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Recognition of DNA, RNA, and Proteins by Circular Oligonucleotides

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Introduction

While the backbone organization of DNA and RNA is normally quite regular, there are many structural forms which these molecules can adapt by undergoing various secondary and tertiary helical and folded interactions, by being subjected to twisting and bending, and by interconversion between various topological and knotted variations. Probably the most common structural form of DNA in nature is a long double helix in which the ends are joined into a circle. The genomes of most lower organisms are organized in this way,[1] and clearly this circular structure lends some important advantage to these organisms or evolution would not have selected for it.

Most chemists are chiefly interested in structure and reactivity of molecules smaller than entire bacterial genomes; such large circular DNAs will not be discussed herein for that reason. Of particular interest, then, is the question: what are the smallest cyclic DNAs or RNAs which exist in nature? For DNA, the answer appears to be several hundred to perhaps 1500 nucleotides (nt), which is still rather large. For RNA, probably the smallest known circular structures are the viroid RNAs, which are single-stranded and as small as 246 nucleotides in size.[2] However, this is far from the smallest possible cyclic nucleic acid structure. Duplex DNA can exist in circles at least as small as 125 bp (base pairs);[3] cyclic structures smaller than this are difficult to achieve because of the rigidity of the double helix.[4] Single-stranded DNA and RNAs do not have this problem, and rings as small as two nucleotides are known.[5] Thus, the realm of possible cyclic DNA and RNA structures falls easily into the size range most palatable to chemists.

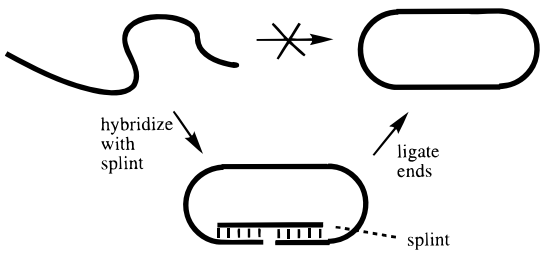

FIGURE 1. Illustration of how an oligonucleotide template or "splint" can be used to organize the ends of a precursor oligonucleotide for cyclization. Ligation chemistries (or ligase enzymes) can be used to close the circle under dilute conditions. Nontemplated cyclizations fare poorly for oligonucleotides greater than ca. 20 nucleotides in length.

Interestingly, although small synthetic circular DNAs had been reported a number of times as early as in 1968,[6] prior to 1990 there were no reported studies investigating the effect of this quite significant structural modification on DNA's molecular recognition properties. Despite this, there was quite reasonable precedent that such a structural alteration might have a large effect on such recognition properties. Indeed, in recent decades it has become widely recognized that macrocyclic molecular structures can have strong advantages in the formation of noncovalent complexes.[7] Among these advantages (relative to noncyclic molecules of similar structure) are tighter binding affinity and greater specificity for binding the target of interest. The advent of simple synthetic approaches to the construction of oligonucleotides has led to an explosion of studies aimed at studying and modifying their noncovalent binding properties. In our early work we proposed that the principle of macrocyclic recognition could also be applied to nucleic acids in small synthetic well-defined systems. The testing of that hypothesis is the subject of this Account.

Recognition of Nucleic Acids

In 1990 when we began our studies it was not trivial to synthesize a circular oligonucleotide from a linear precursor; however, the development of new synthetic methods (Figure 1)[8−12] allowed us to turn our attention toward characterizing the DNA-binding properties of such compounds. The simplest approach to recognition of a single-stranded DNA (ssDNA) or RNA with a cyclic ssDNA ligand is to form a double helical complex using simple Watson−Crick complementarity. However, for macrocycles this size it is not possible to form a complex all the way around the circle because such a duplex would be too distorted. In addition, there is a topological problem with such binding if the target strand is considerably longer than the cyclic ligand: since duplexes are right-handed helices, each turn of a helix would require that the strand pass through the circle once. In a long target this would require that the circle first be threaded onto the end and then make its way down to the binding site from the end, which is highly unlikely if the target is single stranded.

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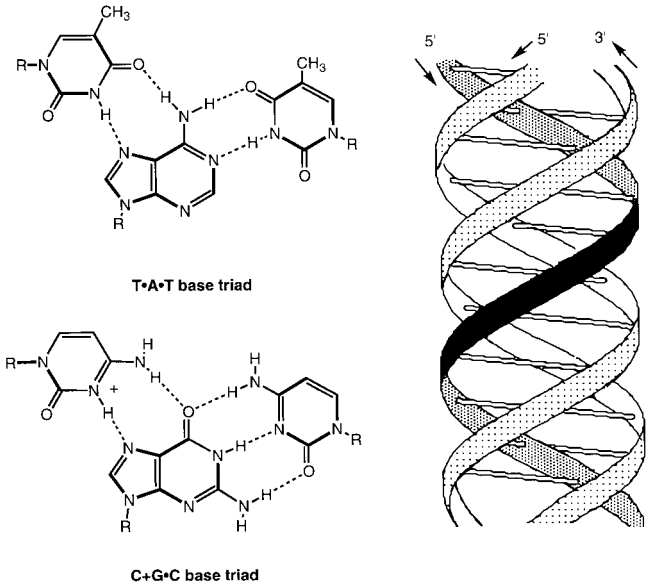


FIGURE 2. Architecture of a pyrimidine−purine−pyrimidine triple helix, showing the base triads and the strand organization.

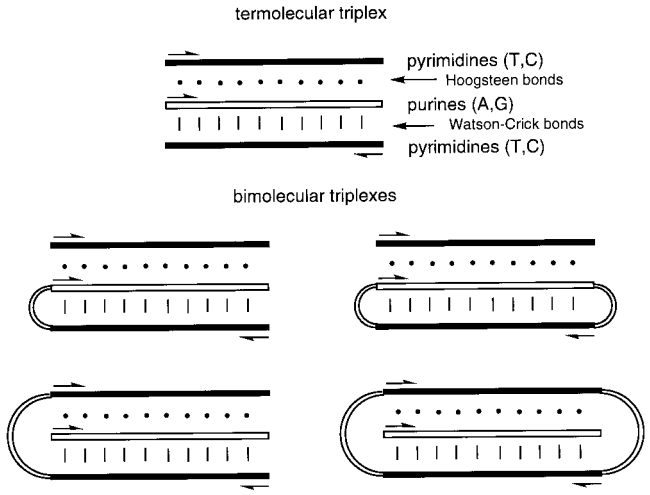
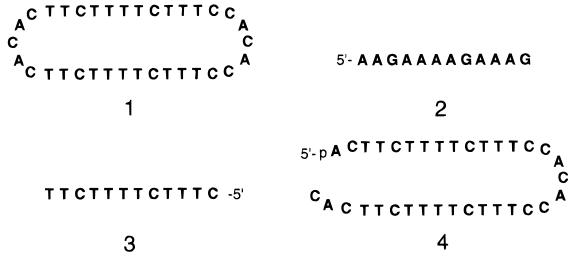


FIGURE 3. Strand orientation of a pyr-pur-pyr triplex and illustration of how a termolecular complex can be made bimolecular by use of various linking strategies. Dashes indicate Watson−Crick complementarity, and dots, Hoogsteen complementarity. Connecting two antiparallel-oriented strands makes a hairpinlike ligand, and connecting both ends makes a circular ligand.

Alternatively, such a complex can be formed by closing the circle in the presence of the single-stranded target, which will have the effect of locking the circle onto the target. Interestingly, this very strategy (using so-called "padlock probes")[13] is under investigation in a number of laboratories as a diagnostic method for identifying specific DNA sequences. However, our own initial goal was to synthesize a cyclic ligand and then use it as a binding agent. For this reason, among others, our studies have focused primarily on triple, rather than double, helical structures.

Triplex Binding of Single-Stranded DNA. DNA triple helices are commonly formed when a linear oligonucleotide is bound to a double-stranded DNA target.[14] A look at the basic structure of a DNA triple helix (Figure 2) suggested to us in 1990, however, that some other possible variants of recognition might exist. The most well-studied DNA triplex is that formed between a pyrimidine ssDNA and a duplex target consisting primarily of purines in one strand and pyrimidines in the other. The directionality of strands in such a pyr-pur-pyr triplex implies that one could connect various pairings of two of the strands in a triplex with each other, resulting in different bimolecular (rather than three-stranded) complexes (Figure 3). One case that seemed particularly interesting was one in which two pyrimidine-rich domains could be connected with a bridging loop of nucleotides to give a hairpinlike ligand for a purine-rich ssDNA target. Such a complex had in fact been reported in 1990 in a study intended to model an unusual folded triple helical DNA structure termed H-DNA.[15] Taking this one step further, we realized that addition of another loop would result in a circular structure which might benefit more greatly from the preorganization of the molecule.

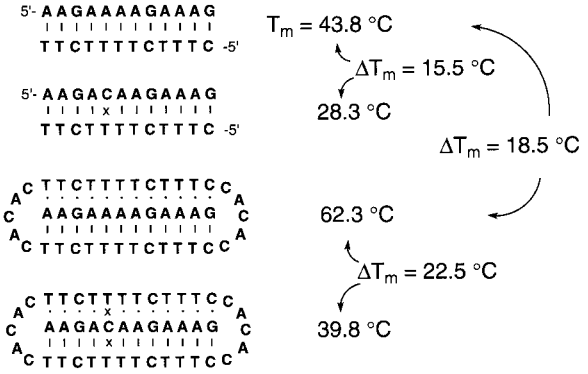
We described one of the first such ligands, circular 34nt oligonucleotide **1**, in 1991.[10a,10b] It was synthesized in greater than 50% yield on a 1 μmol scale and was designed to recognize a 12-base sequence of DNA, **2**, by forming a triple helix in which the purine-rich target strand is bound



between the two opposite sides of the macrocycle in a sandwich- or clamplike complex. Two reports describe binding properties of this molecule, which was studied in aqueous buffers approximating physiological ionic strength and pH.[10a,b] Binding affinity was measured by carrying out UV-monitored thermal denaturation studies, which can generate data such as melting temperature (T_m, a measure of thermal stability) as well as free energy of binding. For comparison we also studied the binding of a standard 12mer linear oligonucleotide, **3**, which is complementary in Watson−Crick sense to the target.

The results (Table 1) showed that this new macrocyclic ligand was greatly improved in its DNA-binding properties. It bound the intended target with a T_m nearly 20 °C higher than the Watson−Crick complement and a with a free energy nearly 7 kcal/mol more favorable.[10a] This corresponds to an association constant 6 orders of magnitude higher. Also quite significant was the ability of the circular ligand to discriminate against target sequences different by only one nucleotide. Placement of a single base mismatch in the target resulted in a loss of 18−22 °C in T_m and 6−8 kcal/mol of binding free energy, whereas a standard complementary strand lost a considerably smaller 10−21 °C in T_m and only 3−6 kcal/mol in binding energy.[10b] Thus, not only did this cyclic ligand demon-

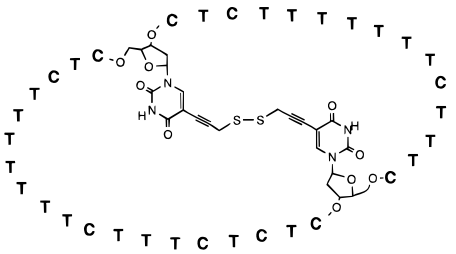
Table 1. DNA-Binding Properties of Linear Watson−Crick Oligonucleotide 3 as Compared to Cyclic Compound 1 with Data Measured at pH 7.0



strate much higher binding affinity than a standard oligonucleotide but it also showed considerably higher sequence selectivity.

It is also notable that, nearly simultaneous with our first reports, independent work by Hélène and co-workers was published describing related hairpinlike triplex-forming ligands which were closed at one end rather than two. Similar advantages in binding were described.[16,17] Thus, the early results in more than one laboratory demonstrated that such a preorganization approach could indeed be applied to oligonucleotides in the binding of DNA. At that time, the macrocyclic DNA ligands were among the tightest-binding of known ligands for DNA and were certainly the most sequence selective.

Is there truly an entropic binding advantage to having a fully closed circular structure? This was investigated by comparing a closed circular oligonucleotide to ones having the same sequence but left open at various positions (such as compound **4**).[10c] All of these should be able to form the same complex with the same noncovalent interactions. However, it was found that the closed cyclic ligand was by far the tightest binding of the three that were directly compared. Thus it was concluded that much of the positive effect on binding was very likely due to preorganization. Indeed, more recent studies have been directed at further preorganization of such a macrocyclic structure. One result was compound **5**, a cyclic 36mer engineered



5

5'AAAGAGAGAGAAA

6

to contain an added disulfide linkage across the center of the macrocyclic ring. This molecular strategy was described by Chaudhuri *et al.*[18] This bicyclic ligand binds its intended DNA target (**6**) with affinity nearly 10 orders of magnitude higher than a standard DNA complement at neutral pH. At the same time it discriminates against single-base mismatches by a full 10−12 kcal/mol, corresponding to 8 orders of magnitude in binding constant. This is, to our knowledge, the highest level of discrimination measured for any DNA-binding molecule to date.

Examination of the gross structure of a bimolecular triplex between a cyclic DNA and its target indicates that, to a first approximation, the bridging loops serve only as spacers to link the two binding domains. We therefore investigated replacing pentanucleotide loops with simpler linkers such as hexaethylene glycol, a linker first used in triplexes by Hélène.[16] Such linkers were shown to still allow favorable binding properties in circular oligonucleotides and to give the advantages of simpler synthesis and greater resistance to degradation by nuclease enzymes.[19] More recently, the length, sequence, and geometry of nonnucleotide linkers have been optimized for such applications as well.[20,21]

It is important to note that a number of variations on this strategy of binding single-stranded targets by a longer pyrimidine-rich oligonucleotide have been carried out in several laboratories since the earliest reports came out (see, for example, compounds **7**, **9**, **11**, **13**, and **15** and their targets **8**, **10**, **12**, **14**, and **16** in Chart 1). Some research groups have added to the studies of hairpin-type triplex formation, including biological and biophysical studies[22−25] as well as in making further synthetic changes and substitutions.[26−31] Alternative structures which can be used as loop replacements have been reported,[26] and alternative methods for closure of cyclic structures have been described.[27,30] Branched structures have also been utilized in such bimolecular triplexes.[31]

Differences between DNA and RNA Binding. While RNA and DNA are seemingly quite similar, and the rules for Watson−Crick complementarity are virtually the same, the two backbones can behave quite differently in triple helical structures. Roberts and Crothers showed in 1992 that pyr-pur-pyr triple helices were quite sensitive in their stability to DNA vs RNA composition of the three strands.[32] Indeed, two of the eight possible triplexes could not even be formed. Consistent with this, we found that a circular triplex-forming DNA oligonucleotide could bind a purine-rich RNA target only weakly, using only Watson−Crick bonds and leaving the Hoogsteen binding domain unbound.[33] We investigated several circular ligands which contained combinations of DNA, RNA, and even 2′-*O*-methyl-RNA backbones, and it was found that replacing one or both binding domains in a circular ligand with RNA resulted in considerably stronger triplex binding of RNA targets (Table 2).[35] Other published studies on this general topic are worth noting.[36]

Multitarget Recognition. An alteration of our first-generation ligand design made it possible to bind more than one target sequence with a single ligand.[37] By designing four binding domains (two opposing pairs) into a cyclic oligonucleotide, one could hope to bind one sequence and use the other set of domains as bridging

Chart 1

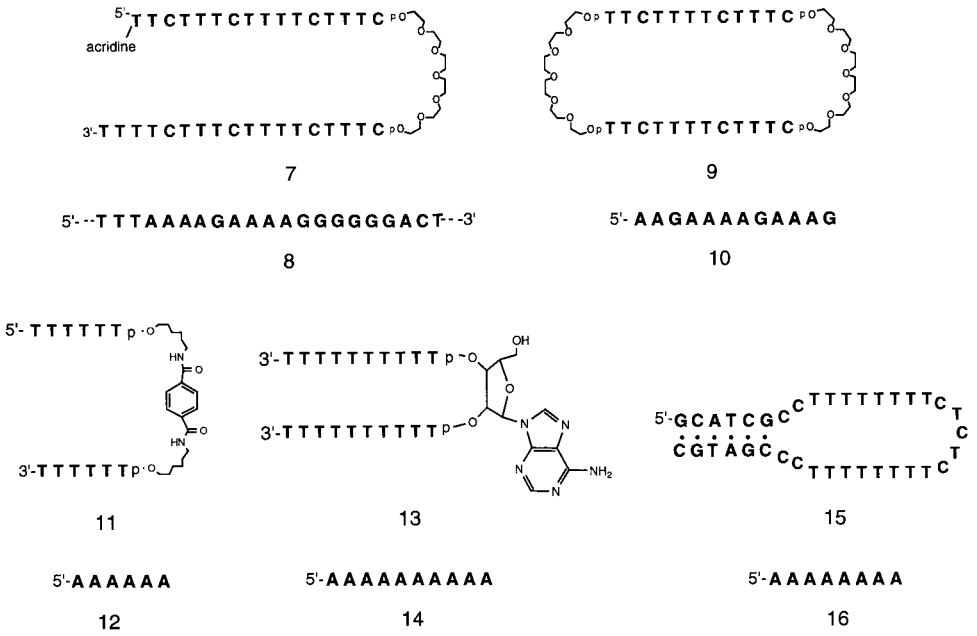


Table 2. Effect of DNA and RNA Backbone Combinations on the Binding of Purine Targets by Circular Oligonucleotides at pH 7

complex	type	T_m (°C)
A C TTCTTTCTTTTC C A C dAAGAAAGAAAAG C A C TTCTTTCTTTTC C A	DNA ligand DNA target	54.5
A C TTCTTTCTTTTC C A C rAAGAAAGAAAAG C A C TTCTTTCTTTTC C A	DNA ligand RNA target	42.8[a]
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	RNA ligand RNA target	51.1
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	RNA ligand DNA target	54.0

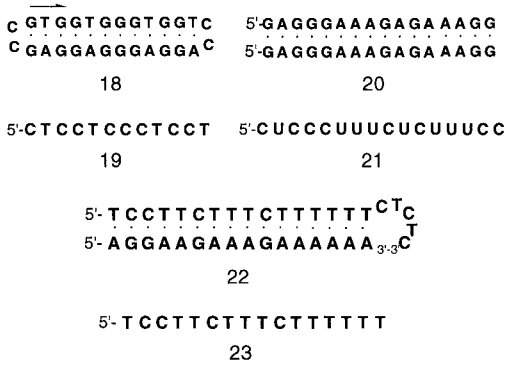
[a] Triple helical complex not formed.

linkers. Binding a second sequence would then involve dissociation, switching conformation, and rebinding the second target with the alternate binding domains.

The first attempt at such multisite recognition resulted in compound **17**, a cyclic 36mer with four separate 9mer binding domains (Figure 4).[37] This compound was shown to bind two separate 9mer target sequences with approximately equal affinity. It was also shown to be able to bind only one of these at a time, thus confirming that dissociation and conformation switching were required for binding a second target. In a more recent study this multiple binding domain strategy was further refined and extended to a cyclic 35mer which binds strongly and specifically to six different 8-base target sequences.[38]

Binding Pyrimidine-Rich Sequences. The design of the above cyclic triplex-forming ligands, and, indeed, all triplex-forming oligonucleotides targeted to duplexes as well, has a significant limitation: the binding is restricted to targets composed entirely (or nearly so) of purines (A and G). This is a serious limitation when searching for sites to target in specific genes. An alternative approach is possible, however. A 1993 paper by Mirkin described a short purine-rich synthetic hairpin modeled after what happens during replication when a DNA contains two adjacent pyrimidine-rich runs having a pseudomirror symmetry (Figure 5).[39]

We realized that this type of triplex could in principle be formed using a circular purine-rich ligand to bind a pyrimidine-rich target. We therefore synthesized compounds such as **18**, a cyclic 32mer, which was designed



to bind a 12mer target (**19**) composed of pyrimidines C and T.[40]

Binding studies showed that this compound does indeed bind the predicted target, and does so with affinity considerably higher than a simple Watson−Crick complement (Table 3). Moreover, the closed circular structure binds significantly more tightly than does an unclosed hairpinlike ligand. Also notable is the finding that such a

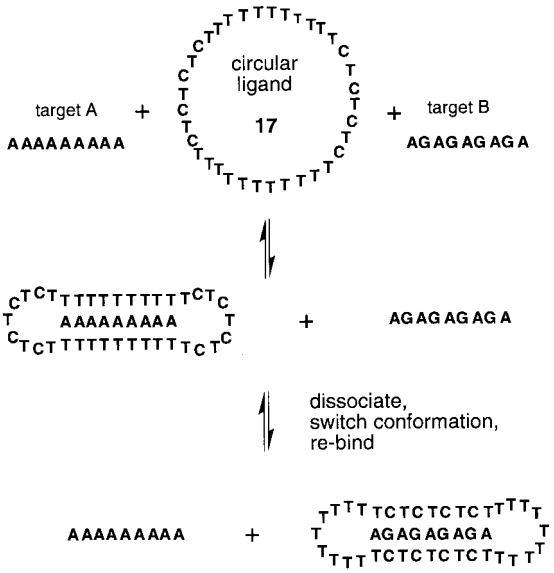


FIGURE 4. Strategy for design of a cyclic oligonucleotide that can bind two different target sequences by conformation switching. Multisite binding can in principle lead to a broader spectrum of activity or application.

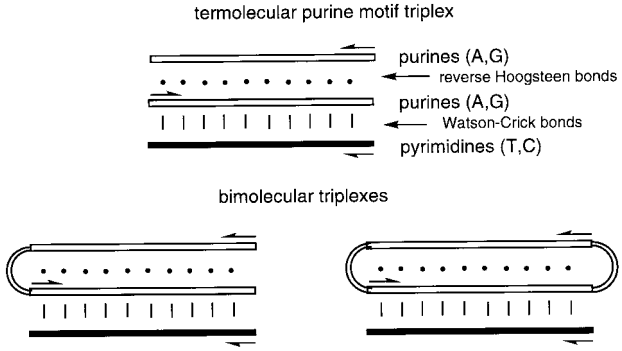


FIGURE 5. The strand orientation of a pur-pur-pyr triplex and illustration of how a termolecular complex can be made bimolecular by use of various linking strategies. This allows the efficient targeting of pyrimidine strands.

DNA ligand can bind both RNA and DNA targets with equally high affinity, unlike the case with purine-rich targets.[40,41]

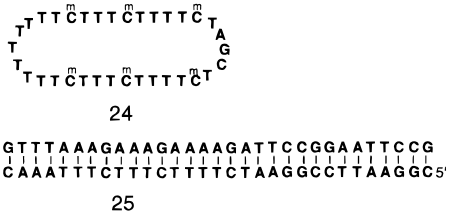
It is also worth mentioning that other strategies which have been, or might be, used for triplex binding of pyrimidine sequences have been described recently,[42−45] and it is possible that strategies such as these could also be applied to macrocyclic ligands as well. Notable examples are the approaches described by Ts'o (see **20**, **21**),[44] by Agrawal (**22**, **23**),[42] and by Switzer.[45]

Binding of Duplex DNA. More recent studies have described the binding of clamp-type pyrimidine-rich triplex-forming oligonucleotide derivatives or analogues to duplex DNAs.[46−48] Because of the greater complexity of the system, a number of binding modes might be envisioned.[47,49] Examination of models led us to believe that triplex binding domains incorporated into circular oligonucleotides might also interact with duplex DNA in interesting (and potentially useful) ways. Thus in 1993 we turned our attention to whether triplex-forming circular structures such as **1** or **24** could bind to a complementary target in duplex DNA and, if so, by what mode of binding.[48,49]

Table 3. Binding of a C,T-Containing Target Strand by Linear and Circular Purine-Rich Duplex- and Triplex-Forming Oligonucleotides at pH 7.0

complex	T_m (°C)
3'- GAGGAGGGAGGA 5'- CTCCTCCCTCCT	56.9
ACGTGGTGGGTGGT C AC GAGGAGGGAGGA-5' 5'-CTCCTCCCTCCT	68.4
ACGTGGTGTGTGGT C AC GAGGAGGGAGGA-5' 5'-CTCCTCCCTCCT	61.9
ACGTGGTGGGTGGTCA C AC GAGGAGGGAGGACAC 5'-CTCCTCCCTCCT	71.0

Our first studies were carried out with synthetic duplexes 36−48 bp in length and containing a 12mer purine-rich target in the center (see sequence **25**). Binding was



evaluated by gel mobility shift, in which complexes travel more slowly in a nondenaturing gel than do the unbound components. It was immediately shown that a compound such as **24** could indeed bind duplex **25** and does so with an affinity (K_{diss}) of ca. 1 μM at pH 7. However, the binding gels nearly always showed two distinct bands of similar intensity, indicating two separate types of complexes being formed. Further studies showed that only one binding domain on the circle was involved in both these complexes.[48] Our initial assumption was that triplex formation could occur either by direct binding of one part of the circle with the purine run in the major groove of the duplex (analogous to standard triplexes) or by strand displacement (Figure 6). However, all attempts to probe for strand displacement in the two observed complexes failed. Thus the question arose: if strand displacement is not occurring, then how can one account for two complexes?

A Novel Threaded Duplex DNA Binding Mode. We were at first stymied in our efforts to imagine a different mode of binding other than these two. However, discussions on this subject and model building led to a sudden insight: perhaps the two bands were simply topological isomers of one another. It was realized that binding of a circle to a full turn or more of the helix has a tendency to create stress on the circular structure which can be

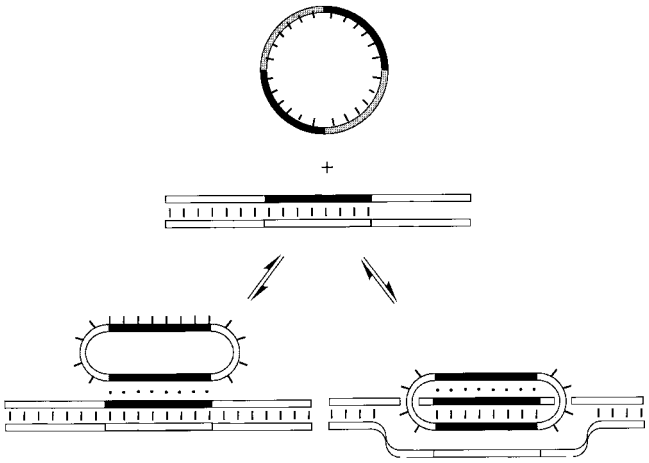


FIGURE 6. Illustrations of two of three possible complexes formed by a triplex-complementary circular oligonucleotide with duplex DNA. The third mode is illustrated in Figure 7.

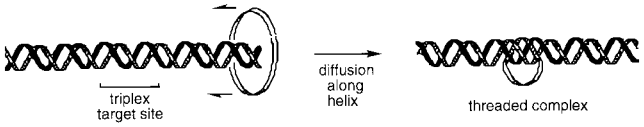


FIGURE 7. Proposed mode of highest-affinity binding of duplex DNA by a circular oligonucleotide containing a triplex-forming domain. The oligonucleotide threads itself over the duplex, forming a pseudorotaxane complex.

relieved by passing the duplex through the circle. The only way that this can happen is for the circle to thread itself onto the end of the duplex, slide down to the target site, and then form the triple helical complex. This hypothesis, that one of the observed bands was a topologically threaded complex, allowed us to begin to design experiments to test it.[49] A number of experiments were found to be consistent with this idea and suggested that the band having greater mobility (the more stable of the two) was in fact a threaded complex.

To gather further evidence, we used this binding mode to construct a catenated superstructure which relied on the self-assembly of a circle onto a short duplex segment.[49] A strategy involving ligation of inherently bent DNA sequences[50] was used to construct a series of circular duplexes, and this was done in the presence of one segment with a circle threaded on it. This created a series of catenanes having a small ssDNA circle threaded on the larger duplex circles (Figure 8), thus confirming our hypothesis. These complexes could be formed two different ways and were reversible by opening the duplex circles at specific sites and reclosing them with ligase enzymes.

This threading mode of binding is unprecedented in synthetic ligands for DNA, although similar pseudorotaxane complexes of small organic molecules have been the subject of much recent investigation.[51] Interestingly, in nature a related mode of DNA binding is utilized by a number of proteins in structures termed "sliding clamps".[52] This novel DNA binding strategy using synthetic macrocyclic ligands might offer some unique possibilities for recognition and reaction on DNA. For example, a threaded

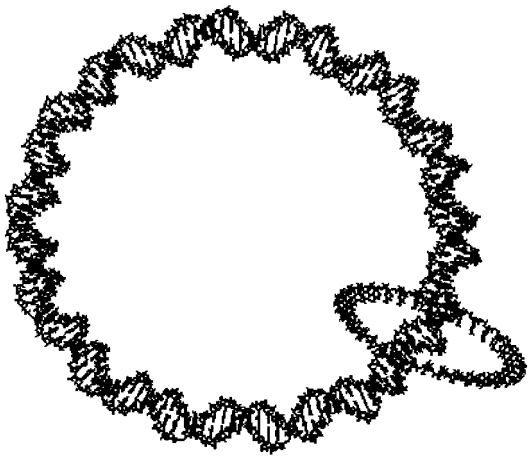


FIGURE 8. Catenane formation by a triplex-forming 34mer circular oligonucleotide trapped on a 147-bp circular duplex DNA. The threading mode of binding (Figure 7) was proven by forming the catenane from a linear duplex on which the smaller circle was threaded.

circular structure might conceivably carry a reactive or catalytic function along the DNA, sliding back and forth rapidly to reach desired target sites.

Recognition of Proteins

Several studies have shown recently that proteins and protein enzymes can recognize small circular single-stranded DNAs and RNAs and that in many cases the recognition and/or enzymatic processing is significantly altered by the circular topology.

Nuclease Enzymes. If oligonucleotides are to be used to exert biological activity in cellular media, then they must evade degradation long enough to have their effect. Nucleases which cleave DNA internally (endonucleases) and from the ends (exonucleases) are ubiquitous in biological fluids. Circular DNAs and RNAs are by definition completely resistant to exonucleases, and this has been shown to add significantly to their lifetime in biological fluids.[19,53,54] However, unless some other secondary structure (or chemical modification) adds resistance,[19] circular nucleic acids are still susceptible to endonuclease cleavage.[55]

Circular Structures as Decoys. A number of laboratories have investigated the use of small circular DNAs and RNAs as targets for capturing specific DNA- or RNA-binding proteins.[56−58] A principal goal has been the use of such compounds as decoys to sequester proteins which cause a disease state (see structure **26** for a DNA example). In another example, RNA decoys such as **27** have been constructed for the binding of HIV-1 transactivating proteins.[62]

Polymerase Enzymes. A broad class of enzymes which have particularly interesting behavior with small circular

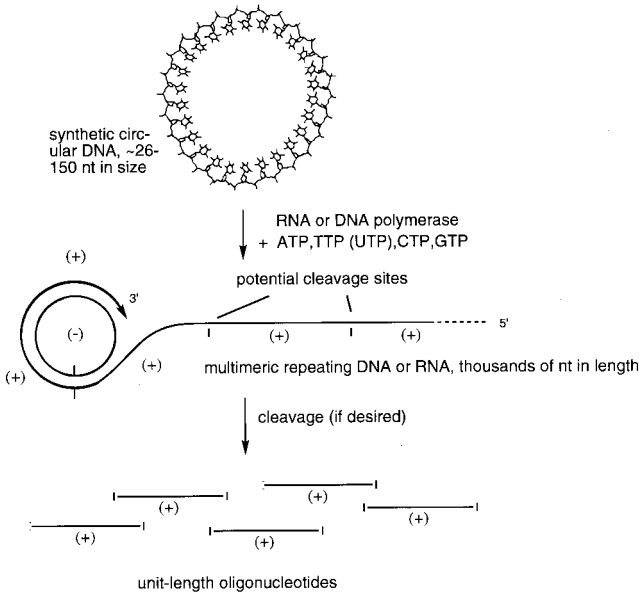


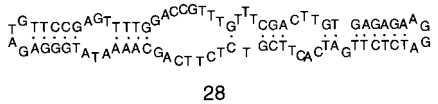
FIGURE 9. Scheme for use of small synthetic circular oligonucleotides as catalysts for the synthesis of long repeating strands of DNA or RNA ("rolling circle synthesis"). Note that for rolling circle DNA synthesis a short primer is also required to initiate each repeating strand; for RNA synthesis, no primer is needed and the enzyme starts at a random "C" in the circle. If a cleavage site is engineered into the repeating strand, the reaction can yield unit-length oligonucleotides in amplified amounts.

DNAs are the polymerases. A DNA polymerase normally copies a DNA strand by starting with a short primer sequence and then elongating it (using deoxynucleoside triphosphates as monomers) using a long template strand to direct the complementary sequence. If the template is a large biologically derived circle, many polymerases simply stall after proceeding around once, because they cannot proceed past the front end of the duplex they have previously synthesized.[59] However, with very small circles it has been found that this is apparently not a problem.[60,61] Since the small diameter prevents duplex formation all the way around, an enzyme can proceed many times around a circle without colliding with its recently synthesized strand, because the DNA must unwind itself as it is made.

The result of this processive synthesis around a small circle is a repeating sequence of DNA (a series of end-to-end complements of the circle) which can be many thousands of nucleotides in length (Figure 9). Quite surprisingly, it has been shown that common polymerase enzymes can handle circular templates at least as small as 26 nucleotides, a diameter considerably smaller than the enzymes themselves.[61] This "rolling circle" strategy is now being studied in a number of laboratories as a method for amplification of DNA sequences and for sensitive detection of specific sequences. It has also been used to generate repeating DNAs which encode the synthesis of repeating peptide sequences.[62]

RNA polymerase enzymes are similar to DNA polymerases in that they also make a sequence by traveling along a template strand of DNA and copying it. However, RNA polymerases do not require primers but instead are directed to start at a particular site by conserved sequences called promoters. We discovered in 1993 that despite the usual requirement for duplex DNA as a template and a promoter, RNA polymerases can in fact efficiently transcribe very small circular single-stranded DNAs.[63] Such unusual templates can be at least as small as 28 nucleotides and do not require a promoter for efficient RNA synthesis. Analogous to the DNA synthesis case, the result is long repeating strands of RNA encoded by the small circular template. These long strands have recently been visualized by atomic force microscopy.[64]

In one recent application, such small synthetic circular DNAs have been used as catalytic templates for the efficient synthesis of catalytic RNAs (ribozymes) in the test tube. For example, circular 83mer DNAs such as **28** were



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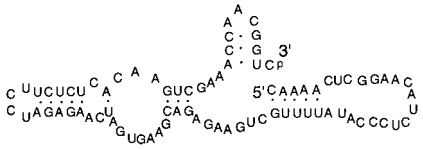

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designed to encode HIV RNA-cleaving hammerhead-type ribozymes as well as their own substrates for cleavage.[65] If such a circular template is incubated with a commercially available RNA polymerase and the four ribonucleoside triphosphates, the result is rapid synthesis of repeating RNAs several thousand bases long. After a brief time, shorter products of regular length begin to appear. These arise from the ribozymes cleaving their own substrates within the repeat units. Ultimately there is virtually a single product from these reactions: a unit-length 83mer hammerhead ribozyme (**29**) which can then cleave HIV-derived sequences. It is notable that this strategy of rolling circle transcription followed by self-processing closely mimics the replication cycle proposed for viroid RNAs,[2] which were mentioned above as the smallest known circular nucleic acids in nature.

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