

17. Been, M. D. & Cech, T. R. *Cell* **50**, 951–961 (1987).
 18. Wells, N. C. & Fersht, A. R. *Nature* **316**, 656–657 (1985).
 19. Turner, D. H., Sugimoto, N., Kierzek, R. & Dreikow, S. D. *J. Am. chem. Soc.* **109**, 3783–3785 (1987).
 20. Quigley, G. J. & Rich, A. *Science* **194**, 796–806 (1976).
 21. Dix, D. B., Wittenberg, W. L., Uhlenbeck, O. C. & Thompson, R. C. *J. biol. Chem.* **261**, 10112–10118 (1986).
 22. Dock-Bregeon, A. C. et al. *J. molec. Biol.* **209**, 459–474 (1989).
 23. Bass, B. L. & Cech, T. R. *Biochemistry* **25**, 4473–4477 (1986).
 24. Perreault, J.-P., Wu, T., Cousineau, B., Ogilvie, K. K. & Cedergren, R. *Nature* **344**, 565–567 (1990).
 25. Yang, J., Perreault, J.-P., Labuda, D., Usman, N. & Cedergren, R. *Biochemistry* **29**, 11156–11160 (1990).
26. Dahm, S. C. & Uhlenbeck, O. C. *Biochimie* **72**, 819–823 (1990).
 27. Michel, F. & Westhof, E. *J. molec. Biol.* **216**, 585–610 (1990).
 28. Wu, T., Ogilvie, K. K. & Pon, R. T. *Nucleic Acids Res.* **9**, 3501–3517 (1989).
 29. Scaringe, S. A., Franklyn, C. & Ussman, N. *Nucleic Acids Res.* **18**, 5433–5441 (1990).
 30. Michel, F., Jacquier, A. & Dujon, B. *Biochimie* **64**, 867–881 (1982).
 31. Waring, R. J., Towner, P., Minter, S. J. & Davies, R. W. *Nature* **321**, 133–139 (1986).

ACKNOWLEDGEMENTS. We thank C. Grosshans and A. Ritter for synthesis of oligonucleotides, and J. Piccirilli, D. Herschlag and O. Uhlenbeck for helpful discussions. This work was supported by the Jane Coffin Childs Fund for Medical Research (A.M.P.). T.R.C. is American Cancer Society Professor and Investigator, Howard Hughes Medical Institute. We thank the W. H. Keck Foundation for support of RNA science on the Boulder campus.

Synthesis from DNA of a molecule with the connectivity of a cube

Junghuei Chen & Nadrian C. Seeman

Department of Chemistry, New York University, New York, New York 10003, USA

A PRINCIPAL goal of biotechnology is the assembly of novel biomaterials for analytical, industrial and therapeutic purposes. The advent of stable immobile nucleic acid branched junctions^{1–4} makes DNA a good candidate for building frameworks to which proteins or other functional molecules can be attached and thereby juxtaposed^{5–7}. The addition of single-stranded ‘sticky’ ends⁸ to branched DNA molecules converts them into macromolecular

valence clusters that can be ligated together¹. The edges of these frameworks are double-helical DNA, and the vertices correspond to the branch points of junctions. Here, we report the construction from DNA of a covalently closed cube-like molecular complex containing twelve equal-length double-helical edges arranged about eight vertices. Each of the six ‘faces’ of the object is a single-stranded cyclic molecule, doubly catenated to four neighbouring strands, and each vertex is connected by an edge to three others. Each edge contains a unique restriction site for analytical purposes. This is the first construction of a closed polyhedral object from DNA.

The synthetic scheme, shown in Fig. 1, is to synthesize ten strands, corresponding to two squares, and then to ligate them together. This scheme permits phosphorylation at three stages, so that intermediate products of a well-defined nature can be analysed: (1) the 80-mer circles, strands 1 and 6 are phosphorylated, cyclized by ligation, and then associated with the other strands that make up their squares; (2) the two squares are

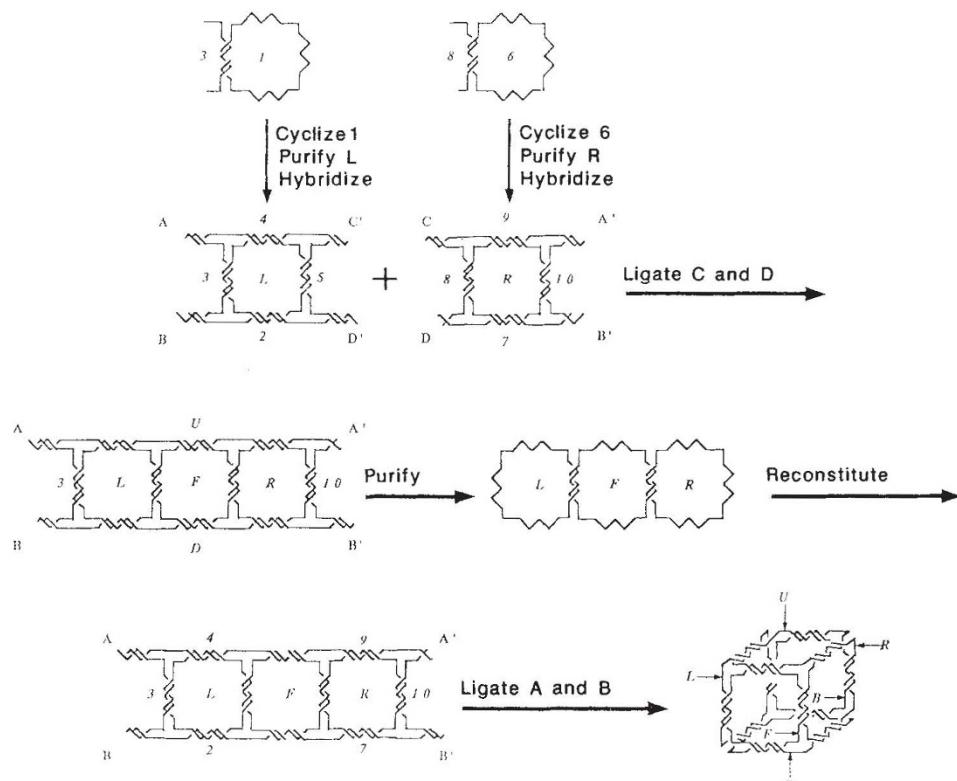


FIG. 1 The synthetic scheme used to synthesize the cube-like object. This diagram illustrates the strand identification used in the text. Numbers refer to strand numbers. We have used the convention that as a new strand is formed by ligation, its identification changes from one or more numbers to a letter corresponding to its position in the final object. For example, strand 1 is synthesized as a linear molecule, but is referred to as L once it is cyclized. The six final strands in the object are referred to as L (left), R (right), U (up), D (down), F (front) and B (back). Depiction of twisting between strands is confined to the central portion of each edge for clarity, following a previous convention⁷. This scheme depicts 5 steps (separating 6 stages) in the synthesis of the three-dimensional, 3-connected DNA object, after individual strands have been purified. The first step has two parts, the cyclization of the full-length strands 1 and 6 to form L and R, respectively. These two cycles are then hybridized with strands 2–5 and 7–10, respectively, to form the constituent squares shown in the second step. These squares are ligated together at the complementary sticky ends C' and C (5 base 5' overlap) and D and D' (4 base 3' overlap). This reaction forms strands U, F and D. U and D are discarded in a purification on a denaturing gel that isolates the L–F–R triple complex. The L–F–R–2–3–4–7–9–10 complex is then reconstituted and the final ligations are performed to close sites A and A' (4 base 5' overlap) and B and B' (5 base 3' overlap), as well as to seal the nicks in U (4–9) and D (2–7). This series of reactions forms the entire 3-D, 3-connected¹⁴ object. Note that each edge is doubly linked to each of its four neighbouring strands. Denoting an edge by the two strands that comprise it, the restriction sites are: LF, *Dde*I; FU, *Bst*EII; RU, *Sau*96I; BU, *Bst*NI; LF, *Rsa*I; FR, *Bst*UI; RB, *Hha*I; BL, *Alu*I; FD, *Hinf*I; RD, *Taq*I; BD, *Sty*I; LD, *Hae*II. The sequences of the individual strands are: strand 1: CTGGCTCATCTGCA-

AGGGGGCGAG; strand 2: CGCTGTGGTCAGGCCTGGCGAATGCGAGGCCAAT-CCTTGG; strand 3: GATTGGCTTAGAGCAAGCTGCCCTCTCGTTAGTT; strand 4: CTGGAACTAACGCTAACCATCTAAGTGAAAGTCTCTTG; strand 5: GTGAC-CAAGAGAGTTGGCGTACACGGTGTACCCACAGCGACTC; strand 6: CGTGCTAACAGGTAGATTCGACGAATTACACAATCGCGCAATACTATCCGACTTGGACCGCGCTTCGCGCATCTCG; strand 7: GTGATTGGTAATTCTCGCAACTCTACCCTGAATGCGAGT; strand 8: GCATTCAGTGTAGCACCGCAGAGATGGCGCTTGACG; strand 9: GTCACCGTCAGAAAAGGCTGTCAGTCGGGGCAGCGCT; strand 10: CCAGGACGCTGCATAGATTGCGCCGATTGTCATCACCCAAG.

1 2 3 4 5 6

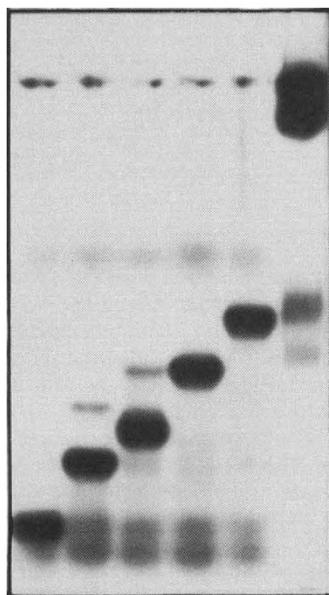


FIG. 2 The association of individual strands 2-5 with strand L to form quadrilaterals. Autoradiogram of a native gel in which strand L has been labelled and cyclized previous to the reactions shown. The first five lanes shown indicate the sequential addition of strands 2-5 to strand L. Lane 1: strand L; lane 2: L + 2; lane 3: L + 2 + 3; lane 4: L + 2 + 3 + 4; lane 5: L + 2 + 3 + 4 + 5. Lane 6 shows the ligation of two quadrilaterals to form the belt-like structure shown in Fig. 1, following ligation of ends C and D. The dark band near the top of the well in lane 6 contains the belt-like intermediate and many failure products as well, indicating that one cannot separate the desired product by native gel electrophoresis. The topmost band (top of the well) contains a complex of smaller species that cannot penetrate the gel. The DNA molecules used are synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support and deprotected using routine phosphoramidite procedures¹⁵. DNA molecules are purified from denaturing gels. Hybridized complexes are formed by mixing a stoichiometric quantity of each strand, as estimated by absorbance at 260 nm; this mixture is heated to 90°C for 5 min and slowly cooled. Errors in stoichiometry are corrected by electrophoresing the complex on a non-denaturing gel, and extracting the appropriate band from the gel. Except where noted in the text, all gels contain 6% polyacrylamide (19:1, acrylamide:bisacrylamide).

annealed with only those phosphates on the end-labelled C, C', D and D'; (3) the last phosphorylation before reconstitution puts a 5' phosphate on all unphosphorylated strands, and is followed by the final ligation. The sequences of the individual strands (see legend to Fig. 1) have been assigned using the program SEQUIN (ref. 9). Each square contains no repeated contiguous stretch of 6 nucleotides^{1,9}. Only 28 of 480 such sequences are duplicated in the entire cube. In principle, only two of the steps used are necessary for the synthesis of the cube: (1) ligation of C and D, simultaneously with the covalent cyclization of strands 1 and 6, followed by (2) the ligation of A and B. The five steps shown in Fig. 1 are a response to the failure of that approach.

Figure 1 shows the strand numbering and nomenclature used. The association of L with strands 2-5 is shown in Fig. 2; similar results are obtained with R and strands 7-10. We show that these fifth-order reactions go largely to completion, but this does not guarantee that all strands are double-helical. For example,

strand 2 might be paired with strands 3 and 5, but not with L. Both here and in the reconstitution steps below, we use susceptibility to restriction endonucleases as an assay for perfect pairing, as most of our sites are recognized by enzymes with exclusively double-helical activity. In each case, complete digestion indicates that the relevant strands have completely hybridized.

Ligation of square L to square R forms the tricyclic belt, shown at the third stage of Fig. 1. The desired product of the ligation is not separable from other products on non-denaturing gels (Fig. 2), but successful double ligation of cohesive ends C and D is identified by a 3-circle catenated intermediate (L-F-R) after treatment with exonuclease III. Figure 3 shows a denaturing gel with numerous exonuclease III-resistant product bands following ligation of C and D. Figure 3 also shows the products obtained when 21 nucleotide pairs per edge are used, rather than 20; the L-F-R band is virtually absent. We purify the catenated L-F-R core of the belt on denaturing gels, and then reconstitute it by the adding the missing six strands, now phos-

FIG. 3 The products of the first ligation (ends C and D). Autoradiogram of a denaturing gel illustrating the products of ligating two quadrilaterals together at ends C and D. Kinase labelling and ligations were as described⁷. The quadrilaterals are radioactively labelled on the F strand. The first two lanes illustrate the ligation of quadrilaterals containing 20 nucleotide pairs per edge, whereas the quadrilaterals used for the second two lanes contain 21 nucleotide pairs per edge. Lanes 1 and 3 show all the products, and lanes 2 and 4 show the products following digestion with exonuclease III. Small products seen in these lanes result from incomplete digestion. The symbols on the left and right indicate the total number of nucleotides in the species in the band with the indicated mobility. L means linear species that degrade upon treatment with exonuclease III. The other symbols indicate circular or catenated species, with two linked circles indicating the L-F or F-R catenane (Fig. 1), and three linked circles denoting the L-F-R catenane (Fig. 1). Note the virtual absence of this material in the lane 4, indicating that 21 nucleotide pairs per edge are inappropriate for closing the central cycle.

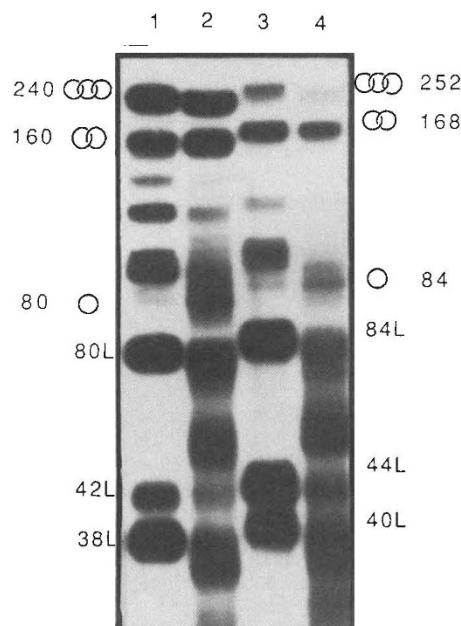
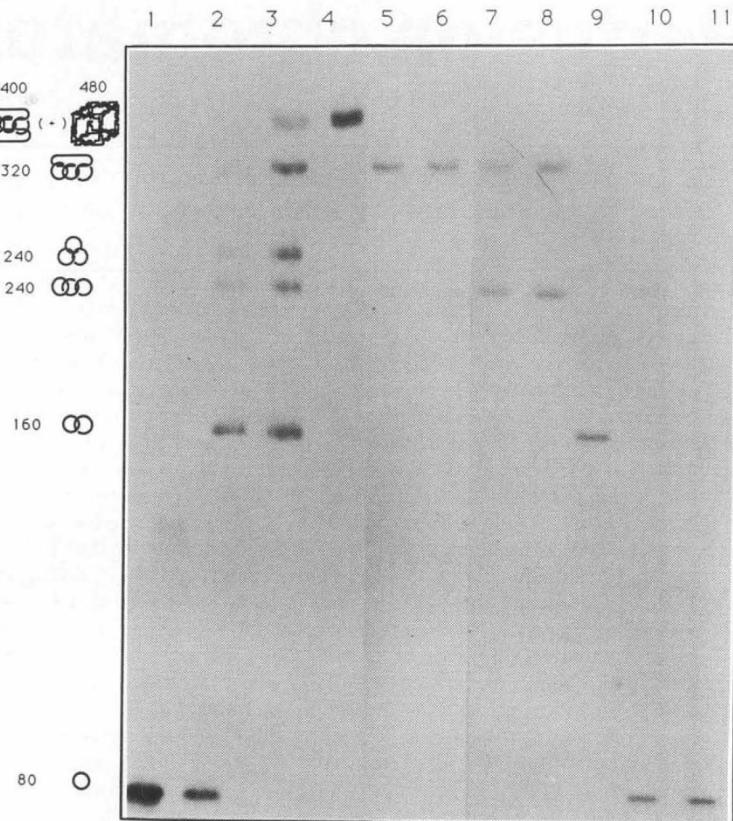


FIG. 4 Products of the final ligation step. Autoradiogram of denaturing gels showing the formation and analysis of the products of the final ligation step. The same labelling conventions apply as in Fig. 3. In addition symbols have been added to indicate other structures: Three cyclic rings correspond to a corner (for example, L-F-U (Fig. 1)); the 4-cycle symbol corresponds to a molecule with the connectivity of L-F-R-U (Fig. 1); the 5-cycle symbol corresponds to the cube lacking a single strand, for example, L-F-R-U-D (Fig. 1); the 6-cycle structure is drawn as the cube representation of Fig. 1. All material has been treated with exonuclease III. Lane 1 contains a cyclic 80-mer marker. Lane 2 contains markers (individually synthesized and then combined) corresponding to the intermediate products (up to four cycles) shown on the left. Lane 3 illustrates the products of the final ligation. The portion of the gel containing the 80-mer circle in this lane was lost during manipulation. The top most band in this lane contains a mixture of the 6-cycle final product and the 5-cycle failure product, as indicated by the (+) symbol. These materials have been separated, and lane 4 contains the purified 6-cycle final product (see text). The digestions in lanes 5–11 were all performed on this purified material, which has been labelled in strand U. Lane 5 illustrates the digestion of the product with *Bst*UI (FR) and lane 6 illustrates the digestion of the product with *Rsa*I (LF) to yield 4-cycle products. Lane 7 illustrates the double digestion of the product with *Apa*I (BL) and *Hha*I (RB) to yield a 3-cycle belt. Lane 8 illustrates the double digestion of the final product with *Bst*UI (BL) and *Rsa*I (LF) to yield another belt. Note that the presence of this belt implies the successful ligation of each bond indicated in the final step. Lane 9 shows digestion with *Apa*I (BL) and *Taq*I (RD) to yield a 2-circle catenane. Note the absence of a doublet, suggesting the absence of mixed (single and double) catenation species. Lanes 10 and 11 are a duplicate experiment in which the product has been digested with *Apa*I (BL), *Hha*I (RB), *Bst*UI (FR) and *Rsa*I (LF). The product is a single 80-mer circle; absence of 160-mer circles indicates that no detectable doubling of the belt has occurred.



phorylated. Once closed, the cube consists of six linked circles, corresponding to the six faces of the object, and labelled left (L), right (R), up (U), down (D), front (F) and back (B). Figure 4 shows a denaturing gel containing the results of the final ligation. The cube comigrates with a 5-strand standard, but can be purified on 13% denaturing gels containing 1.25% bisacrylamide. The yields from square ligation and from final closure are each about 10%.

Restriction of each edge individually results in the production of a 4-circle molecule (see for example Fig. 4, lanes 5 and 6; also data not shown). The final ligation forms the U, B and D circles. Restriction of the L-F and F-R edges results in the U-B-D triple catenane (Fig. 4, lane 8). This is the most robust proof of formation of the cube, as the belt present at the start of the last step is destroyed by restriction. Whether the final product corresponds to a single or multiple cycles of the belt (octagonal prism, dodecagonal prism, and so on) may be determined by the size of the U or D strand: at the concentrations used (10 nM in belt), the cube is the main product

(Fig. 4, lanes 10 and 11).

The side of the belt facing the reader at the branch points (Fig. 1) corresponds to the major groove of DNA, whereas the minor groove is away from the reader. We do not know on which side we have closed the belt, nor whether we have a mixture. Model building¹⁰ suggests that closure behind the page (minor groove) results in a large and more open structure. We have noted that the belt containing 20 nucleotide pairs per edge is much more readily formed than the one containing 21 nucleotide pairs per edge. This finding suggests that the twist around the F square, when attached to the L and R squares, is less than the eight cycles one would expect^{11,12}. Presumably this decreased twist occurs at the site of the junctions. Whether it is shaped like a cube or another parallelopiped, the very tight angles between the object's negatively charged edges are probably more difficult to realize than the angles that characterize better approximations to the sphere¹³. The synthesis of this object establishes that it may be feasible to make larger and more complex objects^{13,14}. □

Received 7 November 1990; accepted 11 February 1991.

1. Seeman, N. C. *J. theor. Biol.* **99**, 237–247 (1982).
2. Seeman, N. C. & Kallenbach, N. R. *Biophys. J.* **44**, 201–209 (1983).
3. Kallenbach, N. R., Ma, R.-J. & Seeman, N. C. *Nature* **305**, 829–831 (1983).
4. Seeman, N. C. *J. Biomol. Str. Dyns* **3**, 11–34 (1985).
5. Robinson, B. H. & Seeman, N. C. *Prot. Engn.* **1**, 295–300 (1987).
6. Hopfield, J. J., Onuchic, J. N. & Beratan, D. N. *Science* **241**, 817–820 (1988).
7. Chen, J.-H., Kallenbach, N. R. & Seeman, N. C. *J. Am. chem. Soc.* **111**, 6402–6407 (1989).
8. Cohen, S. N., Chang, A. C. Y., Boyer, H. W. & Helling, R. B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3240–3244 (1973).
9. Seeman, N. C. *J. biomol. Str. Dyns* **8**, 573–581 (1990).

10. Seeman, N. C. *J. biomol. Str. Dyns* **5**, 997–1004 (1988).
11. Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 200–203 (1979).
12. Rhodes, D. & Klug, A. *Nature* **286**, 573–578 (1980).
13. Williams, R. *The Geometrical Foundation of Natural Structure* (Dover, New York, 1979).
14. Wells, A. F. *Three-dimensional Nets and Polyhedra* (Wiley, New York, 1977).
15. Caruthers, M. H. in *Chemical and Enzymatic Synthesis of Gene Fragments* (eds Gassen, H. G. & Lang, A.) 71–79 (Verlag Chemie, Weinheim, 1981).

ACKNOWLEDGEMENTS. We thank R. Sheardy, S. Fischer, D. Schwartz and N. Kallenbach for discussions and encouragement, and for the use of equipment in their laboratories. This research has been supported by grants from the Office of Naval Research and the NIH to N.C.S., and by a Margaret and Herman Sokol Fellowship to J.C.