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Induced-Fit Recognition of DNA by Small Circular Oligonucleotides

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Abstract: We have investigated the molecular interaction between cyclic and linear oligonucleotides. We have found that short cyclic oligonucleotides can induce hairpinlike structures in linear DNA fragments. By using NMR and CD spectroscopy we have studied the interaction of the cyclic oligonucleotide d<pCCTTCGGT> with d<pCAGTCCCT>, as well as with its two linear analogs d(GTCCCTCA) and d(CTCAGTCC). Here we report the NMR structural study of these complexes. Recognition between these oligonucleotides occurs through formation of four intermolecular Watson–Crick base pairs. The three-dimensional structure is stabilized by two tetrads, formed by facing the minor-groove side of the Watson–Crick base pairs. Overall, the structure is similar to those observed previously in other quadruplexes formed by minor-groove alignment of Watson–Crick base pairs. However, in this case the complexes are heterodimeric and are formed by two different tetrads (G:C:A:T and G:C:G:C). These complexes represent a new model of DNA recognition by small cyclic oligonucleotides, increasing the number of potential applications of these interesting molecules.

Keywords: DNA recognition · NMR spectroscopy · oligonucleotides

Introduction

During the last years, the molecular recognition properties of small circular oligonucleotides have attracted considerable attention.[1,2] These molecules have emerged as promising tools in research, for diagnosis and as therapeutic agents due to their increased nuclease resistance relative to their linear analogues. In addition, in many cases cyclic oligonucleotides exhibit higher binding affinities and greater target specificity than their lineal counterparts. Since the early 1990s, cyclic oligonucleotides have been used to target single-stranded DNA[3] and RNA,[4] by forming triplexes, and double-stranded DNA, by forming either triplexes or more complex structures, named catenanes, in which a short ssDNA chain is threaded on a large circular DNA duplex.[5] This topic has been extensively reviewed by Kool and coworkers.[1,2]

In this paper, we explore a different mechanism for recognition between short linear and cyclic oligonucleotides, which may have applications to target specific regions of genomic DNA. We have found that cyclic octamers can recognize either cyclic or linear oligonucleotides with the appropriate sequence. Recognition occurs by forming four-stranded structures that are stabilized by tetrads resulting from the association of four bases forming two Watson–Crick base pairs. All the previously found structures are homodimers formed by two tetrads connected by four two-nucleotide loops. Such unusual DNA structures were first observed in the crystallographic structure of the linear heptamer d(GCATGCT)[6] and, more recently, in crystallographic[7] and solution studies of several cyclic oligonucleotides.[8,9]

This four-stranded motif is different than the classical G-quadruplex, in which four guanine residues are paired through their Watson–Crick and Hoogsteen sides. In some cases guanine quadruplexes contain unusual tetrads. For example, pure adenine-,[10] thymine-,[11,12] and cytosine-tetrads[13] have been found within the scaffold of parallel gua-

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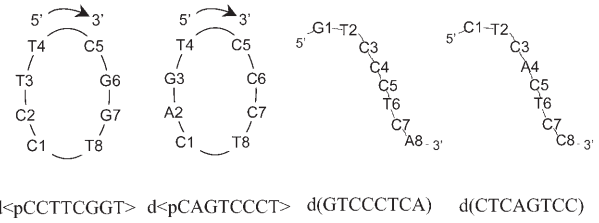
Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author. Supporting information contains a figure showing a NOESY spectrum in D_2O of the complex d<pCCTTCGGT>·d<pCAGTCCCT>.

nine quadruplexes. Mixed tetrads resulting from the association of Watson–Crick base pairs have been found in parallel and fold-back quadruplex structures.[14–18] In contrast, tetrads formed by minor grove alignment of Watson–Crick base pairs have never been found to co-exist with pure guanine tetrads in the same structure.

In this paper, we have investigated the molecular interaction of the cyclic octamer d<pC**CT**TC**GG**T> with the linear oligonucleotides d(**G**TC**C**TC**A**), and d(**C**TC**AG**TC**C**) and their cyclic analogues. The sequences of these oligonucleotides have been designed to explore the formation of heterodimeric structures with this DNA motif. In such heterodimeric structures, nucleotides written in bold letters would be involved in intermolecular base pairs, whereas the others would be part of the loop regions. It has been observed in previous works that some octameric cyclic oligonucleotides can form stable monomeric dumbbell-like structures, consisting of a short stem of two base pairs connected by two miniloops of two residues.[19,20] The sequences studied in this paper have been designed to avoid the formation of such structures. The numbering scheme of these molecules is shown here.



Results

Complex formation: Exchangeable proton spectra of the isolated oligonucleotides do not show any imino signal except in the case of d<pCCTTCGGT>, indicating that this molecule has an appreciable residual structure under the buffer conditions used in the experiment (see Figure 1A). Complex formation of the above-mentioned cyclic octamer with d<pCAGTCCCT> (complex **I**), with d(GTCCCTCA) (**II**), and with d(CTCAGTCC) (**III**) was monitored by observing the changes in this region of the one-dimensional NMR spectra. Upon addition of one equivalent of d<pCAGTCCCT>, the imino signals observed at $\delta = 11.4$, 12.2 and 13.6 ppm disappear and new resonances emerge between $\delta = 13.0$ and 15.0 ppm (Figure 1B). A similar effect is observed when titrating d(GTCCCTCA) or d(CTCAGTCC) with a d<pCCTTCGGT> sample. In these cases, some signals of the isolated species of d<pCCTTCGGT> are still observable when adding an excess amount of linear oligonucleotide, indicating a lower affinity of d<pCCTTCGGT> for linear DNA than for the cyclic analogue (Figure 1C, D). In the three complexes, the presence of imino resonances between $\delta = 12.0$ and 15.0 ppm suggests that recognition occurs through formation of intermolecular Watson–Crick base pairs.

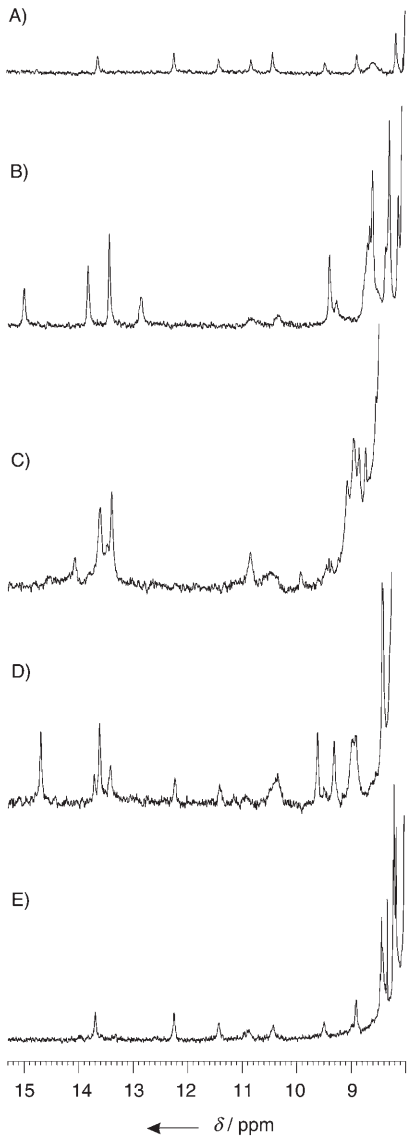


Figure 1. Imino region of the one-dimensional spectra in H_2O at 1 °C of A) d<pCCTTCGGT>, B) d<pCCTTCGGT>·d<pCAGTCCCT> 1:1 (complex **I**), C) d<pCCTTCGGT>·d(GTCCCTCA) 1:5 (complex **II**), D) d<pCCTTCGGT>·d(CTCAGTCC) 1:5 (complex **III**), and E) d<pCCTTCGGT>·r(GUCCCUCA) 1:5.

Interestingly, this effect is not observed when the linear oligonucleotide is RNA. The addition of up to five equivalents of r(GUCCCUCA) leaves the exchangeable proton spectra of d<pCCTTCGGT> virtually unaffected (Figure 1E).

CD spectra of the different complexes are shown in Figure 2, and exhibit a characteristic maximum around 270 nm and a minimum around 240 nm. The common features of the CD spectra suggest a similar structure in the three complexes.

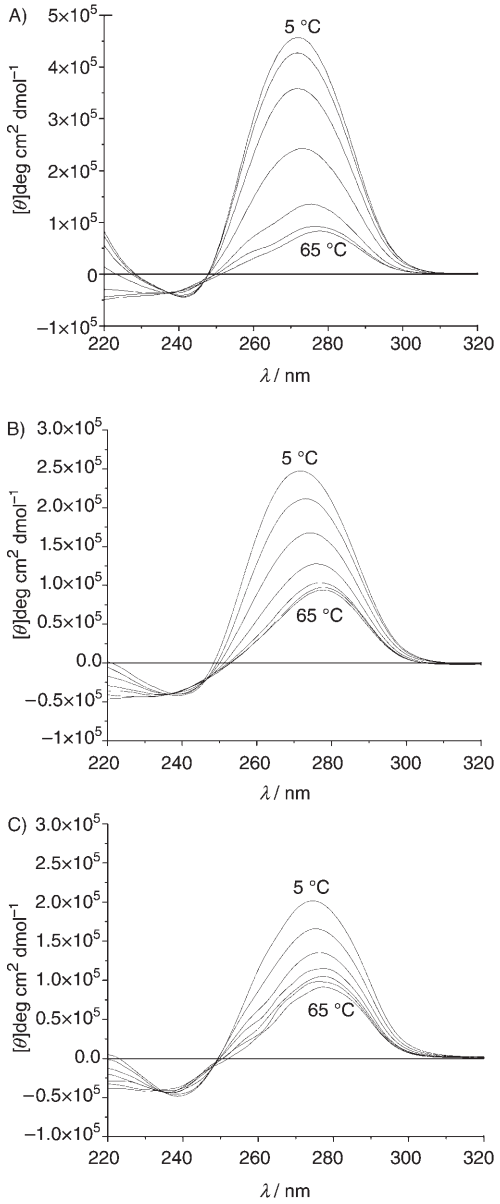


Figure 2. CD spectra of complexes **I**, d<pCCTTCGGT>·d<pCAGTCCCT> (A), **II**, d<pCCTTCGGT>·d(GTCCCTCA) (B), and **III**, d<pCCTTCGGT>·d(CTCAGTCC) (C), at different temperatures.

NMR assignment: The NMR spectra of the three complexes contain severe signal overlapping. Nevertheless, an almost complete sequential assignment could be carried out for d<pCCTTCGGT>·d<pCAGTCCCT>, following standard methods. The assignment list can be found in Table 1. A fragment of the two-dimensional NOESY spectrum of this complex in D_2O is shown in the Supporting Information (Figure S1). All intranucleotide H1′-base NOE correlations are medium or weak, indicating that the glycosidic angle in all the nucleotides is in an *anti* conformation. In both molecules we observed strong sugar–base sequential connections between residues 2→3, and 6→7, but not many NOE correlations could be assigned for the residues in the loops. The exchangeable proton spectra of this complex were particularly informative for the assignment (see Figure 3). Amino protons of all cytosines were assigned from their NOE cross-peaks with the H5 protons. In the case of C1 and C5, these resonances are broad and completely degenerate; however, in the other cases, the amino protons present strong cross-peaks with the three imino signals observed between $\delta = 12.5$ and 15.0 ppm. Imino and amino guanine resonances were sequentially assigned by following the connection from H5 of C through the G·C base pair (H5C→HN4C→H1G→HN2G). This NOE pattern is characteristic of GC Watson–Crick base pairs. It is interesting to note the large downfield shift of the two amino protons of G3 of d<pCAGTCCCT> and G7 of d<pCCTTCGGT>. The imino signal at 15.0 ppm presents cross-peaks with the adenine H2 and its two amino protons (Figure 3). This signal was assigned to T3 of d<pCCTTCGGT>. Two more imino signals are observed in the $\delta = 10$–11 ppm region, indicating that the other thymine residues are not base-paired. Overall, it can be concluded from the exchangeable proton spectra that the complex is stabilized by formation of three G·C and one A·T Watson–Crick base pairs.

In complex **II**, d<pCCTTCGGT>·d(GTCCCTCA), and **III**, d<pCCTTCGGT>·d(CTCAGTCC), the broad lines and large signal overlapping prevent the complete sequential assignment of the spectra. However, many spectral features are common to all cases, indicating that the structures are very similar overall. In particular, in the case of **II**, three G.C Watson–Crick base pairs were clearly identified (see Figure 4), and all the glycosidic angles are in an *anti* conformation, as shown by the intranucleotide H1′-base NOE cross-peaks. Some guanine amino protons also have downfield-shifted signals in these complexes. The imino resonance arising at $\delta = 14.0$ ppm (see Figure 1C) indicates the presence of a base-paired thymine, most probably T3, but no NOE cross-peaks with H2 A8 were found. This probably reflects an enhanced flexibility of the 3′-terminal base. A similar effect is observed in the terminal base pair of the complex **III**.

Solution structure of d<pCCTTCGGT>·d<pCAGTCCCT>: To get further insight into the solution structure of these complexes, a restrained molecular dynamics calculation of the structure of d<pCCTTCGGT>·d<pCAGTCCCT> was carried out based on 132 experimental distance constraints, 46 of which were intermolecular, derived from a complete relaxation matrix analysis of the NOE intensities. In addition, torsion angle restraints derived from the *J* coupling constants for deoxyriboses were also used. Structure calculations were carried out by using the program DYANA and further refined with the molecular dynamics package AMBER. A summary of the experimental constraints and structural statistics is shown in Table 2. The final structures exhibit low residual violations, which show that they are consistent with the experimental constraints. Also, the final AMBER energies and NOE terms are reasonably low in all the structures. Although the overall structure is not very well-defined (root mean square

Table 1. Assignment of the proton resonances [δ in ppm] of the complex d<pCCTTCGGT>·d<pCAGTCCCT> (100 mM NaCl, 10 mM $MgCl_2$, phosphate buffer pH 7, $T=5$ °C).

Residue	NH	$NH_2(2)$	$NH_2(1)$	H6/H8	H2/H5/Me	H1′	H2′	H2″	H3′	H4′
d<pCCTTCGGT>										
1Cyt		–	–	8.00	6.08	6.48	2.20	2.56	4.56	4.20
2Cyt		8.65	7.60	7.88	6.08	6.52	2.25	2.84	4.65	4.34
3Thy	15.01			7.61	1.90	6.34	2.55	2.33	5.08	4.12
4Thy	[a]			7.21	1.84	5.87	1.86	2.40	4.57	3.43
5Cyt		–	–	8.04	6.14	6.48	2.25	2.61	4.55	4.22
6Gua	12.85	8.51	6.64	8.14		5.80	2.92	2.92	4.59	4.13
7Gua	13.83	8.71	8.33	8.13		6.35	2.67	3.02	5.20	4.69
8Thy	[a]			7.64	1.87	5.98	1.95	2.38	4.69	3.80
d<pCAGTCCCT>										
1Cyt		–	–	7.97	6.14	6.49	2.27	2.60	4.64	4.19
2Ade		7.85	7.10	8.61	8.30	6.00	2.95	2.95	5.07	4.65
3Gua	13.44	9.39	8.37	8.07		6.34	3.02	2.65	5.20	4.68
4Thy	[a]			7.64	1.85	5.98	1.95	2.39	4.69	3.89
5Cyt		–	–	8.02	6.14	6.48	2.23	2.61	4.68	4.20
6Cyt		9.27	7.71	7.90	6.16	6.50	2.32	2.70	4.93	4.67
7Cyt		8.72	7.38	7.80	5.94	6.52	2.28	2.63	5.14	4.25
8Thy	[a]			7.14	1.67	5.86	1.87	2.43	4.57	3.15

[a] Imino protons of T4 and T8 of both molecules resonate at $\delta=10.83$ and 10.34 ppm, but the sequential assignment of these signals could not be carried out.

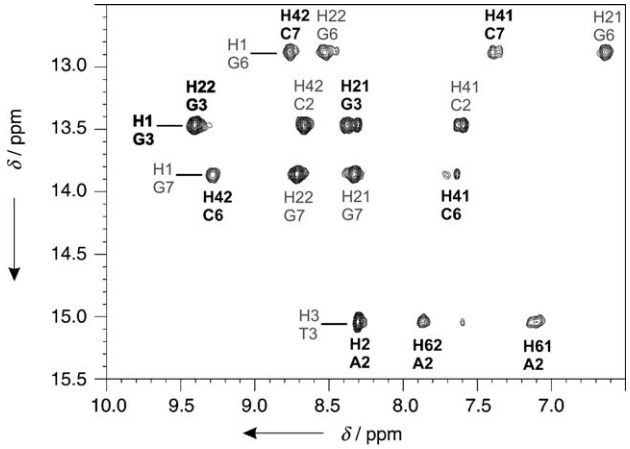


Figure 3. Region of the NOESY spectrum (150 ms mixing time) of the complex **I**, d<pCCTTCGGT>·d<pCAGTCCCT>, in H_2O (2 mM complex concentration, 100 mM NaCl, 10 mM $MgCl_2$, $T=5$ °C, pH 7). Watson–Crick base pairing can be established from the intermolecular NOE cross-peaks H1G1-H42C8, H1G5-H42C4, and H1G4-H42C5 for GC pairs, and H3T1-H2 A8, H3T1-H62 A8, and H3T1-H61 A8 for the AT pair. Proton resonances of d<pCAGTCCCT> are labeled with bold letters, and those of d<pCCTTCGGT> with plain letters.

distance (RMSD) around 2.5 Å for all heavy atoms), this is mainly due to the lack of constraints in the loop residues. When only base-paired residues are considered, the RMSD decreases to 0.6 Å. This is clearly illustrated in Figure 5. The overall superposition, shown in the top panel, indicates a poorly defined structure. However, the core nucleotides are fairly well-defined.

As can be seen in Figure 5, the resulting structure of this complex consists of two oligonucleotides arranged in an antiparallel fashion. The two octamers associate by forming four intermolecular Watson–Crick base pairs, which are arranged in two tetrads. In both cases the tetrads result from the association of the Watson–Crick base pairs through their minor groove sides. In one case, the tetrad is formed by the alignment of a GC and an AT base pair, forming a G:C:A:T tetrad, and in the other case, by two GC base pairs, forming a G:C:G:C tetrad. All glycosidic angles in these tetrads are *anti*, with values ranging from −98° to −140°, and deoxyribose rings are in the general S-domain. In addition to the Watson–Crick hydrogen bonds, the G:C:G:C tetrad is stabilized by two additional intermolecular hydrogen bonds between the amino protons that are not involved in the Watson–Crick base pairs, and the N3 of the neighboring guanine, as shown in Figure 6. These hydrogen bonds account for the unusual chemical shifts observed in the exchangeable proton spectra, in which both amino protons of these two guanine residues show a large downfield shift.

The two-residue loops are poorly defined, due to the lack of distance constraints. The low number of distance constraints involving protons in the loop residues is not a consequence of an enhanced flexibility in this region, but to the severe spectral overlapping between the signals of the four thymines in the first position of the loops. In fact, the H4′ and H5′/H5″ protons of these thymines present a large upfield shift. This unusual values have been observed in the spectra of the homodimeric structures of d<pTGCTCGCT>, d<pCATTCATT> and d<pCGCTCATT>, in which the loops are well-defined, suggesting that the conformation of the loops is probably very similar in this case.

Structure of d<pCCTTCGGT>·d(GTCCCTCA) and d<pCCTTCGGT>·d(CTCAGTCC): Some interesting features in the spectra of the complexes between d<pCCTTCGGT> and the linear octamers d(GTCCCTCA) and d(CTCAGTCC) (complexes **II** and **III**, respectively) can be readily explained in light of the structure of d<pCCTTCGGT>·d<pCAGTCCCT> (complex **I**). Although the spectra of **II** and **III** could not be fully assigned, the observation of Watson–Crick base pairs together with the unusual shifts of some amino resonances suggests that complexes **II** and **III** are also stabilized by a G:C:G:C tetrad. Under these experimental conditions, the G:C:A:T tetrad appears to be disrupted in both complexes, with the terminal base pair of the linear oligonucleotide partially disordered. The upfield shift of some protons of the thymine residues in the first position of the loops suggests that these residues adopt a similar conformation as that in complex **I**.

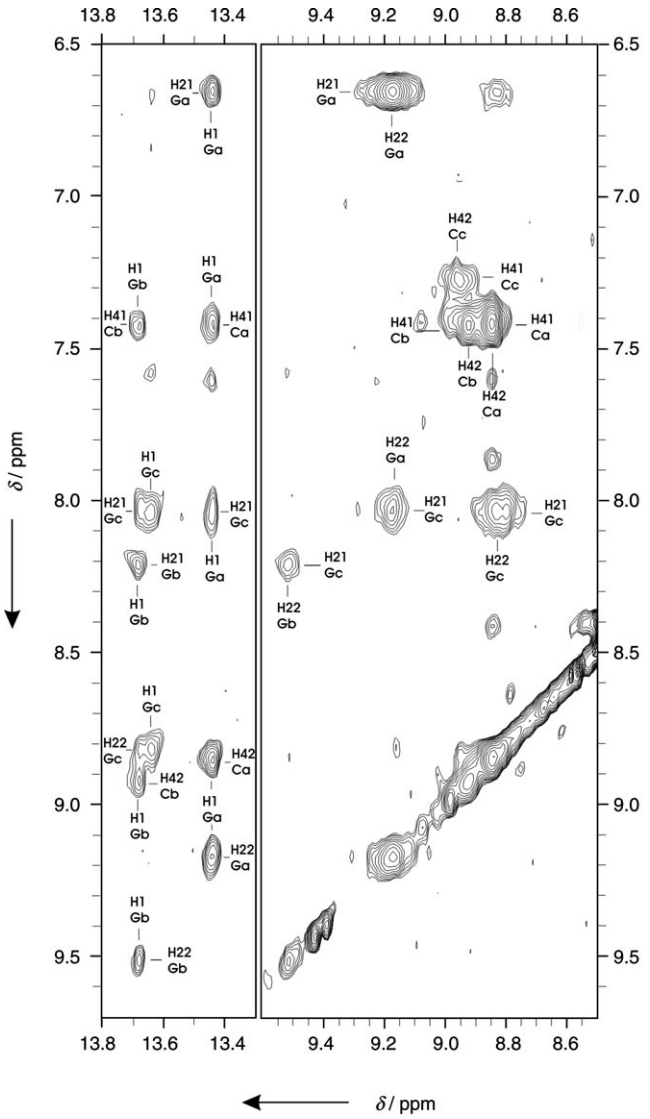


Figure 4. Region of a NOESY spectrum (300 ms mixing time) of the complex **II**, d<pCCTTCGGT>·d(GTCCCTCA)(1:1), in H_2O (0.6 mM of each oligonucleotide, 100 mM NaCl, 10 mM $MgCl_2$, $T=5\,^{\circ}C$, pH 7). Subindexes a, b, and c indicate the three G·C base pairs observed.

Table 2. Experimental constraints and calculation statistics.

Experimental distance constraints		
total number	132	
intraresidue	49	
sequential	25	
range >1	58	
intramolecular	86	
intermolecular	46	
RMSD [Å]		
all well-defined bases[a]	0.6±0.1	
all well-defined heavy atoms[a]	1.1±0.2	
backbone	1.5±0.4	
all heavy atoms	2.5±0.5	
Residual violations	**average**	**range**
sum of violation [Å]	2.7	2.4–3.1
max violation [Å]	0.2	0.16–0.25
NOE energy [kcal mol^{-1}]	18	13–19

[a] Residues 2, 3, 6, and 7.

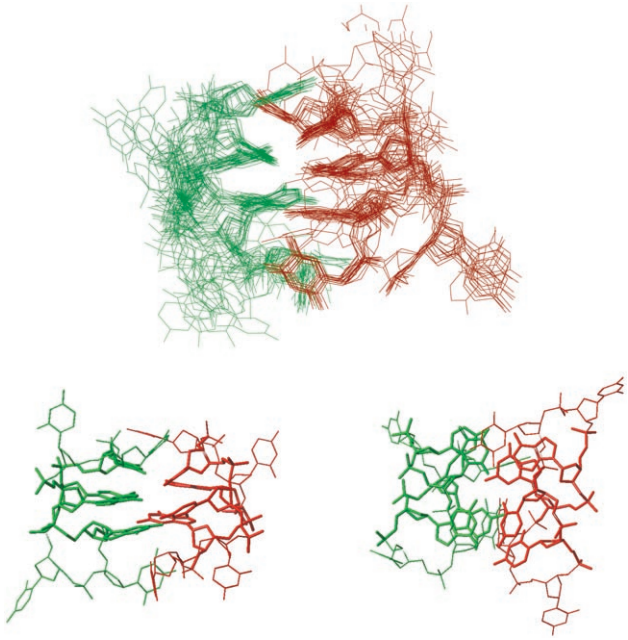


Figure 5. Superposition of the ten refined structures (top), and two views of the average structure of the complex d<pCCTTCGGT>·d<pCAGTCCCT> (bottom).

The overall structure of the three complexes is shown schematically in Figure 7.

Discussion

It is well known that cyclic oligonucleotides can recognize ssDNA by forming stable triplexes. Cyclic oligonucleotides can be designed to bind either purine-rich[3] or pyrimidine-rich[21] sequences with high affinities. The results described in this paper establish a novel mode of binding between cyclic oligonucleotides and ssDNA. Although the affinity of d<pCCTTCGGT> for its targets is not comparable with the affinities observed in triplex-forming cyclic oligonucleotides, this mode of recognition may have interesting applications when targeting sequences that are not susceptible of forming triplexes. Especially attractive is the possibility of targeting miniloop DNA hairpins, since these structures may play roles in gene regulation, recombination, or mutagenesis.[22,23] In this sense, the structure of the complex d<pCCTTCGGT>·d<pCAGTCCCT> can be envisioned as a model of recognition between two miniloop DNA hairpins, analogous to the kissing complexes found between some RNA and DNA hairpins with larger loops.[24–26] In particular, some DNA sequences containing short nucleotide repeats may be interesting targets, since they tend to adopt hairpin structures.[27] It has been postulated that these hair-

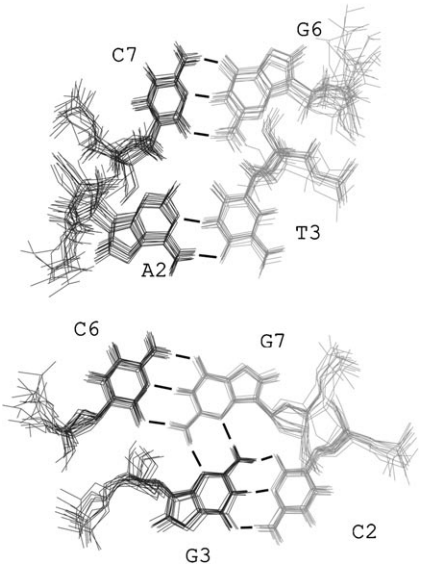


Figure 6. Detail of the two tetrads forming the core of the heterodimeric structure.

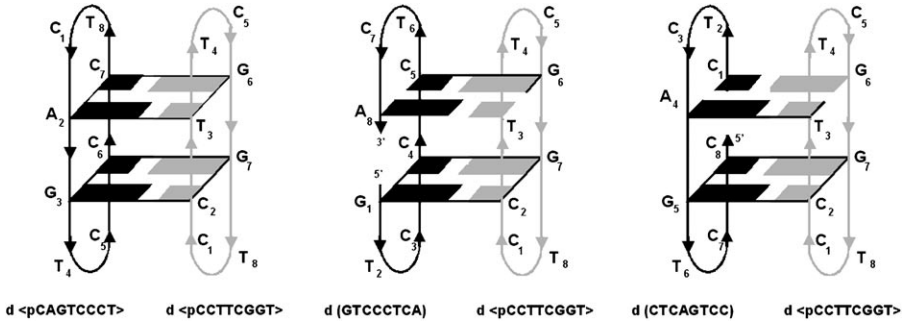


Figure 7. Scheme of the three complexes, indicating the intermolecular base pairs.

pins provoke instabilities during replication, causing the expansion of the repeat. There are around twenty hereditary neurological diseases that are linked to the expansion of these repeat sequences.[28]

It is interesting to compare the structure of the complex d<pCCTTCGGT>·d<pCAGTCCCT> with other structures stabilized by minor groove aligned tetrads. Minor groove G:C:G:C tetrads have been observed in the crystallographic structure of the linear heptamer d(GCATGCT)[6,29] and in the solution structure of the cyclic octamer d<pTGCTCGCT>.[8] Similar tetrads formed by minor groove alignment of two AT base pairs[7,8] and one AT and one GC base pairs[9] have been also found in the dimeric structures of d<pCATTCATT> and d<pCGCTCATT>, respectively. In all these cases, the structures are homodimeric, and are stabilized by two identical tetrads. Interestingly, the complex d<pCCTTCGGT>·d<pCAGTCCCT> is the first case reported in which this DNA motif is found in a heterodimeric structure. Moreover, this is the first case where two different minor groove tetrads co-exist in the same structure.

As in previous cases, the structure is formed by two stacks of base pairs with a mutual inclination of 30–40 degrees. In all homodimeric structures, the oligonucleotide sequence in the stacks was 5′-Pur–Pyr (pur = purine, pyr = pyrimidine). However, in this case the stack sequence is the 5′-Pur–Pur and 5′-Pyr–Pyr. This shows that an alternating purine–pyrimidine sequence is not an absolute requirement for the formation of the motif, although it might affect its stability.

The G:C:A:T tetrad found in this complex is very similar to that observed in the dimeric structure of d<pCGCTCATT>.[9] However, there are significant differences between the G:C:G:C tetrad observed in this structure and other minor groove G:C:G:C tetrads found in previous studies. In the G:C:G:C tetrad of the dimeric structures of d(GCATGCT) and d<pTGCTCGCT>, the two G·C pairs align directly opposite each other. The interaction between the two base pairs is stabilized by two additional intramolecular hydrogen bonds between one of the guanine amino protons and the O2 of the cytosine (see Figure 8A in reference [8]). In the G:C:G:C tetrad shown in Figure 6, the two base pairs are shifted along the axis defined by the Watson–Crick base pairs. This tetrad is also stabilized by two additional hydrogen bonds, but in this case they are formed between the guanine amino protons and the N3 atom of the neighboring guanine.

The heptamer d(GCATGCT) is the only case reported to date in which a linear oligonucleotide adopts a quadruplex structure stabilized by minor groove aligned tetrads. The dimeric structure of that DNA hairpin has been solved by crystallographic methods under different conditions.[6,29] Such result suggests that the motif is plausible in cellular DNA. The dimeric structures of d<pTGCTCGCT>, d<pCATTCATT>, and d<pCGCTCATT>, as well as the structure of complex **I** in this paper, suggest that the conformational restriction caused by cyclization is playing an analogous role to crystal packing forces in the case of d(GCATGCT). In the light of our results with complexes **II** and **III**, cyclization of only one of the chains confers enough stability to the motif to be observed in solution. A detailed thermodynamic study of the dimeric structures formed by cyclic oligonucleotides of different sequences, and their affinity for lineal oligonucleotides is now in progress.

Finally, it is worth mentioning that the cyclic oligonucleotide d<pCCTTCGGT> exhibits no affinity for r(GUCCCUCA), as shown in Figure 1E. The different binding properties of this cyclic octamer for linear RNA or DNA may be a consequence of the close proximity between the sugar moieties of the tetrads aligned in the minor groove. Such contacts may lead to favorable hydrophobic

contacts in the case of DNA, but to unfavorable interactions when the 2′OH group is present.

In conclusion, we have shown that cyclic oligonucleotides can form complexes with other cyclic and linear DNAs by inducing quadruplex structures based on tetrads aligned in the minor groove. The structures of these complexes belong to a four-stranded motif that had been found earlier in self-associated structures of linear and cyclic oligonucleotides. In this report, we show that this DNA motif may serve as a template for an induced-fit recognition of DNA oligonucleotides with a minimum requirement of sequence complementarity. This structure may be a model of recognition between DNA stem-loop hairpins. Such a recognition process suggests that small cyclic oligonucleotides might be good candidates to target small DNA hairpins, which are known to be present in regulatory regions of the DNA, and are sites of preferential interactions of numerous proteins.[22,30,31]

Experimental Section

Sample preparation: The cyclic octamers were synthesized as reported by Alazzouzi et al.[32] The stoichiometry for complex formation was determined by UV absorbance. Samples for NMR were suspended (in Na^+ salt form) in either D_2O or 9:1 H_2O/D_2O (100 mM NaCl, 10 mM $MgCl_2$, sodium phosphate buffer, pH 7).

Circular dichroism: Circular dichroism spectra were collected on a Jasco J-810 spectropolarimeter fitted with a thermostatted cell holder. CD spectra were recorded at concentrations of the complexes ranging from 300 to 600 μM. The spectra were normalized to facilitate comparisons.

NMR spectroscopy: All NMR spectra were acquired on Bruker spectrometers operating at 600 MHz or 800 MHz, and processed with the XWIN-NMR software. For the experiments in D_2O, presaturation was used to suppress the residual H_2O signal. A jump-and-return pulse sequence[33] was employed to observe the rapidly exchanging protons in one-dimensional H_2O experiments. NOESY[34] spectra in D_2O were acquired with mixing times of 100, 200, and 300 ms. TOCSY[35] spectra were recorded with the standard MLEV-17 spin-lock sequence and a mixing time of 80 ms. In two-dimensional experiments in H_2O, water suppression was achieved by including a WATERGATE[36] module in the pulse sequence prior to acquisition. NOESY spectra in H_2O was acquired with a mixing time of 150 ms. The spectral analysis programs XEASY[37] and SPARKY[38] were used for semiautomatic assignment of the NOESY cross-peaks, and quantitative evaluation of the NOE intensities.

Structure calculation: Distance constraints were obtained from NOE cross-peak intensities by using a complete relaxation matrix analysis with the program MARDIGRAS.[39] Final constraints were obtained by averaging the upper and lower distance limits obtained in different calculations with several initial models, mixing times, and correlation times. In addition to these experimentally derived constraints, Watson–Crick hydrogen-bond restraints were used. Target values for distances and angles related to hydrogen bonds were set as described from crystallographic data. Torsion angle constraints for the sugar moieties were derived form the analysis of *J* coupling data obtained from DQF-COSY experiments. No constraints for backbone angles were used.

Structures were calculated with the program DYANA 1.4[40] and further refined with the SANDER module of the molecular dynamics package AMBER 5.0[41] following standard annealing protocols used in our group.[8,42] Analysis of the average structures as well as the molecular dynamics trajectories was carried out with the programs Curves V5.1[43] and MOLMOL.[44]

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