Triplex-directed self-assembly of an artificial sliding clamp on duplex DNA

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Background: Circular triplex-forming oligonucleotides (CTFOs) have previously been shown to bind tightly to short single-stranded homopurine DNAs in a sequence-specific manner. In view of the importance of double-stranded DNA as a target in the development of gene-specific therapeutic and diagnostic agents, we have investigated the binding of CTFOs to double-helical DNA.

Results: DNA-binding experiments show that a CTFO can recognize its homopurine target when the target is embedded in a long duplex. Unlike their linear counterparts, CTFOs bind the double helix in two topologically distinct forms. The more stable of the two complexes is found to be a pseudorotaxane, having the same topology as the sliding clamp protein subunits associated with some DNA and RNA polymerases.

Conclusions: Circular triplex-forming oligonucleotides have been shown to bind the DNA double helix in a topological manner which is unprecedented among synthetic ligands. This novel binding motif allows a synthetic CTFO to be irreversibly locked onto a circular double-stranded DNA target without covalently modifying the target.

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Key words: catenane, DNA tracking, DNA triple helix, pseudorotaxane, sliding clamp

Received: **23 October 1997**
Revisions requested: **11 November 1997**
Revisions received: **8 December 1997**
Accepted: **15 December 1997**

Published: **10 February 1998**

Chemistry & Biology February 1998, **5**:59–67
http://biomednet.com/elecref/1074552100500059

 ISSN 1074-5521

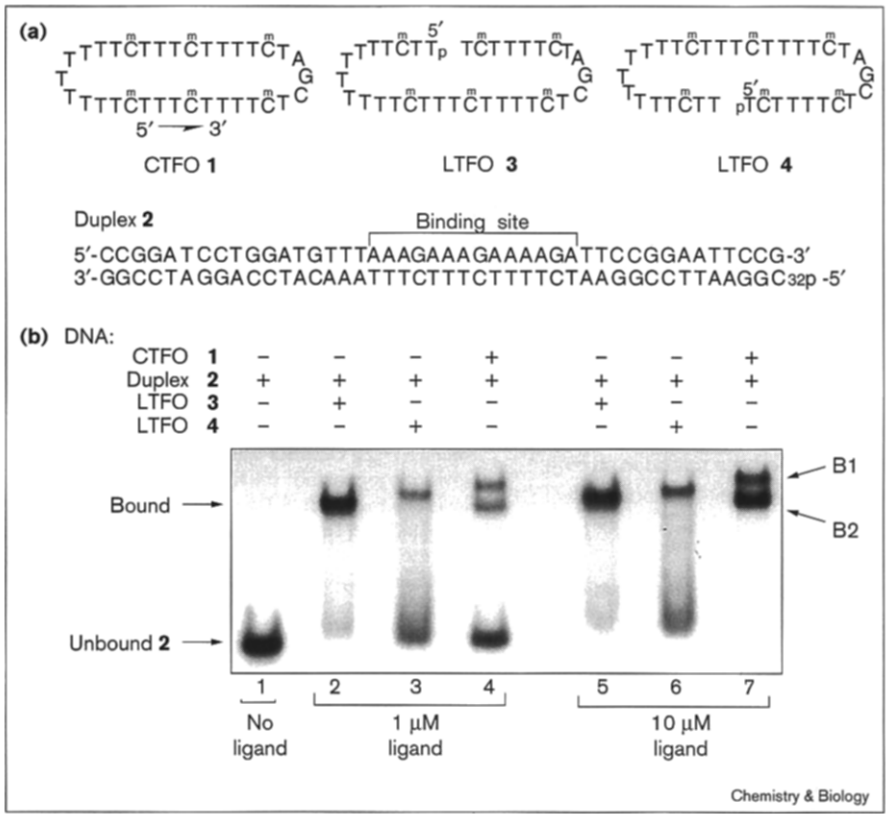
Introduction

During the past decade much progress has been made in the design of agents that bind double-stranded DNA with high affinity and sequence specificity. This progress has been motivated in part by the hope of artificially controlling the expression of specific genes at the level of transcription [1]. Among the strongest and most specific ligands are linear triplex-forming oligonucleotides (LTFOs) and modified analogs, which bind in the DNA major groove [2], synthetic oligoamides, which bind in the minor groove [3], and peptide nucleic acids (PNA), which can locally unwind the double helix to form a triplex or duplex with one of its strands [4]. The double helix is a grooved cylindrical molecule and each of these classes of sequence-specific ligands recognizes a different facet of the three-dimensional shape of the helical sequence.

One aspect of the design of DNA-binding ligands that has been overlooked, however, is the overall one-dimensional rodlike character of the helix. Chemists have long recognized that linear rodlike molecules can be encircled by macrocyclic molecules to form structures known as pseudorotaxanes [5], where the topological relationship between the two is that of a bead on a string. The ends of the linear molecule in a pseudorotaxane may be blocked to form a true rotaxane, or joined to form assemblies of interlocked rings known as catenanes. Catenanes and rotaxanes bring the strength of the covalent bond to otherwise noncovalent intermolecular interactions even though, paradoxically, no electrons are shared between the molecules involved.

The strategy of topologically linking molecules for a specific purpose has evolved in nature. The kinetoplast DNA (K-DNA) found in the mitochondria of the trypanosomatids consists of a large network of catenated double-stranded DNA minicircles (0.5–2.5 kilobases (kb)) and maxicircles (19–39 kb) [6]. It has been hypothesized that by physically linking this set of information-bearing molecules the chances of losing small but essential bits of information during cell division are minimized [7]. Another natural example of a topological linkage with DNA is found in the form of the sliding-clamp subunits used by some DNA [8] and RNA [9] polymerases. In this case, clamp-loader proteins assemble polymerase subunits onto a double-stranded DNA template. This molecular strategy confers extraordinary processivity on the polymerization by minimizing polymerase dissociation from the template while allowing the holoenzyme to move freely along it. Both of these examples demonstrate how nature has exploited the rodlike shape of the double helix to keep it in high local concentration with other molecules. This form of molecular recognition is unique in that the bound molecules are not obliged to remain fixed at specific points on their respective molecular surfaces, but are free to move locally in relation to, but without separating from, one another.

Figure 1



Gel shift experiment to show the binding of different sequences to a double-stranded DNA target. **(a)** Sequences used in the gel mobility shift assay. A superscript m denotes 5-methylcytidine residues. **(b)** Autoradiograph of a typical gel mobility shift assay. Linear triplex-forming oligonucleotides (TFOs) **3** and **4** bind the radiolabeled duplex **2** to form a single complex, while the circular TFO **1** binds to form two complexes, denoted B1 and B2. The equilibrium dissociation constants (K_d values) of each association using the gel shift technique [27] are estimated to be: **1**, 0.5–1 μM; **3**, 10–50 nM; **4**, >10 μM. In the case of **1** this value is not a true K_d as more than one product is formed.

A number of unusual DNA-binding properties of circular triplex-forming oligonucleotides (CTFOs) have been described recently [10,11]. For example, small (24–46 nucleotide) pyrimidine-rich single-stranded DNA macrocycles can recognize short single-stranded homopurine DNA sequences by forming a bimolecular triple helix. Such CTFOs bind their target sequences more strongly and with greater sequence specificity than do the natural Watson–Crick complements. The strength of this interaction provides sufficient thermodynamic driving force to enable a CTFO to replace the Watson–Crick complement of a short 12 nucleotide homopurine target strand [12].

As DNA occurs in nature most often in the form of long double-helical sequences, we have investigated the possibility that CTFOs might also recognize their target when the homopurine sequence is buried in a long double-helical molecule. The results establish that a CTFO does indeed recognize its target in the context of a double helix. Furthermore, in contrast to standard linear triplex-forming oligonucleotides, we have found that a CTFO can bind to a double helix in two topologically distinct forms, the more stable of which is a pseudorotaxane. The unusual topology of this complex has been unequivocally deduced by experiments in which the pseudorotaxane complex is selectively converted into a series of synthetic [3]-catenanes.

Results

A CTFO binds double-stranded DNA to form two distinct complexes

We initially employed a gel mobility shift assay to investigate the binding of a typical CTFO to a relatively long double-stranded DNA target containing a homopurine–homopyrimidine (pur–pyr) sequence. Incubation of the 34-mer CTFO **1** with radiolabeled duplex **2** (Figure 1a) at pH 7.0 led to the formation of two lower-mobility bands, B1 and B2 (Figure 1b). Linear versions of the same sequence, LTFOs **3** and **4**, led as expected to the formation of only one product [2], which is expected to be a parallel triplex. This result demonstrates that a CTFO can bind a homopurine sequence as it occurs in a double helix, and that the circular nature of the ligand leads to the formation of more than one complex. This same behavior was also observed for two other CTFOs 26 and 38 nucleotides in size and containing the same putative triplex-binding domain (data not shown).

We had initially anticipated that the 14 nucleotide Hoogsteen domain of **1** would bind the duplex in a manner completely analogous to the way that standard parallel-motif LTFOs bind in the major groove of a double-stranded target [2]. The non-Hoogsteen portion of the CTFO (the remaining 20 nucleotides) was at first envisioned as simply

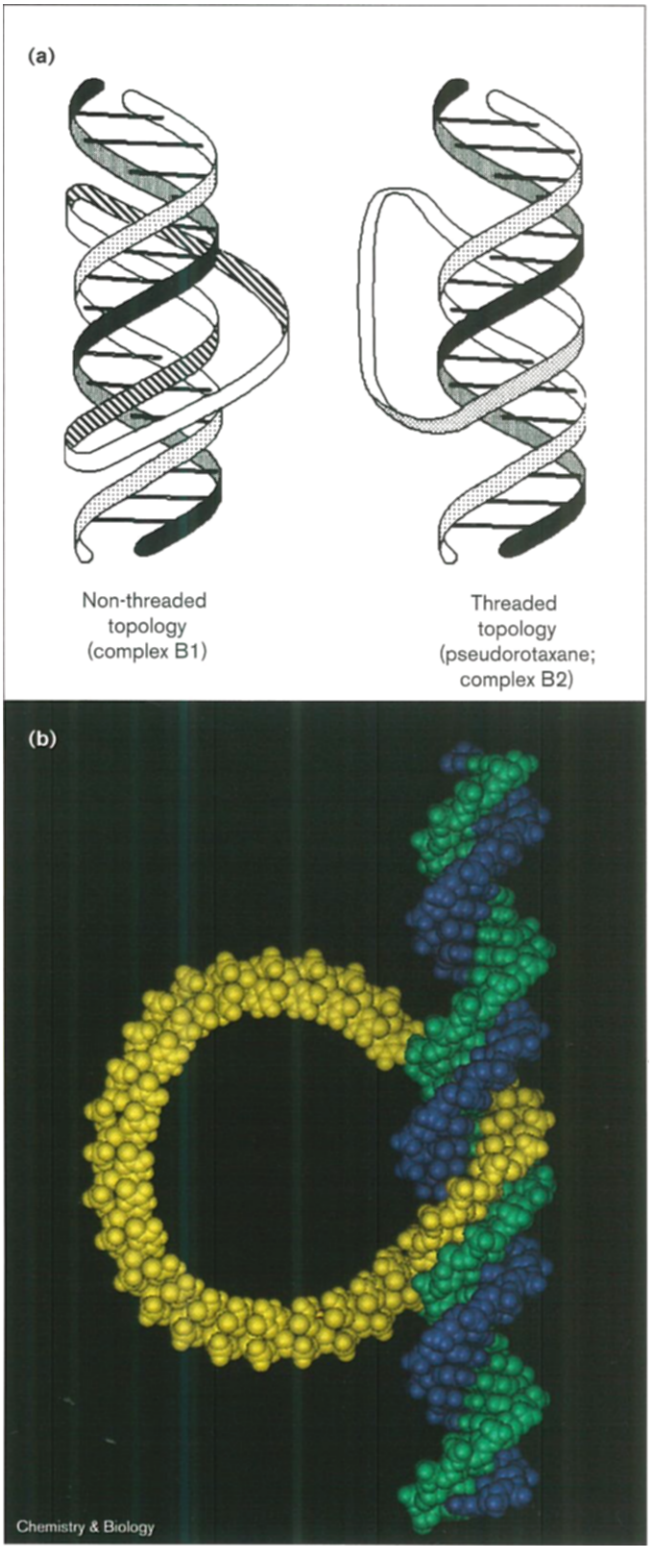
a linker that would unite the opposite ends and perhaps confer exonuclease resistance [13]. The unexpected appearance of two complexes during parallel-motif triplex formation, however, showed that the CTFO and duplex could associate in two distinct ways, including at least one form that was not anticipated. One possibility was that the CTFO could bind the double helix by locally displacing the pyrimidine-rich complement of the homopurine sequence. This type of recognition, known as strand invasion, has been found to occur in the case of PNAs [4]. Nuclease S1 and potassium permanganate probing of isolated samples of B1 and B2 indicated, however, that in both complexes the target duplex remained intact. Standard dimethyl sulfate interference footprinting [14] experiments verified that the CTFO **1** was bound only at the intended pur–pyr site near the center of duplex **2** in both complexes B1 and B2 (data not shown). In addition, a battery of chemical probing experiments using potassium permanganate, diethylpyrocarbonate and dimethylsulfate on saturated complexation mixtures of B1 and B2 (unique label in either strand in separate experiments) revealed no abnormalities in the bound duplex outside of the pur–pyr site. Furthermore, because CTFO **1** was observed in a UV melting experiment not to self-associate, and because it is improbable that two CTFOs could occupy the same central site at the same time, it was likely that in both complexes only one CTFO was bound per duplex. These initial investigations indicated that a CTFO is able to bind a double-stranded pur–pyr sequence in two distinct ways without locally unwinding the duplex by displacing one strand.

A proposed pseudorotaxane triplex structure

The puzzle of how one CTFO could bind one double-stranded sequence to form two different products was solved by recognizing that there are two topologically distinct ways that the linear duplex and circular ligand molecules could come together to form a triplex, as depicted schematically in Figure 2a. In these two structures the same covalent and non-covalent bonds are formed. The complexes differ only by the topological relationship between the duplex and the CTFO: in the linked (pseudorotaxane) configuration the CTFO encircles the double helix, whereas in the non-linked configuration it winds around it. A space-filling model of the pseudorotaxane structure is shown in Figure 2b.

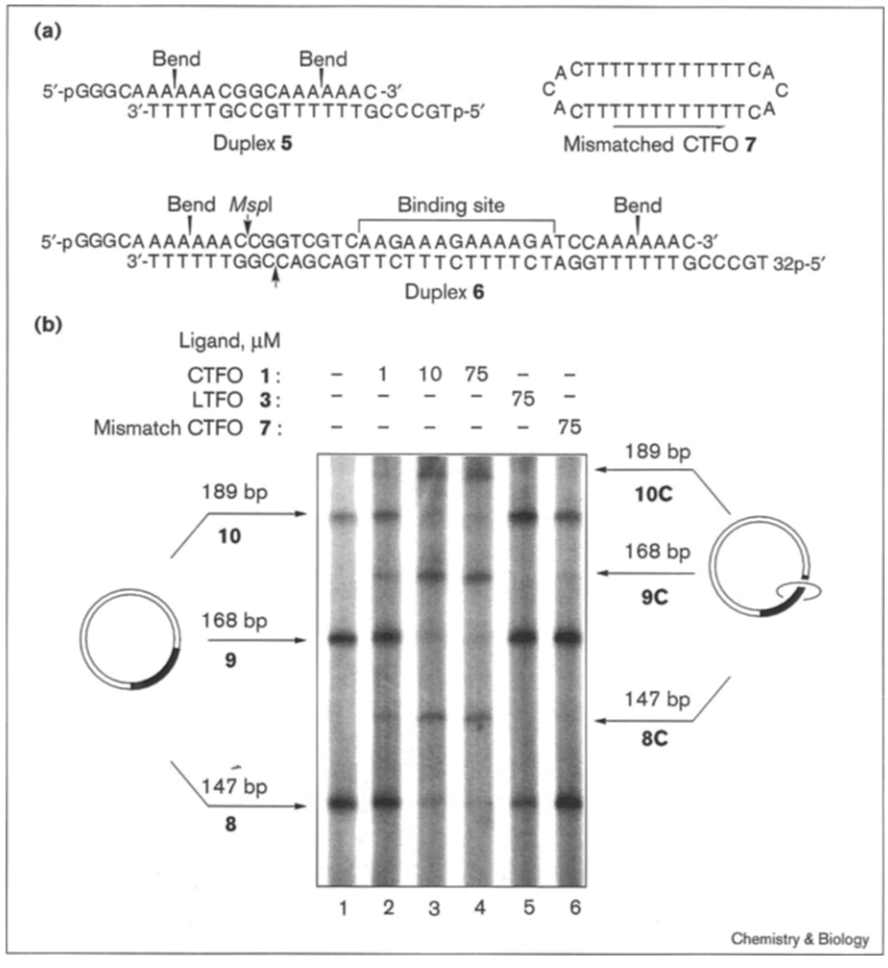
To form such a linked complex, the duplex must first thread the CTFO which then diffuses in one dimension along the helix to the triplex-forming site. In order to loop over the end of the duplex, the CTFO ligand must be large enough to adopt an open conformation with an inside diameter significantly larger than the ~20 Å cross-sectional diameter of the DNA double helix. Molecular models of CTFO **1** in an open circular conformation indicated that it can, in principle, form a central cavity as much as 55 Å in diameter (not shown).

Figure 2



A CTFO can bind to double-stranded DNA in two topologically distinct ways. **(a)** Ribbon diagrams showing the two topoisomeric complexes formed between a CTFO and approximately one turn of a double helix. **(b)** A space-filling model of the pseudorotaxane complex B2.

Figure 3



A-tract cyclization and catenation experiment. **(a)** Sequences used in the catenation experiment. Duplex **5** was taken directly from [16]. Duplex **6** contains the triplex-forming pur–pyr sequence. The global centers of the sequence-dependent bends are indicated by the wedges, and the *Msp*I restriction endonuclease cleavage site is also indicated. **(b)** Autoradiograph of the catenation experiment (6% denaturing polyacrylamide gel). Lane 1, assembly of the double-stranded circles using oligomers **5** (16.3 μM) and radiolabeled **6** (0.2 μM) in the absence of any triplex-forming ligand. Lanes 2–4, double-stranded circle formation after **6** was allowed to bind with increasing concentrations of CTFO 1. Lane 5, double-stranded circle formation after **6** was allowed to bind to LTFO **3**. Lane 6, control reaction using a non-complementary CTFO **7**.

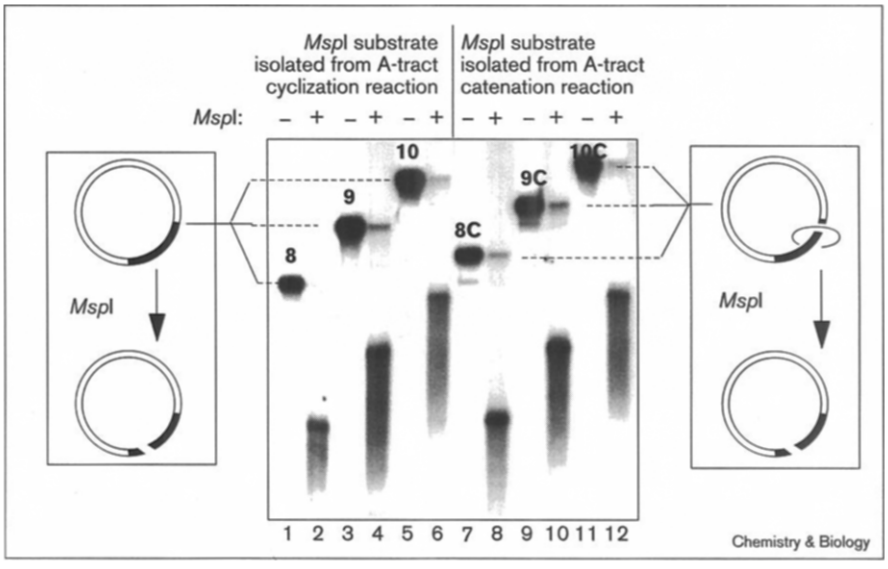
A topological experiment unequivocally demonstrates the pseudorotaxane structure

In order to obtain definitive evidence for or against a possible threaded complex (Figure 2a), we devised an experiment that would reveal the presence of a topological link between the CTFO and the duplex. After initially allowing the two complexes B1 and B2 to form, we envisioned extending the ends of the bound target duplex in such a way that they would meet to form a circular double-stranded molecule. Manipulation of the pseudorotaxane in this manner might be expected to trap the bound CTFO in the form of a [3]-catenane (the CTFO around the intertwined two strands of the circular duplex) whereas, in the case of a non-threaded complex, the CTFO will not become linked to the double-stranded molecule. In the latter case, denaturation of the triplex will separate the CTFO from the double-stranded circle. In the case of the catenane, however, the CTFO and the now circular double-stranded target will have become covalently interlocked and they can only be separated by the breaking of covalent bonds. The outcome of the experiment is revealed by a denaturing electrophoresis gel.

In order to extend the linear target duplex to circular form we employed the method of A-tract-induced double-stranded DNA cyclization [15,16]. We chose to adopt the procedure described by Crothers [16] who used the method to study the intrinsic bending property of A-tract DNA. In this procedure, the double-stranded oligomer **5** (Figure 3a), which contains two carefully phased dA_6–dT_6 tracts, is subjected to the action of DNA ligase. The in-phase dA_6–dT_6 sequences of **5** impart a well-characterized bend to this oligomer [16,17] and, as the ligation reaction proceeds, the global curvature grows with the length of the multimers formed by the ligase. A final intramolecular ligation results in a series of double-stranded circles between 105 and 210 base pairs (bp) in size [16]. We found that we could incorporate the central triplex-forming sequence of duplex **2** (Figure 1a) into an A-tract cyclization by replacing it with sequence **6** (Figure 3a). In this oligomer the pur–pyr sequence of duplex **2** is flanked by phased dA_6–dT_6 sequences and ends in single-stranded extensions which can be ligated to **5**. Radiolabeled duplex **6** was first combined with ligase and an excess of unlabeled **5** (in the absence of CTFO **1**) to form

Figure 4

*Msp*I restriction endonuclease treatment (even-numbered lanes) of isolated catenated products (lanes 7–12) and non-catenated products (lanes 1–6). The catenated CTFO in assemblies **8C**, **9C** and **10C** is released by linearization of the underlying double-stranded circle. Thus, **8** and **8C**, **9** and **9C**, and **10** and **10C** are seen to share the same labeled double-stranded DNA and to differ (before treatment) only by the presence or absence of catenated CTFO **1**, denoted by C. The polyacrylamide gel (6%) was run under triplex-denaturing conditions (see the Materials and methods section). Only the triplex was denatured in order to allow the small amount of nicked A-tract cyclization products common to this type of enzymatic synthesis [15] to migrate with the non-nicked material.



double-stranded circles of 147, 168 and 189 bp (designated **8**, **9** and **10**, respectively; Figure 3b), each containing one pur–pyr site (sequence **6**) and five, six or seven oligomer **5** units (Figure 3b, lane 1).

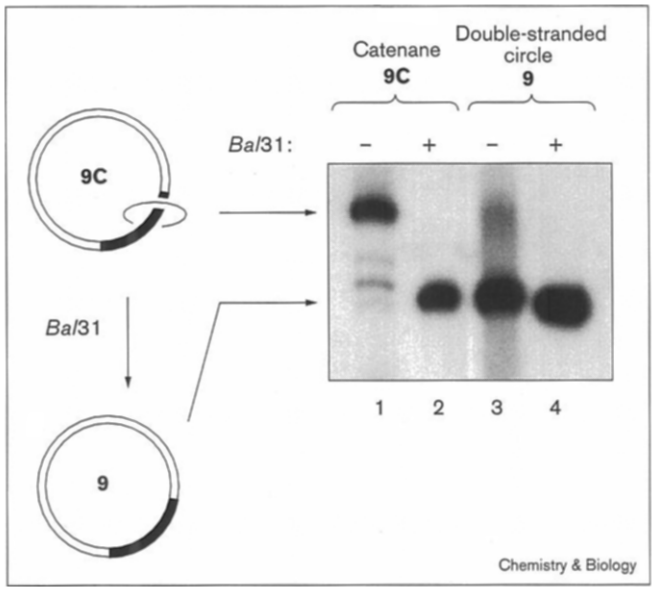
Once we had established that the triplex-forming pur–pyr sequence could be extended into circular double-stranded molecules we performed the cyclization after incubation of **6** with either linear or circular ligands. As shown in Figure 3b, lanes 2–4, allowing CTFO **1** to bind sequence **6** prior to addition of DNA ligase and the curve-inducing oligomer **5** led in a CTFO-concentration dependent fashion to a new set of products (designated **8C**, **9C** and **10C**) which appeared to form at the expense of double-stranded circles **8**, **9** and **10**. When the reaction was performed in the presence of LTFO **3** these new products were not formed (Figure 3b, lane 5), indicating that the closed circular nature of **1** was required for their formation, and that triplex formation at the pur–pyr site did not in any way alter the course of the ligation reaction. Carrying out the ligation reaction after incubation with the mismatched CTFO **7** (lane 6), which does not form a triplex with duplex **6**, showed that the sequence-specific triple-helical interaction was also required for their formation. Subsequent experiments (described below) demonstrated that the labeled component of the CTFO-dependent products was double-stranded and circular. As the CTFO slowed the mobility of the radiolabeled double-stranded circles under denaturing gel conditions it must have become physically linked to these products. (The mobility of linear intermediates formed in the ligation reaction was unaffected by the CTFO. This portion of the gel is not shown.) Thus, **8C**, **9C** and **10C** must be catenanes formed between CTFO **1** and the double-stranded circles, and a pseudorotaxane must be formed when **1** binds the triplex binding site of **2** and **6**.

Characterization of the triplex-directed DNA [3]-catenanes

We subsequently confirmed that bands **8C**, **9C** and **10C** were the [3]-catenanes formed between CTFO **1** and the double-stranded circles **8**, **9** and **10**, respectively. This was established in two ways. First, purified samples of the catenanes and the double-stranded circles were treated separately with the restriction endonuclease *Msp*I, whose recognition sequence is present in the pur–pyr-containing oligomer **6**. The *Msp*I linearizes the double-stranded circle and, in the case of the [3]-catenane, releases the intact CTFO (Figure 4). At CTFO concentrations well below the K_d and/or under triplex denaturing conditions the CTFO dissociates irreversibly. When treated in this way, **8** and **8C** produced the same linear double-stranded fragment (Figure 4). Likewise, **9C** was revealed to be the CTFO catenane of **9** and **10C** the catenane of **10**. No other radiolabeled fragments were released by *Msp*I treatment, indicating that there is only one copy of **6** per double-stranded circle and therefore that the catenanes contain only one CTFO. The identity of the linearized products was also confirmed by comparison on a denaturing polyacrylamide gel with single-stranded DNA of known length (data not shown).

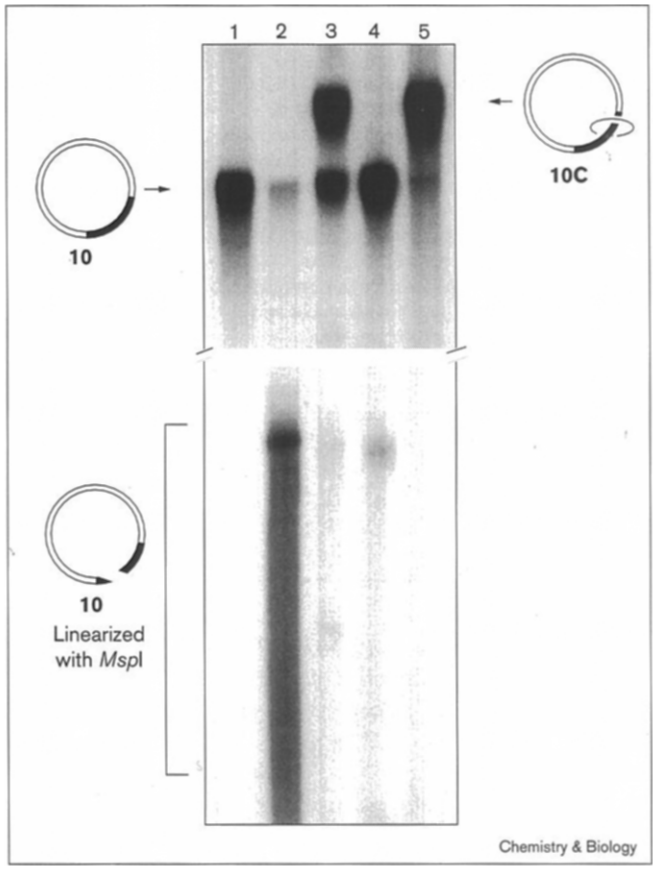
In the second method, *Bal*31 nuclease was used to remove the CTFO from the intact double-stranded circles of the catenane. This nuclease has both single-stranded endonuclease and double-stranded exonuclease activity [18]. *Bal*31 cleaves the (single-stranded) catenated CTFO into fragments that dissociate from the double-stranded circles, which, being resistant to both nuclease activities, are left

Figure 5



Representative *Bal*31 nuclease treatment of a [3]-catenane involving CTFO **1** and a double-stranded circle, and of a double-stranded circle alone. *Bal*31 has both double-stranded exonuclease and single-stranded endonuclease activities and therefore only affects the catenated single-stranded CTFO. Lane 1, isolated catenane **9C**; lane 2, **9C** after treatment with *Bal*31; lane 3, isolated double-stranded circle **9**; lane 4, **9** after treatment with *Bal*31. A 6% denaturing polyacrylamide gel.

Figure 6



Topoisomerization experiment to allow determination of the relative thermodynamic stabilities of the pseudorotaxane and non-pseudorotaxane topologies. Lane 1, double-stranded circle **10**; lane 2, double-stranded circle **10** after treatment with *Msp*I; lane 3, material from the lane 2 reaction was incubated with CTFO **1** (10 μM) and then re-closed with ligase; lane 4, as in lane 3, except LTFO **3** (10 μM) was used in place of **1**; lane 5, authentic sample of **10C** isolated from a scaled-up synthesis and purification of the A-tract catenation reaction shown in Figure 4c. A 6% non-denaturing polyacrylamide gel under triplex denaturing conditions. As in Figure 5, the non-duplex-denaturing conditions were used to avoid separation of the nicked double-stranded circles typical of A-tract-induced double-stranded circle synthesis [15].

intact. A representative treatment of isolated samples of **9** and **9C** with *Bal*31 is shown in Figure 5.

Using these two methods the assignments of all the major cyclic ligation products shown in Figure 3b were made. The two other double-stranded circles expected [16] in the series (126 and 210 bp) appeared to form in relatively small amounts and were therefore not isolated for characterization. In addition, linear intermediates and nicked circular products were also formed (data not shown) in amounts characteristic of this type of circularization [15].

Identification of the more stable complex topology

Catenation as observed here can only proceed through the intermediate pseudorotaxane in which the circular ligand assembles itself onto the duplex via a threading mechanism. The formation of catenanes in this experiment proves unequivocally that at least one of the products formed when a CTFO binds to a duplex has the pseudorotaxane topology. Figure 3b shows that under saturating CTFO concentrations (10 μM) a small amount of the uncatenated double-stranded circularization products are formed, while the major product is the catenated product. Of the two CTFO–duplex binding products observed in the gel shift experiments, B2 was always observed to form in excess over B1 at binding equilibrium. From this we tentatively concluded that a complex analogous to B2 was converted to the (major) catenation products (**8C**, **9C** and **10C**). In order to address specifically the question of which topoisomer (linked or unlinked) is thermodynamically preferred, we designed a topological isomerization experiment involving the double-stranded circle **10** and CTFO **1**. As shown in Figure 6, an isolated sample of **10** was opened at the unique *Msp*I restriction site and then allowed to bind CTFO **1** (or LTFO **3** as a control). Once binding equilibrium had been established, T4 DNA ligase was added to re-close the opened double-stranded circle sequence. In the reaction that included CTFO **1** (Figure 6, lane 3) the ratio of catenane **10C** to reformed double-stranded circle **10** was approximately 3:1 as quantitated by phosphorimaging. Under the same saturating binding conditions with the

analogous linear duplex **2**, the ratio of B2 to B1 was found to range from 3:1 to 5:1 in repeated experiments. As the religation reaction is irreversible and occurs on a time-scale that is expected to be much faster (< 1 h, see [16]) than the half-life of either B1 ($t_{1/2} \approx 6.4$ h, 22°C) or B2 ($t_{1/2} \approx 173$ h, 22°C) we conclude that the outcome of the catenation reaction faithfully represents the ratio of topoisomers at binding equilibrium. Thus, the major binding product analogous to B2 must have led directly to the major reclosure product **10C** and corresponds to the pseudorotaxane structure, the thermodynamically preferred topoisomer in the sequences studied here.

Discussion

The initial motivation for this work was to see if a CTFO could recognize its cognate homopurine target sequence in the context that would be found in nature, that is, in a relatively long double-helical molecule. Our binding experiments show that a CTFO does indeed associate with duplex DNA in a sequence-dependent manner. Unexpectedly, the association was found to result in two topologically different complexes, the more stable of which was identified as a pseudorotaxane. To form the pseudorotaxane complex, the CTFO must first diffuse over the end of the double helix, and then track along the helix until it reaches the triplex-forming site where it is immobilized by the Hoogsteen interaction. The result is a complex in which a sequence-specific ligand surrounds its target in a covalently continuous manner.

In nature, double-stranded DNA occurs in circular chromosomes and plasmids [19], as well as in linear chromosomes whose ends are blocked by telomere structures and associated proteins [20]. Thus, in principle, natural DNA targets provide the basis for catenane and rotaxane formation with circular ligands such as CTFOs. In order to form a threaded complex with such natural targets, however, either the target DNA must be modified to allow access to an unblocked end or the CTFO must be loaded onto the target *in situ* by chemical or enzymatic cyclization of a linear precursor to the CTFO sequence. The latter has been demonstrated using circular oligonucleotides closed around circular single-stranded DNA plasmids [21], but it remains to be seen whether this can be accomplished with triplex binding of a duplex DNA target.

The topological threading of a ligand on duplex DNA, while unprecedented in synthetic molecules, has been characterized for DNA-binding proteins. One well-studied example is the sliding clamp assembly of *Escherichia coli* DNA polymerase III [8]. In this case at least four polypeptides — the γ, δ and two β subunits — have evolved to load the two β subunits onto the double helix [22]. These subunits bind each other noncovalently to form a donut-like clasp. In effect, they are cyclized *in situ* around the helix. The assembled β subunits, which can then move freely along the ensnared helix, are further associated with the catalytic core of the holoenzyme whose motion is restricted to the one dimension it needs for processive polymerization — that of the template double helix.

Although the topology of the CTFO binding described here does resemble that of the β subunit dimer on DNA, there are significant differences between the two modes of binding. Whereas the protein sliding clamp is relatively easily removed by dissociating a noncovalent association of the two subunits, the CTFO cannot be removed from an end-blocked target duplex without breaking bonds, because it is covalently closed. In addition, the CTFO and β subunit dimer have quite different electrostatic charge distributions. Such differences suggest that a CTFO threading mode of binding might be more valuable as a new strategy for DNA binding by synthetic ligands, than as a model of protein sliding clamps. The development of multiple strategies for DNA binding [1–4] can lead to new and potentially useful ways to alter gene expression. The utility of a sliding clamp strategy in particular has recently been demonstrated with engineered proteins. For example, the sliding clamp of *E. coli* DNA polymerase III was engineered into a fusion protein that enhanced transcription by a non-*E. coli* RNA polymerase [23].

The oligonucleotide sliding clamp we describe here does resemble natural sliding clamp proteins in several significant ways. First, it encircles the double helix to form a pseudorotaxane structure which can diffuse in one dimension along the helix. Second, the assembly is sequence specific, requiring the triple-helical interaction to take place. If an LTFO such as **3** were to be loaded via *in situ* cyclization [24], the triplex-forming sequence would provide a DNA entry site which, in the case of the natural sliding clamp, is provided by a priming sequence [25] or sequence-specific nick [9]. And third, the bound CTFO is capable of further supramolecular assembly (by Watson–Crick hybridization to non-triplex portions; data described elsewhere) [24] which might allow the recruitment of other molecules to the assembly. In general, the new binding strategy studied here may provide a small synthetic surrogate for sliding clamp proteins in molecular applications where artificially induced DNA tracking could be of use.

Significance

We have found that circular triplex-forming oligonucleotides prefer to bind double-stranded DNA by looping over the end of the helix and then tracking along the DNA to the triplex-binding site. The formation of catenanes using this strategy furnished unequivocal proof of the linked triplex-binding topology. Topological linkage of circular molecules is a strategy that can keep supramolecular assemblies together even after all noncovalent attractive forces between them have been disrupted. The linear rodlike shape of the DNA double helix is well suited to

this strategy for maintaining high local concentration, as evidenced in nature by the linked networks found in kinetoplast DNA and the recently characterized polymerase sliding clamp assemblies. The present results establish a novel and previously unknown mode of binding between a synthetic ligand and DNA, mimicking the topology of the sliding clamp class of DNA-binding proteins.

Materials and methods

Gel mobility shift assay

The duplex **2** (approximately 5 nM, 16,000 cpm by Cerenkov counting in a plastic Eppendorf tube, 5′-end-labeled by T4 polynucleotide kinase in the pyrimidine-rich strand by standard procedures [26]) was mixed with either **1**, **3** or **4** (concentrations indicated in Figure 1b) in 10 µl of a solution containing 70 mM Tris-borate pH 7, 10 mM $MgCl_2$ and 5.5% (v/v) glycerol. After 16 h at 22°C the reactions were loaded onto a pre-chilled (4°C) 15% non-denaturing polyacrylamide gel run in a continuously recirculated buffer of the same electrolyte composition. For K_d determinations the incubation was extended to 120 h to insure that equilibrium had been reached. Migration of the bands was visualized by autoradiography and/or phosphorimaging using a Molecular Dynamics Phosphorimager.

A-tract cyclization/catenation reactions

Duplexes **5** (48.9 µM) and **6** (5′-^{32}P labeled in the pyrimidine-rich strand, 0.6 µM) were incubated (22°C, 120 h) in 200 mM Tris–HCl pH 7, 50 mM $MgCl_2$ along with the appropriate ligand at three times the concentration indicated in Figure 4c. Water, ATP, dithiothreitol (DTT) and T4 DNA ligase (Gibco-BRL, 6.6 Gibco units, in 1.1 µl) were then added to the following final concentrations: ATP 1 mM, DTT 5 mM, Tris-HCl pH 7 70 mM, $MgCl_2$ 10 mM, duplex **5** 16.3 µM, duplex **6** 0.2 µM, ligase 0.35 units/µl. The ligase was added last, 1.4 h after addition of the other reagents was completed. The final reaction volume was 19 µl. After 20 h the reactions were phenol–chloroform extracted, ethanol precipitated, resuspended in formamide loading buffer, heated to 92°C for 3 min, chilled on ice and loaded onto a 6% denaturing polyacrylamide gel (7 M urea).

*Isolation of A-tract cyclization reaction products **8, 8C, 9, 9C, 10 and 10C***

The A-tract cyclization reactions with and without CTFO 1 (10 µM) were scaled up ten times and the double-stranded cyclized products separated on a 6% denaturing gel. The bands were located by autoradiography, excised, eluted in ammonium acetate (2.5 M) overnight and ethanol precipitated. The products were resuspended in 10 mM Tris–HCl pH 7.6, 1mM EDTA and stored at −70°C.

MspI restriction endonuclease treatment

A 5000–10,000 cpm sample of each of the double-stranded circles **8–10** and **8C–10C** was incubated in the buffer provided by the vendor (New England Biolabs) with 50 units of the enzyme in 10 µl at 37°C for 2 h. Reactions were phenol–chloroform extracted and ethanol precipitated. For electrophoresis on a native gel with triplex denaturation the precipitated product was resuspended in 30 mM Tris–HCl pH 8.1, 5 mM EDTA, and heated to 45°C for 30 min followed by 1 min at 55°C. Glycerol (to 10% by volume) and tracking dyes were added before loading onto a room temperature 6% non-denaturing polyacrylamide gel run in 70 mM Tris–borate pH 8.9. For completely denaturing gel experiments, a portion of the reaction mixture was mixed with an equal volume of 15 mM EDTA/8 M urea and heated to 92°C for 3–5 min, then quickly chilled on ice before loading onto a denaturing polyacrylamide gel.

Bal31 nuclease treatment

A sample of **9** and **9C** or a crude A-tract cyclization/catenation reaction (not shown) was taken up in the buffer provided by the vendor (United States Biochemical) and 2 units enzyme (from a vendor stock of 1unit/µl) was added. After 1.5 h at 37°C the reaction was heated to 70°C for 10 min, allowed to cool slowly and then ethanol precipitated. Approximately equal amounts of radioactivity were then loaded onto a 6% denaturing polyacrylamide gel. As a control, an 84 bp linear intermediate from the A-tract cyclization reaction was treated with the enzyme in the same way, phenol–chloroform extracted and loaded onto the gel. Under these conditions this linear double-stranded fragment was completely degraded (data not shown).

Topoisomerization experiments

The 189 bp double-stranded circle **10** was cut with *MspI* as described above. The precipitated pellet was resuspended in 10 mM Tris–HCl pH 7.6, 1 mM EDTA and divided into three portions, one of which was set aside for the gel. The other two portions were incubated (22°C, 120 h) in 106 mM Tris–HCl pH 7.0, 15 mM $MgCl_2$ with either CTFO **1** (three times final concentration, Figure 6b, lane 3) or LTFO **3** (three times final concentration, Figure 6b, lane 4), and then buffer, $MgCl_2$, ATP, DTT and T4 DNA ligase were added to the final concentrations listed in the A-tract cyclization procedure. After 20 h the reactions were phenol–chloroform extracted, ethanol precipitated and resuspended in 30 mM Tris-HCl pH 8.1, 5 mM EDTA, heated to 45°C for 30 min, and then to 55°C for 1 min. Glycerol was added to 6% by volume and the reactions were loaded onto a non-denaturing 6% polyacrylamide gel. Under these gel conditions the triplex was selectively denatured, allowing any nicked products to migrate with the completely intact products, and thus giving a true representation of the topological outcome.

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