Ligation of Triangles Built from Bulged 3-Arm DNA Branched Junctions

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Abstract: An important stepping stone to controlled construction on the nanometer scale is the development of rigid components, building blocks whose directions of bonding bear fixed angular relationships to each other. We are exploring nanoconstruction with branched DNA junctions. Branched junctions with three and four arms are known to be flexible, but 3-arm branched junctions containing bulges are known to have a preferred stacking direction. Here, we have tested the rigidity of equilateral triangles whose vertices are all bulged 3-arm branched junctions. There are two kinds of equilateral triangles with an integral number of turns per edge that can be made from these components: those with the bulges on the inside strand, and those with the bulges on the outside strand. Alternating the two species generates a reporter strand. Each triangle is assembled and purified as a topologically closed species. Assembly of the triangle with the bulges on the inner strand results in two topoisomers that can be separated. Sticky ends are generated on the triangles by restricting their external arms. Incompletely restricted triangles and completely restricted hairpins are removed by binding magnetic streptavidin beads to biotin groups incorporated in the hairpins. Assembly of rigid triangles would result in a closed hexamer of triangles. Ligation experiments have been performed, in which twisting was varied about the expected values. In all cases, the primary intact product is seen to be the cyclic tetramer. Thus, triangles with bulged 3-arm branched junctions at the vertices do not constitute a rigid component for nanoconstruction.

Introduction

The control of the structure of matter on the nanometer scale is a central goal of many chemists today.[1] Ideally, such control would enable the construction of both individual objects and periodic matter. There are at least three elements necessary for the control of three-dimensional structure in molecular construction:[2] (1) the predictable specificity of intermolecular interactions between components; (2) the structural predictability of intermolecular products; and (3) the structural rigidity of the components. The first two of these elements allow for topological control over the products of assembly, in the senses both of the connectivity of the molecular graph and of the linking of substructure chains that wrap around each other. The third element, structural rigidity, is necessary to assemble molecules on the nanoscale with the same geometrical certainty enjoyed when building objects and devices on the macroscopic scale. Such structural integrity appears to be needed when fabricating targets that contain symmetry, so as to ensure the proper association of components; hence it seems to be particularly important for the construction of periodic networks, whose components exhibit translational symmetry.

For several years, our laboratory has investigated the use of DNA branched junction molecules as components for nanoscale construction. The ligation of branched junctions leads to stick figures, in which the edges consist of double helical DNA, and the vertices correspond to the branch points of the branched junctions.[3] DNA branched junctions are good building blocks from the standpoint of the first two requirements: (1) ligation directed by Watson−Crick base pairing between sticky ended DNA molecules has been used successfully by biotechnologists to direct intermolecular specificity for over two decades,[4] and (2) the ligated product is double helical B-DNA, whose local structural parameters are well-known.[5] These first two rules appear sufficient for the fabrication of individual objects whose assembly entails making a finite number of edges, because it is always possible to specify unique complementary pairs of sticky ended arms that can be ligated to form a set of target edges. If the vertices of the molecular graph represent the branch points of junctions, and the edges represent helix axes, there is no chance that the vertices will be connected by incorrect edges, so long as the Watson−Crick complementarity of the sticky ends is not violated. Likewise, the linkage between DNA strands is likely to generate the target topoisomer, so long as the separations between vertices are appropriate on both strands.

We have found it possible to exploit this system to generate target constructions that have been characterized by their molecular topologies. Several years ago, a DNA molecule whose helix axes have the connectivity of a cube was constructed in solution.[6] More recently, a solid support-based methodology has been developed,[7] and it has been used to assemble a DNA molecule whose helix axes have the connectivity of a truncated octahedron.[8] These constructions have relied on the high specificity of associations directed by DNA sticky ends. The assembly of individual objects can rely on this type of high specificity, because there is no inherent requirement for sequence symmetry associated with these molecules. However, if high sequence symmetry is involved,

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the components either must be rigid[9,10] or must contain protecting groups that are removed at every step of the assembly.[7]

The constructions of the cube and of the truncated octahedron have been predicated implicitly on the flexibility of their branched junction components. The cube construction scheme assumes that eight 3-arm branched junctions can fold to generate a cube or a rhombohedron. Likewise, the truncated octahedron assembly scheme assumes that twenty-four 4-arm branched junctions could be deformed from their solution structures[11,12] to generate the vertex geometries needed for this polyhedron. Ligation studies show that both 3-arm[13] and 4-arm[14] branched junctions are flexible on the time scales (ca. 10 h) used in ligation experiments. The presence of a large number of cyclic species as the products of these ligations suggests that the angles between the branches are highly variable; hence, they do not fulfill condition 3 above. By contrast, short double helical pieces of DNA are likely to provide stiff edges.[15] We regard a rigid branched DNA component as one in which *the vectors of double-helix axes (and hence the angles between them) vary within limits of flexibility no greater than those of linear DNA.* Thus, connections between two such components generate predictable and unique structures, *not* a series of products.

One of the key goals of DNA nanotechnological construction is the assembly of three-dimensional periodic arrays. In addition to the potential assembly of interesting and useful materials,[16] it has been proposed that such periodic structures could facilitate diffraction studies of biological macromolecules;[17] furthermore, the suggestion has been made that they could be used in the development of nanoscale memory devices.[18] Useful diffracting objects should contain at least $10^{10}-10^{11}$ unit cells (the smallest diffracting macromolecular crystal of which we are aware[19]), useful memory units should be larger yet (10^{15} unit cells is the minimum size envisioned[18]), and practical materials may need to be very large indeed. By its very nature, periodic matter is a high-symmetry state; the large sizes of the most interesting and useful periodic target assemblies preclude the stepwise removal of protecting groups. Although one can envision hierarchical schemes[20] for the assembly of large constructs, the most desirable means of constructing a large object is assembly from a single building block, preferably in a single step. To achieve this goal, it is necessary to seek DNA molecules whose branches bear a fixed angular relationship to one another.

Recently, we have characterized the ligation properties of bulged 3-arm DNA branched junctions.[2] Leontis and his colleagues have shown that these molecules are more stable than conventional 3-arm branched junctions.[21] The structural basis of this finding has been confirmed by NMR[22−24] to be a stacking arrangement between two of the arms of the junction.

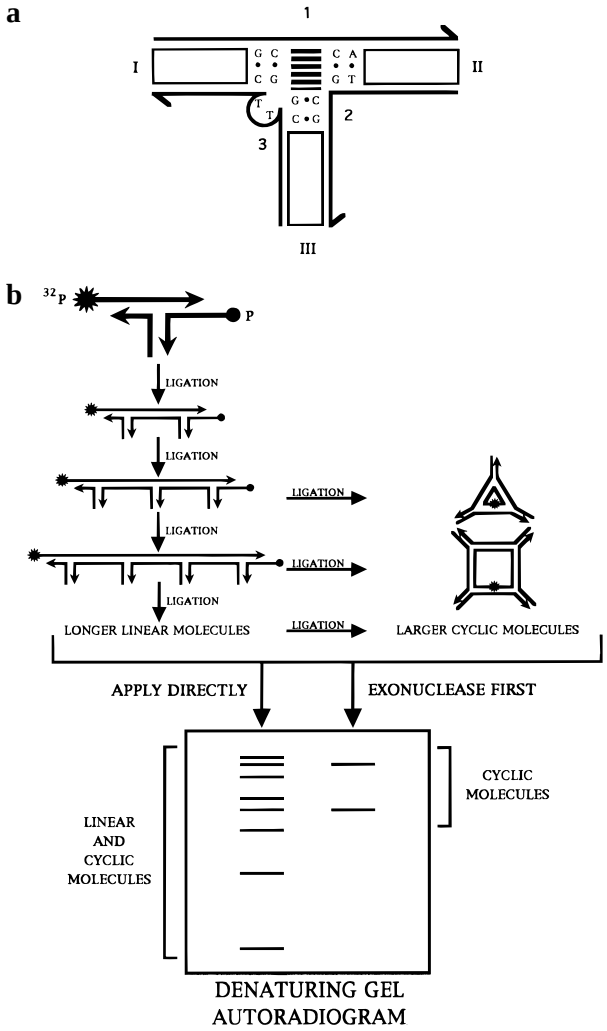

Figure 1. The components and strategies used in these experiments. (a) The bulged 3-arm branched junction. The three strands of this junction are indicated by Arabic numerals, and the arms are indicated by Roman numerals. The half-arrows indicate the 3′ ends of each strand. The $(dT)_2$ bulge is shown on strand 3. The two nucleotides closest to the Leontis-type junction are shown. The sequence beyond the junction-flanking dinucleotides is indicated schematically by a blank rectangle; although each of the junctions used in these experiments has the same junction-flanking nucleotides, each junction differs beyond that point. The stacking of arm I on arm II is indicated by the striped region. (b) The reporter strand cyclization experiment. The basic component is indicated in the upper left hand corner of this drawing. Arrows indicate the 3′ ends of the strands; a radioactive phosphate is indicated by the sunburst figure, and a non-radioactive phosphate is indicated by the filled circle. Ligation is shown through the level of the linear tetramer. In addition, the linear trimer and the linear tetramer are shown to form cyclic trimers and cyclic tetramers. Note that each product can be identified by the identification of the radioactive strand. If it is linear, its size indicates the extent of oligomerization. The same is true if it is cyclic. The bottom portion of the drawing shows how the cyclic strands are identified: The products of ligation are run on a denaturing gel by being applied directly, or after treatment by exonucleases, such as exo I and exo III, which are used here; only the reporter strands are visible in the autoradiogram. The cyclic molecules are resistant to treatment by exonucleases, whereas the linear strands are digested.

In the Leontis-type junction, the stacking occurs between the two arms that are 3' to the bulge in the cyclic sense (see Figure 1a). Whereas unbulged 3-arm branched junctions in which all possible base pairs are formed do not have a backbone long

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enough to stabilize stacking between any of the helices, the addition of two nucleotides at the junction furnishes 12 extra backbone bonds to permit stacking. Our ligation study found that cyclization occurs through this pair of arms far less often than through the other pairs of arms.[2] Thus, it is useful to test the rigidity of bulged components in DNA nanoconstruction.

Here, we examine by means of a ligation-cyclization study the utility of placing bulged 3-arm branched junctions at the vertices of equilateral triangles. In addition to using the most stable components for the branch points, it is sensible to make the target structure (a future component itself) as rigid as possible. It can be shown that a convex polyhedron is rigid if and only if each of its faces is a triangle.[25] Thus, we have coupled a potentially rigid junction with an object whose edges bear a fixed angular relationship to each other; such triangles ought to provide a good test system to examine the structural properties of bulged 3-arm branched junctions. An additional positive feature of the system is that only a single possible stacking alternative exists for each junction, in addition to the one nominally assigned by selecting the location of the bulge; this is in contrast to the free bulged junction, where there are two alternatives to the designated stacking direction.

Materials and Methods

Synthesis and Purification of DNA. All DNA molecules in this study have been synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected, using routine phosphoramidite procedures.[26] Biotin-Virtual-Nucleotide phosphoramidites were purchased from Clontech. DNA strands have been purified by electrophoresis; bands are cut out of 12−20% denaturing gels and eluted in a solution containing 500 mM ammonium acetate, 10 mM magnesium acetate and 1 mM EDTA.

Enzymatic Reactions. A. Kinase Labeling. One picomole of an individual strand of DNA is dissolved in 10 μL of a solution containing 50 mM Tris−HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol and mixed with 1 μL of 2.2 μM γ-^{32}P-ATP (10 mCi/mL) and 3 units of polynucleotide kinase [U. S. Biochemical (USB)] for 3 h at 37 °C. The reaction is stopped by heating the solution to 90 °C, which eliminates kinase activity.

B. Ligations. Ligations are performed in the kination buffer, to which 1 mM ATP has been added. The other strands are added, and the solution is heated to 90 °C and cooled slowly to room temperature; 10 units of T4 polynucleotide ligase (USB) are added, and the ligation proceeds at 16 °C for 16 h. Particular care is taken to ensure that a stoichiometric mixture is used; it is important that the labeled strand not be in excess. Stoichiometry is established on non-denaturing gels.

C. Exonuclease Treatment. Fifty units of exonuclease III (exo III) (USB) are added directly to the ligation mixture, and the reaction is allowed to proceed for 30 min at 37 °C. The solution is heated to 90 °C for 5 min and cooled on ice for 2 min to generate single-stranded DNA. Ten units of exonuclease I (exo I) (USB) are added, and the digestion is continued for 30 min at 37 °C. The reaction is stopped by phenol extraction.

D. 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 50 units of Bgl I and PflM I or 10 units of Rsa I, Nla III, Bfa I, ScrF I, Dpn II, and Mae II.

E. Topoisomerase I Treatment. Approximately 10 fmol of purified DNA are dissolved in 50 μL of a buffer containing 50 mM Tris−HCl (pH 7.5 at 25 °C), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, and 30 μg/mL bovine serum albumin (BSA). One microliter (10 units) of calf thymus topoisomerase I (BRL) is added to the DNA, mixed gently by tapping, spun for 2 s, and incubated at 37 °C for 30 min. The reaction is stopped by phenol extraction.

Streptavidin Bead Treatment. Streptavidin beads (50−100 μL) (Promega), stored at 4 °C in a solution containing Phosphate-buffered saline (PBS), 1 mg/mL BSA, and 0.02% sodium azide are put into a siliconized eppendorf tube on a magnetic stand and allowed to settle for 5−10 min. The buffer is then removed and the beads are rinsed three times with 50−100 μL of fresh PBS buffer. The beads are then rinsed twice with a solution containing 100 mM NaCl, 50 mM Tris−HCl, 10 mM $MgCl_2$, and 1 mM DTT, at pH 7.9 and 25 °C (New England Biolabs restriction buffer 3). The biotinylated DNA which has been restricted in 50−100 μL of buffer 3 is added to the beads, mixed well, and allowed to sit at room temperature for 15−30 min. The solution is separated from beads by allowing it to settle on the magnetic stand. Streptavidin particles are supplied as a 1-mg/mL suspension with a binding capacity of 0.75−1.25 nmol/mg. The amount used in this protocol is 10−20 times greater than that recommended by the manufacturer.

Polyacrylamide Gel Electrophoresis. A. Denaturing Gels. Standard gels contain 6−20% acrylamide (19:1, acrylamide−bisacrylamide) and either 8.3 M urea or up to 7 M urea plus 20% or 40% formamide. The running buffer consists of 89 mM Tris−HCl (pH 8.0), 89 mM Boric acid, and 2 mM EDTA (TBE). The sample buffer consists of 10 mM NaOH, 1 mM EDTA, and 0.1% Xylene Cyanol FF tracking dye. Gels are run on a Hoefer SE 620 Electrophoresis Unit at 63 °C at 500 V/32 cm or 500 V/16 cm, constant voltage, dried onto Whatman 3MM paper, and exposed to Kodak X-OMAT AR film for up to a week. Autoradiograms are scanned with a Hoefer GS300 densitometer in transmission mode in order to estimate relative yields of products.

B. Nondenaturing Gels. Gels contain 5% acrylamide (19:1, acrylamide−bisacrylamide). DNA is suspended in 10 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). When triangle restriction is shown, the sample buffer used is the restriction buffer. Tracking dye containing TAEMg, 50% glycerol, and 0.02% each of Bromophenol Blue and Xylene Cyanol FF is added to the sample buffer. Gels are run on a Hoefer SE-600 gel electrophoresis unit at 16 V/cm at 4−37 °C and are stained with Stainsall dye or exposed to Kodak X-OMAT AR film.

Results

Design of the Triangles. The goal of the experiments performed here is to determine whether triangles whose vertices are bulged 3-arm branched junctions can function as rigid components in DNA nanoconstruction. We evaluate them by means of a ligation closure experiment, as done previously with individual junctions.[2,13,14] In previous experiments, we have used the presence of reporter strands to indicate the ultimate results of the experiment: If the reporter strands are linear, the product is linear; if the reporter strands are cyclic, then a cyclic product has formed. The two species are distinguished by their susceptibilities to exonucleases. This strategy is illustrated in Figure 1b.

This experiment requires that a reporter strand traverse the entire cycle that is to be formed from the ligation of the bulged triangles. The easiest experiment to design involves equilateral triangles with an integral number of turns between vertices, although it is possible to design other triangles, as well.[2] In such triangles, there are two strands, an inside strand, and an outside strand, as shown in Figure 2. Figure 2 indicates that there are two varieties of equilateral triangles, those with the bulges on the inside strand (I-type) and those with the bulges on the outside strand (O-type).[2] Although it is not obvious from inspection of two-dimensional projections, model building[27] reveals that a reporter strand will traverse the central cycle only if the two types of triangles alternate around the periphery of the product. This point is illustrated in Figure 3. Previously, we have shown that it is easiest to work with DNA geometrical

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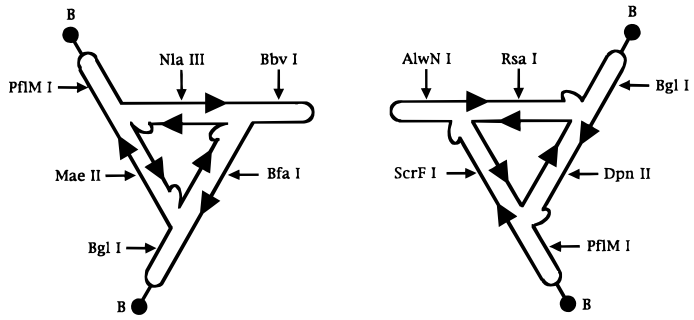

Figure 2. Equilateral triangles built from three bulged 3-arm DNA branched junctions. Two triangles are illustrated. Each contains a bulged 3-arm branched junction at each vertex. The arrowheads indicate the strand polarities, and the bulges are indicated schematically as rounded segments of the chain. The two triangles shown are 3-fold symmetric equilateral triangles, with the same number of double helical turns in each edge. The strand structure has been simplified so that it is drawn as parallel lines. This is valid, so long as each edge contains an integral number of double helical turns. These are not the only triangles possible; asymmetric equilateral triangles and 3−4−5 right triangles appear to be valid alternatives.[2] The triangles are drawn as closed catenanes. The two triangles differ in the placement of the bulges, they are on the inside strand on the left, and on the outside strand on the right. The right-most junction in the triangle on the left and the left-most junction in the triangle on the right are viewed from the same side: The horizontal stacking helices are parallel, and they differ because the bulge is enclosed by a 60° angle on the left and by a 120° angle on the right. Whereas the triangles are closed catenanes, they could not be ligated together unless the ends of the helices were restricted. Restriction sites are indicated on each of the triangles; they are used both for purposes of ligation and for purposes of structure analysis. The exocyclic arm containing the AlwN I site also contains a Bbv I site. The **B** on two of the extended arms indicates the presence of a pair of biotin groups at each site.

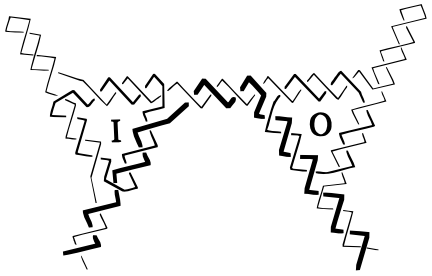

Figure 3. A helical diagram representing the reporter strand formed by combining I-type and O-type bulged triangles. An I-type triangle is shown on the left, and an O-type triangle is shown on the right, as indicated by the **I** and the **O** at their centers. Each triangle is composed of three separate strands: (1) a very thick strand that constitutes the reporter strand; (2) a cyclic strand of intermediate thickness that forms the central core of the each triangle; and (3) a strand containing a hairpin drawn in a thin line. The two triangles have been ligated together to indicate that the reporter strand is associated with both sticky ends, as it must be if it is to fulfill its role.

objects that are topologically closed.[7] Thus, we have designed the triangles to be prepared as cyclic molecules to be restricted, thereby generating cohesive ends to be used in the ligation.

The ultimate product molecules are complex catenanes, such as the hexamer illustrated in Figure 4a; the hexamer is shown schematically, along with its restriction enzyme sites in Figure 4b. This is a cyclic trimer of the I-type:O-type triangle dimer illustrated in Figure 3. In order to establish its identity, the reporter strand would have to be liberated by restricting an edge of each of the six triangles shown. Restriction at those positions would cleave the cyclic molecules (drawn with a line of intermediate thickness in Figures 3 and 4) associated with each triangle, as well as the long external strand. Thus, each of the

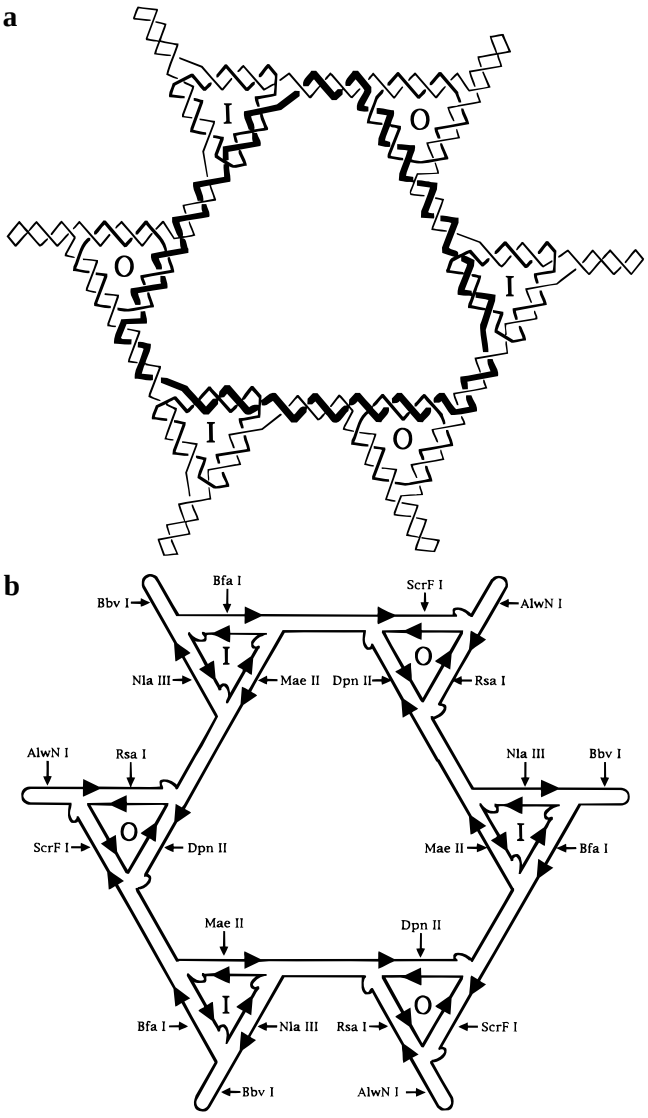

Figure 4. The target molecule to be formed if the bulged triangles are rigid components. (a) A helical diagram of the target molecule. This molecule is clearly a cyclic trimer of the pair of molecules illustrated in Figure 3. The same conventions apply here; in particular, the reporter strand is thicker than the other strands. The I-type and O-type triangles are indicated by the **I** and the **O** symbols at their centers. Note that the very thin strand at the outside could, in principle, also be used as a reporter strand. Note that this structure is a complex catenane of 8 strands, and that the reporter strand can only be freed from the catenane by cleavage of the triangular central strands, along with cleavage of the outer strand. (b) A schematic diagram indicating restriction sites. This is a schematic diagram of the structure shown in part a. The bulges are shown at each of the vertices. The arrowheads indicate strand polarities. The counterclockwise 3-fold symmetric strand at the center is the reporter strand. Regardless of the size of the cycle, this strand could be freed from the complex catenane by cleavage by ScrF I and/or Rsa I, accompanied by cleavage by Nla I and/or Bfa I. Note that each triangle has two vertices that abut the reporter strand so that all twelve vertices would have to maintain their stacking structures for this structure to form.

triangles contains restriction sites on each of its edges, although only two edges need be cleaved for the analysis (Figure 2).

The restriction sites used for ligation product analysis are conventional "palindromic" (2-fold symmetric) restriction sites. By contrast, one gains the greatest control over intermolecular reactions if the restriction sites do not produce symmetric sticky ends.[7,8] Consequently, we have used Bgl I and PflM I sites to generate the sticky ends, to be used for ligation sites. The sticky end exposed by Bgl I scission on the I-type triangle is

Table 1. Sequences of Bulged Triangles

I-TYPE-10 TRIANGLE

Inside Strands

Strand 1:

5'-T C G G T C G T T G C A G G T T-3'

Strand 2:

5'-A C C A C A T G G A G C G T T G C T T C A C G T A G C C-3'

Strand 3:

5'-T G G T C T A G G T C C G T T G C C T A C A-3'

Outside Strands

Strand 1:

5'-A G A C C A A A C C T G C C A C C G C C A C A G T G G C T C T T C C A A G T C A C C G T T T B T T T B T C G G T G A C T T G G A A G A G C C A C T G T G G C G G T G C G A C C G A G G C T A C-3'

Strand 2:

5'-G T G A A G C C A T A C C A T C G C T T G G A A C T C A C T A C C A T T T T B T T T B T A T G G T A G T G A G T T C C A A G C G A T G G T A T G C G C T C C A-3'

Strand 3:

5'-T G T G G T T G T A G G C C A A C T T C G T C C A C G A G C T T G C T G C T A T G T T C C T C T T T T G A G G A A C A T A G C A G C A A G C T C G T G G A C G A A G T T G C G G A C C T-3'

I-TYPE-11 TRIANGLE

Inside Strands

Strand 1:

5'-T C G G T C G T T G C A G G T T-3'

Strand 2:

5'-A C C A C A T G G A G C G T T G C T T C A C G T A G C C-3'

Strand 3:

5'-T G G T C T A G G T C C G T T G C C T A C A-3'

Outside Strands

Strand 1:

5'-A G A C C A A A C C T G C C A C C G G C C A C A G T G G C T C T T C C A A G T C A C C G T T T B T T T B T C G G T G A C T T G G A A G A G C C A C T G T G G C C G G T G C G A C C G A G G C T A C-3'

Strand 2:

5'-G T G A A G C C A T A T C C A T C G C T T G G A A C T C A C T A C C A T T T T B T T T B T A T G G T A G T G A G T T C C A A G C G A T G G A T A T G C G C T C C A-3'

Strand 3:

5'-T G T G G T T G T A G G C C A A C T T C G T C C A C G A G C T T G C T G C T A T G T T C C T C T T T T G A G G A A C A T A G C A G C A A G C T C G T G G A C G A A G T T G C G G A C C T-3'

I-TYPE-11 TRIANGLE

Inside Strands

Strand 1:

5'-T C G G T C G T T G C A G G T T-3'

Strand 2:

5'-A C C A C A T G G A G C G T T G C T T C A C G T A G C C-3'

Strand 3:

5'-T G G T C T A G G T C C G T T G C C T A C A-3'

Outside Strands

Strand 1:

5'-A G A C C A A A C C T G C C A C C G G C C A C A G T G G C T C T T C C A A G T C A C C G T T T B T T T B T C G G T G A C T T G G A A G A G C C A C T G T G G C C G G T G C G A C C G A G G C T A C-3'

Strand 2:

5'-G T G A A G C C A T A T C C A T C G C T T G G A A C T C A C T A C C A T T T T B T T T B T A T G G T A G T G A G T T C C A A G C G A T G G A T A T G C G C T C C A-3'

Strand 3:

5'-T G T G G T T G T A G G C C A A C T T C G T C C A C G A G C T T G C T G C T A T G T T C C T C T T T T G A G G A A C A T A G C A G C A A G C T C G T G G A C G A A G T T G C G G A C C T-3'

O-TYPE-11

Inside Strands

Strand 1:

5'-T C A T C T G C G G A C C T-3'

Strand 2:

5'-T G G T G T A C C A G T G C G C T A T C G T A G C C-3'

Strand 3:

5'-T G G A A T G C G C C G T A A C C G G A-3'

Outside Strands

Strand 1:

5'-G A T A G C G T T G C C G C T T G C A G G C G C T G T T G C T G C T T T T G C A G C A A C A G C G C C T G C A A G C G G C C A C T G G T-3'

Strand 2:

5'-A C A C C A A G G T C C G T T G C G A C C G C C A G C G A G G C A C A T A G T G T C A T T T T B T T T B T A T G A C A C T A T G T G C C T C G C T G G C G G T C G C C A G A T G A T C C G G T-3'

Strand 3:

5'-T A C G G C G T T G C A C T A C C A C C T G A T G G C T T C T A C A A A C T C C T T T B T T T B T G G A G T T T G T A G A A G C C A T C A G G T G G T A G T G C C A T T C C A G G C T A C-3'

complementary to the sticky end exposed by PflM I on the O-type triangle, and *vice versa*. These enzymes recognize interrupted palindromes that produce 3-nucleotide sticky ends that can be designed to be asymmetric. It has been our experience that restriction at these sites is often incomplete.[7] In previous synthetic work,[8] incomplete restriction has led to decreased yields. However, in this analytical experiment, unrestricted triangles will act as a poison in the system, because they will terminate ligations. In order to remove any unrestricted molecules, we have attached biotin groups to the ends of the hairpins to be removed by restriction. If a hairpin is unrestricted or only nicked, its entire triangle can be removed by treating the solution with streptavidin-coated magnetic beads.

The sequences of the triangles were designed by use of the program SEQUIN:[28] Each of the restriction sites was incorporated as needed. The two nucleotides closest to the Leontis bulged junction site (Figure 1a) were used for each junction, in agreement with previous studies indicating that the nucleotides closest to the junction determine its structure.[29] T_4 loops were used for the external arms, and the biotin groups were incorporated into nine-nucleotide loops of the sequence T_3BT_3-BT. All other sequences were chosen with the aid of the program to minimize sequence symmetry. The inter-triangular segments were chosen to be 20, 21, or 22 nucleotides long, as a hedge against the possible variability of the helicity of the DNA. Thus, two types of I-type triangles, I-type-10 and I-type-11, were synthesized, as well as two types of O-type triangles, O-type-10 and O-type-11; the combination of I-type-10 and O-type-10 generates 20 nucleotide pairs between the triangles, and the combination of I-type-11 and O-type-11 triangles generates 22 nucleotide pairs between the triangles; and combining either type-10 with either type-11 generates 21 nucleotide pairs between the triangles. Unless otherwise noted, triangles are type-10 triangles. The sequences of the strands used to make the triangles are listed in Table 1.

Construction and Analysis of the Triangles. The initial components of each triangle consist of individual 3-arm junctions composed of a single strand (to form the inside strand of the triangle) and a hairpin-containing strand that will hybridize to it to form the junction; the three hairpin-containing strands form the outside strand of the triangle. Figure 5a

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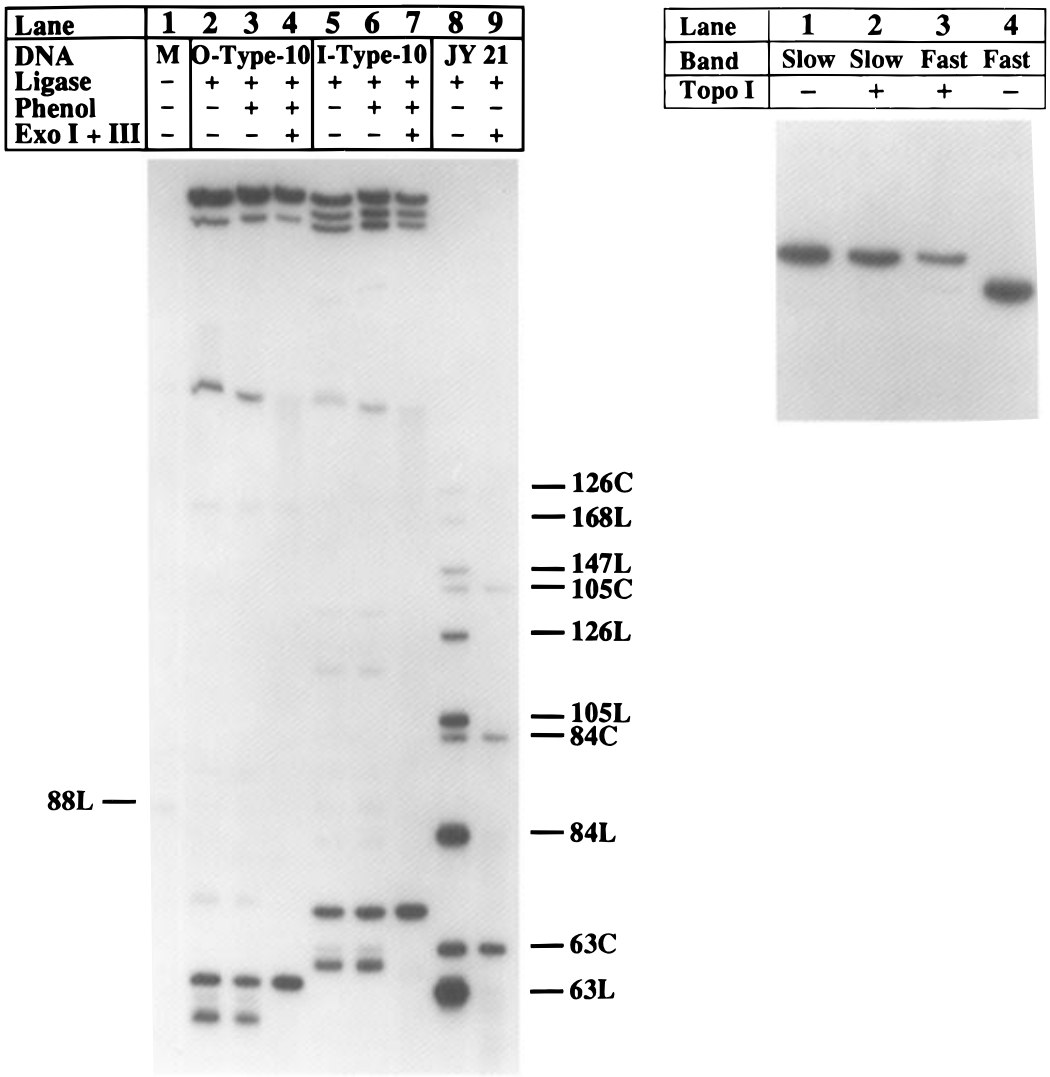

Figure 5. Assembly of the triangles. (a, left) Ligation of the components. This is an autoradiogram of a denaturing gel, containing 20% formamide, which shows the products of triangle ligation. The contents of each lane are indicated above the lane. Lane 1 contains a linear marker, lane 8 contains previously characterized linear (**L**) and cyclic (**C**) markers derived from a 3-arm branched junction called JY21,[34] and lane 9 contains the material in lane 8 treated with exonucleases I and III. Lane 2 contains the raw ligation products for the O-type triangle, lane 3 contains the products of phenol-extracting the material in lane 3, and lane 4 contains the results of treating the material in lane 3 with a combination of exo I and exo III. Lanes 5−7 respectively contain the same products for the I-type triangle. The target triangles are the top bands. The band directly below the O-type triangle lacks the inner circle. The band two below the I-type triangle also lacks the inner circle. (b, right) Characterization of the extra band in the I-type triangle assembly. The top band is labeled the slow band, and the band directly below this band in the I-type ligation is called the fast band. Treatment of the slow band (lane 1) generates no new product (lane 2). However, treatment of the fast band (lane 4) shows that it can be converted to the slow band (lane 3). Thus lane 4 is a topoisomer of the material in lane 1.

illustrates the assembly of the individual triangles. Lanes 2−4 show the results of ligating the O-type triangle, and lanes 5−7 show the results of ligating the I-type triangle. Lanes 4 and 7 illustrate the exonuclease-resistant products of these ligations. It is clear that three species are present in lane 4; restriction analysis (data not shown) indicates that they are the inner 60-mer circle, the outer 262-mer circle, and the product triangle. The two circles are expected failure products, seen when one or the other of the two circles fails to ligate. In addition to these three species, lane 7 contains a fourth species, which moves faster than the target triangle. When restricted, this band produces the same products as the slower-migrating target triangle (data not shown).

This finding suggests that the extra band is a topoisomer of the target triangle. The linking number of two chains is the number of times that one chain must be passed through the other in order to separate the chains: The expected linking number between the inner and outer strands is 6, but the material in this band moves faster than the presumptive target, suggesting that its linking number is greater.[30] In order to test this hypothesis, we have treated it with calf thymus topoisomerase I (topo I), and the results of the experiment are shown in Figure 5b. Lanes 1 and 4 in the gel respectively contain the target triangle and the extra band. Lane 2 contains the results of treating the target triangle with topo I and lane 3 contains the results of treating the extra band with topo I. No reaction is seen to occur in lane 2, but the material in lane 3 is seen to be converted to the slower moving material, corresponding to a decrease in linking number. *E. coli* topo I is ineffective in promoting this reaction, possibly because there is not enough single-stranded DNA available to act as a binding site.[31] The inner strand contains an extra six nucleotides, which could lead to the generation of this topoisomer. It is possible to purify the target molecule from its topoisomer, and this has been done in all work involving the I-type triangle.

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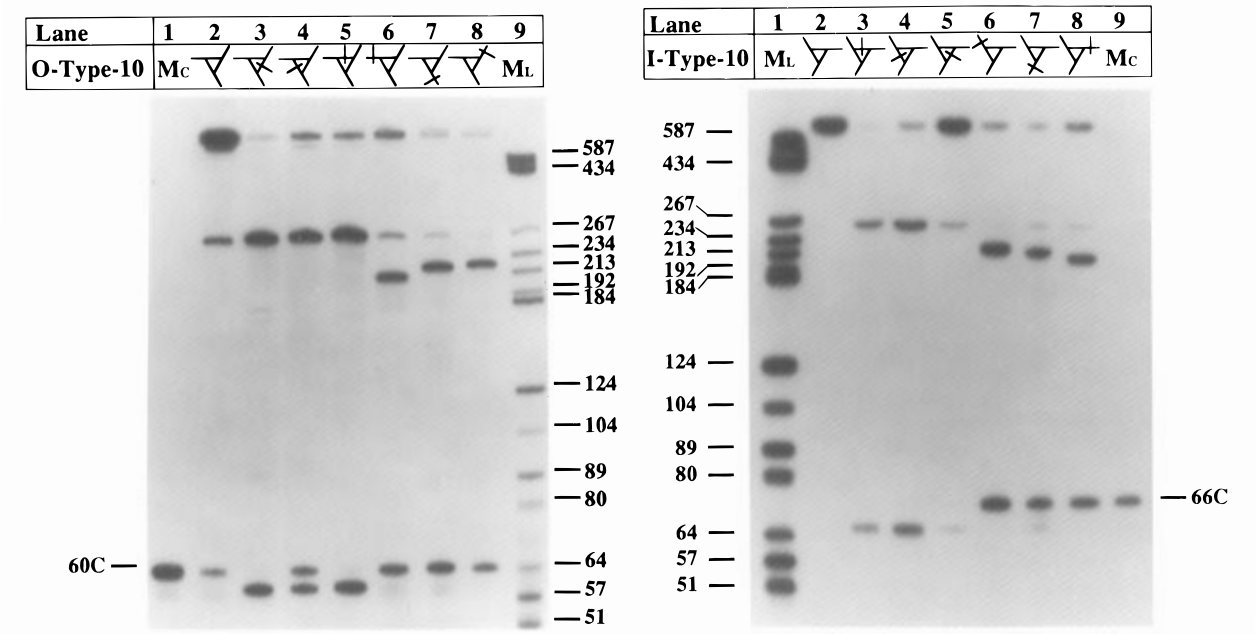


Figure 6. Characterization of the Triangles. These are both autoradiograms of 20% formamide denaturing gels. Circular markers are in a lane labeled "Mc" and linear markers are in a lane labeled "ML". Above each lane a triangle, including its external arms is drawn schematically (e.g., lane 2). The site of scission by a restriction endonuclease is indicated by a line perpendicular to the triangle, either through an edge (lanes 3−5), or through an external arm (lanes 6−8). (a, left) The O-type triangle. Lane 1 contains a 60-mer circular marker, and lane 9 contains a pBR322-Hae III digest that generates linear markers. The material in lane 2 is undigested O-type triangle. Some breakdown of the triangle is seen in this lane. Lanes 3−5 respectively contain the digestion of this triangle by the edge-cleaving restriction enzymes Dpn I, ScrF I, and Rsa I. Lanes 6−8 respectively contain the digestion of this triangle by external-arm digestion enzymes Bbv I, Bbs I, and BsmA I. These sites were later modified to those shown in Figure 2, when the enzymes were found to be ineffective for purposes of ligation. Note that the products of edge-cleavage are all the same, a linear 60-mer and a linear 262-mer. Some of the circle remains intact upon cleavage by ScrF I, because it is only nicking. Cleavage of the external arms leads to 60-mer circles and linear molecules of various lengths, depending on the site of scission. These enzymes are also seen to nick somewhat. (b, right) The I-type triangle. Lane 1 contains a pBR322-Hae III digest, lane 2 contains undigested material, and lane 9 contains a 66-mer cyclic marker. Lanes 3−5 respectively contain the products of the edge-cleaving enzymes Nla III, Mae II, and Bfa I. Lanes 6−8 respectively contain the products of the external arm cleaving enzymes BsmA I, Bbs I, and Bbv I. The same results seen in part a are seen here: edge-cleavage produces linear molecules of the same length (66 and 256 nucleotides), and external arm cleavage generates circles and variable-length linear molecules.

We have analyzed the constructions of the two triangles by means of restriction analysis. Figure 6a shows the restriction of the O-type triangle by six restriction enzymes. Lanes 3−5 correspond to restriction of the edges of the triangle, resulting in the same length linear outer molecule and the same length linear inner molecule. Lanes 6−8 show the restriction of the three hairpins, generating outer strand fragments of different lengths (as a function of the placement of the restriction sites), and 60-mer circles, corresponding to the inner strands. The incompleteness of cleavage results in a substantial amount of circle in lane 4, and a small amount of singly nicked outer strand in lanes 6−8.

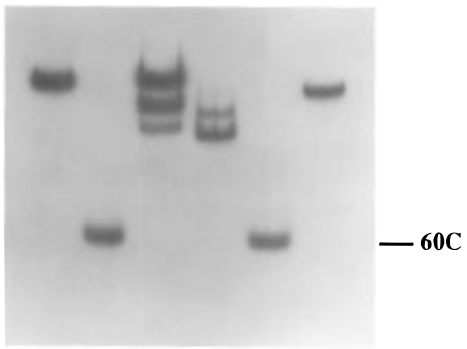
Figure 6b shows the analogous experiments for the I-type triangle. Lanes 3−5 illustrate the cleavage of the triangle's edges, and lanes 6−8 contain the products of digesting the external hairpins. Again, the restriction of the edges produces bands of equal length, and restriction of the hairpins produces variable-length linears and constant-size 66-mer circles. Cleavage of the inner circles is more efficient in this gel, but a substantial amount of uncleaved material remains from the restriction of the hairpins.

Generation of Cleaved Triangles. Having demonstrated that it is possible to generate the triangles, and to purify away improper topoisomers, it is now necessary to show that it is possible to generate triangles with sticky ends. Two issues are involved: (1) it must be possible to produce triangles that remain intact without dissociating after cleavage; and (2) incompletely cleaved molecules must be removed from the solution, because they will poison the reaction. Figure 7a illustrates the first point. This is a non-denaturing gel, in which the outer lanes contain the I-type (lane 1) and the O-type (lane 6) triangles. Their inner circles are shown in the adjacent lanes, the 66-mer circle in lane 2 and the 60-mer circle in lane 5. The products of digesting each triangle with both PflM I and Bgl I are contained in lanes 3 (I-type) and 4 (O-type). The bottom band in each of these lanes corresponds to the doubly restricted product. There is clearly no material beneath these bands in either of lanes 3 and 4, demonstrating that the triangles have not dissociated. Thus, the two doubly-cleaved triangles are stable species, ready for use in a ligation experiment.

By contrast to the lack of dissociated material in lanes 3 and 4, there is a large amount of incompletely digested material in each of those lanes. Lane 3 contains substantial material that is undigested, and although lane 4 is largely free of undigested triangles, both lanes contain material that is only singly digested. It is necessary to remove this material, to remove nicked but uncleaved molecules, and to remove the hairpins that result from successful scissions. Any of these species can act as a poison for the closure experiment. Treatment of restricted molecules by streptavidin beads permits us to purify all these materials away from the triangles that are going to be ligated together.

Figure 7b illustrates the removal of unwanted material by biotin-streptavidin bead treatment. This denaturing gel illustrates the streptavidin bead purification of four mixtures of restricted I-type and O-type triangles. Lanes 1, 4, 7, and 10 contain respectively I-type-10, O-type-10, I-type-11, and O-type-11 triangles before restriction. Lanes 2, 5, 8, and 12 contain respectively the restricted combinations of triangles [I-type-10

Lane	1	2	3	4	5	6
I-Type-10	+	−	+	−	−	−
O-Type-10	−	−	−	+	−	+
Bgl I + PflM I	−	−	+	+	−	−



Lane	1	2	3	4	5	6	7	8	9	10	11	12
I-Type-10	+	+	+	−	−	−	−	−	−	−	+	+
O-Type-10	−	+	+	+	+	+	−	−	−	−	−	−
I-Type-11	−	−	−	−	+	+	+	+	+	−	−	−
O-Type-11	−	−	−	−	−	−	−	+	+	+	+	+
Bgl I + PflM I	−	+	+	−	+	+	−	+	+	−	+	+
Streptavidin	−	−	+	−	−	+	−	−	+	−	+	−

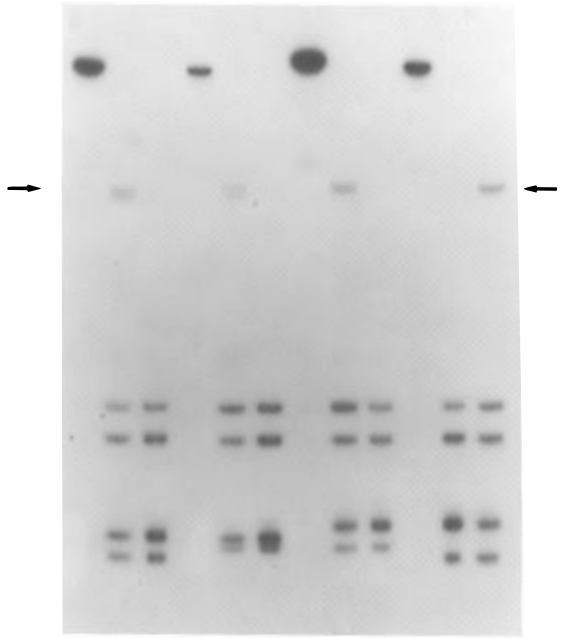


Figure 7. Cleavage of the triangles. The material in each lane and its treatment are indicated above it. (a, left) Intact nature of cleavage products This is a non-denaturing gel illustrating that the products of cleavage do not decompose visibly on gels. Lane 1 contains the I-type triangle, and lane 6 contains the O-type triangle. Lane 2 contains the 66-mer circle at the center of the I-type triangle, and lane 5 contains the 60-mer circle at the center of the O-type triangle. Lane 3 contains the products of digesting the I-type triangle with its two exocyclic arm restriction enzymes, PflM I and Bgl I, and lane 4 contains the products of digesting the O-triangle with the same enzymes. In each case, the lowest band present is the double-digestion product, and no circle is seen on the gels, indicating that the triangles are intact after cleavage. Note that a fair amount of material remains undigested. (b, right) Removal of undigested material by streptavidin. This is an autoradiogram of a 40% formamide denaturing gel. Lane 1 contains the I-type-10 triangle, lane 4 contains the O-type-10 triangle, lane 7 contains the I-type-11 triangle, and lane 10 contains the O-type-11 triangle. Lanes 2, 5, 8, and 12 contain respectively the Bgl I and PflM I digestion products of [I-type-10 + O-type-10 triangles], [I-type-11 + O-type-10 triangles], [I-type-11 + O-type-11 triangles], and [I-type-10 + O-type-11 triangles]. Prominent in each of these lanes is a certain amount of incompletely digested material, as indicated by the arrows on the sides of the figure. Lanes 3, 6, 9, and 11 contain the products of treating the material in the adjacent lanes (2, 5, 8, and 12) with streptavidin beads. The incompletely digested material is completely removed. An earlier version of triangles containing a single biotin in the hairpin loops left about 2−3% of the incompletely digested material.

+ O-type-10], [I-type-11 + O-type-10], [I-type-11 + O-type-11], and [I-type-10 + O-type-11]. The molecules contain radioactive labels in the inner circles and in the strand that will be part of the reporter strand, but the rest of the triangle is unlabeled; consequently, there should be four bands visible in each of these lanes, corresponding to the two circles and the two reporter strands. However, there is extra labeled material in each lane, marked by the arrows that flank the gel. Lanes 3, 6, 9, and 11 contain the material in lanes 2, 5, 8, and 12, but treated with streptavidin beads. It is clear that the bead treatment eliminates these unwanted molecules, and permits us to perform the ligation experiment.

Ligation of the Triangles. Following restriction and treatment with the streptavidin beads, the I-type and O-type triangles were ligated together. Figure 8a is a denaturing gel that illustrates the results of ligating the triangles together. Lanes 3, 4, and 5 contain ligation products that have been treated with a combination of exo I and exo III; the material in lane 5 is concentrated by ethanol precipitation before ligation. Lanes 1 and 2 contain a ladder of linear reporter strands from unclosed products. Exonuclease resistant bands are labeled with the first six letters of the Greek alphabet (α, β, γ, δ, ϵ, ζ), in order of increasing mobility. With the possible exception of lane 4, the most prominent band is the second band from the top of the gel (β). As will be demonstrated below, this band contains a tetramer of the triangles. This is a hexacatenane, consisting of an outer circle, an inner reporter circle, and four circles from the inner circles of the triangles. The four exonuclease-resistant bands below it (γ, δ, ϵ, ζ) are missing one or more of the cyclic molecules. For example, restriction of the major band by AlwN I, to remove the outer circle, results in band δ; the band below it (ϵ) is a minor product of that same restriction. Likewise, the lowest product band (ζ) is the cyclic dimer of triangles, because cleavage with AlwN I and Dpn II results in a linear 82-mer reporter molecule.

The key point is to determine the size of the major cyclic product molecule. Restriction of the major band by a combination of the edge-cleaving restriction enzymes (Nla III and Bfa I for the I-type triangles, ScrF I and Rsa I for the O-type triangles) frees the cyclic reporter strand, which can be sized on a denaturing gel. We have done this ligation with four molecular pairs, to generate different spacings between the triangles: 20 nucleotides, 21 nucleotides (11 from an O-type triangle), 21 nucleotides (11 from an I-type triangle), and 22 nucleotides. These products are shown in lanes 1, 3, 5 and 7 of Figure 8b. Their restriction products are shown respectively

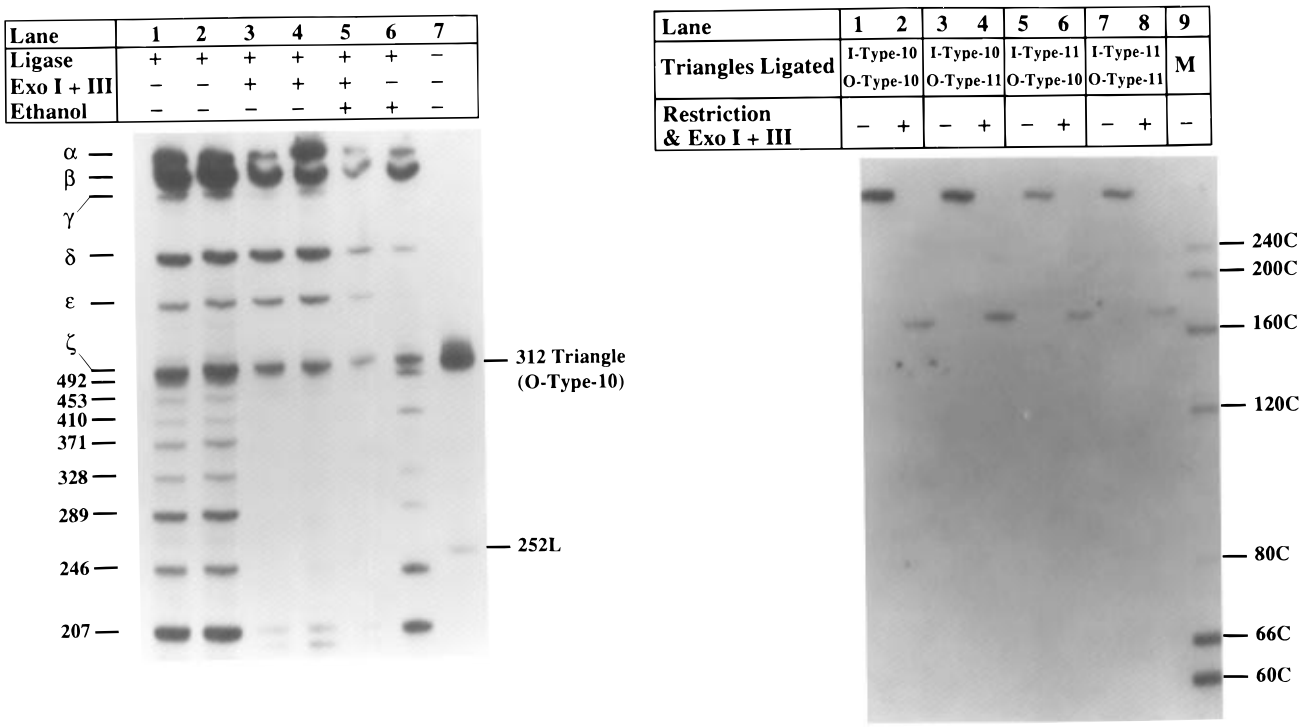


Figure 8. Ligation of the triangles. Both gels are autoradiograms of 40% formamide denaturing gels. The material in each lane and its treatment are indicated above it. (a, left) The ligation reaction. Lane 7 contains an O-type triangle marker and the 252 linear breakdown molecule corresponding to its outside strand. Lanes 1 and 2 contain the crude ligation products of the triangular ligation. The linear molecules formed generate a ladder of reporter strand bands, whose sizes are indicated on the left. Lanes 3 and 4 contain the material in lanes 1 and 2, but treated with exonucleases I and III. Lanes 5 and 6 have been concentrated by ethanol precipitation. Lane 5 has been treated with exo I and exo III, but lane 6 has not been treated with exonucleases. Exonuclease resistant bands are labeled with the first six letters of the Greek alphabet (α, β, γ, δ, ϵ, ζ), in order of increasing mobility. Among the exonuclease resistant bands, β corresponds to a complete product containing four triangles. (b, right) Characterization of the major cyclic product. Lanes 1, 3, 5, and 7 contain the major cyclic products that result respectively from ligation of [I-type-10 + O-type-10 triangles], [I-type-10 + O-type-11 triangles], [I-type-11 + O-type-10 triangles], and [I-type-11 + O-type-11 triangles]. Lanes 2, 4, 6, and 8 contain the reporter strands obtained by restricting the material in the lanes to their left (1, 3, 5, and 7). Lane 9 contains a series of cyclic markers.[14] It is clear that in each case, the major cyclic product is the tetramer, with a size near 160 nucleotides. The expected sizes for these molecules are 164, 168, 168, and 172 nucleotides.

in lanes 2, 4, 6, and 8. In each case, the band migrates similarly to circular markers[14] of about 160 nucleotides. The expected lengths of the cyclic tetramer reporter strands are respectively 164, 168, 168, and 172 nucleotides. This series of experiments is a control for possible undertwisting of the system by using I-type-10 and O-type-10 triangles. In addition, we have controlled for possible overtwisting of the system, by adding ethidium (41:1 to 51:1 ethidium−triangle, assuming saturation in non-reporter regions of the triangle) to the ligation mixture. In no case did we change the major product, the tetramer of triangles.

Discussion

Cyclization of the Triangles To Form a Tetramer as the Primary Product. The goal of this work was to establish whether or not the combination of bulged triangles with bulged 3-arm junctions would constitute a rigid component for nanoconstruction. Had this been the case, the primary product would have been the hexamer of triangles drawn in Figure 4. The main product of the ligation of intact molecules is clearly a tetramer, rather than a hexamer, so the material does not appear to have properties required of a rigid component in nanoconstruction. A band slower than the tetramer band is seen as a ligation product; this band (α, Figure 8a) may correspond to the target hexamer of triangles. However, the question asked here is the identity of the major product, so this material has not been characterized.

We do not know the direction of flexibility, although it is easiest to imagine that its axis is perpendicular to the plane of the triangle. However, we cannot exclude the possibility that the final tetramer is pyramidal, rather than planar. Nevertheless, it is important to point out that this was a very rigorous test. Each triangle contains two points where the molecule could bend. Thus, all eight of the potential bending sites in the tetramer had to remain in their intact conformations in order for ligation not to occur.

A recent study by Leontis and his colleagues[32] points out that adding extra nucleotides to 3-arm and 5-arm junctions stabilizes them, whereas adding them to 4-arm junctions destabilizes them. In each case, the more stable species is able to form stacking arrangements, and the less stable species is less able to do this. Consequently, the bulge provides the 3-arm junction (and the 5-arm junction) with stacking capabilities already present in the 4-arm junction. The 4-arm junction has already been shown to be flexible,[14] so it appears that DNA molecules with greater structural integrity will need to be utilized in order to generate components for nanoconstruction. In this regard, the work of Li *et al.*[33] offers hope that DNA components unmodified by cross-links or other perturbations can be used as components for nanoconstruction.

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Purification of Incompletely Restricted Materials by Streptavidin Bead Treatment. An important feature of this work is that incompletely restricted material can be removed because of its biotin label. Treatment of the restriction digest with streptavidin allows for the purification of restricted material away from partially restricted and unrestricted material. In addition, completely restricted hairpins, which are removed readily from solid supports,[7,8] can also act as a poison for solution ligation of restricted objects. Although no label was present to detect hairpins, we have no evidence that they interfered with subsequent ligations. We found that a single biotin group per hairpin was inadequate to purify the system completely (data not shown); that is the reason we used two in each loop.

In a future scenario of lattice construction in solution, one can imagine building complex objects, such as topologically closed deltahedra (polyhedra whose faces are all triangular),[33] on solid supports, releasing them into solution, and annealing them to closure.[7] They could then be purified under the denaturing conditions that work best for this purpose.[6−8] Following purification, they could be restricted in solution, and have their hairpins removed by streptavidin treatment. Solution-phase ligation in the presence of restricted hairpins often results in their reattachment (Y. Zhang, JQ and NCS, unpublished). Thus, it will be possible to perform a solution-based lattice construction with freshly restricted materials, utilizing the streptavidin purification of restricted objects that has been developed here.

Topoisomers of Bulged Triangles. An important assumption about the assembly of DNA objects has been shown to be unreliable here. The unique expected topoisomer of DNA objects has always been obtained from constructions of DNA objects in the past.[6−8] However, in the assembly of the I-type triangle, two different topoisomers were detected. The constructions performed here are the first ones in which the same number of nucleotides were not used on both strands of every edge of an object. This experience should be taken as a warning that one must always check for the correct topoisomers in DNA construction, as well as for the correct stoichiometry of strands. It is encouraging that denaturing gel electrophoresis is able to detect this subtle difference between two synthetic objects of identical mass, ca. 100 kd.

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