in multiples of 21 nucleotides.[41] Lane 2 contains the products of digesting the material in lane one with a mixture of exo I and exo III (termed exo I−III), to leave nothing but cyclic molecules. Lanes 3−8 all contain an equal amount of the cyclic single-stranded 42-mer, so as to permit calibration of the densities in each lane. Lanes 3 and 5 contain the products of ligating the DAE molecule without the extra junction (molecule α, Figure 1b), and lane 4 contains the products that result when the material in lane 3 is digested with exo I−III. The ligation ladder seen in lane 3 extends much longer than ligation ladders seen previously, involving the ligation of branched junctions. It appears to show products representing as long as 17-mers or 18-mers of the fundamental repeat. Nevertheless, treatment with exo I−III shows that all of these products are linear, in the sense that they are not cyclic molecules. True cyclic molecules in this system are complex catenanes that cannot be detected until product molecules are treated with Hha I restriction endonuclease to cleave strands 1 and 3 (the GCGC site one nucleotide pair from the right crossover in Figure 3). Lane 6 contains the products of treating the material in lane 5 with Hha I, in addition to exo I−III. No

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new exonuclease resistant material is seen in this lane. Thus, no detectable cyclic material is seen for this ligation experiment. Lane 7 contains the products of ligating the same molecule, but modified so that strand 3 is composed of two parts, strands 3a and 3b, and the nick on strand 1 has been rephased by 10 nucleotides to be opposite the gap between them (molecule γ, Figure 1b). Thus, the molecule ligated here is effectively two branched junctions, rather than a single DAE molecule. Exo I−III treatment of the material in lane 7 reveals the ladder of cyclic products in lane 8. The cyclic molecules begin with the dimer (60 nucleotides) and proceed at least to the heptamer. This marker lane also shows that large cyclic molecules would not be detected in the body of the gel but only in the region near the wells.

Figure 4b illustrates a parallel and identical series of experiments, but from now on the molecule contains a bulged, branched junction in the central region of the molecule (molecule ζ, Figure 1b). Inspection of lane 4 now shows a small amount of closed cyclic product, beginning with the dimer. The material noted in this lane is necessarily an artifact; until treatment with Hha I, single-stranded circles should not be seen in ligations where both strands are sealed. Lane 6 shows a trace of cyclization for this material, beginning with the dimer. The nicked double junction (molecule γ, Figure 1b) experiment from Figure 4a is repeated as a marker control in lanes 7 and 8.

Discussion

Lack of Cyclization. We find that DAE-type DNA double crossover molecules appear to cyclize minimally, if at all, in a ligation assay. It is possible to estimate the persistence length of DNA molecules from ligation assays,[42] but only if one has closed cyclic products in sufficient yield that it is possible to calculate j values[43] (the ratio of cyclic to linear products) for a series of the ligation products. Whereas we do not have those products, that calculation will have to await further work in this system. In our hands, DAO molecules have behaved previously as well as, or better than DAE molecules,[25,27,28] so we expect that their rigidities are unlikely to be inferior to those of comparable DAE molecules. In addition, they are a much more convenient system with which to work, because they do not include the extra strand in the center of the molecule.

An incommensurate twist can decrease the probability of cyclization markedly.[44] The doubly-nicked DAE molecule only represents a partial control in this regard, because the two junctions it contains might well have twisting flexibility that compensates for an incommensurate twist. We have shown that the twisting flexibility of branched junctions is less than the bending flexibility, but it still appears greater than in linear duplex DNA.[18] In order to see whether the 30-mer sequence that we are polymerizing is capable of cyclizing at all, we have performed a control ligation experiment[45] on just that double helical sequence. The results of that experiment are shown in Figure 5. Figure 5a is a two-dimensional denaturing gel, in which the products of the ligation reaction were electrophoresed first in 6% acrylamide and then in 10% acrylamide in a direction perpendicular to the first. In this type of gel, linear material runs on the diagonal, single-stranded circles run in an arc above the diagonal, and duplex circles run in a second arc somewhat above the first.[46] We have sized the duplex circles by boiling them for about 10 min, so that some of them break to yield linear molecules and single circles, whose sizes can be compared with markers, as shown in Figure 5b. We find that not only do we make a readily detectable amount of duplex 150-mer circle, as expected,[45] but also we even make a trace of 120-mer duplex circle. Thus, unless the duplex is more flexible than the DAE molecule in the twisting direction, we are not seeing an artifact due to incommensurate twisting.

Extent of Ligation. One of the features of the reactions reported here is that the ligations are far more extensive than those noted in previous reactions of a similar nature.[14,17,18] It was rare in those studies of more flexible DNA molecules to see ligation products beyond the heptamer, yet here ligation has proceeded to generate 17-mers or so. Given that the reaction conditions used here are similar to those used previously, there are at least two possible reasons for this finding: [1] The highly cohesive sticky end used here CGGC:GCCG may provide a better substrate for the ligase; or [2], the relatively inflexible substrate examined in this work could produce sticky ends that are available for interactions that lead to ligation for a greater fraction of the time.

Nanoconstructions with DAE and DAO Molecules. We have made a key step in the construction of nanometer-scale objects and lattices from DNA: We have found what appears to be a branched DNA motif at least as rigid as linear duplex DNA. *A rigid DNA component must be able to specify the vectors of DNA double helical axes (and hence the angles between them) within limits of flexibility no greater than those of linear duplex DNA.* It would be pointless in this system to seek a molecule stiffer than linear DNA: That would be the logical equivalent of attaching a steel anti-auto theft device across a steering wheel made of styrofoam. To our knowledge, this is the first time that a DNA motif of this stiffness has been identified in a DNA molecule containing branch points. Now this motif must be made amenable to DNA assembly methodologies. In what way can the stiffness of DAE or DAO molecules be used in the construction of complex DNA materials? One could imagine the construction of a lattice of DAE or DAO molecules that would contain parallel and antiparallel helix axis vectors; its direct physical applications are not obvious, although it has been suggested for use in DNA computing.[47]

We have pointed out previously[14] that triangles in two dimensions form appropriate building blocks with fixed angles at their vertices. Likewise, it can be shown for the three-dimensional case that a convex polyhedron is rigid if and only if each of its faces is a triangle.[48] Thus, triangles and deltahedra (polyhedra whose faces are exclusively triangles) seem to be the obvious instruments through which double crossovers should be used in nanoconstructions that involve high symmetry. Figure 6 shows our suggestion for using them. It should be noted that these designs are different from an edge-sharing proposal made earlier.[49] In Figure 6a, we suggest orienting a series of double crossover molecules to form a trigonal set of vectors by means of their attachment to a triangle. The triangles are connected, so as to tile a plane. Thus, it appears possible to use DAE molecules to form a two-dimensional DNA lattice. This suggestion is predicated on the ability of a DNA triangle

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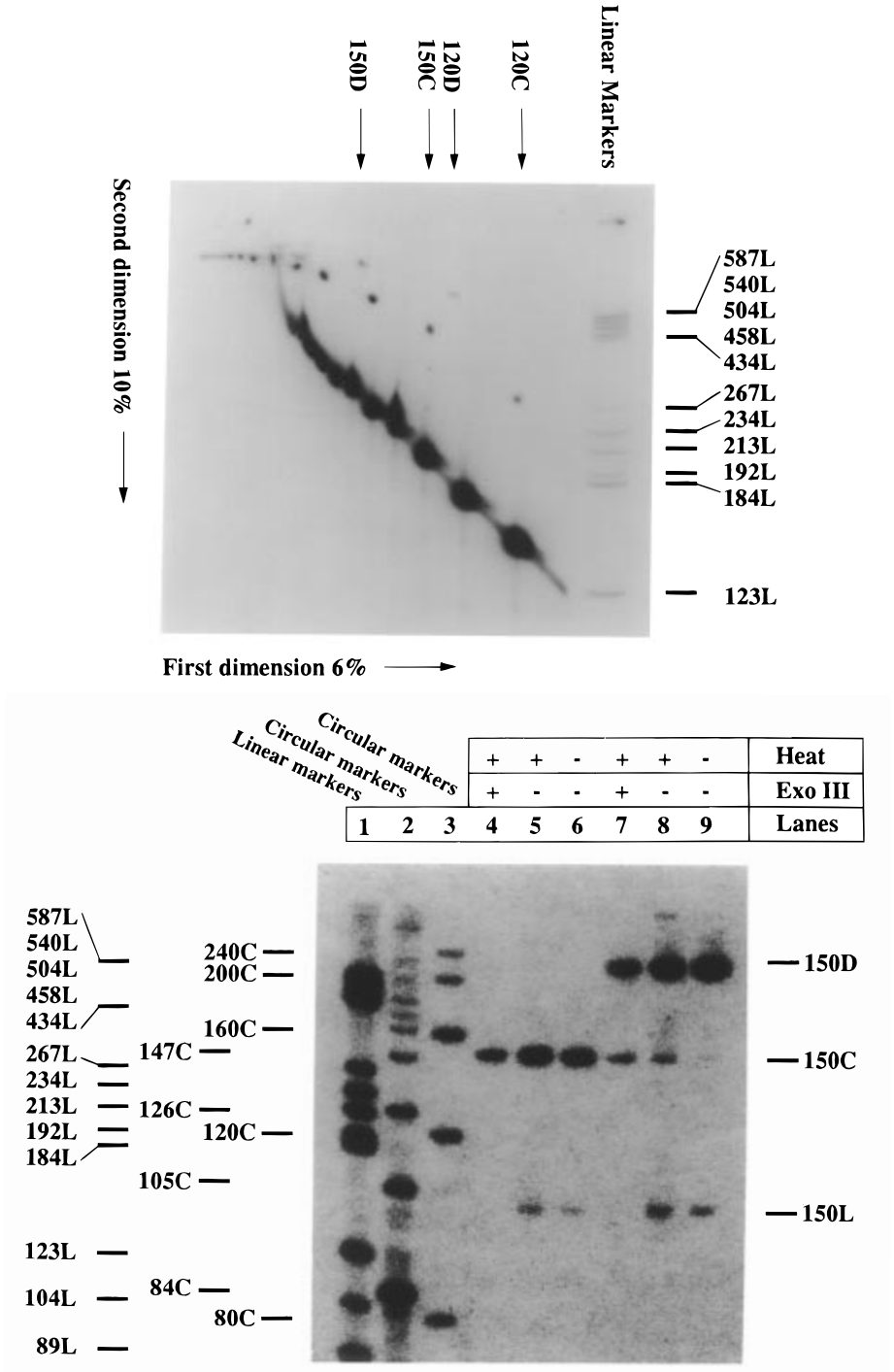

Figure 5. Ligation of a 30-nucleotide duplex with the same sequence as the ligation domain of the DAE molecules. (a) The products of ligation. This is an autoradiogram of a two-dimensional (6%, then 10%) polyacrylamide denaturing gel. A ladder of linear markers has been run in the second dimension. Linear molecules run on the diagonal, single-stranded circles in the arc above it, and duplex circles in the second arc above the first one. Molecular identities are marked as follows: Linear DNA, "**L**", single-stranded circles, "**C**", duplex circles, "**D**". The faint spot at the right of the duplex arc is a 120-mer circle. The spot to its left is a 150-mer circle, whose size is characterized in (b). The 120-mer circle is reproducible, indicating that this sequence has no problem forming small circles. (b) Characterization of the 150-mer circle. This figure represents a denaturing gel analyzed on a BioRad GS-250 Molecular Imager. The labeling conventions of *(a)* are retained. Lanes 1−3 contain ladders of linear markers, the 21-mer single-stranded circular ladder of Figure 4, and a 40-mer single-stranded circular ladder.[18] Lanes 4−6 contain a single-stranded 150-mer circle extracted from the gel in (a). The material in lane 6 is untreated, the material in lanes 4 and 5 has been heated to 90 °C for 10 min, and the material in lane 4 has also been treated with exonucleases I and III. The material in lanes 7−9 corresponds to the putative 150-mer circle of (a). The material in lane 9 is untreated, the material in lanes 8 and 7 has been heated to 90 °C for 10 min, and the material in lane 7 has also been treated with exonucleases I and III. It is clear that the material in lane 9 breaks down to single-stranded circle (in lanes 8 and 7), similar to the material in lane 6, as well as generating some linear material. This shows that this band is a 150-mer duplex circle.

to maintain its shape, an assumption that appears warranted, but which will require testing. In our hands, DAO molecules appear to be much better behaved than DAE molecules, so it is likely that they can be used even more effectively than DAE molecules for this purpose, so long as a reporter strand is not needed to ascertain the results of the construction.

Figure 6b illustrates the same lattice shown in Figure 6a, but now incorporating DAE molecules containing an extra junction, as in the second class of molecules tested here. In the case shown, every third molecule contains this type of DAE molecule, so that an extra DNA arm has been added to each hexagon, one that is not directly involved in the integrity of

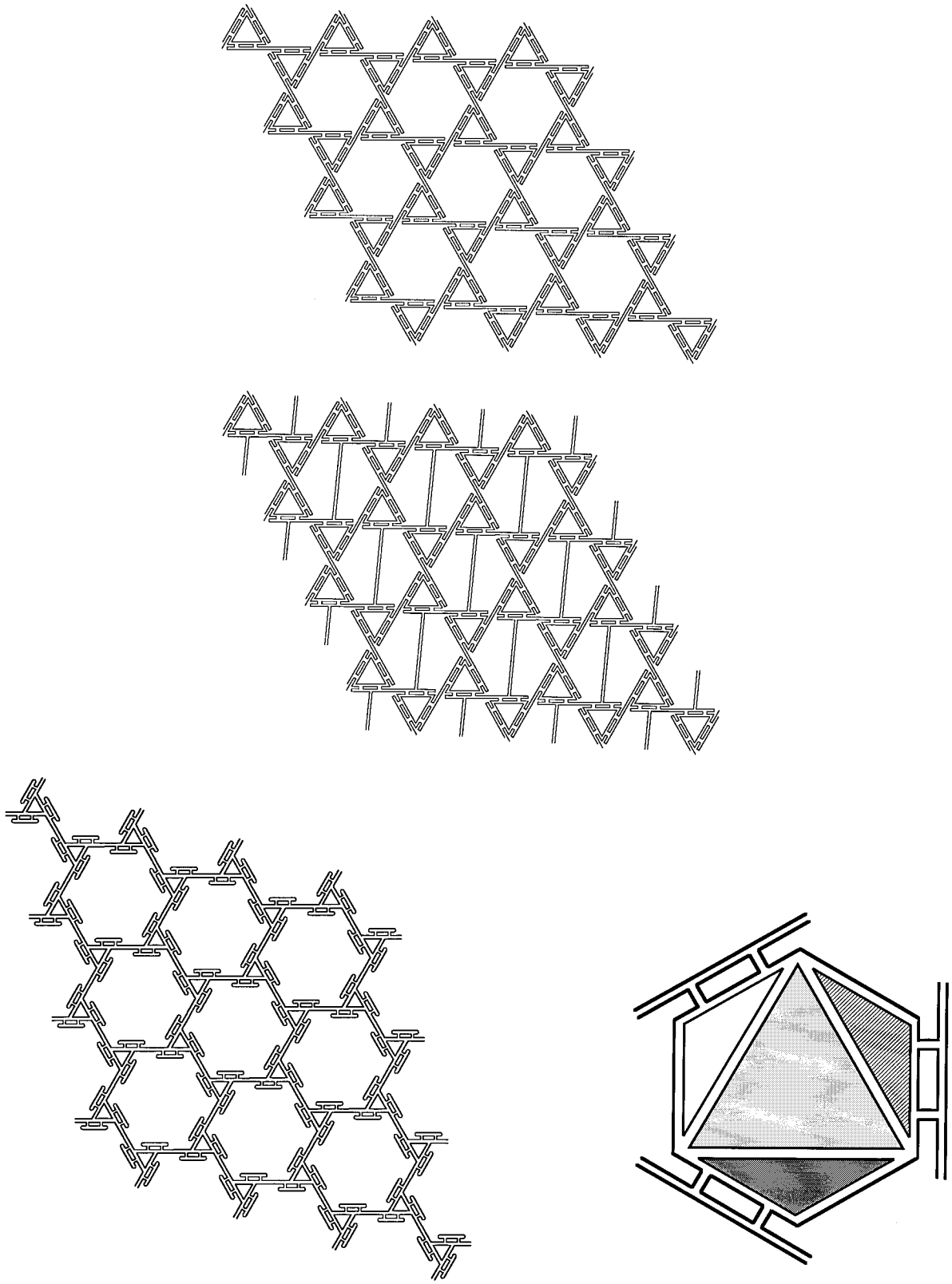

Figure 6. Constructions that could be made with double crossover molecules. (a) A two-dimensional lattice. This diagram shows a series of equilateral triangles whose sides consist of double crossover molecules. These triangles have been assembled into an hexagonally-symmetric two-dimensional lattice. The basic assumption here is that triangles will retain their angular distributions here, so that they represent eccentric trigonal valence clusters of DNA. (b) A lattice containing a DAE molecule with an extra three-arm bulged junction. This is the same lattice shown in (a), but every third arm contains a DAE structure, just like the one studied here containing the extra junction. It can be seen that the symmetry of the lattice decreases, but the extra arm associated with each triangle is not a fundamental part of the lattice and can be distorted as needed by an appropriate guest. (c) A lattice constructed from buttressed junctions formed into triangles. The same motif is used here that is used in (a). However, the triangles are formed using the extra junction, so that it is part of the lattice. The junction is buttressed by the double crossover molecule, so that the edge remains straight. (d) An octahedron containing three edges made from double crossovers. This drawing of an octahedron down one of its three-fold axes shows only four of its eight equilateral triangular faces. The three edges shown constructed from DAE molecules are not coplanar but span a three-dimensional space. An enantiomorphous set also exists. Connecting their outside domains to similar domains in other octahedra would yield a lattice resembling the octahedral portion of a face-centered cubic lattice but of lower symmetry.

the lattice. Such an arm could be used to attach other molecules of interest, such as site-specific binding molecules, possibly for purposes of structural analysis.[6]

Figure 6c illustrates a second means of utilizing double crossover molecules that contain an extra junction. This figure shows the same lattice employed in Figure 6 (parts a and b). Here, however, the extra junction is used to form the triangles, and the other domain of the double crossover molecule is used to buttress the edge and to keep its helix axis linear. The slightly greater flexibility noted above for these double crossover molecules does not lead us to believe that this motif is likely to be formed as readily as the one shown in Figure 6a.

Figure 6d shows the extension to three dimensions of the scheme illustrated in Figure 6a. A single octahedron is drawn, containing three double crossover molecules whose free helical domains span a three-dimensional space. The three arms chosen will not intersect each other, no matter how far they are extended. An enantiomorphous set of three arms could also be chosen. If each of the three arms were connected to its corresponding arm in another octahedron, the resulting structure would nucleate a structure resembling the arrangement of octahedral subunits in cubic close packed structures (face-centered cubic structures). The structure would be somewhat distorted from that ideal, however, because of the connections through the extra helical domains. The structures shown in Figure 6 are all goals for DNA construction that appear possible and potentially useful because of the results obtained above. It is possible that other multiply-connected networks[50,51] may be amenable to construction via this motif.

Acknowledgment. We would like to thank Drs. Jason Kahn and Mikhail Livshits for valuable discussions about the results of these experiments. This work has been supported by Grants N00014-89-J-3078 from the Office of Naval Research and GM-29554 from the NIH to N.C.S.

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