Key Experimental Approaches in DNA Nanotechnology

Nanotechnology is concerned with controlling the structure of matter on the nanometer scale. There are many different chemistries that can lead to this endpoint. One that has proved to be very effective is DNA nanotechnology. The chemical features of DNA that make it such a successful molecule when functioning as the genetic material of living organisms can be exploited conveniently to produce a tractable system for nanotechnology. Of course, double-helical DNA is a linear molecule, and it would be hard to produce interesting or useful structural species from it. However, it is possible to design synthetic sequences that hybridize to produce unusual motifs based on DNA branching. DNA nanotechnology is an endeavor that combines these unusual DNA motifs with cohesive (sticky) ends to yield nanoscale structures (Seeman, 1982). These structures include stick polyhedra (Chen and Seeman, 1991a; Zhang and Seeman, 1994), knots (Mueller et al., 1991; Du et al., 1995a), links (Mao et al., 1997), molecular devices (Mao et al., 1999a; Yurke et al., 2000), and both crystalline (Winfree et al., 1998; Liu et al., 1999; Mao et al., 1999b; LaBean et al., 2000; Sha et al., 2000a) and aperiodic (Mao et al., 2000) arrays in one and two dimensions.

DNA is clearly a nanometer-scale object. Its diameter is ~20 Å, and its helical repeat consists of 10 to 10.5 nucleotide pairs spaced 3.4 Å apart. It should be clear that this scale is roughly an order of magnitude larger than the scale that is exploited typically by synthetic organic chemists. What purposes could be served by construction on this scale? The key targets that have been described so far utilize DNA as architecturally robust and convenient scaffolding for other species; the expectation is that this system will be used in the crystallization of biological molecules that cannot otherwise be crystallized (Seeman, 1982) and in the construction of extremely dense nanoelectronic devices (Robinson and Seeman, 1987). Molecular devices are being developed from DNA with the goal of nanorobotics, and aperiodic assembly is likely to contribute to DNA-based computation.

Figure 12.1.1 is a schematic illustrating a branched DNA molecule, similar to a Holliday (1964) junction in genetic recombination. The methods currently used to select sequences to produce stable branched molecules are described below. The molecule is shown with four sticky ends, and the right half of the diagram illustrates four of these branched molecules cohering to produce a quadrilateral. The quadrilateral contains sticky ends on its periphery, so the quadrilateral could serve as the nucleus of an infinite two-dimensional lattice. Most readers of this series are familiar with the fact that in appropriate conditions complementary sticky ends will cohere to bring two DNA molecules together. Less well appreciated is the fact that sticky ends cohere to produce B-DNA when they pair (Qiu et al., 1997). Thus, not only does DNA generate motifs that can serve to produce connected networks (Wells, 1977) and objects with predictable topology, but sticky ends lead to locally predictable geometry when they cohere. The extent of predictability available from DNA is contrasted usefully with that available from, for example, antibodies. Although the structures of these proteins have been well established, the relative orientations of an antibody and its antigen cannot be predicted reliably and must be determined from experiments such as X-ray crystallography.

In addition to the remarkable predictability of the geometry of intermolecular interactions available from DNA, these interactions have numerous other advantages as a nanotechnological system. Prominent amongst these is the ease with which DNA may be synthesized using extremely robust chemistry (Caruthers, 1985); in addition to standard DNA, many unusual bases and derivatized bases are readily available for special purposes. It is easy to purify targets and to troubleshoot experiments using the DNA-modifying enzymes that are also commercially available, including exonucleases that digest all but cyclic molecules, restriction enzymes that cleave at specific sequences, ligases that can cement sticky-ended cohesion to covalency, and topoisomerases that can solve linkage issues. The existence of these enzymes makes it easy to troubleshoot experiments. Other backbones, such as RNA or peptide nucleic acids (PNAs; Neilsen, 1995; *UNIT 4.11*), may ultimately be superior for specific applications, but the absence of similar enzymes for these systems militates at present for concentrating on DNA. In addition, DNA is locally a stiff molecule: its persistence length

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is ~500 Å under standard conditions (Hagerman, 1988), and the typical lengths used in DNA nanotechnology are two to three turns between branch points (~70 to 100 Å). A further feature of DNA is that it has an externally readable code, even when its strands are wrapped tightly into double helices (Seeman et al., 1976).

THE GENERATION OF NEW DNA MOTIFS

Branching of DNA Molecules

It was noted above that the DNA molecule is inherently linear because its helix axis is unbranched. However, it is easy to produce DNA molecules branched at the level of their secondary structures by performing reciprocal cross-overs between two double helices. Figure 12.1.2A illustrates the nature of the reciprocal cross-over operation that leads to DNA branching (Seeman, 2001). The effects of the reciprocal cross-over event between two duplex molecules are shown in Figures 12.1.2B and 12.1.2C. The antiparallel orientation of the DNA strands leads to two possible ways to perform the operation: between strands of the same polarity, leading to a parallel molecule (panel B), and between strands of opposite polarity, leading to an antiparallel molecule (panel C). Parallel and antiparallel can be understood as terms that describe the phasing of the connections between helices. In the case of a single exchange event, the molecules in panels B and C are identical. However, if one performs the operation twice, five isomers are formed, including features that distinguish the fusion of parallel or antiparallel strands and the separation of the cross-over points (Figure 12.1.3; see legend for abbreviations describing cross-over events). Although the single-cross-over molecules shown in Figures 12.1.1 and 12.1.2 are structurally flexible in the angles around their branchpoints (Petrillo et al., 1988), the double-cross-over (DX) molecules (Fu and Seeman, 1993) shown in Figure 12.1.3 have been shown to be quite stiff (Li et al., 1996). The DAE+J species in Figure 12.1.3, which contains an extra helical domain, is also stiff and can be incorporated into periodic arrays (Winfree et al., 1998) to produce patterns that are visible by atomic force microscopy (AFM) if its extra helix axis is directed normal to the plane containing the other helices.

In addition to molecules containing double-cross-overs, DNA motifs have been developed with three co-planar helices, known as triple-cross-over (TX) molecules (LaBean et al., 2000), and even a six-helix bundle has been reported (Mathieu et al., 2001). The antiparallel molecules with short separations between their cross-over points are much more robust than parallel molecules (Fu and Seeman, 1993); however, a parallel species in which every possible cross-over is present, known as paranemic cross-over (PX) DNA (Seeman, 2001), is well behaved. Paranemic DNA refers to the interaction of DNA duplex molecules with single- or

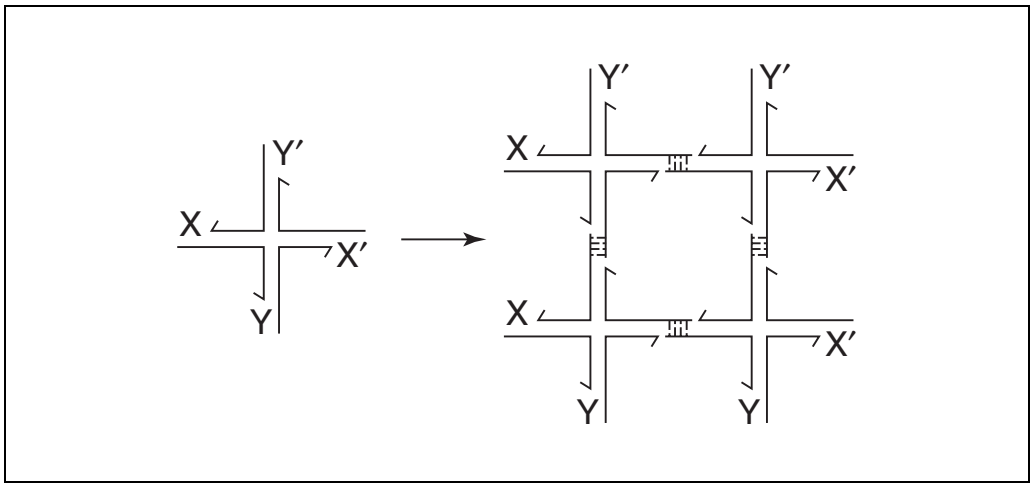

Figure 12.1.1 Formation of a two-dimensional lattice from a four-arm junction with sticky ends. A sticky end and its complement are indicated by X and X′, respectively. The same relationship exists between Y and Y′. Four of the monomeric junctions on the left are complexed in parallel orientation to yield the structure on the right. DNA ligase can close the gaps left in the complex. Note that the complex has maintained open valences, so that it could be extended by the addition of more monomers.

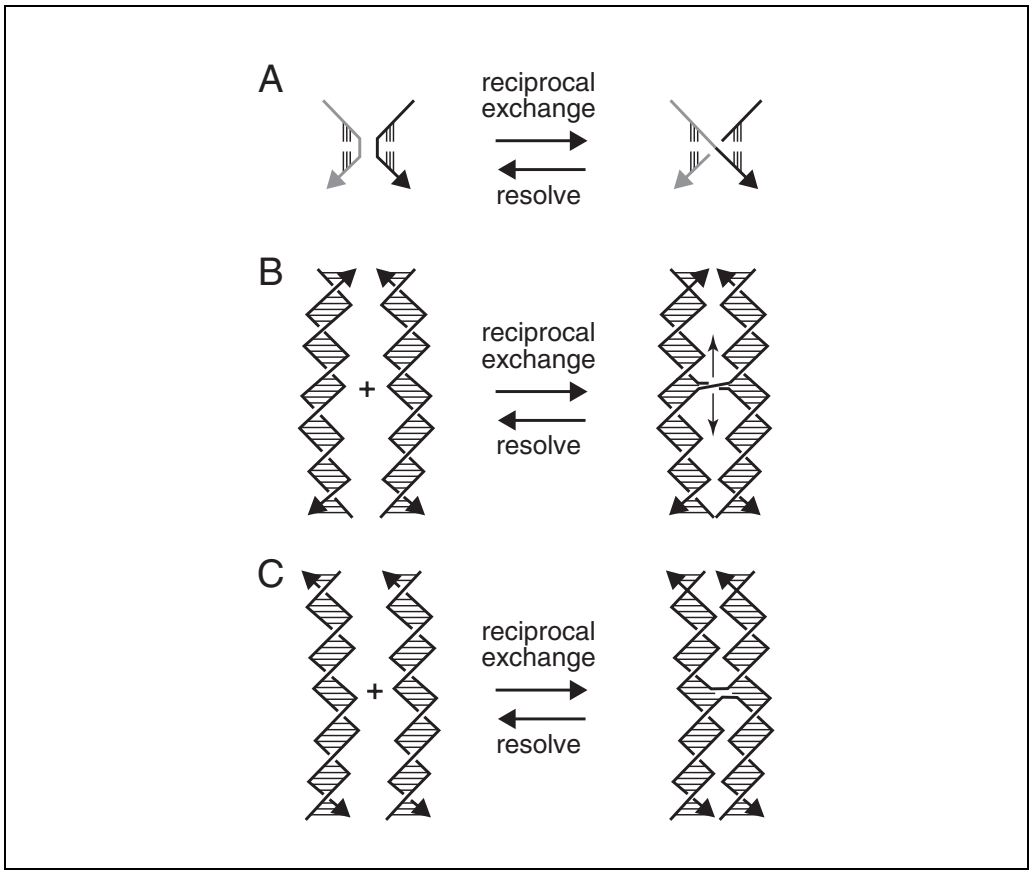

Figure 12.1.2 Reciprocal exchange between DNA strands. (**A**) Reciprocal exchange between two juxtaposed helical half turns. A black and a gray hairpin are shown. The helix axis is horizontal in this view and the dyad axis is vertical. Arrowheads on strands indicate the 3′ ends. A negative node is formed in the rightward reaction, where the strands have retained their initial shading. (**B**,**C**) Reciprocal exchange generates a four-arm junction from two double helices. Panel B shows exchange between strands of the same polarity, and panel C shows exchange between strands of opposite polarity. Symmetry elements are indicated by arrows in B.

double-stranded molecules without strand exchange.

Sequence Selection

The concept of complementary DNA base pairing—A with T and G with C—is a familiar one. It is also well known that a strand with a given sequence will pair with a strand that is complementary to it to form a double helix. How is a branch point formed? The procedure is predicated on the assumption that DNA molecules will form Watson-Crick base pairs in double-helical structures in preference to all other types of base pairing. However, the concept of complementarity can be broadened to include more than a single strand (Seeman, 2000). If the way that a strand can maximize its Watson-Crick base pairing is to form a branch point, it seems that it will do so (Kallenbach et al., 1983; Wang et al., 1991).

Sequences are designed according to the approach illustrated in Figure 12.1.4. Each strand of the structure is broken up into a series of overlapping elements. In Figure 12.1.4, each of the 16-mer strands of the branched junction shown is divided into thirteen elements of four nucleotides (black boxes). Each element is required to be unique. In addition, no element that is designated to go around a corner (e.g., the boxed CTGA) is permitted to have its complement present. A further restriction is that twofold symmetry cannot flank the branch point of a four-arm junction, so that branch migration cannot occur (Seeman, 1982).

With these constraints in place, competition with the target octamer structures shown in each of the arms of the junction in Figure 12.1.4 comes only from trimers or smaller, such as the ATG sequences drawn in gray boxes. This approach is based on the cooperativity of DNA hybridization, and it has a thermodynamic basis (Seeman and Kallenbach, 1983). However, the application of thermodynamic criteria has been largely abandoned in recent years (Seeman,

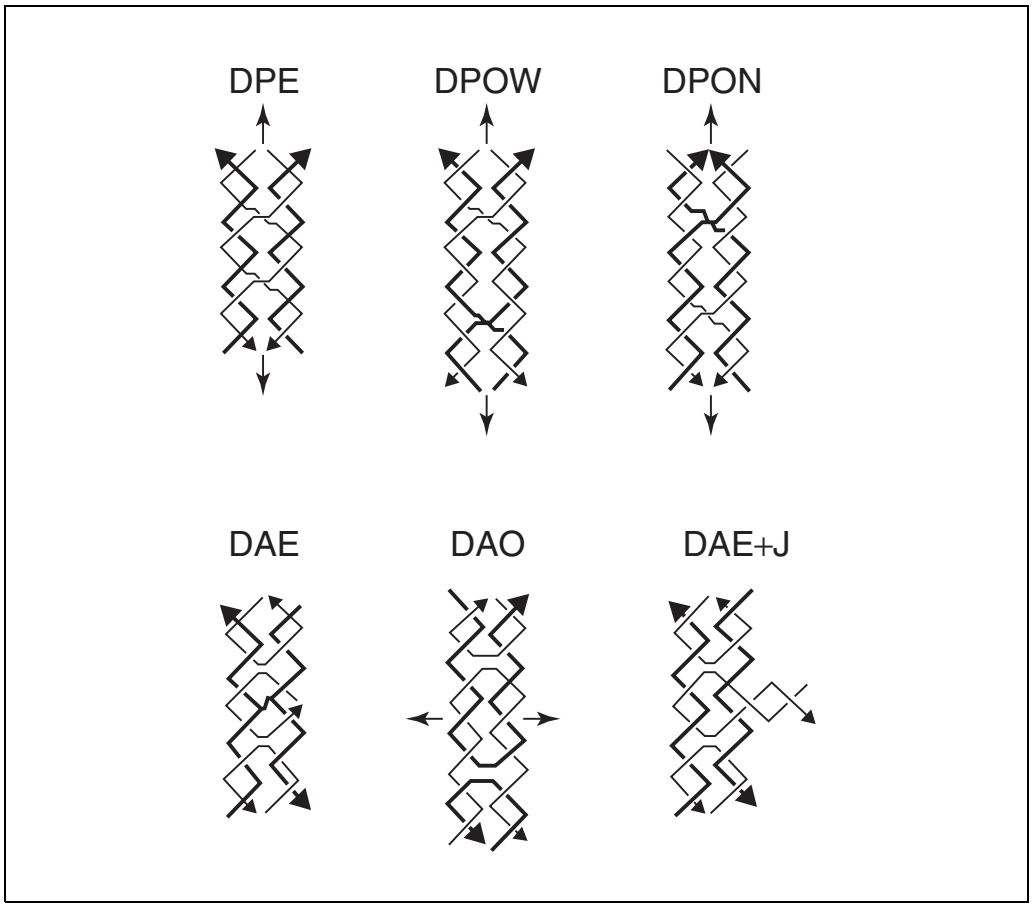

Figure 12.1.3 The structural isomers of DNA double-cross-over (DX) molecules. DPE, DPOW, and DPON are the three parallel DX molecules. The DAE and DAO molecules are the antiparallel isomers. Symmetry in DAO is between the thick and thin lines. DAE+J is a molecule in which the cyclic strand of DAE is extended to form a three-arm bulged junction. Arrowheads indicate 3′ ends. Symmetry elements are shown by arrows, and line thickness is related by symmetry in each drawing. In abbreviations, A is antiparallel, P is parallel, E and O refer to even and odd numbers of double-helical half turns between cross-overs, W and N indicate wide or narrow groove spacings for the odd half turn, and J is junction.

1990), because the systems used have been sufficiently robust to sustain the numerological approach described above. A program that applies this approach, SEQUIN, is available from the author.

CONSTRUCTION METHODS

Synthesis and Purification

Solid-support methods for the synthesis of DNA containing designated sequences (Caruthers, 1985) are the central enabling methodology for the pursuit of DNA nanotechnology. This field is still at the stage where the key principles are being elucidated. Consequently, it is both economical and convenient to perform syntheses on relatively small scales (e.g., 200 nM). On a well-tuned synthesizer, one can achieve apparent trityl-based step yields of ~99.2%, leading to an ~45% yield of crude target product in the synthesis of a 100-mer. Despite numerous attempts at alternative approaches, purification by denaturing gel remains the most reliable (and tedious) method of separating target molecules from failure products. It is worth pointing out that DNA of this length is frequently damaged during the synthesis, so reproducing (and amplifying) it with PCR may be advisable in cases where high chemical purity is an issue (Wang et al., 1998). Future issues involving synthesis will likely entail scaling up to larger quantities for specific applications.

Hybridization

In the author's experience, the design algorithm is very reliable in producing unusual DNA motifs. However, it is predicated on estimates of equilibrium structures, and it is key that kinetic traps be avoided. Consequently, all

samples should be heated to 90°C for 5 min and then cooled slowly. Relatively quick protocols entail a number of stages in heating blocks, such as 20 min at 65°, 45°, 37°C, room temperature, and perhaps a lower temperature. To form simpler arrays, the 90°C solution can be put in a Styrofoam box and cooled to room temperature over ~40 hr. For more complex arrays, a thermocycling protocol is often used with preformed tiles. The presence of Mg^{2+} or other multivalent cations in solution appears to be required for the stability of small branched molecules on gels (Seeman et al., 1985).

Phosphorylation and Ligation

Molecules that are to be ligated must contain phosphates on their 5′ ends. Phosphates may be added chemically as a final step in the synthesis or enzymatically using polynucleotide kinase. The author has found that phosphorylation of unpurified DNA with ^{32}P-labeled phosphate may occasionally result in the labeling of a failure sequence (i.e., one whose secondary structure is more accessible to the enzyme than the target strand). Neither chemical nor enzymatic phosphorylation produces completely phosphorylated material. If this is critical to an experiment, the most highly phosphorylated strands can be produced by restricting a molecule containing the sequence of interest (Podtelezhnikov et al., 2000).

Ligation of DNA is central to many aspects of DNA nanotechnology. The author has found that enzymatic ligation is superior to chemical ligation (Du et al., 1995b), but it is still not very effective (Chen and Seeman, 1991a). Virtually stoichiometric quantities of ligase are often needed to produce relatively limited yields of product. The origin of this problem is not well understood and may arise from the presence of unphosphorylated ends or branch points titrating out the ligase. Minimizing the number of ligations of branched molecules in a preparative protocol is strongly advised.

The topological state of a ligation product is often a key feature of a target molecule. If the molecule contains unpaired segments, it can be useful to employ topological protection (Seeman, 1992; Fu et al., 1994a). This is a technique whereby the single-stranded regions

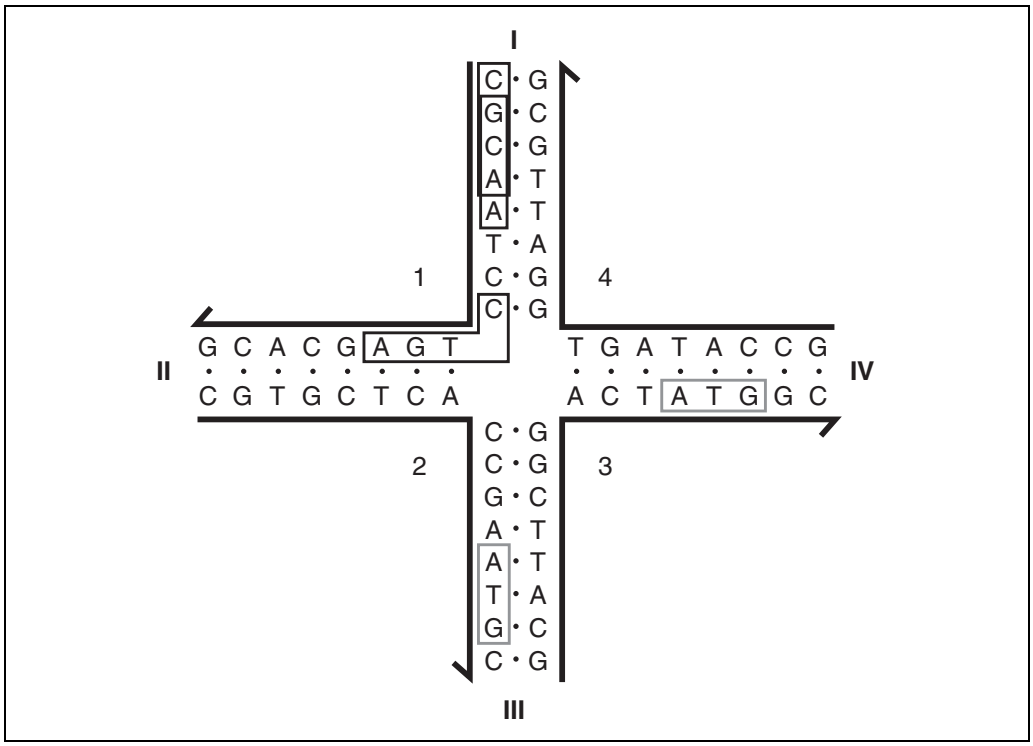

Figure 12.1.4 The design of a stable four-arm branched junction. The junction shown is composed of four strands of DNA, as indicated by Arabic numerals. The 3′ end of each strand is indicated by a half arrow. Each strand is paired with two other strands to form double-helical arms, labeled with Roman numerals. There is no homologous two-fold sequence symmetry flanking the central branch point, thereby stabilizing its position. Tetrameric elements are boxed in black; trimeric elements are boxed in gray. The tetramers are all unique, and there is no complement to any tetramer flanking the junction. Competition with the target octamers can only occur from trimers, such as the ATG sequences.

are paired temporarily with complements during ligation, thus eliminating any braiding they may undergo. This procedure has been successful in the synthesis of specific catenanes, but has not been as useful in the synthesis of knots (Du and Seeman, 1994), because the single-stranded segments consist of oligo(dT); the addition of oligo(dA) leads to the formation of DNA triplexes, whose rotation defeats the purpose of topological protection.

Solid-Support Methodology

Control over the products of DNA ligation is critical for the construction of complex motifs. Our initial complex object, a DNA cube, was synthesized entirely in solution (Chen and Seeman, 1991a). This approach afforded only limited control over the edges that were designed to ligate at any particular step: they could be phosphorylated or not. After the first partial products were obtained, even this level of control was lacking, because there was no control over which 5′ ends were phosphorylated during the second step. A solution to the lack of control was the development of a solid-support methodology, allowing successive double-helical edges of the target molecule to be ligated individually (Zhang and Seeman, 1992). The starting unit was added to the solid support, with all of its sticky ends protected as DNA hairpins. These could be deprotected individually by restriction enzymes and then ligated to another molecule whose only unclosed helix contained the complementary sticky end. Thus, every target intermediate is topologically closed, permitting intermediate purification by exonuclease treatment.

One major advantage of the solid-support approach is that intermolecular reactions can be conducted at high concentrations, but cross-linking between growing objects during intramolecular reactions can be minimized. It is worth noting that sticky ends should not be self-complementary, as this unacceptably decreases control. Figure 12.1.5 illustrates the synthesis of a quadrilateral using this overall approach, which has been utilized to construct a truncated octahedron (Zhang and Seeman, 1994).

CHARACTERIZATION IN DNA NANOTECHNOLOGY

Characterization of Motifs

DNA nanotechnology has produced a large variety of complex motifs derived by merging

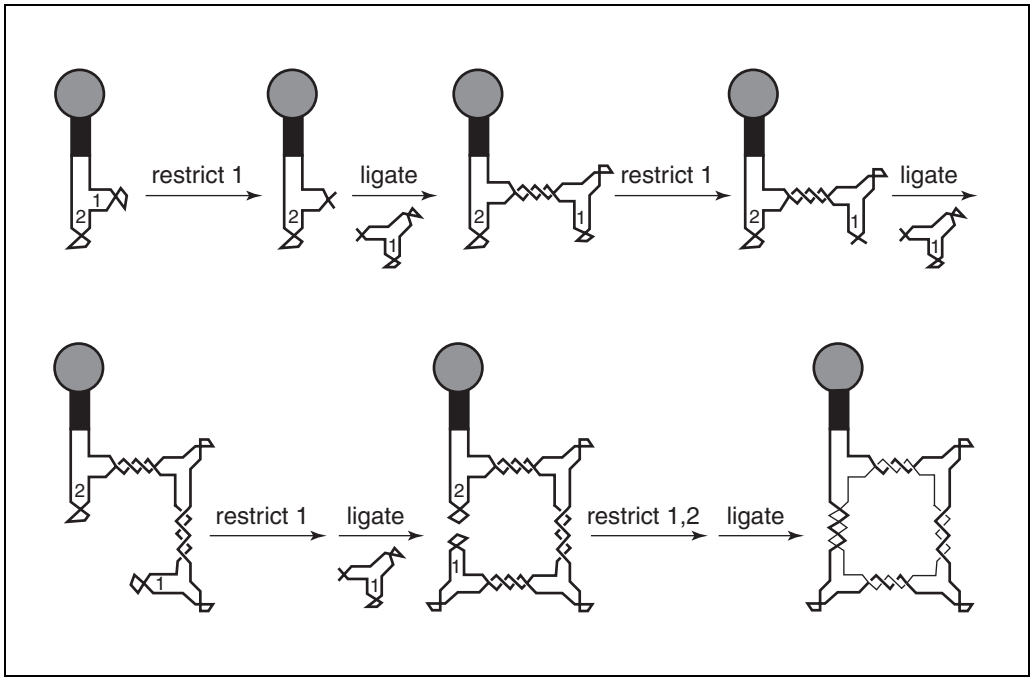

Figure 12.1.5 Protocol for the solid-support synthesis of a quadrilateral. Beginning with the support containing a closed strand, alternate cycles of restriction and ligation are performed, always at the position indicated as site 1. Selection of the target product (e.g., triangle, quadrilateral, pentalateral) is determined by the point at which one chooses to restrict at site 2, exposing a sticky end complementary to that exposed by restriction at site 1. Note that the final closure converts a simple cyclic molecule to a catenane.

the notions of reciprocal exchange between strands (Seeman, 2001) and the generalization of complementarity (Seeman, 2000). If one produces a set of strands designed to form such a motif, one must demonstrate that the target motif actually has been obtained. These species are quite large and are not readily tractable to techniques that produce unambiguous structural results, such as X-ray crystallography or NMR. Consequently, a battery of analytical procedures has been developed or adapted to establish the features of these unusual DNA motifs.

Partial-product gels

The very first thing to do with a new motif is to demonstrate that it forms cleanly. A routine way to do this is to prepare a nondenaturing gel containing the stoichiometric complex and its subcomplexes; for example, for a four-arm junction, such a gel would have lanes containing each strand, plus each of the six strand pairs, the four three-strand complexes, and the four-strand target complex (Kallenbach et al., 1983). It is important to work out the stoichiometry of the strands first in a pairwise fashion. Titrating each strand against one of its partial complements on gels is straightforward: the end point is the ratio that shows no excess of either strand. It is not uncommon for individual strands to migrate as multimers, and this occurrence should not be regarded as a problem. The SEQUIN algorithm is designed for the final complex, not the partial products.

The successful assembly of a motif is characterized by a single band of roughly the expected molecular weight. Migration under nondenaturing conditions is a function of many factors, including surface area, molecular weight, and shape. Nevertheless, target motifs tend to migrate roughly in the vicinity of linear duplex markers of similar molecular weight. Ferguson analysis (see discussion of shape analysis, below) should always be performed to ensure that the results are not affected by the choice of gel percentage.

There are three ways that a motif can fail this analysis. (1) It can be unstable, producing bands that migrate more rapidly than the target band. (2) It can produce bands that correspond to multimers of the target complex. Multimers can arise from a system that is stressed by torsional or electrostatic features of the molecular design; for example, parallel DX molecules generate eight-strand dimers, twelve-strand trimers, and sixteen-strand tetramers at high concentrations (Fu and Seeman, 1993). Multimers can be avoided at high concentrations if the target complex is converted to a topologically closed form (Fu et al., 1994b). Sticky ends can be an artificial source of multimer bands (Li et al., 1996). In this case a new motif with blunt ends should be assayed, or the gels used for analysis should be run at temperatures that preclude intermotif cohesion. (3) A third form of failure is a smear, resulting from the formation of an open complex (i.e., strand 1:2:3:4:1:2:3...) rather than a closed complex (i.e., strand 1:2:3:4). This behavior was seen with the four-strand antijunction complex at high concentrations (Du et al., 1992).

In addition to knowing the constituents of a new motif, it is also important to establish the stoichiometry of the complex. This is easily done for an n-strand complex by titrating an $(n-1)$-strand stoichiometric complex with the missing strand. For example, in a four-strand motif, a 1:1:1 three-strand complex can be titrated with the fourth strand (Kallenbach et al., 1983). The titration process is monitored readily on a nondenaturing gel. Assuming 1:1:1:1 stoichiometry, a 1:1:1:0.5 mixture will show bands for both the partial complex and the target motif. A 1:1:1:1 mixture should show a band containing only the target motif, and a 1:1:1:2 mixture should show bands corresponding both to the target motif and to the excess single strand.

Shape analysis

The qualitative shape of a DNA motif can be compared usefully and conveniently with standards by means of a Ferguson plot (Rodbard and Chrambach, 1971). This is a plot of log(mobility) versus acrylamide concentration, and its slope is proportional to the friction constant of the molecule. Ferguson analysis has been used to characterize the stacked character of four-arm junctions in comparison to three-, five-, and six-arm junctions (Wang et al., 1991) and to demonstrate that the addition of helices to linear duplex and DX molecules results in similar changes in the friction constant (LaBean et al., 2000). Likewise, the qualitative structure of PX DNA has been compared usefully to that of DX molecules to establish their similar shapes. Both denaturing and nondenaturing Ferguson analyses have been applied to a DNA cube-like molecule and its subcatenanes (Chen and Seeman, 1991b).

Cooper and Hagerman (1987) have developed a method for the analysis of DNA branched junctions. By adding long reporter double helices pairwise to the arms of four-arm

junctions, they established the basic structural features of DNA branched junctions. Their results suggest that gel mobility is a monotonically increasing function of the angle between the two extended arms. This approach was modified by Lilley and colleagues (Duckett et al., 1988) for the conventional Holliday junction, and was utilized effectively as well for Bowtie junctions containing 3′,3′ and 5′,5′ linkages in the cross-over strands of the branched species (Sha et al., 1999). The same pairwise approach has been applied to junctions using transient electric birefringence (Cooper and Hagerman, 1989) and fluorescence resonance energy transfer (FRET) to establish details of the branched-junction structure and dynamics (Murchie et al., 1989; Eis and Millar, 1993).

Thermal analysis

The relative stabilities of new motifs are often usefully analyzed by melting them while monitoring a feature such as OD_{260} (Kallenbach et al., 1983) or circular dichroism (Seeman et al., 1985). Melting a complex DNA motif

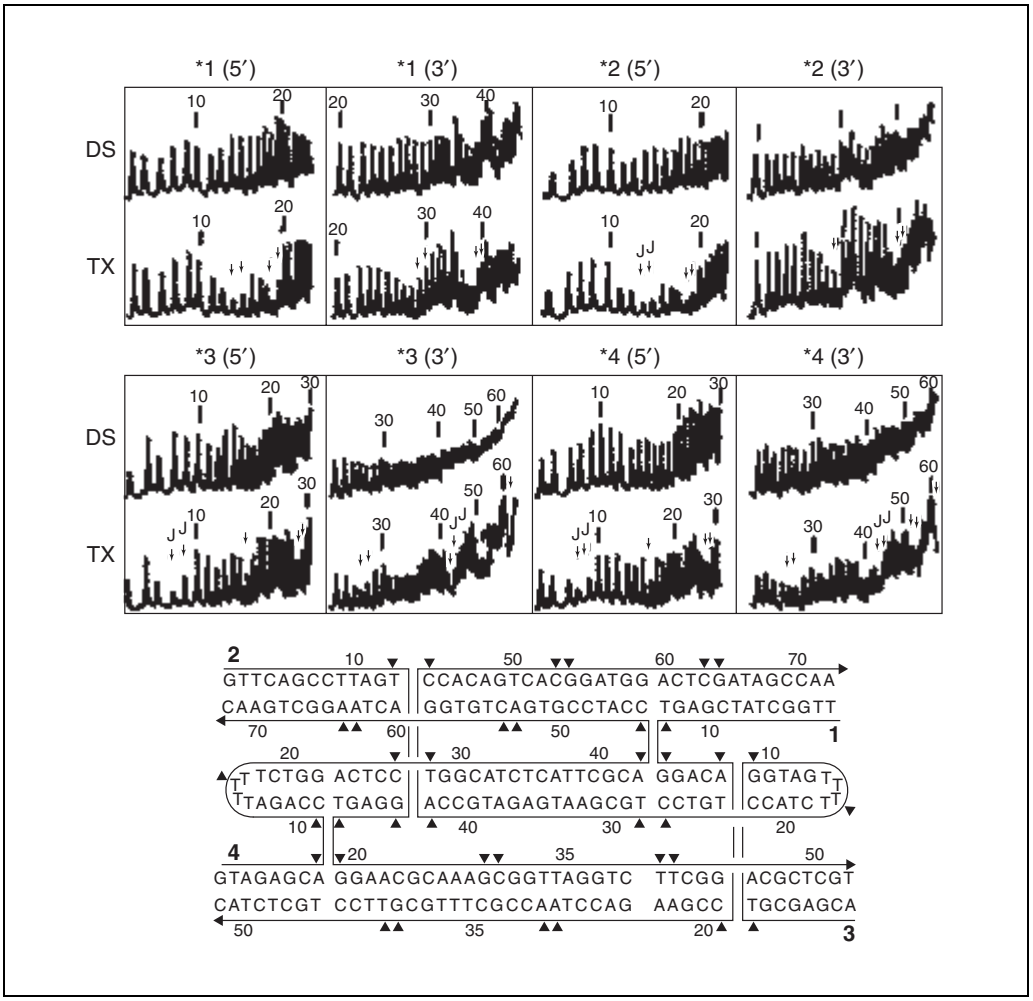

Figure 12.1.6 The hydroxyl radical autofootprinting pattern of a DNA triple-cross-over (TX) molecule. The top portion of the figure contains densitometer scans of autoradiograms for each strand of the TX molecule. The data for each strand are shown twice, once for its 5′ end and once for its 3′ end, as indicated above the appropriate panel. Susceptibility to hydroxyl radical attack is compared for each strand when incorporated into the TX molecule (TX) and when paired with its traditional Watson-Crick complement (DS). Nucleotide numbers are indicated above every tenth nucleotide. The two nucleotides flanking expected cross-over positions are indicated by two Js. Note the correlation between the Js and protection in all cases. Additional protection is seen at further locations (arrows), indicating occlusion a turn away from the cross-over points on the cross-over strands, and about four nucleotides 3′ to the cross-overs on the helical strands, as noted previously. The data are summarized on a molecular drawing below the scans. Sites of protection are indicated by triangles pointing towards the protected nucleotide; the extent of protection is indicated qualitatively by the sizes of the triangles. Asterisks indicate labeled strands.

often reveals features that are more characteristic of the compositions of individual helical domains than of the complex itself. It is often useful to pay particular attention to premelting transitions, because they may be more diagnostic of features of the complex that differentiate it from linear duplex DNA or other topologies of the same composition. Another technique of great value is analysis of the motif on a perpendicular denaturing gradient gel (Fischer and Lerman, 1979). This method enables one to see parts of the complex fall apart, rather than monitoring the details of the intradomain destacking transition. Calorimetry has also been applied usefully to DNA motifs (Marky et al., 1987).

Hydroxyl radical autofootprinting

The highest-resolution technique that is used conveniently to characterize the structures of unusual DNA motifs is hydroxyl radical autofootprinting. Hydroxyl radical autofootprinting (Churchill et al., 1988) is a variation on the technique of hydroxyl radical footprinting, which is used to establish the binding sites of proteins on DNA. Branched junctions (Churchill et al., 1988; Wang et al., 1991), tethered junctions (Kimball et al., 1990), antijunctions and mesojunctions (Du et al., 1992), and DX (Fu and Seeman, 1993) and TX (LaBean et al., 2000) molecules have all been analyzed by this method. The analysis is performed by labeling a component strand of the complex and exposing it to hydroxyl radicals.

The key feature noted at cross-over sites in these analyses is decreased susceptibility to attack when comparing the pattern of the strand as part of the complex relative to the pattern of the strand derived from linear duplex DNA. Decreased susceptibility is interpreted to suggest that access of the hydroxyl radical may be limited by steric factors at the sites where it is detected (Balasubramanian et al., 1998). Likewise, similarity to the duplex pattern at points of potential flexure is assumed to indicate that the strand has adopted a conventional helical structure in the complex, whether or not it is required by the secondary structure.

In studies of junctions, DX molecules, and mesojunctions, protection has been seen particularly at the cross-over sites, but also at non-cross-over sites where strands from two adjacent parallel or antiparallel domains appear to occlude each other's surfaces, thereby preventing access by hydroxyl radicals (Kimball et al., 1990; Fu and Seeman, 1993; LaBean et al., 2000). Thus, cross-over sites can be located reliably by hydroxyl radical autofootprinting analysis, but it is not always possible to distinguish them unambiguously from juxtapositions of backbone strands. The technique is particularly powerful as a method to establish whether

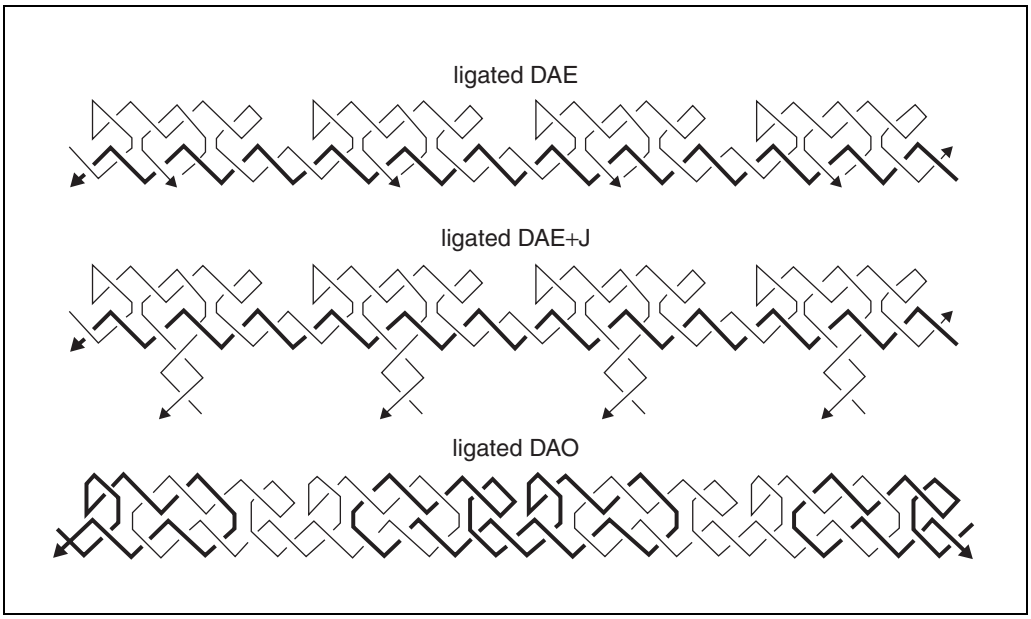

Figure 12.1.7 Ligation products of antiparallel double-cross-over molecules DAE, DAO, and DAE+J. One domain has been capped by hairpins. Ligation of the DAE molecule leads to a reporter strand, which is drawn more darkly. Ligation of the DAE+J molecule also leads to a reporter strand, similar to the one in DAE. However, ligation of the DAO molecule produces a polycatenated structure.

cross-overs occur where they are expected to occur. Those who wish to construct new motifs are advised strongly to ascertain by hydroxyl radical autofootprinting that they have formed the molecular topologies that they have designed. An example of the hydroxyl radical protection pattern of a DNA TX molecule is shown in Figure 12.1.6.

Characterization of Ligation Targets

Denaturing gels

Ligated species are extremely difficult to analyze. The simplest ligated species are linear multimers of a given motif. Junctions (Ma et al., 1986; Petrillo et al., 1988), DX molecules (Li et al., 1996; Winfree et al., 1998), and DNA triangles (Yang et al., 1998) are all examples of unusual DNA motifs that have been oligomerized in one dimension. The trick to analyzing these systems is for them to contain a reporter strand whose fate reports the fate of the complex. Examples of reporter strands are shown in Figure 12.1.7, where the ligation products of DAE, DAE+J, and DAO molecules are shown. The thick strand in the DAE and DAE+J ligations is a reporter strand, but there is no reporter strand in the DAO ligation. It is important to extract the reporter strands from other strands in the ligation product to which they might be catenated; this is usually done by restriction. The reporter strand can be sized in comparison with linear and cyclic markers (e.g., Li et al., 1996). In addition to providing an estimate of the products, ligation experiments can be used to estimate the stiffness of a given motif, either qualitatively (Ma et al., 1986; Petrillo et al., 1988; Li et al., 1996) or quantitatively (Podtelezhnikov et al., 2000).

More complex target ligation products are likely to be catenanes or perhaps knots. This is a consequence of the plectonemic (interwound) nature of the DNA strands; indeed, DNA is almost the ideal synthon for topological construction. Catenanes are usually separable on denaturing gels of one (Chen and Seeman, 1991a,b) or two (Wang, et al., 1998) dimensions. Catenanes of greater linking number migrate more rapidly on denaturing gels than similar catenanes of lower linking number. This seems to be true of knots as well, although it is key to ensure that a fortuitous acrylamide percentage has not been selected for the analysis. A reliable way to ensure independence from gel artifacts is to perform a denaturing sedimentation analysis in comparison with markers. If material appears to behave anomalously on denaturing gels, it is useful to check that the sample is completely denatured. The Fischer-Lerman 100% denaturing conditions (7 M urea and 40% formamide; Fischer and Lerman, 1979) often denature samples that misbehave on conventional denaturing gels.

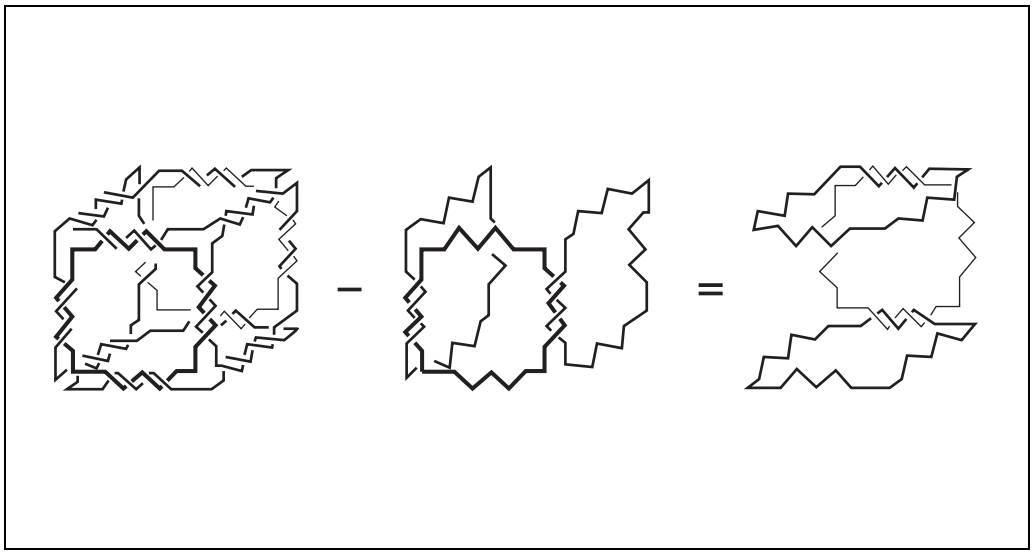

Figure 12.1.8 Restriction analysis of a DNA cube. The cube is drawn with strands of three different thicknesses, with the darkest in front and the lightest at the rear. The linear triple catenane shown at the center was the starting material for the last step of the construction. It corresponds to the left, front, and right sides of the cube. Its removal from the cube by restricting at the left-front and right-front edges leaves the top-back-bottom linear triple catenane as a product. Each edge of the cube contains a unique restriction site.

Restriction analysis

Complex constructions, such as DNA polyhedra (Chen and Seeman, 1991a; Zhang and Seeman, 1994) or Borromean rings (Mao et al., 1997), must be designed carefully from the start, so that proof of construction can be obtained. As noted above, crystallography is not readily available to characterize these species, and molecular weights of 150 to 800 kDa are beyond the range of current NMR capabilities. At present, the best characterizations of these systems are topological rather than structural. The analysis entails the judicious insertion of restriction sites in the molecules. These sites are utilized to break the products down to smaller catenanes that can be synthesized independently and used for comparison. Thus, the DNA cube, a hexacatenane (Chen and Seeman, 1991a), was built from a linear triple catenane corresponding to the ultimate left-front-right faces of the target. Figure 12.1.8 shows that cleavage of the left-front and right-front edges of the cube leads to the top-back-bottom linear triple catenane. Likewise, the cleavage of any of the strands of the Borromean rings leads to two single strands (Mao et al., 1997).

Characterization of Devices

DNA nanomechanical devices hold great promise in the developing field of nanorobotics. Devices may consist of ligated (Mao et al., 1999a) or annealed (Yurke et al., 2000; Yan et al., 2002) components. The requirements for characterization of the system are no less stringent for a device than for a static target. In addition, one must demonstrate that the device responds to the triggers for which it has been designed. Mechanical motion is the hallmark of a device, and this intramolecular motion leads to a change in some distance within the molecule that can be monitored by FRET. FRET has been used successfully to demonstrate the action of devices predicated on the B-Z transition (Mao et al., 1999a) and on the binding and removal of a specific strand (Yurke et al., 2000). A sequence-specific device based on hybridization topology has also been demonstrated recently by AFM. An edge-sharing half hexagon attached roughly perpendicular to the device provides a 17-nm lever arm whose repositioning is readily visible at the 7- to 10-nm resolution of the AFM (Yan et al., 2002).

Characterization of DNA Arrays

Periodic arrays

The use of reporter strands to establish the extent that a system has ligated in one dimension was described above. However, such an approach is of less utility in two dimensions (or three), because it will not reveal the nature or extent of faults very well. Direct observation of the array is necessary. The technique used to date is AFM. Periodic arrays of DX molecules (Winfree et al., 1998), TX molecules (LaBean et al., 2000), and conventional (Mao et al., 1999b) and Bowtie (Sha et al., 2000a) DNA parallelograms have all been characterized by this method. As with all microscopy, the investigator can usually find the structure that is sought; consequently, any observations must be challenged by making a molecular-level change that leads to a predictable change in the AFM pattern. For example, DAE molecules can be interspersed with DAE+J molecules (Figure 12.1.3) whose extra helical domain is oriented out of the plane of the array. For DAE molecules of dimensions 4×16 nm, alternating DAE molecules with DAE+J molecules leads to a striped feature every 32 nm; if there is only a single DAE+J molecule in every four molecules, then the stripes will be separated by 64 nm. Likewise, differently shaped cavities can be designed for arrays made up of DNA parallelograms. To ensure reliable results, changes in molecular design must lead to predictable changes in AFM patterns.

Studies of periodic DNA arrays using other microscopic techniques such as transmission electron microscopy, scanning transmission electron microscopy, or near-field scanning optical microscopy are ongoing, but no data have yet emerged in the literature. Similarly, attempts are going forward to produce three-dimensional crystalline materials, but no successes have been reported. X-ray diffraction clearly will be required to characterize three-dimensional periodic DNA constructions.

Aperiodic arrays

The founding of experimental DNA-based computation by Adleman (1994) has led to an interest in other types of DNA self-assembly. Winfree (1996) pointed out that branched DNA motifs could be used to do logical computation. The idea is that sticky ends can be assigned a logical value, and that their assembly can then be used to perform the computation. This suggestion has been implemented successfully in one dimension, where a reporter strand ap-

proach was used to extract the answer to the computation (Mao et al., 2000). Algorithmic assembly can also be used to program patterns in two or three dimensions, but patterns of this complexity have not yet been obtained. Characterization in two dimensions may be amenable to AFM analysis, but new methods, beyond AFM and diffraction, will be needed in three dimensions to confirm the presence of designed aperiodic patterns.

DNA NANOTECHNOLOGY AS A TOOL IN BIOCHEMISTRY

The applications of DNA nanotechnology are certainly not limited to technological goals like solution of the macromolecular crystallization problem or the facilitation of nanoelectronics or nanorobotics. DNA nanotechnology has already served as a valuable tool in the characterization of components of biological systems. DX molecules have been used to establish the thermodynamics of Holliday junction cross-over isomers (Zhang and Seeman, 1994) and branch migratory isomers (Sun et al., 1998). They have also been used to establish Holliday junction cross-over topology (Fu et al., 1994a), the cutting sites of resolvases (Sha et al., 2000c), and the spontaneity of both Holliday cross-over isomerization (Li et al., 1997) and antiparallel branch migration (Sha et al., 2000b). Two-dimensional parallelogram arrays have been used to measure the interdomain angles in Holliday (Mao et al., 1999b) and Bowtie (Sha et al., 2000a) junctions; V-shaped parallelogram arrays were used to confirm the parallel orientation of Bowtie junction domains (Sha et al., 2000a). Similarly, an RNA knot was used to demonstrate that *E. coli* DNA topoisomerase III is an RNA topoisomerase, although topoisomerase I is not (Wang et al., 1996, 1998). Other applications certainly exist and are not just limited to the analysis of Holliday junctions; they are limited only by the imagination.

CONCLUSIONS

This unit is intended to give a sense of the nature, goals, and experimental techniques that characterize DNA nanotechnology. DNA nanotechnology that utilizes the detailed geometry that results from the predictable molecular structures formed by sticky-ended cohesion has been emphasized. Other approaches to nanotechnology using DNA have been performed utilizing only the complementarity aspects of DNA, without the concomitant structural features inherent in sticky-ended interactions (e.g., Niemeyer et al., 1994; Alivisatos et al., 1996; Mirkin et al., 1996; Shi and Bergstrom, 1997). However, these investigators have brought an important new component to DNA nanotechnology: the combination of DNA and heteromolecules, such as proteins and nanocrystals. It is likely that as more investigators enter this field, heteromolecules will be added to geometrically precise DNA constructs in a way that will incorporate the functionality that this system requires if it is to have a major impact.

LITERATURE CITED

Adleman, L. 1994. Molecular computation of solutions to combinatorial problems. *Science* 266:1021-1024.

Alivisatos, A.P., Johnsson, K.P., Peng, X., Wilson, T.E., Loweth, C.J., Bruchez, M.P., and Schultz, P.G. 1996. Organization of 'nanocrystal molecules' using DNA. *Nature* 382:609-611.

Balasubramanian, B., Pogozelski, W.K., and Tullius, T.D. 1998. DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. *Proc. Natl. Acad. Sci. U.S.A.* 95:9738-9743.

Caruthers, M.H. 1985. Gene synthesis machines: DNA chemistry and its uses. *Science* 230:281-285.

Chen, J. and Seeman, N.C. 1991a. The synthesis from DNA of a molecule with the connectivity of a cube. *Nature* 350:631-633.

Chen, J. and Seeman, N.C. 1991b. The electrophoretic properties of a DNA cube and its substructure catenanes. *Electrophoresis* 12:607-611.

Churchill, M.E.A., Tullius, T.D., Kallenbach, N.R., and Seeman, N.C. 1988. A Holliday recombination intermediate is twofold symmetric. *Proc. Natl. Acad. Sci. U.S.A.* 85:4653-4656.

Cooper, J.P. and Hagerman, P.J. 1987. Gel electrophoretic analysis of the geometry of a DNA four-way junction. *J. Mol. Biol.* 198:711-719.

Cooper, J.P. and Hagerman, P.J. 1989. Geometry of a branched DNA structure in solution. *Proc. Natl. Acad. Sci. U.S.A.* 86:7336-7340.

Du, S.M. and Seeman, N.C. 1994. The construction of a trefoil knot from a DNA branched junction motif. *Biopolymers* 34:31-37.

Du, S.M., Zhang, S., and Seeman, N.C. 1992. DNA junctions, antijunctions and mesojunctions. *Biochemistry* 31:10955-10963.

Du, S.M., Stollar, B.D., and Seeman, N.C. 1995a. A synthetic DNA molecule in three knotted topologies. *J. Am. Chem. Soc.* 117:1194-1200.

Du, S.M., Wang, H., Tse-Dinh, Y.-C., and Seeman, N.C. 1995b. Topological transformations of synthetic DNA knots. *Biochemistry* 34:673-682.

Duckett, D.R., Murchie, A.I., Diekmann, S., von Kitzing, E., Kemper, B., and Lilley, D.M.J. 1988. The structure of the Holliday junction, and its resolution. *Cell* 55:79-89.

Eis, P.S. and Millar, D.P. 1993. Conformational distributions of a four-way DNA junction revealed by time-resolved fluorescence resonance energy transfer. *Biochemistry* 32:13852-13860.

Fischer, S.G. and Lerman, L.S. 1979. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell* 16:191-200.

Fu, T.-J. and Seeman, N.C. 1993. DNA double crossover structures. *Biochemistry* 32:3211-3220.

Fu, T.-J., Tse-Dinh, Y.-C., and Seeman, N.C. 1994a. Holliday junction crossover topology. *J. Mol. Biol.* 236:91-105.

Fu, T.-J., Kemper, B., and Seeman, N.C. 1994b. Endonuclease VII cleavage of DNA double crossover molecules. *Biochemistry* 33:3896-3905.

Hagerman, P.J. 1988. Flexibility of DNA. *Annu. Rev. Biophys. Biophys. Chem.* 17:265-286.

Holliday, R. 1964. A mechanism for gene conversion in fungi. *Genet. Res*. 5:282-304.

Kallenbach, N.R., Ma, R.-I., and Seeman, N.C. 1983. An immobile nucleic acid junction constructed from oligonucleotides. *Nature* 305:829-831.

Kimball, A., Guo, Q., Lu, M., Kallenbach, N.R., Cunningham, R.P., Seeman, N.C., and Tullius, T.D. 1990. Conformational isomers of Holliday junctions. *J. Biol. Chem.* 265:6544-6547.

LaBean, T., Yan, H., Kopatsch, J., Liu, F., Winfree, E., Reif, J.H., and Seeman, N.C. 2000. The construction, analysis, ligation and self-assembly of DNA triple crossover complexes. *J. Am. Chem. Soc.* 122:1848-1860.

Li, X., Yang, X., Qi, J., and Seeman, N.C. 1996. Antiparallel DNA double crossover molecules as components for nanoconstruction. *J. Am. Chem. Soc.* 118:6131-6140.

Li, X., Wang, H., and Seeman, N.C. 1997. Direct evidence for Holliday junction crossover isomerization. *Biochemistry* 36:4240-4247.

Liu, F., Sha, R., and Seeman, N.C. 1999. Modifying the surface features of two-dimensional DNA crystals. *J. Am. Chem. Soc.* 121:917-922.

Ma, R.-I., Kallenbach, N.R., Sheardy, R.D., Petrillo, M.L., and Seeman, N.C. 1986. Three-arm nucleic acid junctions are flexible. *Nucl. Acids Res.* 14:9745-9753.

Mao, C., Sun, W., and Seeman, N.C. 1997. Assembly of Borromean rings from DNA. *Nature* 386:137-138.

Mao, C., Sun, W., Shen, Z., and Seeman, N.C. 1999a. A DNA nanomechanical device based on the B-Z transition. *Nature* 397:144-146.

Mao, C., Sun, W., and Seeman, N.C. 1999b. Designed two-dimensional DNA Holliday junction arrays visualized by atomic force microscopy. *J. Am. Chem. Soc.* 121:5437-5443.

Mao, C., LaBean, T., Reif, J.H., and Seeman, N.C. 2000. Logical computation using algorithmic self-assembly of DNA triple crossover molecules. *Nature* 407:493-496.

Marky, L.A., Kallenbach, N.R., McDonough, K.A., Seeman, N.C., and Breslauer, K.J. 1987. The melting behavior of a nucleic acid junction: A calorimetric and spectroscopic study. *Biopolymers* 26:1621-1634.

Mathieu, F., Mao, C., and Seeman, N.C. 2001. A DNA nanotube based on a six-helix bundle motif. *J. Biomol. Struct. Dyn.* 18:907-908.

Mirkin, C.A., Letsinger, R.L., Mucic, R.C., and Storhoff, J.J. 1996. A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature* 382:607-609.

Mueller, J.E., Du, S.M., and Seeman, N.C. 1991. The design and synthesis of a knot from single-stranded DNA. *J. Am. Chem. Soc.* 113:6306-6308.

Murchie, A.I., Clegg, R.M., von Kitzing, E., Duckett, D.R., Diekmann, S., and Lilley, D.M.J. 1989. Fluorescence energy transfer shows that the four-way DNA junction is a right-handed cross of antiparallel molecules. *Nature* 341:763-766.

Neilsen, P.E. 1995. DNA analogs with non-phosphodiester backbones. *Annu. Rev. Biophys. Biophys. Chem.* 14:167-183.

Niemeyer, C.M., Sano, T., Smith, C.L., and Cantor, C.R. 1994. Oligonucleotide-directed self-assembly of proteins. *Nucl. Acids Res.* 22: 5530-5539.

Petrillo, M.L., Newton, C.J., Cunningham, R.P., Ma, R.-I., Kallenbach, N.R., and Seeman, N.C. 1988. The ligation and flexibility of four-arm DNA junctions. *Biopolymers* 27:1337-1352.

Podtelezhnikov, A., Mao, C., Seeman, N.C., and Vologodskii, A.V. 2000. Multimerization-cyclization of DNA fragments as a method of conformational analysis. *J. Biophys*. 79:2692-2704.

Qiu, H., Dewan, J.C., and Seeman, N.C. 1997. A DNA decamer with a sticky end: The crystal structure of d-CGACGATCGT. *J. Mol. Biol.* 267:881-898.

Robinson, B.H. and Seeman, N.C. 1987. The design of a biochip: A self-assembling molecular-scale memory device. *Protein Eng.* 1:295-300.

Rodbard, D. and Chrambach, A. 1971. Estimation of molecular radius, free mobility, and valence using polyacrylamide gel electrophoresis. *Anal. Biochem.* 40:95-134.

Seeman, N.C. 1982. Nucleic acid junctions and lattices. *J. Theor. Biol.* 99:237-247.

Seeman, N.C. 1990. De novo design of sequences for nucleic acid structure engineering. *J. Biomol. Struct. Dyn.*8:573-581.

Seeman, N.C. 1992. The design of single-stranded nucleic acid knots. *Mol. Eng*. 2:297-307.

Seeman, N.C. 2000. In the nick of space: Generalized nucleic acid complementarity and the development of DNA nanotechnology. *Synlett* (2000):1536-1548.

Seeman, N.C. 2001. DNA nicks and nodes and nanotechnology. *NanoLetters* 1:22-26.

Seeman, N.C. and Kallenbach, N.R. 1983. Design of immobile nucleic acid junctions. *J. Biophys.* 44:201-209.

Seeman, N.C., Rosenberg, J.M., and Rich, A. 1976. Sequence specific recognition of double helical nucleic acids by proteins. *Proc. Natl. Acad. Sci. U.S.A.* 73:804-808.

Seeman, N.C., Maestre, M.F., Ma, R.-I., and Kallenbach, N.R. 1985. Physical characterization of a nucleic acid junction. *In* Progress in Clinical and Biological Research, Vol. 172A: The Molecular Basis of Cancer (R. Rein, ed.) pp. 99-108. Alan R. Liss, New York.

Sha, R., Liu, F., Bruist, M.F., and Seeman, N.C. 1999. Parallel helical domains in DNA branched junctions containing 5′,5′ and 3′,3′ linkages. *Biochemistry* 38:2832-2841.

Sha, R., Liu, F., Millar, D.P., and Seeman, N.C. 2000a. Atomic force microscopy of parallel DNA branched junction arrays. *Chem. Biol.* 7:743-751.

Sha, R., Liu, F., and Seeman, N.C. 2000b. Direct evidence for spontaneous branch migration in antiparallel DNA Holliday junctions. *Biochemistry* 39:11514-11522.

Sha, R., Iwasaki, H., Liu, F., Shinagawa, H., and Seeman, N.C. 2000c. Cleavage of symmetric immobile DNA junctions by Ruv C. *Biochemistry* 39:11982-11988.

Shi, J. and Bergstrom, D.E. 1997. Assembly of novel DNA cycles with rigid tetrahedral linkers. *Angew Chem. Int. Ed. Engl.* 36:111-113.

Sun, W., Mao, C., Liu, F., and Seeman, N.C. 1998. Sequence dependence of branch migratory minima. *J. Mol. Biol.* 282:59-70.

Wang, Y.L., Mueller, J.E., Kemper, B., and Seeman, N.C. 1991. Assembly and characterization of five-arm and six-arm DNA branched junctions. *Biochemistry* 30:5667-5674.

Wang, H., Di Gate, R.J., and Seeman, N.C. 1996. An RNA topoisomerase. *Proc. Natl. Acad. Sci. U.S.A.* 93:9477-9482.

Wang, H., Di Gate, R.J., and Seeman, N.C. 1998. The construction of an RNA knot and its role in demonstrating that *E. coli* DNA topoisomerase III is an RNA topoisomerase. *In* Structure, Motion, Interaction and Expression of Biological Macromolecules (R.H. Sarma and M.H. Sarma, eds.) pp. 103-116. Adenine Press, Schenectady, New York.

Wells, A.F. 1977. Three-dimensional nets and polyhedra. John Wiley & Sons, New York.

Winfree, E. 1996. On the computational power of DNA annealing and ligation. *In* DNA Based Computing (R.J. Lipton and E.B. Baum, eds.) pp. 199-219. American Math Society, Providence, R.I.

Winfree, E., Liu, F., Wenzler, L.A., and Seeman, N.C. 1998. Design and self-assembly of two-dimensional DNA crystals. *Nature* 394:539-544.

Yan, H., Zhang, X., Shen, Z., and Seeman, N.C. 2002. A robust DNA mechanical device controlled by hybridization topology. *Nature* 415:62-65.

Yang, X., Wenzler, L.A., Qi, J., Li, X., and Seeman, N.C. 1998. Ligation of DNA triangles containing double crossover molecules. *J. Am. Chem. Soc.* 120:9779-9786.

Yurke, B., Turberfield, A.J., Mills, A.P. Jr., Simmel, F.C., and Neumann, J.L. 2000. A DNA-fueled molecular machine made of DNA. *Nature* 406:605-608.

Zhang, S. and Seeman, N.C. 1994. Symmetric Holliday junction crossover isomers. *J. Mol. Biol.* 238:658-668.

Zhang, Y. and Seeman, N.C. 1992. A solid-support methodology for the construction of geometrical objects from DNA. *J. Am. Chem. Soc.* 114:2656-2663.

Zhang, Y. and Seeman, N.C. 1994. The construction of a DNA truncated octahedron. *J. Am. Chem. Soc.* 116:1661-1669.

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