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^{1}H NMR of 5′CGCGTATATACGCG3′, a duplex and a four-membered loop

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ABSTRACT

The deoxyribonucleotide 5′CGCGTATATACGCG3′ was synthesized and studied by NMR methods. This short, fully palindromic duplex also forms a hairpin under certain conditions described within. As such, it is considered to be a model for cruciform formation. We show that this sequence forms a four-membered loop and a duplex in solution. The duplex is shown to be a normal, B-DNA like helix, while the hairpin is shown to be a four-membered ATAT loop.

INTRODUCTION

Palindromic sequences with long DNA strands are stable as duplexes, but can sometimes be driven to an alternate cruciform structure containing intrastrand hydrogen bonds. Cruciforms have been found in supercoiled plasmid DNAs *in vitro*[1,2,3] and recently evidence has been presented which indicates their formation *in vivo* as well.[4] In plasmid DNAs cruciform formation is driven by negative supercoiling. For most DNA sequences there is a threshold level of supercoiling required for cruciforms to be seen and highly variable sequence dependent kinetic barriers as well.[5,6] Although a variety of studies have been carried out on these systems some physical studies are limited by the large size of the plasmids. An alternative, which can provide some of the desired information, is the study of short, fully palindromic duplexes. The pathway leading to cruciforms in plasmids will generally lead to separated hairpins in the oligomer models.

The advances in DNA synthesis over the past few years have made preparation of milligram amounts of DNA straightforward.[7] As a consequence it is possible to carry out physical studies such as NMR. The sequences which form structural elements such as hairpins are currently the subject of much interest.[8,9,10] In the present paper we discuss the behavior of the tetradecamer CGCGTATATACGCG, extending earlier studies of CGCGTATACGCG.[11] Although fully self-complementary these sequences undergo duplex to hairpin transitions with increasing temperature, particularly at very low salt concentrations. The central $(TA)_3$ sequence can in principle form a loop of 2, 4, or 6 bases. We show that the stable form in solution is a four base loop, and describe the basic features of its structure.

MATERIALS AND METHODS

The DNA used in these studies was synthesized by the solid-phase phosphite triester method, as described previously.[12] The DNA was prepared with a 10 μmol column and was removed from the automated synthesizer as the fully protected, DMT-oligonucleotide.[13] The protecting groups were removed from the bases and the oligonucleotide was cleaved from the solid support on the 3′ end by treatment with ammonium hydroxide at 55°C for 6 hours. The oligonucleotide was purified on an IBM HPLC using a Magnum-C18 reverse-phase column in a gradient of acetonitrile in 0.1 M TEAA buffer and then desalted by dialysis. The syntheses yielded 5–12 mg of pure material. For analysis of purity the deoxyribonucleotide was ^{32}P-end labelled and electrophoresed on a polyacrylamide gel showing a single band corresponding to a 14-mer. The NMR samples were dialyzed into 10 mM phosphate with the appropriate amount of NaCl as indicated. The NMR samples were prepared by drying the sample from the appropriate buffer and redissolving in 99.9% D_2O (Cambridge or Stohler Isotopes).

The melting behavior of this oligomer was studied at micromolar concentrations by optical melting and at millimolar concentrations by ^{1}H NMR. The NaCl concentration was varied from 'no added' salt to 2 M salt. The NMR melts were carried out over a 90°C temperature range. Collecting NMR spectra at 5° intervals clearly indicated that equilibrium is reached in no more than five minutes at each temperature and salt condition. The optical melt data is discussed elsewhere.[14] The melt data was used to select the conditions for the studies of both the all-duplex and the duplex/hairpin using 2D NMR spectroscopy.

The concentration of DNA used for the NMR studies was 1.5 mM, measured by absorbance at 260 nm. The extinction coefficient was estimated from nearest neighbor and base-pairing interactions.[15] NMR spectra were taken on a General Electric GN-500 spectrometer equipped with a Nicolet 1280 computer.

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The NMR data sets were transferred to magnetic tape and processed on a VAX/11-780 or a μVAX using the FTNMR program supplied by Dr Dennis Hare. COSY spectra were obtained in the absolute value mode, while the NOESY spectra were obtained in the phase sensitive mode using TPPI.[16] In a typical experiment 1024 complex t_2 data points were collected for each of about 450 t_1 values, using a spectral width of 5000 Hz, giving a digital resolution of 4.8 Hz/pt after zero filling in t_1. 64 scans were usually taken with a recycle time of 2 seconds. The mixing time used in the duplex NOESY experiment was 300 ms. Mixing times of 100, 300, 600 and 1200 ms were used for the duplex-hairpin equilibrium NOESYs. The chemical shifts were referenced to internal TSP.[17] The imino, amino and adenine C2H protons were assigned using the NOE in samples which were in 90%H_2O/10%D_2O as previously described.[18] The 1331 solvent suppression sequence was employed to suppress the solvent signal.[19]

RESULTS AND DISCUSSION

The assignment of the ^1H chemical shifts of the nonexchangeable hydrogens for the 5'CGCGTATATACGCG-3' 14-mer was performed using the two-dimensional COSY and NOESY NMR experiments as previously described.[20,21,22] The exchangeable imino protons were assigned using the one dimensional ^1H NMR inter-base NOEs.[23] The 14-mer was assigned in the all-duplex form by collecting NMR spectra at 1.5 mM strand concentration, 10 mM phosphate, pH 7.0 (of sample), 0.3 M NaCl, and at 25°C. Under these conditions practically no hairpin is present. This condition was chosen after observation of the melting behavior at millimolar concentrations for NMR studies and at micromolar concentrations for UV spectrophotometry under a number of different salt and strand concentrations. A typical optical melt is shown in Figure 1.[14] The lower transition has a melting point which is strand-concentration dependent and can be attributed to the duplex to hairpin transition. The upper transition has a melting point which is not dependent on strand concentration and can be attributed to the hairpin to random coil transition. At optical concentrations the hairpin is strongly favored as is discussed elsewhere.[14] At NMR concentrations the duplex is strongly favored. Even at the lowest concentrations feasible for NMR studies we were not able to convert to pure hairpin.

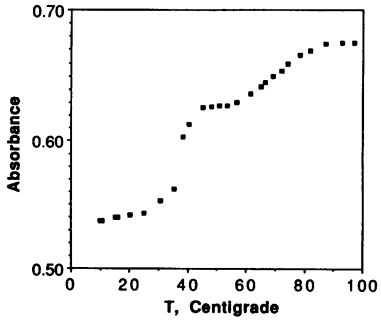


Figure 1. Absorbance versus temperature profile for d(CGCGTATATACGCG) in 10 mM phosphate at pH 7 at an additional salt concentration of 0.05 M NaCl. The strand concentration was 4.5 μM. As the DNA unmelts the bases unstack which results in the observed hyperchromicity with increasing temperature. Further experimental details and analysis of such profiles are described elsewhere (D.A. Kallick and D.E. Wemmer, in preparation).

A portion of the phase sensitive NOESY is shown in Figure 2. Use of the primary sequence of the oligodeoxyribonucleotide and verification of each base type from the COSY data allows assignment of most of the protons in the duplex (Table 1). There are no ambiguities in the sequential resonance assignments, and the relative positions and intensities of the cross peaks are consistent in each spectral region with those seen for related

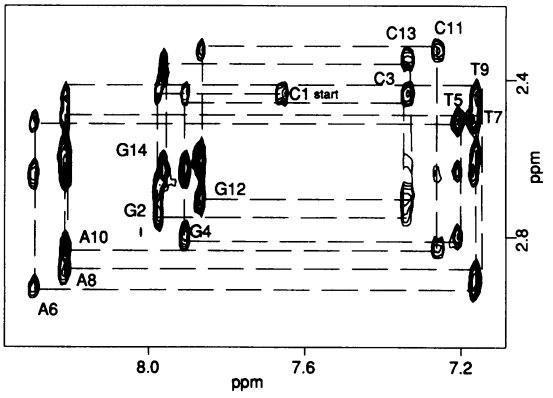


Figure 2. Expansion of part of the aromatic C8H, C6H to C2H'', C2H' sugar proton region of the NOESY spectrum of d(5'CGCGTATATACGCG3') at 25°C in 10 mM phosphate, 0.3 M NaCl at pH 7 (of sample). The sequential assignment path is traced as indicated with the cross peaks between aromatic and sugar C2H'' protons of the same residue labelled with that residue. The peaks not connected by lines represent the aromatic to sugar C2H' cross peaks.

Table 1. Chemical shifts of assigned resonances in duplex and hairpin forms of d(5'CGCGTATATACGCG) at 25°C.

residue	C8H	C6H	C5H	CH3	C2H	C1H'	C2H''	C2H'
1C		7.64a)	5.92			5.77	2.43	2.00
		7.66b)				5.83	2.46	
2G	7.96					5.92	2.67	2.00
	8.00					5.98	2.80	2.72
3C		7.34	5.38			5.74	2.43	2.08
		7.37				5.97		
4G	7.87					5.97	2.80	2.63
	7.90					5.97	2.68	2.55
5T		7.21		1.50		5.72	2.50	2.13
		6.91		1.52		5.96	2.69	2.28
6A	8.26				7.03	6.24	2.92	2.64
	8.16				7.90	6.20	2.81	2.66
7T		7.16		1.42		5.68	2.50	2.10
		7.21		1.68		5.80	2.66	2.10
8A	8.20				6.92	6.19	2.89	2.64
	8.06				8.10	6.09	2.58	
9T		7.14		1.36		5.62	2.43	2.02
		7.36		1.78		5.90		
10A	8.19				7.15	6.17	2.83	2.59
	8.33				8.00	6.11	2.86	
11C		7.24	5.22			5.59	2.32	1.95
		7.37				5.64		
12G	7.84					5.86	2.70	2.60
	7.92					5.96		
13C		7.34	5.42			5.74	2.34	1.92
		7.36				5.82		
14G	7.94					6.17	2.63	
	7.96					6.19		

a) chemical shift of B form b) chemical shift of hairpin

sequences.[24] It is clear from the pattern of NOE cross peaks that this 14-mer forms a normal, B-DNA structure under these conditions.

We illustrate in Figure 2 part of the aromatic C8H or C6H to sugar C2H′, C2H″ region of the phase-sensitive NOESY for the duplex. Each intra-residue C6H or C8H cross peak to C2H″ cross peak is downfield from C2H′ as has been previously observed.[25] The intra-residue C8H, C6H to C2H″ cross peaks are all of approximately equal intensity. In addition sequential $C6H_n$ or $C8H_n$ cross peaks to $C2H''_{n-1}$ and $C2H'_{n-1}$ are seen. The C2H′ cross peaks are weaker for purines than for pyrimidines, probably reflecting some sequence dependent structural variation in this alternating purine–pyrimidine sequence as suggested by Calladine's rules.[26] The aromatic C8H or C6H to sugar C1′H region is shown in Figure 3. The pyrimidine aromatic to purine C1′H cross peaks are all of approximately equal intensity and as a group, are less intense than all the purine aromatic to pyrimidine sugar C1′H cross peaks.

Similar to what is observed for the related 12-mer, 5′-CGCGTATACGCG-3′, as the temperature is raised or as either the salt or strand concentration is decreased, we observe additional peaks in the ^{1}H NMR spectrum which can be assigned to the hairpin-loop conformation of the 14-mer.[24] As shown in Figure 4 (bottom) at low temperature and moderate salt (100 mM NaCl; 10°C) only three thymine methyl peaks appear in the ^{1}H NMR spectrum corresponding to the duplex form. The small peak at 1.49 ppm is attributable to a salt effect and disappears when the sample is dialyzed extensively. At low salt, however, and as the temperature is raised (see Figure 4 legend), three additional peaks appear in the upfield region which can be assigned to the three hairpin thymine methyl resonances. This equilibrium in which the duplex and hairpin are in slow exchange on the chemical shift time scale is fully reversible at NMR concentrations. The hairpin to duplex conversion is exceedingly slow (on the order of hours) at optical (μM) concentrations. The conditions for which the duplex and hairpin coexist were chosen to obtain another NOESY, an expansion of which is shown in Figure 5. The duplex and hairpin are again in slow exchange on the chemical shift time scale under these conditions. A long mixing time was used for the hairpin sequential resonance assignments, but intermediate and short mixing times were used to corroborate conclusions drawn from the relative intensities of the NOE cross peaks. Only at the very long mixing time does the set of hairpin cross peaks become nearly complete as required for assignment. At mixing times of 100 ms and 300 ms the spectrum is dominated by duplex peaks but several hairpin peaks are visible. These hairpin peaks at the 100 ms mixing time were assigned by overlaying the long mixing time NOESYs on the short mixing time NOESYS. At short mixing times the NOESY cross peak intensities are still proportional to the inverse sixth power of the distances between spins.[27] The requirement for the longer mixing time may be explained by the

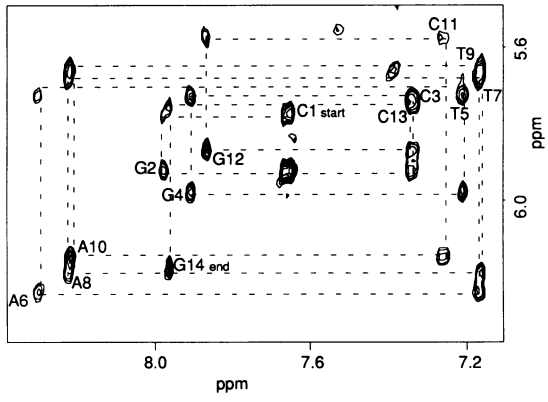


Figure 3. Expansion of the all-duplex aromatic C8H, C6H to sugar C1′H proton region of the NOESY spectrum of d(5′CGCGTATATACGCG3′) obtained under the same conditions as Figure 1. The sequential assignment path is traced with the cross peaks between aromatic and sugar C1′H protons for the same residue labelled with that residue.

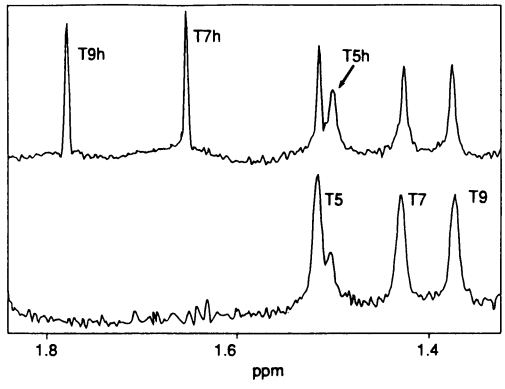


Figure 4. The all-duplex form exists over a range of temperature and salt conditions, one of which is represented in the bottom ^{1}H NMR spectrum. This lower spectrum shows three duplex thymine methyl peaks obtained at 10°C, 100 mM NaCl. The shoulder at 1.49 ppm (bottom) is attributable to a salt effect and disappears when the sample is dialyzed. The upper ^{1}H NMR spectrum, obtained at 35°C and 1 mM added NaCl, shows an additional three methyl peaks which are assigned to the hairpin. Both samples were at pH 7. All resonances were assigned using the 2D NMR experiments described in the text.

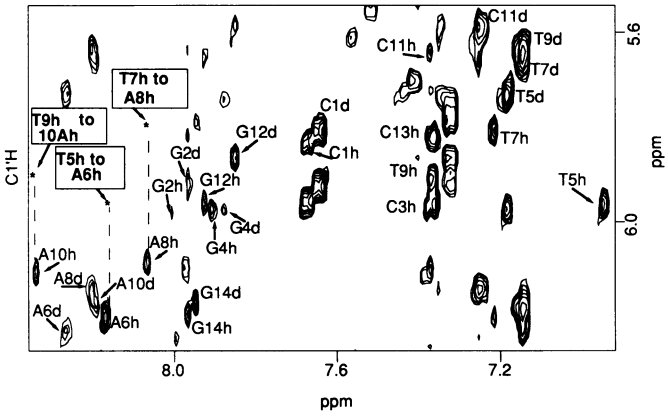
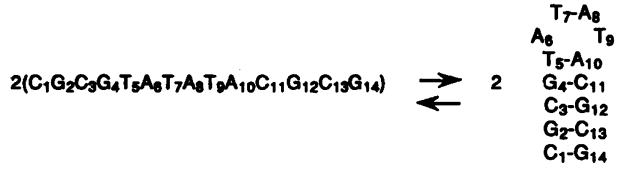


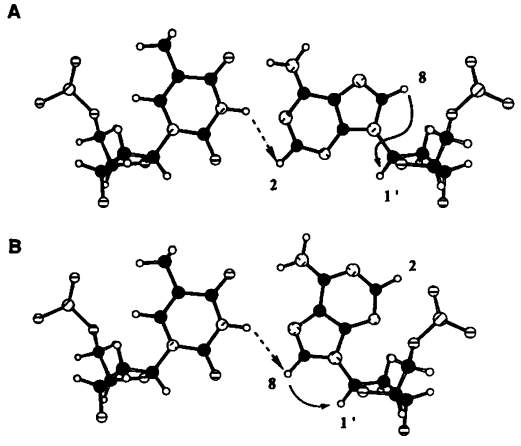
Figure 5. Expansion of the duplex/hairpin aromatic C8H, C6H to sugar C1H′ proton region of the NOESY spectrum of d(5′CGCGTATATACGCG3′) at a mix time of 1200 ms, obtained at 25°C with no added NaCl, 10 mM phosphate at pH 7 (sample). The strand concentration was the same as the all-duplex NOESY, 1.5 mM. Every cross peak expected in this region for both the duplex and the hairpin is observed, except for each adenosine C8H to the thymidine $C1H'_{n-1}$, as indicated in the boxes, and the 1-cytidine to 2-guanosine cross peak. The remaining labels refer to the NOE from each purine or pyrimidine to its own sugar C1H′. The unlabeled cross peaks are the sequential n aromatic to (n−1) sugar protons.

fact that the hairpin is half the molecular weight of the duplex, and hence the rotational correlation time is also approximately half that of the duplex. Since the initial cross relaxation rate scales with correlation time, the NOE builds up about a factor of two more slowly. As a result, at short mixing times the duplex cross peaks dominate the NOESY spectrum. At the longer mixing times used there are essentially two complete sets of resonances. Almost certainly there is spin diffusion at the long mixing time but this does not invalidate the sequential assignments. Only the shortest mixing time NOESY is used to draw conclusions from the relative intensities of the cross peaks.

The aromatic to sugar C1H′ region from the phase sensitive NOESY of the duplex/hairpin is shown in Figure 5. In the following discussion we consider just the hairpin conformation. In the stem the NOESY cross peak patterns (and intensities as derived from the short mixing time data) indicate a normal B-DNA conformation, from 1C through 4G in the 5′ end and from 11C through 14G in the 3′ end, in agreement with what has been observed in solution for related hairpins.[8] With regard to the loop, there are several unusual features. In this region we observe that three cross peaks are missing in the hairpin conformation — each adenosine C8H to the preceding thymidine C1H′. This is accompanied by the presence of a very strong cross peak from the A6C8H to its own C1H′, validated at a mixing time of 100 ms. This observation implies that *none* of the adenines in the hairpin conformation are in the normally observed B-DNA *anti* configuration about the sugar. In the *anti* conformation, the distance from any adenosine C8H to its own C1H′ is 3.7 Å (Scheme 2a). In the NOESY with the 100 ms mixing time the 6-adenosine C8H to C1H′ cross peak is as intense as the aromatic cytidine C5H to C6H cross peaks, the protons of which are 2.5 Å apart (data not shown). The 10-adenosine C8H to C1H′ cross peak appears at the 300 ms mixing time NOESY (data not shown), which implies that the 10AC8H and the 10C1H′ are somewhat farther apart than the same two protons in the sixth residue, but likely closer than 3.7 Å. The same situation exists for the 8-adenosine C8H with respect to its own C1H′ cross peak.



Scheme 1. Numbering is from the 5′ end of the oligodeoxyribonucleotide.



Scheme 2. Thymidine-adenosine base pairs. The dashed arrows indicate the adenine proton which is close enough to the N3H imino proton to be observed by an NOE in H_2O using ^1H NMR. The curved arrows indicate the AC8H to sugar C1H′ distance. (A) Idealized B-DNA T-A base pair in which the AC8H to AC1H′ distance is 3.7 Å and the thymidine imino is ca. 2.8 Å from the adenosine C2H. (B) Hypothetical T-A base pair in which the adenine is *syn* to the sugar. The AC8H to C1H′ distance is ca. 2.2 Å. The thymidine imino is in this case also within NOE distance from the adenosine C8H.

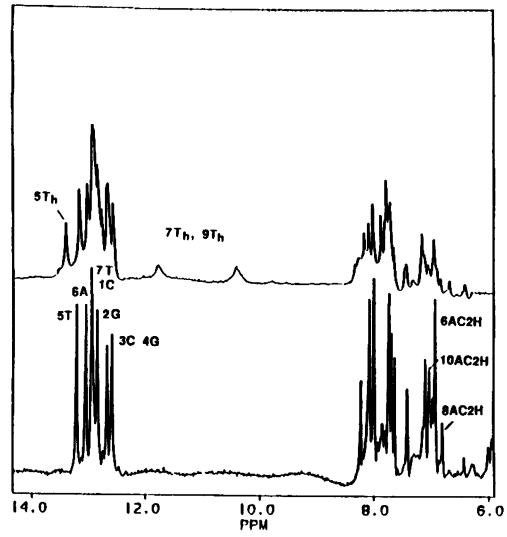


Figure 6. Proton NMR spectrum in H_2O (see text for details) showing the aromatic region and the downfield imino proton region for the all-duplex conformation (bottom) and the duplex/hairpin conformation (top) at 25°C, pH 7 (of sample), at a strand concentration of 1.5 mM for both spectra. Each spectrum was obtained in 90% H_2O/10%D_2O with 10 mM phosphate. The upper spectrum had no additional salt; the lower spectrum had an additional 0.1 M NaCl. The broad, upfield peaks at 10.6 and 11.9 ppm we attribute to the 7-thymidine and the 9-thymidine hairpin imino protons. The most downfield peak is assigned to the 5-thymidine in the hairpin conformation.

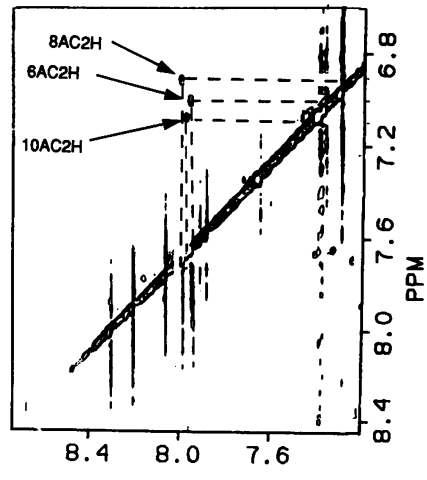


Figure 7. Expension of a region of the exchange NOESY at 53°C. The cross peaks represent a saturation transfer between the resonances of protons in the duplex and hairpin forms. The duplex adenosine C2H protons were identified from 1D ^1H NMR NOEs as described in the text; in addition each duplex A-C2H chemical shift moves downfield without crossing each other as the temperature is raised to 53°C. Each hairpin adenosine C2H shifts downfield from the duplex as shown.

A near-normal intensity cross peak from the 10-adenosine C1H′ to the 11-cytidine C6H is observed, implying that the 10-adenosine is stacked normally on the cytidine. Another unusual cross peak is observed from the 5-thymidine C6H to the 7-thymidine CH_3, also confirmed at the shorter mixing time. Thus the short mixing time, NOESY spectra confirm what is seen in the longer mixing time NOESY data, although caution must be used in any qualitative interpretation.

The pattern of NOEs seen in the aromatic to C1H′ region suggest that the loop in the center of the TA repeat is four bases in length, comprised of A6T7A8T9. This is confirmed by one dimensional NMR NOE difference studies of the imino protons under the same conditions as the duplex/hairpin NOESY. Shown in Figure 6 is the downfield ^1H NMR spectrum in H_2O of the all-duplex form (bottom) compared to the duplex/hairpin spectrum (top). The assignment of the all-duplex imino and aromatic protons was unambiguous and is described in the experimental section. The duplex-hairpin spectrum illustrates two new resonances which appear upfield of the duplex A-T imino resonances and a new resonance which appears downfield of the duplex A-T imino resonances. The two broad peaks at 10.6 and 11.9 ppm could not be individually assigned because no NOEs were observed in the NOE difference spectra. Based on their chemical shifts they are likely the two loop iminos of thymidines which are non-based-paired.[28] In 1D NOE difference spectra the downfield 5-thymidine resonance shows one G-C neighbor, and the usual strong connection to the A2H within the base pair. There is no evidence for the 5-thymidine-10-adenosine base pair to be a *syn* base pair as in Scheme 2B. This base-paired 10AC2H resonance is observed in one-dimensional NMR temperature studies to move downfield to a chemical shift of 8.0 ppm at 53°C (the 10AC8H resonance moves to 8.33 ppm, see Table 1), observable since the hairpin and random coil are in fast exchange on the chemical shift time scale. Further proof of the identity of this H2 resonance in the hairpin conformation is provided by observation of exchange cross peaks in the NOESY spectrum at 53°C, Figure 7. At 53°C a considerable amount of hairpin is present (see Figure 1; this transition is strand concentration-independent) and the duplex and hairpin are still in slow exchange on the chemical shift time scale. The exchange cross peaks however indicate that the exchange rate for the interconversion of duplex to hairpin is fast compared to the longitudinal relaxation.[29] The duplex to hairpin transition has a high activation energy[14] and thus a strong temperature dependence, so the emergence of the exchange cross peaks under these conditions is not surprising. The spectrum in Figure 7 establishes the base pair closing the loop as a standard Watson–Crick type rather than a Hoogsteen type as has been formed for a TTTA loop.[10]

The NOESY data also indicate that the structure formed in the ATAT loop is significantly different from that found in several other four base loops, particularly TTTT characterized in some detail by Hare and Reid. The most prominent feature is the strong intra-residue cross peak for A6, observed at a 100 ms mix time. By comparison with the cytosine C5H-C6H cross peaks it appears that the A8H–H1′ distance is close to 2.5 Å, putting it in the *syn* glycosidic angle range. The NOEs from the A8H to the C2H′, C2H″, however, are not particularly weak, so it is likely that the A6 is close to *syn*, but perhaps not rotated the entire 180° required for an idealized *syn* conformation. The lack of a cross peak between the A6H8 and the T5H1′ and the low intensity of the A6H8 to T5H2′ indicate that A6 is not stacked onto T5. Hilbers and Haasnoot have studied a number of different hairpin loops, both experimentally and in modelling calculations, and have concluded that stacking of the first loop residue onto the 3′ side of the stem should occur, contrary to the observation in this case. The 3′ stacking has been observed for a number of different loop sequences including TTTT, TAAT and TTTA, and seems to be independent of the base pair which closes the stem. These workers also explored the effect of larger bases in the loop, specifically TAAA. In this case they report the loop expands to six bases. Our finding that the TATA loop (the bulky base in the second position) is stable with four residues, even with TAAT containing loops, with the stem closed by a GC base pair, shows that there can be bulky bases at both central positions.

The ^{31}P NMR spectrum of the sample under conditions where duplex and hairpin coexist is not significantly different from the all duplex spectra (spectrum not shown). In both cases resonances are dispersed about 1 ppm from the average chemical shift of −4.25 ppm.[30] Since the ^{31}P NMR chemical shifts are sensitive to the backbone angles, especially α and ζ[31], we conclude that the formation of the loop does not force these angles to unusual values as has been seen with two base loops.[32] It therefore appears that there are no unusual torsion angles in the backbone of this hairpin.

These results represent our effort at understanding the rules which govern the influence of sequence on DNA hairpin loop structure. The picture emerging in the last several years is that the conformation of nucleic acid loops appears to be exquisitely sensitive to the sequence and probably depends upon the stem sequence as well. Our sequence highlights this sequence sensitivity in at least two ways. It differs somewhat from the aforementioned model of Blommers *et al.* (ref. 10) with respect to the base stacking in the loop. Furthermore, it differs from the recently observed DNA hairpin[33] in that it does not appear to have any unusual torsion angles in the backbone, as evidenced by the B-DNA-like ^{31}P NMR spectrum. We are currently further investigating the conformation of the loop using molecular modelling.

ACKNOWLEDGEMENT

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13. Abbreviations: DMT, Dimethoxytrityl; TEAA, Triethylammonium acetate; NMR, Nuclear Magnetic Resonance; NOE, Nuclear Overhauser Effect.
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