A Synthetic DNA Molecule in Three Knotted Topologies†

Shou Ming Du,§ B. David Stollar,‡ and Nadrian C. Seeman*,§,⊥

Contribution from the Department of Chemistry, New York University, New York, New York 10003, and Department of Biochemistry, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, Massachusetts 02111

Received June 17, 1994®

Abstract: The construction of knotted topologies is a key goal of stereochemistry. In order to measure the chiral properties of knotted molecules, it is necessary to produce both enantiomers of a knot from the same molecule. A molecule containing the same backbone structure that is an amphichiral knot can provide a useful control molecule for such measurements. In the case of molecules with chiral backbones, configurational chirality, exclusive of the chirality due to knotting, must be measured from the circle of the same sequence. Trefoil knots of both chiralities, an amphichiral knot, and an unknotted circular molecule have all been constructed by enzymatic closure of the same linear DNA molecule. The molecule contains two double helical domains that can be induced to assume the right-handed B conformation or the left-handed Z conformation under selected solution conditions. The molecules expected to contain left-handed DNA have been shown to bind an anti-Z-DNA antibody in gel-retention assays.

Knotted topologies have a long history in both biology[1−5] and chemistry,[6−9] but it is only recently that it has been possible to construct particular knotted molecules. Sauvage and his colleagues have reported the synthesis of a mixture of both chiralities of trefoil knots[10,11] from small molecules, and we have reported the synthesis of trefoil[12−14] and figure-8[15] knots from single-stranded DNA. The physical properties of knots are of great interest.[16] However, they are not readily available from chemical species, because of the difficulties associated with their syntheses. In order to use physical means to probe chirality resulting from knots of opposite handedness, it is necessary to produce and isolate both enantiomers of the same molecule. Liang and Mislow[17] have used the term amphicheiral, to describe knots that are topologically achiral; in discussing knots constructed from chiral components (D-nucleotides), we use the term "cheirality" here to describe the chirality that refers to the knotting of the molecule, regardless of the local backbone configuration. Topologically negative nodes are produced by normal right-handed B-DNA, so that the trefoil knots we have reported previously have always been negative trefoil knots. In order to construct an amphicheiral figure-8 knot, containing both positive and negative nodes, we used a strand containing a proto-Z-DNA domain, a sequence capable of forming left-handed Z-DNA under appropriate solution conditions; we then performed the ligation in conditions that promote the formation of Z-DNA.[13,15] Recently, we have suggested that it is possible to construct any designated knot from a single-stranded DNA molecule.[18] This suggestion is predicated on the apparent equivalence between a half-turn of double helical DNA and a node in a knot.

In order to measure the cheiral properties of knotted DNA molecules, it is necessary to prepare cheiral knots of both hands, and the circle of the same sequence from a single molecule. As discussed below, the preparation of an amphicheiral knot from this molecule can provide a useful control. Here, we report the synthesis of trefoil knots of both cheiralities, the circle and the amphicheiral figure-8 knot, all from the same DNA molecule. We do this by using a motif that we have used previously,[12,13,15] containing two double helical domains. We have continued to use the formation of left-handed Z-DNA[19,20] as a means of generating positive nodes. Both of the domains contain proto-Z sequences, but their propensities for forming Z-DNA are different. Thus, under different solution conditions, both domains are in the B conformation, one is B-DNA and one is Z-DNA, or both are Z-DNA. DNA knots are most readily distinguished from each other by their gel mobilities, because knots with more nodes are known to migrate faster than knots with fewer nodes under appropriate conditions.[21,22] Whereas the two species of trefoil knots both contain three nodes, we have distinguished these target molecules by gel-retention assays that use antibodies to Z-DNA.

* Address correspondence to this author.

† Keywords: DNA branched junctions, DNA knots, DNA topology, control of polymer structure.

‡ Tufts University.

§ New York University.

⊥ This paper is dedicated to Alexander Rich on the occasion of his 70th birthday.

® Abstract published in *Advance ACS Abstracts*, January 1, 1995.

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Materials and Methods

Synthesis and Purification of DNA. The 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.[23] Molecules are synthesized with a 5′ phosphate added chemically, using 2-[[2-[(4,4′-dimethoxytrityl)-oxy]ethyl]sulfonyl]ethyl 2-cyanoethyl *N,N*-diisopropylphosphoramidite, purchased from Glen Research (Sterling VA). DNA strands are purified by denaturing gel electrophoresis.

Enzymatic Reactions. *A. Kinase Labeling.* 2 pmol of an individual strand of DNA are dissolved in 10 μL of a solution containing 66 mM Tris·HCl, pH 7.6, 6.6 mM $MgCl_2$, and 10 mM dithiothreitol (DTT), and mixed with 1 μL of 2.2 μM γ-^{32}P-ATP (10 mCi/mL) and 3 units of polynucleotide kinase (U.S. Biochemical) for 2 h at 37 °C. The reaction is stopped by heating the solution to 90 °C. We find that previous chemical phosphorylation results in increased specific activity of the radioactively labeled material; this is because it is not necessary to follow the radioactive phosphorylation step with a second phosphorylation step using unlabeled ATP, thereby avoiding exchange of labeled phosphate with unlabeled phosphate.

B. Ligations. Ligations are performed using a DNA concentration of 100 nM, in the kination buffer, which is brought to 66 μM ATP and 1 μM DNA linker. T4 polynucleotide ligase (10 units) (U.S. Biochemical) is added, and the ligation proceeds at 16 °C for 10−16 h. The solution is brought to 3 mM in $Co(NH_3)_6Cl_3$ (Aldrich) when 3_1^+ knots are synthesized. Samples are purified by formamide-containing polyacrylamide gel electrophoresis. The corresponding bands are cut out and electroeluted in the Elutrotrap System (Schleicher & Schull) for 2 h at 200 V. Then the samples are microdialyzed against water overnight in the cold room and later precipitated by alcohol.

C. Exonuclease III Treatment. Exonuclease III (100 units) (U.S. Biochemical) is added directly to the ligation mixture, and the reaction is allowed to proceed for 2 h at 37 °C.

Antibody Preparation. Monoclonal antibody Z22,[24,25] from a mouse immunized with brominated poly(dG-dC), was purified from ascites fluid with a protein A-Sepharose affinity column. Antibody Z22 binds Z-DNA of varying sequence.[25]

Gel Retention Assay. The indicated knot (0.1 pmol) is used in each determination. The amount of Z22 antibody is varied as indicated. Gels containing 4% acrylamide (29:1, acrylamide/bisacrylamide) are buffered with 44.5 mM Tris·HCl, pH 8.0, 44.5 mM boric acid, and 1 mM EDTA. The sample buffer is the same as the running buffer, except that it contains 5% glycerol and 0.1% xylene cyanol FF tracking dye. Electrophoresis is performed on a Hoefer SE-600 unit at 4 °C for 4 h at 8.3 V/cm. The gels are electrophoresed for 4 h under the same conditions prior to loading. Gels are dried onto Whatman 3MM paper and are exposed to Kodak X-OMAT AR film for up to 15 h.

Denaturing Polyacrylamide Gel Electrophoresis. Gels contain 8.3 M urea and 10% acrylamide (19:1, acrylamide:bisacrylamide), except as indicated. Gels may contain 20−40% formamide, as indicated. 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, and 1 mM EDTA, containing 0.1% xylene cyanol FF tracking dye. Gels are run on an IBI Model STS 45 electrophoresis sequencing gel unit at 70 W (50 V/cm, constant power) or on a Hoefer SE 600 electrophoresis unit at 60 °C (28 V/cm, constant voltage); gels are then dried onto Whatman 3MM paper and are exposed to Kodak X-OMAT AR film for up to 15 h. For Ferguson analysis, absolute mobilities (cm/h) are measured from denaturing gels containing 7 M urea and 30% formamide run at 59 °C; logarithms are measured to base 10.

Results

Strand Design. The molecule used in this study is based on DNA knots synthesized previously[12,13,15] that contain two different double helical domains. These knots contain 104 nucleotides, whose cyclic sequence may be represented as **X-T-Y-T-X′-T-Y′-T-**. In this representation, **X** and **Y** represent 11- or 12-nucleotide segments that are designed to form Watson−Crick base pairs with **X′** and **Y′**, respectively, and **T** represents linker segments that consist of dT_{14} or dT_{15}. In order to construct the three target knots from the same strand, one must be able (1) to ensure that the two domains form separately, so that, for example, **X** does not pair with **Y′** and (2) to control individually the B → Z transitions in the two domains. Including synthesis of the circle, there are four independent synthetic "windows" on the system that must be defined. One cannot construct both domains from dCpdG components exclusively, because all four pairing sequences would be complementary, the first condition would not be met, and only circular molecules would be likely to result. However, specificity can be achieved by including two A−T pairs in one of the domains. Behe and Felsenfeld[28] have shown that methylation of the 5-position of cytosine facilitates the formation of Z-DNA in mild solution conditions. Therefore, one route to the target molecules is to methylate eight of the cytosines in the first domain, whose sequence is $[(dCpdG)_6]_2$, and all ten cytosines in the second domain, whose sequence is 5′-C·G·C·A·C·G·C·G·C·A·C·G-3′ paired with 5′-C·G·T·G·C·G·C·G·T·G·C·G-3′. We find DNA ligase to be ineffective in ligating Z-DNA; therefore, the 5′ and 3′ ends of the molecule have been rephased from previous knotted molecules,[13,15] to occupy a site in one of the regions between helical domains. Hence, a complementary template molecule is employed to promote the ligation. The use of a complementary template requires a 20 nucleotide linker region between helical domains. Therefore, in order to keep the length of the molecule 104 nucleotides, as in previously synthesized molecules, available as markers, we have decreased the lengths of the other linker regions to dT_{12}.

The synthetic scheme is summarized in Figure 1. Knot formation requires the presence of at least 300 μM Mg^{2+}, because the polyanionic DNA backbones are forced into close proximity with one another, when the molecule is knotted. Ligation in lower concentrations of Mg^{2+} produces circles, rather than knots (top of Figure 1). When the Mg^{2+} concentration is increased threefold, the product is a trefoil knot with negative nodes (upper central part of Figure 1). The first domain converts readily to Z-DNA in the presence of 10 mM Mg^{2+}, but the A−T-containing domain converts to Z-DNA only when 3 mM $Co(NH_3)_6Cl_3$ is added to the ligations mixture containing 10 mM Mg^{2+}. Thus, ligation in the presence of 10 mM Mg^{2+} generates the figure-8 knot (lower central part of Figure 1), and ligation in the presence of both 10 mM Mg^{2+} and 3 mM $Co(NH_3)_6^{3+}$ produces the trefoil knot with positive nodes (bottom of Figure 1). The first domain is methylated so that it will undergo the B → Z transition in the absence of $Co(NH_3)_6^{3+}$. Methylation of the second domain overcomes the inhibitory influence of the A−T base pairs on the B → Z transition. The sequence of the molecule, termed **K1**, is shown in Table 1.

Construction of the Knots. Figure 2 shows denaturing gels demonstrating that the **K1** molecule forms the different target species. Two gels are shown, containing different percentages of polyacrylamide and formamide; these two gels allow us to differentiate the trefoil knot with positive nodes (3_1^+) from both the figure-8 knot (4_1) and the trefoil knot with negative nodes (3_1^-). The treatment of the DNA is indicated above each lane

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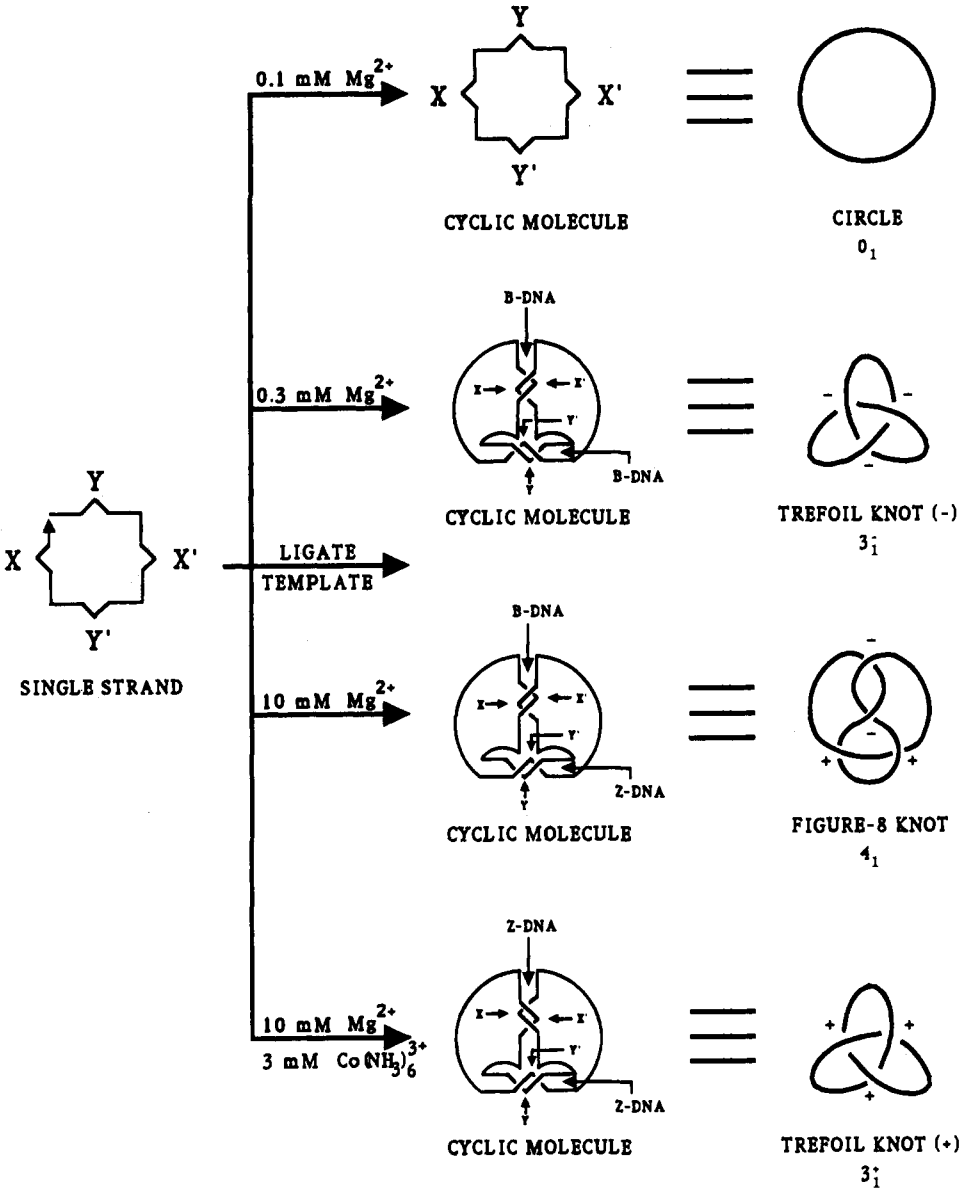

Figure 1. The synthetic schemes used to produce the target molecules: The left side of this synthetic scheme indicates the molecule from which the target products are produced. The four pairing regions, **X** and its complement **X'**, and **Y** and its complement **Y'**, are indicated by the bulges from the square. The 3′ end of the molecule is denoted by the arrowhead. Note that unlike previous designs,[12,13,15] the 3′ end is between helical domains and therefore requires a linker complementary to the 3′ and 5′ ends of the strands, in order to promote the ligation. The four independent solution conditions used to generate the target products are shown to the right of the basic structure. The pairing and helical handedness expected in each case is shown to the right of these conditions, and the molecular topology of the products is shown on the far right of the figure.

in the two panels shown. The third lane from the left in Figure 2a shows that the exonuclease resistant circle is the major product when ligation takes place in the presence of only 100 μM Mg^{2+}. The two lanes to the right show that increasing the concentration of Mg^{2+} to 300 μM enables the molecule to form the trefoil with negative nodes (3_1^-) from two domains containing B-DNA. Raising the concentration of Mg^{2+} to 10 mM permits the first domain to convert to Z-DNA. The knot that results is the figure-8 knot, the amphicheiral knot with two positive nodes and two negative nodes. A small amount of the 3_1^- knot is also formed. Proceeding to the right, the addition of 3 mM $Co(NH_3)_6Cl_3$ results in the conversion of the other domain to Z-DNA, generating the trefoil knot with positive nodes (3_1^+). The mobility of this knot is slightly slower than that of the 4_1 knot under these conditions, but the differences in mobility can be seen more readily in Figure 2b. This gel contains different percentages of both formamide and acrylamide, which results in different mobilities for the species containing Z-DNA. The first two lanes of the gel in Figure 2b show the ligation of **K1** in the presence of 10 mM Mg^{2+}, and the next two lanes show the ligation of **K1** when the mixture is brought to 3 mM in $Co(NH_3)_6Cl_3$. The ligation products are compared with previously characterized[13] marker knots constructed from a different 104 nucleotide molecule, termed **K2**, in lanes 5 (3_1^-) and 6 (4_1); linear and circular markers are shown in lanes 7 and 8. Note the similar mobilities of the circular traces in lanes 1−6 to the mobility of the 105-mer marker circle. Note also the absence of exonuclease III-resistant K1 ligation products whose mobility is lower than that of the circle (0_1). It is clear from this gel that the major exonuclease III-resistant product of **K1** ligation in the presence of 10 mM Mg^{2+} has the same mobility as the **K2** 4_1 knot and that the major product switches to a molecule of lower mobility, suggesting a trefoil knot (3_1^+), when the ligation solution is brought to 3 mM in the Z-promoting reagent, $Co(NH_3)_6Cl_3$.

The Binding of Anti-Z-DNA Antibodies to the 3_1^+ Knot.

Table 1. The Sequences of the Molecules Studied Molecule Region Sequence

molecule	region	sequence
K1	5′ template complement	CTCT
	linker 1	T_5
	domain 1 (5′)	$(^{m5}CG)_4(CG)_2$
	linker 2	T_{12}
	domain 2 (5′)	$^{m5}CG^{m5}CA(^{m5}CG)_2{}^{m5}CA^{m5}CG$
	linker 3	T_{12}
	domain 1 (3′)	$(CG)_2(^{m5}CG)_4$
	linker 4	T_{12}
	domain 2 (3′)	$^{m5}CGTG(^{m5}CG)_2TG^{m5}CG$
	linker 5	T_5
	3′ template complement	CTGGAC
K1a	5′ template complement	CTCT
	linker 1	T_7
	domain 1 (5′)	$(^{m5}CG)_6$
	linker 2	T_{11}
	domain 2 (5′)	$(^{m5}CG)_2CACA(^{m5}CG)_2$
	linker 3	T_{11}
	domain 1 (3′)	$(^{m5}CG)_6$
	linker 4	T_{11}
	domain 2 (3′)	$(^{m5}CG)_2TGTG(^{m5}CG)_2$
	linker 5	T_6
	3′ template complement	CTGGAC
K1b	5′ template complement	CTCT
	linker 1	T_7
	domain 1 (5′)	$(^{m5}CG)_4(CG)_2$
	linker 2	T_{11}
	domain 2 (5′)	$(^{m5}CG)_4CACA$
	linker 3	T_{11}
	domain 1 (3′)	$(CG)_2(^{m5}CG)_4$
	linker 4	T_{11}
	domain 2 (3′)	$TGTG(^{m5}CG)_4$
	linker 5	T_6
	3′ template complement	CTGGAC

[a] ^{m5}C signifies a nucleotide containing 5-methylcytosine.

The two trefoil knots are expected to have similar mobilities. Therefore, it is critical that we be able to demonstrate unequivocally that they are different molecules. Under native conditions, the 3_1^+ knot will contain two domains in the Z-conformation, and the 4_1 knot will contain a single Z-DNA domain; however, Z-DNA will be lacking in the 3_1^- knot. Hence, we have performed a series of gel retention experiments on these knots with an anti-Z-DNA antibody, Z22.[24,25] These experiments are shown in Figure 3. The three panels present a qualitative picture of the binding of the antibody to each of the purified knots. A trace of binding is seen when 20 ng of antibody is added to both the 4_1 knot and the 3_1^+ knot (band 1), and substantial binding is seen when 70 ng of antibody is added to these two knots. By contrast, no binding is seen with the 3_1^- knot. Binding to the 4_1 knot results in two bands, band 1 and a second band (band 2) of lower mobility; the region between them is smeared. Binding to the 3_1^+ knot results in a third band (band 3), of yet lower mobility, as well as a smear that extends from this band through band 2, to band 1. A further retarded band (band 4) is seen for both species when 180 ng of antibody is added. It is possible to make a tentative assignment of these bands as **Ab-K** (band 1), **Ab-K$_2$** (band 2), **Ab$_2$-K** (band 3), and **Ab$_2$-K$_2$** (band 4), where "**Ab**" represents an antibody molecule and "**K**" represents a knot molecule. A single antibody is capable of binding to a single Z-domain of a knot, but the **Ab-K$_2$** species is possible because Z22 is a bivalent antibody. The appearance of band 3 (**Ab$_2$-K**) only in the case of 3_1^+ agrees with the assignment, because that knot contains two Z-domains. A plot of log(MW) vs mobility supports this assignment (data not shown).

Binding to the 3_1^- knot is eventually seen, when 180 ng of antibody is added. The binding of the 3_1^- knot may be due to the conversion of its domains to a Z-like conformation. The presence of an unretarded knot and the absence of the intermediate species seen with the 4_1 and 3_1^+ knots suggests that this species forms cooperatively. We have shown elsewhere that 104-nucleotide knots containing four linkers, each 14 nucleotides long, are far from the tightest knots that can be made from a similar motif.[15] There is more linker DNA in this knot than helical DNA, thereby providing sufficient flexibility for the domains to be converted to the Z-form, while retaining the 3_1^- topology. It has been shown previously that antibody binding can drive the conversion of B-DNA to Z-DNA.[26,27] Two μg of Z22 can be shown to retard the mobility of 0.1 pmol of a previously reported 3_1^- 104-nucleotide knot (**K2**) containing a proto-Z domain[13] (data not shown). Thus, it is likely that the binding of Z22 to the 3_1^- knot entails conversion of the B-DNA to Z-DNA.

Ferguson Analysis. We have compared the molecules constructed here by means of Ferguson plots, in which the logarithm of the mobility is measured as a function of the gel concentration. The slope of the Ferguson plot is proportional to the friction constant of the molecule.[29] . The Ferguson plots of the three knots, the circle, and the linear monomer and dimer are shown in Figure 4. We are interested in the properties that depend on the topologies of the molecules, and not their structures, so we have measured the mobilities under denaturing conditions. The slopes of the 4_1 and 3_1^+ knots are identical to each other and to the circle. The 3_1^- knot is the unusual member of the group, with a slope about 6% lower. The linear monomer has parameters very different from those of the cyclic molecules, but the linear dimer has a friction constant similar to that of the 3_1^- knot. In previous measurements, the slope of the 4_1 knot was also found to be larger than that of the 3_1^- knot.[13] The measuring error of the Ferguson plot is about 0.5 mm, which translates to approximately ± 0.003 in the value of the slope which would lead to ± 0.02 in the value of the intercept for the range of acrylamide concentrations considered.

Discussion

Proof of Synthesis. It is useful to detail the logic by which the data presented above constitute proof that the exonuclease III-resistant ligation products of K1 are actually the four target molecules. Part of the proof rests on the similarity in gel mobility between previously characterized molecules and the new molecules. In this regard, it is important to point out that Figure 2b contains two different 4_1 knots of equal mobility, the target 4_1 knot from this construction (**K1**), and a 4_1 knot (**K2**) that has been characterized previously by electrophoretic and restriction analysis.[13] It is well-known that knots containing more nodes migrate more rapidly on gels.[22] Thus, the molecule identified as the 4_1 knot has the highest mobility of all the species we have generated from **K1**, the circle has the lowest mobility, and the trefoil knots are intermediate. However, there is not a simple increase in electrophoretic mobility with an increase in the Z-promoting nature of the ligation conditions: The mobility of the 3_1^+ knot is lower than the mobility of the 4_1 knot, as would be expected for this target knot.

What other topologies are available to this system? The products we infer are the simplest knots possible; more complex knots would entail five or more nodes. Nevertheless, we have pointed out that these are not the tightest knots available to this motif,[15] so it is conceivable that knots containing more nodes could be formed from **K1** molecules. Indeed, in an earlier construction, we noted trace bands moving faster than target trefoil knots that might correspond to 5_2 knots.[12] The extra nodes would need to be formed by fortuitous braiding of the

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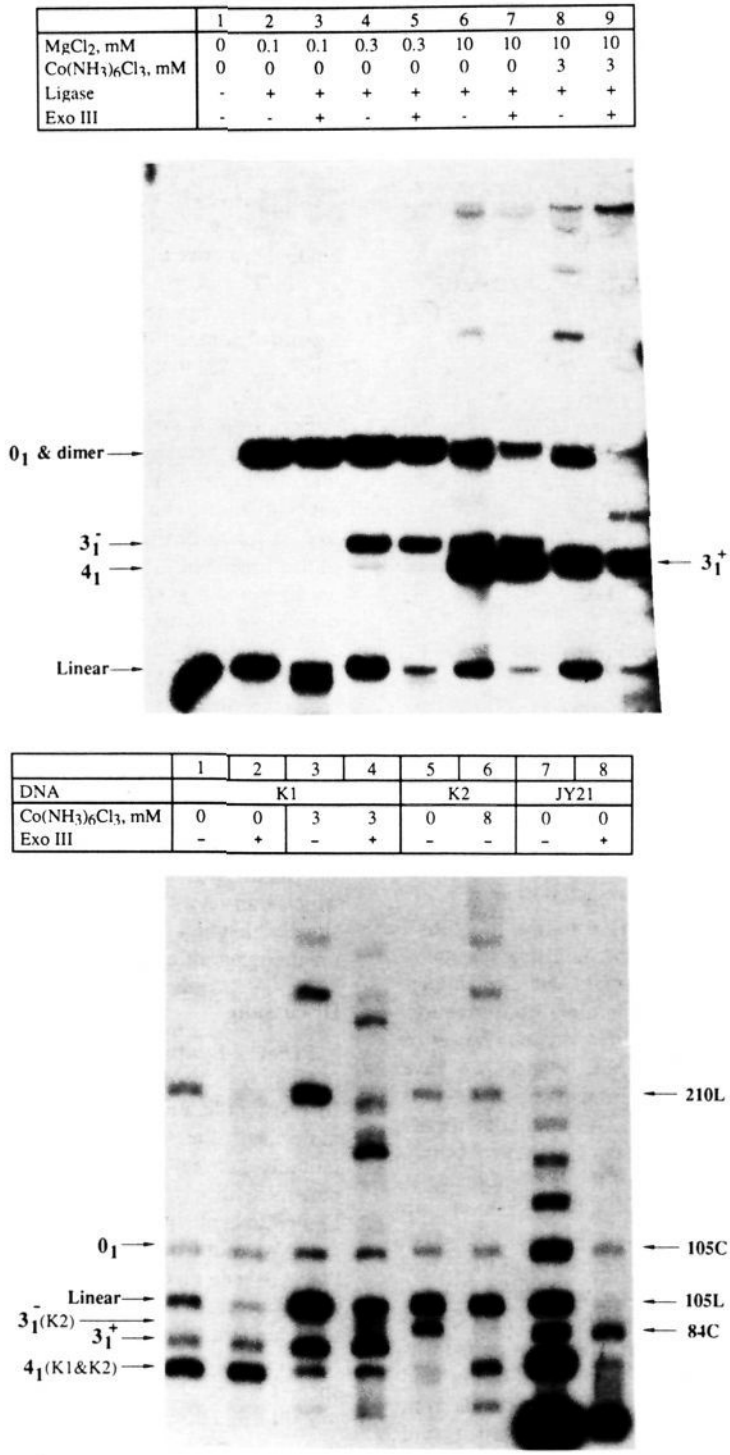

Figure 2. The products of the ligation under different Z-promoting conditions: (a, top) Ligations resolved on an autoradiogram of a denaturing 12% polyacrylamide gel containing 40% formamide: Lanes 2, 4, 6, and 8 contain the products of the **K1** strand ligated under the conditions indicated at the top of the panel. Lanes 3, 5, 7, and 9 contain the same material digested with exonuclease III. Known migratory positions are indicated at the left of the gel. Note the resistance of the circle (lane 3), 3_1^- knot (lane 5), 4_1 knot (lane 7), and 3_1^+ knot (lane 9) to digestion by exonuclease III. (b, bottom) An autoradiogram of a denaturing 6% *polyacrylamide gel containing 20% formamide:* The separation and distinct identities of each of the bands is clarified in this panel. Marker and experimental species are indicated on the sides of the gel. The contents of each lane are indicated at the top. Lanes 1 and 3 contain material ligated under conditions designed to produce the figure-8 and trefoil (3_1^+) knots from **K1**, respectively, and lanes 2 and 4 contain the same material, but digested with exonuclease III. The material in lanes 5 and 6, labeled **K2**, corresponds to marker trefoil (3_1^-) and figure-8 (4_1) 104-mer knots reported previously.[13] The sequence of K2 is **C·T·C·T·T$_{14}$·(C·G)$_6$·T$_{15}$·A·G·A·G·G·T·C·C·A·G·T·T$_{14}$·(C·G)$_6$·T$_{15}$·A·C·T·G·G·A·C·** The material in lanes 7 and 8, labeled **JY21**, corresponds to circular and linear markers previously characterized.[12] The clear separation of trefoil knot (3_1^+) from figure-8 knot is seen here. Likewise, the different mobilities of the positive and negative trefoil knots are evident.

linkers, because our varied solution conditions only affect the linking of the double helical regions. Whereas the longest linker region is paired to the template during ligation, we do not regard linker braiding as likely. We have observed no species here

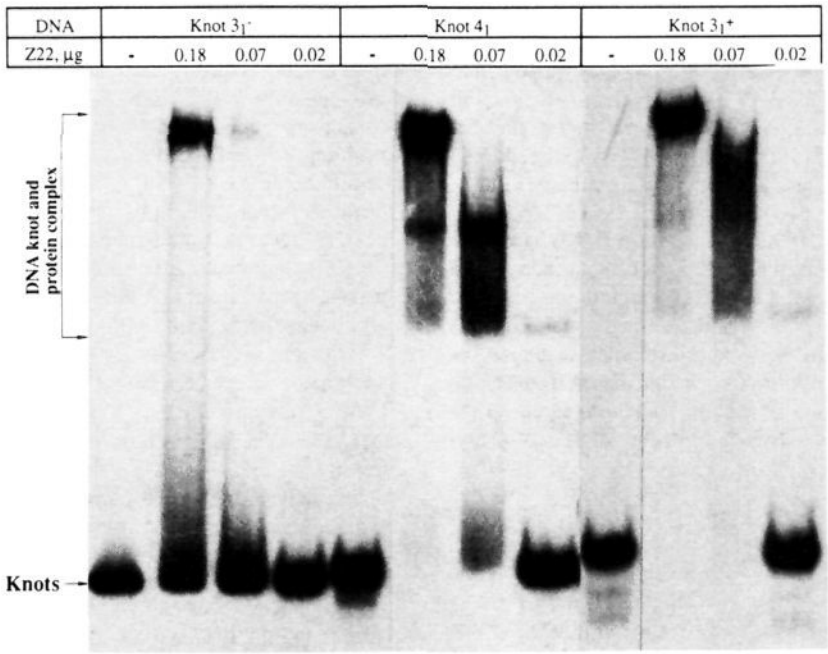

Figure 3. The binding of the knots by an anti-Z-DNA antibody: This figure shows a gel retention assay that illustrates the binding of the anti-Z antibody, Z22 to the knots. Each section of the gel is labeled by the knot employed, 3_1^-, containing exclusively B-DNA in its helical domains, 4_1, containing both B-DNA and Z-DNA, and 3_1^+, containing exclusively Z-DNA in its helical domains. Note that the antibody binds to both the 4_1 and 3_1^+ knots at a much lower concentration of antibody than does the 3_1^- knot.

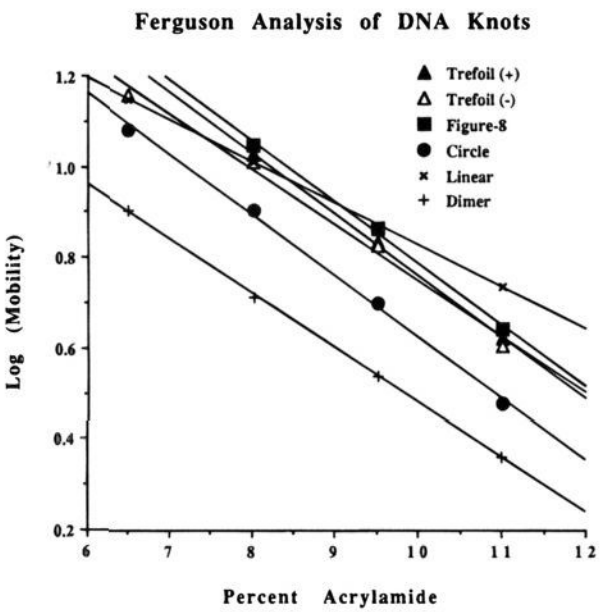

Figure 4. Ferguson analysis of all species produced: The 3_1^+ knot is represented by the filled triangles, and the 3_1^- knot by the open triangles. The 4_1 knot is represented by the filled squares and the circle by the filled squares. The linear monomer molecule is represented by the **X** symbols and the linear dimer by the + symbols. The intercepts and slopes of the plots are as follows: 3_1^+ knot, 2.1140, −0.13433; 3_1^- knot, 1.9793, −0.12307; 4_1 knot, 2.1383, −0.13533; circle, 1.9725, −0.13500; linear monomer, 1.7475, −0.09200; linear dimer, 1.6830, −0.12040.

that migrate more rapidly than the 4_1 knot (even in Figure 2a, which is somewhat overloaded), so there appear to be no higher knots present. The similarity of the mobility of our putative 4_1 knot to that of the marker 4_1 knot, combined with the fact that this is a dominant product (not a minor knot) generated by middle-strength Z-promoting conditions, lead us to reject the possibility that this knot contains more than four nodes. We have demonstrated previously[30] that catenated dimers migrate much more slowly than knots of the same cycle length, so it is unlikely that we have produced catenanes. In work to be reported elsewhere, we have shown that *E. coli* DNA topoisomerase I is capable of catalyzing the interconversion of all four of these species *via* strand passage operations that generate no additional topological species; the interconversions are driven by varying the solution environment (S.M.D., H. Wang, Y.-C. Tse-Dinh, and N.C.S. *Biochemistry*, in press, 1995). This finding rules out the possibility that any one of these bands represents a highly knotted multimeric molecule.

The increase in gel mobility in progressing from the circle through the trefoil knots to the figure-8 knot agrees with behavior expected from the products obtained under the relative ionic strength and Z-promoting conditions used: The circle (lowest mobility), the trefoil knots (intermediate mobilities that are slightly different from each other, but clearly different from the other products) and the figure-8 knot (highest mobility) are certainly distinct migratory species and hence are identified according to the number of nodes (zero, three, or four) contained in markers that migrate similarly. We had no expectation that the 3_1^+ knot would show slightly different migratory behavior from the 3_1^- knot (see below). *Therefore, we have relied on the binding of the Z22 antibody to differentiate the 3_1^- knot from the 3_1^+ knot.* The behavior of the target knots is seen clearly in Figure 3 in the lanes containing 0.07 μg of Z22 antibody. The two knots expected to contain Z-DNA, 3_1^+ and 4_1 are retarded by the antibody, but the 3_1^- knot is *not* retarded by the antibody. This finding demonstrates that this concentration of Z22 antibody is incapable of inducing the B → Z transition in these molecules. The retardation of the 4_1 knot shows two bands (1 and 2), which can be assigned as an **Ab-K** complex and as an **Ab-K$_2$** complex, formed by the bivalent Z22 antibody. *The 3_1^+ knot is expected to contain two domains of* Z-DNA, and a third band (3) arises that can be ascribed to an

(30) Fu, T.-J., Tse-Dinh, Y.-C; Seeman, N. C. *J. Mol. Biol.* **1994**, *236*, 91−105.

Ab_2-K complex. Bands 2 and 3 for both knots are followed by large smears that may result from the dissociation–reassociation of the antibody during migration, a phenomenon that has been noted previously.[31] Thus, the antibody binds to these two target molecules in explicable ways, but the differentiation between the 4_1 knot and the 3_1^+ knot is predicated on gel mobility, not on antibody binding: Antibody binding serves to distinguish the two trefoil knots.

The Measurement of Chiral Properties. The ability to produce four topologies from the same molecule implies that it will be possible to probe the cheiral properties of knotted molecules. The inherently chiral nature of single-stranded DNA backbones makes it somewhat more difficult to examine the cheiral properties of DNA knots than knots formed from achiral backbones. It is necessary to subtract the chiral signal of the backbone from the signal derived from the knot. Thus, it is expected that

$$CP(K^+) - CP(O) = CP(O) - CP(K^-) \quad (1)$$

where CP represents a chiral property, such as optical rotation or circular dichroism at a given wavelength, K^+ is the positive knot, K^- is the negative knot, and O is the circle of the same sequence. It is clearly necessary that all molecules be completely denatured for eq 1 to hold, for otherwise features of the native structures can perturb the signal. In addition, it is useful to have an amphicheiral knot of the same sequence, as an experimental control in limiting systematic errors; this knot should not demonstrate the putatively cheiral features found in the other two species

$$CP(K^0) = CP(O) \quad (2)$$

under denaturing conditions, where K° represents the amphicheiral knot. For example, for a spectral feature, eq 2 should apply at all wavelengths.

Construction of the Knots. The data presented demonstrate that it is possible to construct a circle, positive and negative trefoil knots, and a figure-8 knot, all from the same strand. This opens the way to performing experiments on the cheiral properties of these knots and on the transformations between them. Scale-up experiments are in progress, in order to synthesize enough material to characterize the cheiral properties of the knots by optical means. As noted above, topological transformation studies of **K1**, catalyzed by single-strand-specific topoisomerases, will be reported elsewhere (S.M.D., H. Wang, Y.-C. Tse-Dinh, and N.C.S. *Biochemistry*, in press, 1995).

It should not be surprising that we have had to do a certain amount of experimentation to design a molecule with the properties we seek. Table 1 lists two other sequences (**K1a** and **K1b**) that did not yield all the target knots. They differ from the successful sequence in the domain that is most difficult to convert to Z-DNA. In **K1b**, the 3′ end of one domain contains the sequence ACA, and in **K1a** that sequence occupies positions 6–8 of the domain. These sequences were unsuccessful because it was not possible to generate more than a trace of the 3_1^+ knot, even in the presence of 10 mM $Co(NH_3)_6Cl_3$; at such high concentrations of the Z-promoting reagent, DNA ligase is inhibited. In the successful molecule, the two A–T pairs are separated from each other by five nucleotide pairs and are flanked by 5-methyl-C's. Thus, the promotion of the B → Z transition is a function of the placement of non-G-C base pairs within several sequences of the same nucleotide composition.

To our surprise, it is possible to separate the two species of trefoil knot by means of gel electrophoresis. The basis for the differential mobilities of the two knots is obscure. However, the sensitivity of mobility to formamide concentration suggests that the two molecules are not both completely denatured. The native structures of the two molecules are likely to differ, because the twists of the Z-domains are expected to be roughly −360°, but the B-domains will have twists near +411°, if they contain 10.5-fold DNA.

Antibody Binding. We have used the binding of antibodies to the knots to demonstrate that they contain Z-DNA. The DNA knots in this study consist of two double helical domains linked by single-stranded DNA spacers. The 3_1^- knot is expected to contain B-DNA in both domains, the 3_1^+ knot is expected to have Z-DNA in both domains, and the 4_1 knot is designed to contain one domain of each type. They are topologically constrained, so the handedness of each domain should be locked into the 3_1^+ and 4_1 knots, even under B-promoting conditions. Although the anti-Z-DNA antibody can promote the B → Z transition, we have noted binding differences between the 3_1^- knot and the 3_1^+ and 4_1 knots, suggesting that the double helical domains contain Z-DNA. This is the first time that the presence of Z-DNA has been shown unambiguously in DNA knots. In previous studies,[14,15] we have assumed that the positive nodes were to be found in the double-helical Z-DNA domain, but it has not been possible previously to exclude the possibility that the positive nodes were in the linker regions of the knot.

Acknowledgment. This research has been supported by grants GM-29554 (to N.C.S.) and GM-32375 (to B.D.S.) from the NIH. The support of Biomolecular Imaging on the NYU campus by the W. M. Keck Foundation is gratefully acknowledged.

JA941918R

(31) Jang, Y. J; Stollar, B. D. *J. Immunol.* **1990**, *145*, 3353–3359.