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DNA Hairpin Structures in Solution: 500-MHz Two-Dimensional ^{1}H NMR Studies on d(CGCCGCAGC) and d(CGCCGTAGC)†

Goutam Gupta, Mukti H. Sarma, and Ramaswamy H. Sarma*
Institute of Biomolecular Stereodynamics, State University of New York at Albany, Albany, New York 12222
Rolf Bald, Ute Engelke, Shiao Li Oei, Reinhard Gessner, and Volker A. Erdmann
Institut für Biochemie, Fachbereich Chemie, Freie Universität Berlin, 1000 Berlin 33 (Dahlem), FRG
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ABSTRACT: A hairpin structure contains two conformationally distinct domains: a double-helical stem with Watson–Crick base pairs and a single-stranded loop that connects the two arms of the stem. By extensive 1D and 2D 500-MHz ^{1}H NMR studies in H_2O and D_2O, it has been demonstrated that the DNA oligomers d(CGCCGCAGC) and d(CGCCGTAGC) form hairpin structures under conditions of low concentration, 0.5 mM in DNA strand, and low salt (20 mM NaCl, pH 7). From examination of the nuclear Overhauser effect (NOE) between base protons H8/H6 and sugar protons H1′ and H2′/H2″, it was concluded that in d(CGCCGCAGC) and d(CGCCGTAGC) all the nine nucleotides display average (C2′-endo,anti) geometry. The NMR data in conjunction with molecular model building and solvent accessibility studies were used to derive a working model for the hairpins.

Self-complementary DNA oligomers, in general, adopt double-helical structures. Double helices are stabilized by Watson–Crick base pairing, intra- and interstrand stacking, and favorable backbone and base–backbone conformations. The double-helical structures of self-complementary DNA oligomers are formed only when negatively charged phosphate ions are neutralized by counterions of salt and when the concentration of DNA is sufficiently high such that every oligomer finds its complementary partner. But high salt and DNA concentration are not merely enough to form a double helix; the temperature should also be significantly lower than the melting temperature T_m of the corresponding oligomer. Thus, salt, DNA concentration, and temperature are three physical features that are often altered to monitor helix → coil transitions. It has been recently (Haasnoot et al., 1983, 1986; Orbons et al., 1986) reported that in the pathway of helix → coil there could be a structural intermediate for self-complementary DNA oligomers. In other words, at a particular salt and DNA concentration and within a small range of temperature it has been observed that oligomers display a novel kind of structure called the "hairpin" (Haasnoot et al., 1983, 1986; Orbons et al., 1986). A hairpin structure contains two distinct domains—a double-helical stem with Watson–Crick paired bases and a single-stranded loop that joins the two arms of the stem and the stacking interaction of the bases in the loop and at the junctions between the stem and the loop (Haasnoot et al., 1986). The possibility of the formation of a hairpin structure as a stable thermodynamic entity is markedly enhanced for non-self-complementary DNA oligomers because mismatch pairs significantly lower the stability of a rigid duplex under ordinary conditions of salt and DNA concentration. In this paper, we discuss the identification and the stereochemical characterization of hairpin structures as obtained for two non-self-complementary DNA segments each nine nucleotide long; these DNA segments are DNA analogues

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of the helix II sequence of 5S RNA from *Escherichia coli* and *Thermus thermophilus* (Digweed et al., 1985). The sequences are d(CGCCGCAGC) (*T. thermophilus*) and d(CGCCGTAGC) (*E. coli*).

Comments on the pH Used in This Study

In the preceding paper (Sarma et al., 1987) we showed that the oligomer d(CGCCGCAGC) remains as an intact double helix under high oligomer concentration and high salt, pH 4.5 and $T = 5$ °C. In this accompanying paper we show that the same oligomer, as well as its analogue d(CGCCGTAGC), assumes the shape of a monomeric hairpin when the concentration of the oligomer was reduced by a factor of 16 and the salt concentration by a factor of 25, $T = 15$ °C and neutral pH. A referee has raised a point on the effect of pH on the stability of the hairpin. The dominant forces that convert the duplex to hairpin are lowering the concentration of the oligomer and that of salt. In the present case, lowering the pH can facilitate the formation of a hairpin with three base pairs in the stem, but this will leave only two bases in the loop; i.e., the stem will contain C9-G2, G8-C3, and A^+7-C4 pairs, and the loop will contain C6 and G5. Haasnoot et al. (1986) has shown that a hairpin is most stable when the loop part contains four to five bases.

Materials and Methods

For synthetic and purification procedures of the oligomers, see Materials and Methods of the preceding paper. All NMR studies reported in this paper were for dilute soutions of DNA (0.5 mM in DNA strand) in low salt (20 mM NaCl, 10 mM phosphate buffer, pH 7.0). 1D NMR spectra of DNA in water were recorded with a time-shared long pulse sequence with a notch filter at 3750 Hz and a 2K data size with about 5000 transients (relaxation delay RD = 1.5 s). The two-dimensional correlated spectroscopy (COSY) spectra were recorded with the pulse sequence (RD–90°–t_1–90°–Acq)$_n$. For each of 512 t_1 values, 64/128 transients were recorded with 1024 data points with RD = 1.5 s; HDO was presaturated. Two-dimensional NOE (NOESY) spectra were collected under the same solution conditions of the COSY experiment. For NOESY experiments the pulse sequence was (RD–90°–t_1–90°–τ_m90°–Acq)$_n$ with a mixing time τ_m = 500–100 ms, other parameters being the same as in the COSY experiment. Both COSY and NOESY spectra were recorded in the pure absorption mode (States et al., 1982). Time domain data of COSY and NOESY experiments were processed on a VAX-11/750 computer; free induction decays (FIDs) were weighed with an exponential multiplication factor (EM) of 10 Hz before Fourier transformation.

Identification of the Hairpin Structure: 1D NMR Spectra of d(CGCCGCAGC) and d(CGCCGTAGC) in Water

Figure 1 shows the 1D NMR spectra of d(CGCCGCAGC) (spectrum A) and d(CGCCGTAGC) (spectrum B) in 90% H_2O + 10% D_2O at 15 °C for a low DNA concentration (0.5 mM in DNA strand) in low salt (20 mM NaCl, 10 mM phosphate buffer, pH 7.0). It is well-known that in a duplex structure the NH protons (i.e., N1-H of G and N3-H of T) appear in the water spectrum. In the single-stranded coil state the protons are totally exposed to the solvent and exchange rapidly resulting in the loss of signals. However, in the loop segment of the hairpin structure even though the bases are not involved in Watson–Crick pairing the exchangeable NH protons are not completely accessible to H_2O presumably due to intrastrand stacking interactions. As a result, the exchangeable NH protons belonging to the loop segment of the hairpin structure of an oligomer do appear in the water spectrum (Haasnoot et al., 1983, 1986; Orbons et al., 1986). The NH signals in the double-helical stem and in the loop segment are easily distinguished from each other because the former appears at a lower field than the latter [as demonstrated by Haasnoot et al. (1983, 1986) and Orbons et al. (1986)]. Therefore, the hairpin structure of d(CGCCGCAGC) in water should show three NH signals: two from Watson–Crick pairs G2-C9 and C3-G8 and one from G5 in the loop segment (see inset of Figure 1), the first two signals occurring at a lower field than the third. Similarly, the hairpin structure of d(CGCCGTAGC) in water should show four NH signals: two from G2-C9 and C3-G8 and two from G5 and T6 (see inset of Figure 1). Notice that spectra A and B bear the signatures of hairpin structures for d(CGCCGCAGC) and d(CGCCGTAGC). For d(CGCCGCAGC) the N1-H signals of G2 (from G2-C9) and G8 (from C3-G8) appear within 13.2–13.0 ppm while N1-H signals of G5 in the loop occur at ~11.0 ppm. For d(CGCCGTAGC) the N1-H signals of G2 (from G2-C9) and G8 (from C3-G8) appear within 13.2–13.0 ppm, and N1-H of G5 and N3-H of T6 appear within 10.9–10.7 ppm.

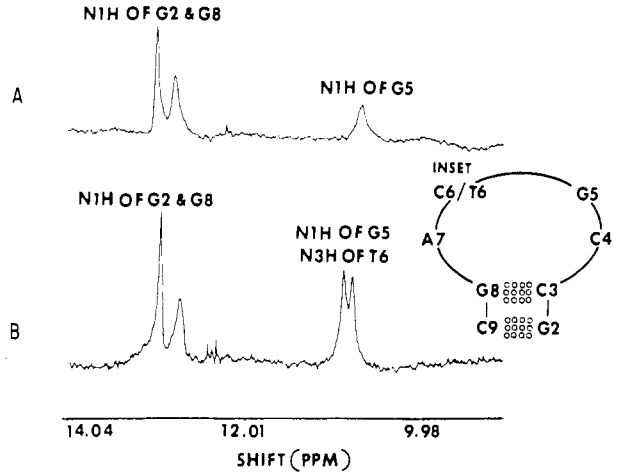
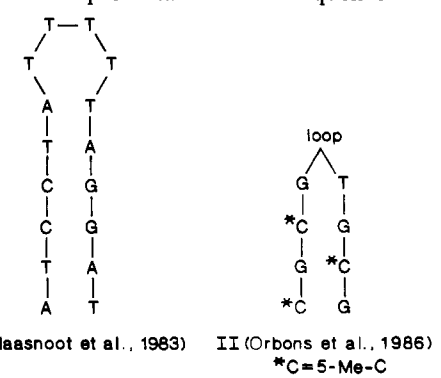


FIGURE 1: Low-field region of the 500-MHz ^{1}H NMR spectra of (A) d(CGCCGCAGC) and (B) d(CGCCGTAGC) in 90% H_2O + 10% D_2O at 15 °C with 0.5 mM DNA in strand in 20 mM NaCl–10 mM phosphate buffer, pH 7.0. TSP was used as an internal standard. The spectra were recorded with a time-shared long pulse sequence with a notch filter at 3750 Hz. (A) For d(CGCCGCAGC), two NH protons appear within 13.2–13.0 rpm resulting from the double-helical stem of the hairpin structure (as described in the inset). The signal at 11.0 ppm is contributed by the NH proton of the guanine residue in the loop (i.e., G5) of the hairpin structure (see the inset). (B) For d(CGCCGTAGC), two NH protons appear within 13.2–13.0 ppm resulting from the double-helical stem of the hairpin and two more signals from the NH protons of G5 and T6 in the loop (see the inset).

Two of the most well-characterized hairpin structures have been observed for representative DNA sequences:



The monomeric nature of the hairpin I was established by computing the melting temperature T_m by NMR methods (for which the DNA concentration was 1 mM in strand) and comparing that with the T_m obtained from UV studies by Albergo et al. (1981) for which the DNA concentration was 0.01 mM in strand. From the similarity in T_m from UV and NMR measurements, it was concluded that over a concentration range of 0.01–1 mM in DNA strand, d-(ATCGTATTTTTACGAT) adopted a monomeric hairpin structure. In the case of d(*CG*CGTG*CG), Orbons et al. (1986) showed that at 0.4 mM in DNA strand the molecule exclusively adopted the hairpin structure. For the two DNA oligomers we studied, the concentration of DNA (0.5 mM in DNA strand) was well within the bounds so that a monomeric hairpin would be the only expected ordered structure, signatures of which are seen from the NMR data (Figure 1). We have not measured the T_m of the hairpin structures in Figure 1 by NMR methods because our major interest was to characterize the hairpin structures in terms of the conformations of each constituent nucleotide unit (and not their stability with respect to temperature). We have conducted UV melting studies on d(CGCCGCAGC) and d-(CGCCGTAGC) with DNA samples diluted 80–400 times compared to our NMR samples at pH 7.0 (10 mM sodium cacodylate, 20 mM NaCl). The melting temperature T_m for d(CGCCGCAGC) was 59 ± 2 °C, and T_m for d-(CGCCGTAGC) was 58 ± 3 °C. Under the same solution conditions, the DNA oligomers without any flanking C, i.e., d(GCCGCAGC) and d(GCCGTAGC), have T_m's very close to the corresponding nonamers, thus indicating that the flanking C had very little effect on the stability of the hairpin structure. In addition to the water NMR spectra, the following observations support that the sequences that we have used under the solution conditions stated adopt hairpin motifs:

(i) We monitored the area of NH signals vs temperature (from G·C pairs and from G and T in the loop). As suggested by Haasnoot et al. (1983), the NH's in the loop of the hairpin showed a higher exchange rate than those in a G·C pair in the stem.

(ii) We performed 1D NOE experiments on the hairpin structure of d(CGCCGTAGC) in 90% H_2O + 10% D_2O. Irradiation of the NH's of G·C pairs (within 13.2–13.0 ppm) for a presaturation time τ_m of 300 ms showed no NOEs at the NH signals of G5 and T6 in the loop. In a hairpin structure, NH's of G5 and T6 are far away (>4 Å) from the NH's of G·C pairs in the stem (Figure 1).

(iii) We have monitored the NH region of d-(CGCCGTAGC) in water at pH 8.5. We observed that the NH signal from the loop region, i.e., N3-H of T6 and N1-H of G5, does disappear at higher pH as expected from a hairpin structure (Haasnoot et al., 1983, 1986; Orbons et al., 1986).

In summary, it may be concluded that d(CGCCGCAGC) and d(CGCCGTAGC) at 15 °C under conditions of low concentration (0.5 mM in DNA strand) and low salt (20 mM NaCl, pH 7.0) adopt hairpin structures.

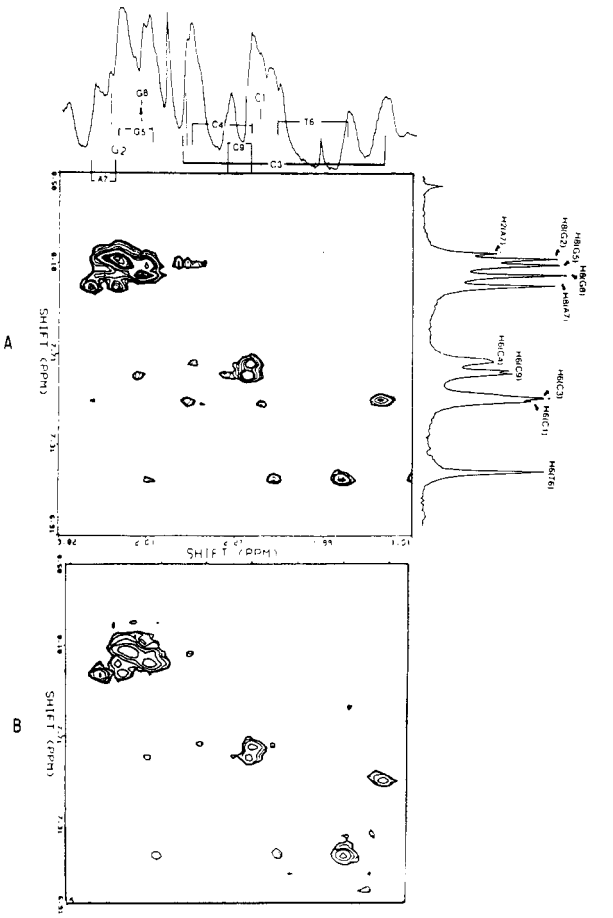


FIGURE 2: (A) NOESY spectrum showing H8/H6---H2′/H2″ cross section of the d(CGCCGTAGC) hairpin in D_2O at 15 °C under the condition of low DNA concentration (0.5 mM in strand) and low salt (20 mM NaCl, pH 7.0). The mixing time τ_m was 500 ms; the number of scans NS = 64, and relaxation delay RD = 1.5 s. Notice that for each base proton H8/H6, NOEs are observed at its sugar H2′ and H2″. This along with the fact that H8/H6(i)---H3′(i) NOEs (data not shown) were either very weak or absent suggested that all the nine residues in the d(CGCCGTAGC) hairpin adopted an average C2′-endo,anti conformation (Gupta et al., 1985, 1986; Sarma et al., 1986a). When nucleotide units with C2′-endo,anti conformations are right-handedly stacked, they can show internucleotide NOE H2″(i)---H8/H6(i + 1), the distance being 2.0–3.2 Å. With H6 of T6 as a marker, the protons H8/H6, H2′, and H2″ of the nine residues were sequentially assigned. Following were the internucleotide NOESY cross-peaks: H2″(C4)---H8(G5), H2″(G5)---H6(T6), and H2″(G8)---H6(C9). (B) NOESY spectrum showing H8/H6---H2′/H2″ cross section for τ_m = 200 ms—other conditions being the same as in (A). In this cross section the prominent intranucleotide NOEs are observed for H8/H6(i)---H2(i), but the NOEs between H8/H6(i) and H2″(i) are either totally absent or very weak. This along with the fact that the NOEs H6(i)---H5(i) and H8/H6(i)---H2′(i) were comparable suggested that for τ_m = 200 ms we essentially observe primary NOEs for a pair of protons separated by a distance ≤2.5 Å. The internucleotide NOEs in this cross section are H2″(G5)---H6(T6) and H2″(G8)---H6(C3). Note that for τ_m = 200 ms the NOE involving the terminal G is absent, and for τ_m = 500 and 200 ms the internucleotide cross-peak H2″(G2)---H6(C3) is absent, implying that the corresponding distance may be ≥3.0 Å.

Assignment of Protons of the Individual Nucleotides in the Hairpin Structures

d(CGCCGTAGC)

The assignment of the protons H8/H6, H1′, H2/H5/CH_3, and H2′,H2″ belonging to the nine individual residues was made by combining the results of NOESY and COSY experiments. NOESY experiments on d(CGCCGTAGC) were conducted at mixing times τ_m = 500 and 200 ms (Figure 2). Examination of the NOESY spectra at τ_m = 500 and 200 ms revealed strong NOE between the base protons H8/H6 and the protons in the H2′,H2″ region but very weak or no NOE between the H8/H6 and the protons in the H3′ region. This suggested that all the nine residues in the hairpin structure of d(CGCCGTAGC) displayed an average C2′-endo,anti conformation (Gupta et al., 1985, 1986a; Sarma et al. 1986a]. The NOESY cross section involving H8/H6---H2′/H2″ cross-peaks can be used for sequential assignment of H8/H6 and H2′/H2″ of the nucleotides in d(CGCCGTAGC) because, when nucleotides in C2′-endo,anti conformation are organized

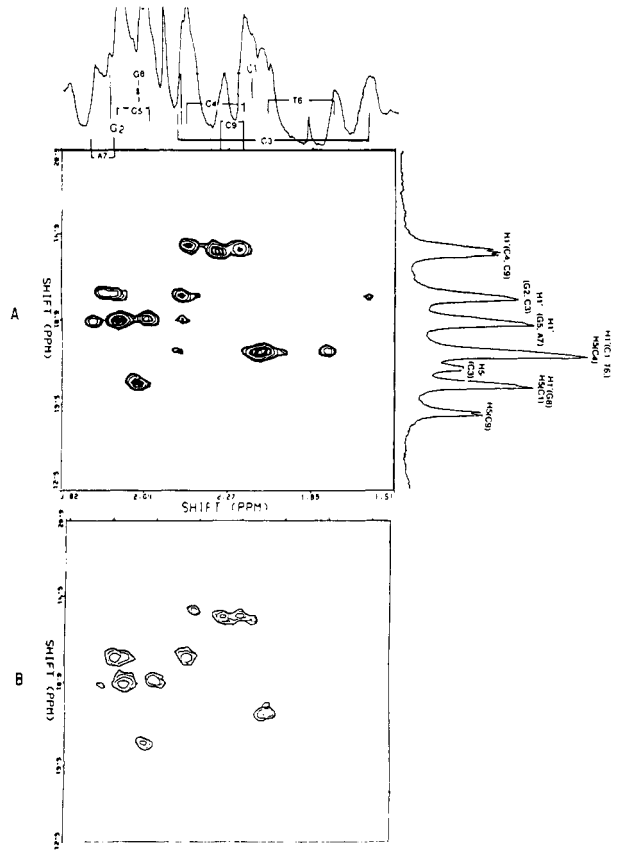


FIGURE 3: (A) NOESY spectrum showing the H1′---H2′,H2″ cross section (τ_m = 500 ms) with conditions being the same as in Figure 2. Notice that for each sugar H1′(i) two NOE sites are the corresponding H2′(i) and H2″(i). For a C2′-endo sugar H2″(i)---H1′(i) = 2.4 Å and H2′(i)---H1′(i) and 3.1 Å. However, H2″(i) and H2′(i) being 1.8 Å apart at τ_m = 500 ms, in addition to primary NOE the higher order NOEs are also observed, viz., H1′(i) → H2″(i) → H2′(i) or H1′(i) → H2″(i) → H2″(i). (B) The NOESY spectrum showing the H1′---H2′,H2″ cross section (τ_m = 200 ms) with conditions being the same as in Figure 2. Lowering the τ_m from 500 to 200 ms results in strong NOEs involving H2″(i)---H1′(i) but only weak or no NOEs for H2′(i)---H1′(i), which is consistent with the fact that all the nine residues adopt C2′-endo,anti conformation. Note that for τ_m = 200 ms the NOE involving the terminal C1 is absent.

in a right-handed stacking, the distances H2″(i)---H8/H6 (i + 1) = 2.0–3.2 Å (Gupta et al., 1985, 1986a; Sarma et al., 1986a).

In d(CGCCGTATC), the base protons H8/H2 and H6 were located within 8.2–7.1 ppm; four H6's were identified within 7.7–7.3 ppm from the four H6---H5 COSY cross-peaks, and the signal at 7.15 ppm was assigned as H6 of T6 because it showed a strong NOESY cross-peak with CH_3 of T6 at the lowest field signal at 1.40 ppm. This meant that H8/H2 of G2, G5, A7, and G8 were located within 8.2–7.9 ppm and H6's of C1, C3, C4, T6, and C9 were located within 7.7–7.1 ppm.

The NOESY cross section of the d(CGCCGTAGC) hairpin involving H8/H6---H2′/H2″ cross-peaks is shown in Figure 2A (for τ_m = 500 ms) and Figure 2B (for τ_m = 200 ms). In figure 2A (τ_m = 500 ms), using H6 of T6 as a marker, the protons H8/H6 and H2′/H2″ of individual residues are sequentially assigned. Notice that the internucleotide cross-peaks H2″(T6)---H8(A7) and H2″(G2)---H6(C3) are absent; however, for each base proton H8/H6 two cross-peaks were obtained on the H2′/H2″ axis—one (the stronger) for H2′ at its own sugar and the other (weaker) for the corresponding H2″ wherever H2′ and H2″ had different chemical shifts. The intranucleotide NOE at H2″(i) from H8/H6(i) originated as

Table I: Chemical Shift Values (ppm) of Protons in the Hairpin Structure of d(CGCCGCAGC) and d(CGCCGTAGC)[a]

residue	H8/H6	H2	H5/CH3	H1′	H2′ and H2″
C1	7.46		5.66	5.82	2.09, 2.09
(C1)	(7.48)		(5.74)	(5.88)	(2.12, 2.12)
G2	8.09			6.20	2.78, 2.72
(G2)	(8.13)			(6.16)	(2.74, 2.74)
C3	7.39		5.64	6.10	1.62, 2.54
(C3)	(7.53)		(5.80)	(6.15)	(1.65, 2.44)
C4	7.73		5.90	6.35	2.20, 2.45
(C4)	(7.67)		(5.89)	(6.38)	(2.19, 2.46)
G5	8.13			6.01	2.73, 2.55
(G5)	(8.11)			(6.05)	(2.73, 2.58)
C6	7.32		5.40	5.99	1.66, 2.20
(T6)	(7.15)		(1.40)	(5.90)	(1.81, 2.06)
A7	8.00	8.13		6.03	2.88, 2.74
(A7)	(8.00)	(8.13)		(6.00)	(2.88, 2.75)
G8	8.05			5.62	2.66, 2.66
(G8)	(8.06)			(5.74)	(2.66, 2.66)
C9	7.61		5.55	6.36	2.21, 2.30
(C9)	(7.59)		(5.60)	(6.35)	(2.21, 2.31)

[a] The specific assignment of H2′ and H2″ for a residue was arrived at based upon C2′-endo,anti nucleotide geometry. All the nine residues belong to C2′-endo,anti domain, and thus there is a strong NOESY cross-peak between base proton H8/H6 and its sugar H2′ (H8/H6---H2′ ~ 2.4 Å). H2″ being 1.8 Å away from the corresponding H2′ in the same sugar, there is bound to be a secondary NOE from base proton H8/H6 to H2″ in C2′-endo,anti conformation. This allows one to assign H8/H6, H2′, and H2″ of a residue. The chemical shift values within parentheses correspond to the protons in d(CGCCGTAGC).

H8/H6(i) → H2′(i) → H2″(i). When τ_m was lowered to 200 ms (Figure 2B), except for terminal C1, we observe H8/H6(i)---H2′(i) NOEs (strong); H8/H6(i)---H2″(i) NOEs are either absent or very weak. Internucleotide NOEs are observed between H2″(G5)---H6(T6) and H2″(G8)---H6-(C9).

Assignment of sugar H1′, H2′, and H2″ of the nine residues in the d(CGCCGTAGC) hairpin was obtained from the NOESY cross-section involving H1′---H2′/H2″ cross-peaks (Figure 3). Figure 3A shows the H1′---H2′/H2″ cross section for τ_m = 500 ms—at this mixing time for each sugar H1′ NOEs are observed at corresponding H2′ and H2″ (wherever H2′ and H2″ have different chemical shifts). Figure 3B shows the H1′---H2′/H2″ cross section for τ_m = 200 ms—at this mixing time for each sugar H1 (except the terminal C1) strong NOEs are at the corresponding H2″ but the NOEs at the H2′ is either weak or absent. This is consistent with the fact that the sugars in d(CGCCGTAGC) have C2′-endo pucker for which H1′---H2″ ~ 2.4 Å and H1′---H2′ ~ 3.1 Å. Thus, the combination of the NOE data in Figures 2 and 3 allows us to assign the protons H8/H6, H2/H5/CH_3, H1′, H2′, and H2″ of the d(CGCCGTAGC) hairpin (see Table I).

The assignment of the spin system H1′, H2′, and H2″ belonging to the nine different residues in d(CGCCGTAGC) hairpin was verified from the COSY cross section showing H1′---H2′/H2″ couplings (Figure 4). If the assignment of the protons as deduced from Figures 2 and 3 and listed in Table I is true, then it should be reflected in the COSY cross section of Figure 4; i.e., for a C2′-endo sugar with the coupling constants $J_{1'2'}$ ~ 10 Hz and $J_{1'2''}$ ~ 5 Hz (Sarma, 1980), one expects the COSY H1′---H2′ cross-peak to be stronger than the corresponding H1′---H2″ cross-peak. Figure 4 shows that, in accordance with the assignment of Table I, H1′---H2′ COSY cross-peaks are stronger than the corresponding H1′---H2″ cross-peaks. Figure 4 also shows the connectivity between the H2′ and the corresponding H2″ of a sugar—H2″, which shows a weak COSY coupling in H1′---H2′/H2″ cross section, manifests as a strong cross-peak in the H2′/H2″--

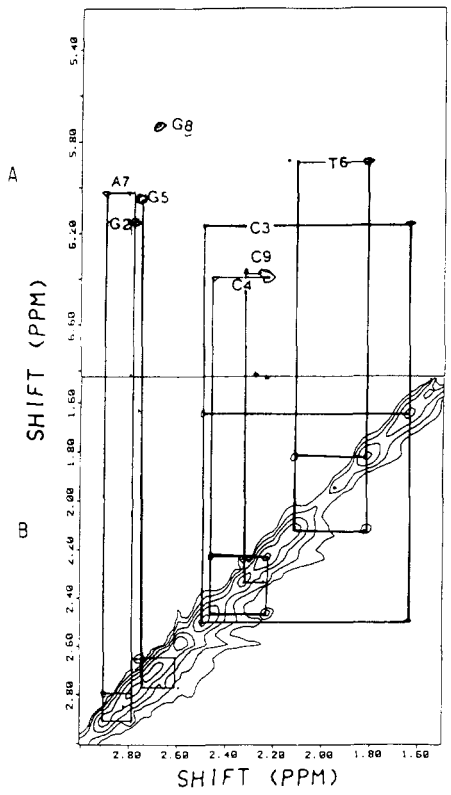


FIGURE 4: COSY experiment was conducted for the d-(CGCCGTAGC) hairpin at 15 °C in D_2O—the other conditions being the same as in Figure 2. NS = 48; RD = 1.5 s. (A) The COSY cross section involving H1′---H2′/H2″ couplings. For a C2′-endo sugar $J_{1'2'} \sim 10$ Hz and $J_{1'2''} \sim 5$ Hz (Sarma, 1980). Thus, a strong cross-peak is expected for H1′---H2′ and a weaker one for H1′---H2″ if the assignment based upon the NOESY data were to be true. This is exactly what is observed in this cross section, thus verifying the assignment in Figures 2 and 3 and Table I. Note that for the terminal C1 no cross-peaks are observed. (B) The COSY cross section showing the H2′---H2″ connectivity ($J_{2'2''} \sim 15$ Hz). H2″ which shows a weak or no peak in Figure 4A is present in this cross section. Thus the assignment for H8/H6, H1′, H2′, and H2″ for the nine residues in the d(CGCCGTAGC) hairpin is based upon the results of Figures 2, 3, and 4A. The corner points of a square give the locations of H2′ and the corresponding H2″.

-H2′/H2″ cross section ($J_{2'2''} \sim 15$ Hz). The squares in the H2′/H2″---H2′/H2″ cross section indicate the J connectivity of H2′ with the corresponding H2″.

d(CGCCGCAGC)

The assignment of the protons H8/H6, H2/H5, H1′, H2′, and H2″ belonging to the nine individual residues was made by combining the results of NOESY and COSY experiments. In d(CGCCGCAGC), the base-protons H8/H2 and H6 were located within 8.2–7.3 ppm; five H6's were identified within 7.7–7.3 ppm from the H6---H5 COSY cross-peaks. This meant that H8/H2 of G2, G5, A7, and G8 were within 8.2–7.9 ppm and H6's of C1, C3, C4, C6, and C9 were within 7.7–7.3 ppm.

The NOESY cross section of d(CGCCGCAGC) involving H8/H6---H2′/H2″ cross-peaks is shown in Figure 5 (τ_m = 500 ms). Notice the striking similarity between the NOESY spectrum in Figure 2B at τ_m = 200 ms for the d-(CGCCGTAGC) hairpin and that in Figure 5 at τ_m = 500 ms for the d(CGCCGCAGC) hairpin. As mentioned earlier, Figure 2B (τ_m = 200 ms) for d(CGCCGTAGC) esentially reflects the first-order NOEs for distances ≤2.6 Å; hence, if we assume that the two hairpins have similar structures (see later), it appears that in the case of the d(CGCCGCAGC) hairpin the motional dynamics is such that even at τ_m = 500

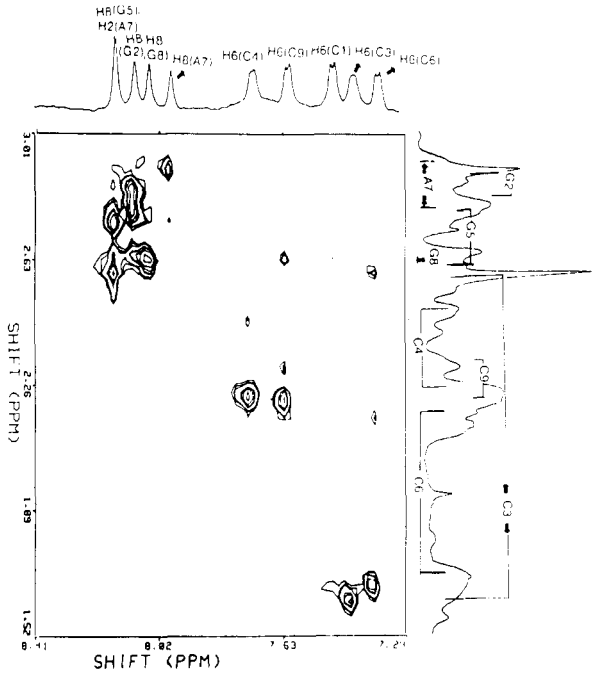


FIGURE 5: NOESY experiment on the d(CGCCGCAGC) hairpin at 15 °C in D_2O was conducted under the condition of low DNA concentration (0.5 mM in strand) and low salt (20 mM NaCl, pH 7.0). NS = 64; τ_m = 500 ms; RD = 1.5 s. Compare the NOESY cross-section H8/H6---H2′/H2″ of the d(CGCCGCAGC) hairpin with that of d(CGCCGTAGC) (Figure 2A) both at τ_m = 500 ms: for the d(CGCCGCAGC) hairpin no NOE is observed for the terminal C1, but for the d(CGCCGTAGC) hairpin the NOE for H6(C1)---H2′(C1) is observed. By comparing H8/H6---H2′/H2″ cross-peak with H6---H5 cross-peaks, it appears that NOEs are observed for distances ≤2.7 Å. And it is very natural that the NOESY (τ_m = 500 ms) cross section H8/H6---H2′/H2″ for the d(CGCCGCAGC) hairpin and the H8/H6---H2′/H2″ cross section for the d-(CGCCGTAGC) hairpin at τ_m = 200 ms show striking similarity (see Figure 2B for comparison). Hence, by comparison the protons H6, H2′, and H2″ of C6 are assigned at 7.32, 1.66, and 2.20 and the rest as follows. This assignment scheme is based upon the assumption that d(CGCCGCAGC) and d(CGCCGTAGC) hairpins are structurally very similar. In this cross-section, NOEs H8/H6(i)---H2′(i) are strong; NOEs H8/H6(i)---H2″(i) are weaker. This along with the fact that H8/H6(i)---H3′(i) are very weak/absent suggests that in the d(CGCCGCAGC) hairpin all the residues belong to C2′-endo,anti domain (Gupta et al., 1985, 1986; Sarma et al., 1986). The internucleotide NOEs in this cross section are H2″(G5)---H6(C6) and H2″(G8)---H6(C9).

ms we essentially observe primary NOEs for distances ≤2.6 Å. By comparison (from Figures 2B and 5), the H6 at the highest field (7.30 ppm) is assigned to C6 so that it showed an internucleotide H2″(i)---H6(i + 1) connectivity with an H2″ of a purine (at 2.55 ppm), which was assigned to G5 (with H8 at 8.13 ppm). Similarly, by comparison of the internucleotide H2″(i)---H6(i + 1) connectivity pattern of protons H8/H6, H2′ and H2″ belonging to G8 and C9 were assigned. Notice that for d(CGCCGCAGC) at τ_m = 500 ms, sugar H2′ of a residue shows a strong NOE with its base proton H8/H6, but the H2″(i)---H8/H6(i) NOE is much weaker in comparison (the protons H6, H2′, and H2″ which show no cross-peak are assigned to C1). This along with the observation that H8/H6(i)---H3′(i) NOEs were either very weak or absent indicated that in the d(CGCCGAGC) hairpin [as in the d(CGCCGTAGC) hairpin] all the residues belong to the C2′-endo,anti domain. Thus, from Figure 5 we obtain the assignment of the protons H8/H6, H2′, and H2″ of the individual residues in the d(CGCCGCAGC) hairpin.

The spin system H1′, H2′, and H2″ of the individual nucleotides in the d(CGCCGCAGC) hairpin is obtained from

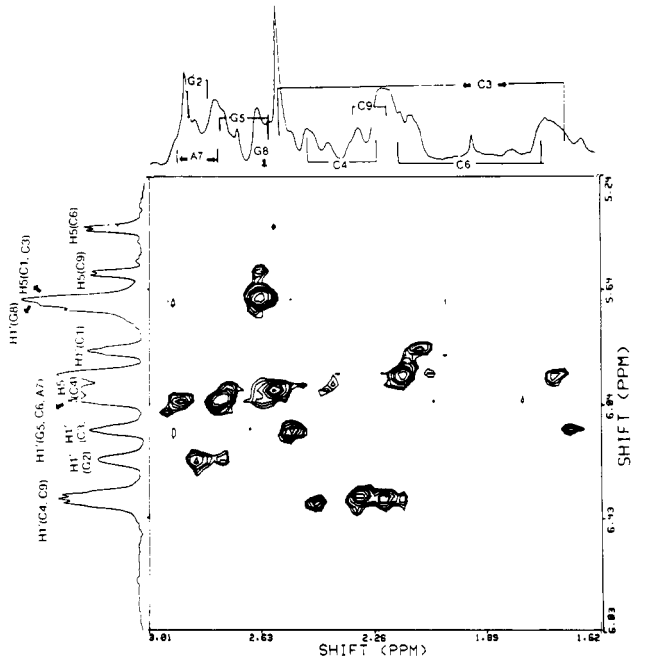


FIGURE 6: NOESY cross section (τ_m = 500 ms) showing the H1′---H2′/H2″ cross-peaks, other conditions being the same as in Figure 5. For a C2′-endo sugar, the distances H1′---H2″ = 2.4 Å and H1′---H2′ = 3.1 Å; hence, a strong NOE is expected for H1′---H2″ and weaker one for H1′---H2′. On the basis of this fact, the protons H1′, H2′, and H2″ of the nine residues of d(CGCCGCAGC) hairpin are assigned.

the NOESY cross section (at τ_m = 500 ms), showing the H1′---H2′/H2″ cross-peaks (Figure 6). Notice that each sugar H1′ shows a strong NOE with the corresponding H2″ and a weaker one with the H2″ which is a characteristic feature of a C2′-endo sugar in which H1′---H2″ ~ 2.4 Å and H1′---H2′ ~ 3.1 Å.

The assignment of the spin system H1′, H2′, and H2″ belonging to nine different residues in the d(CGCCGCAGC) hairpin was verified from the H1′---H2′ and H2′/H2″---H2′/H2″ COSY cross sections (Figure 7). If the assignment of the protons in d(CGCCGCAGC) (as in Table I) is true, then it should be reflected in the COSY spectrum; i.e., for a C2′-endo sugar pucker coupling constant $J_{1'2'} \sim 10$ Hz and $J_{1'2''} \sim 5$ Hz (Sarma, 1980), one expects the H1′---H2′ cross-peak to be stronger than the COSY H1′---H2″ cross-peak. The H1′---H2′/H2 COSY cross section in Figure 4 shows that in accordance with the assignment in Table I H1′---H2′ COSY cross-peaks are stronger than corresponding H1′---H2′ cross-peaks. Figure 7 also shows the connectivity between H2′ and the corresponding H2″ of a sugar H2″ which shows weak coupling in the H1---H2″ cross-section manifests as a strong cross-peak ($J_{2'2''} \sim 15$ Hz) in the H2′/H2″---H2′/H2″ cross-section. The squares in the H2′/H2″---H2′/H2″ cross-sections indicate the J connectivity between H2′ and the corresponding H2″.

It may be pointed out that in the assignment scheme of the protons belonging to the residues in the d(CGCCGCAGC) hairpin it is assumed that this hairpin bears overall structural similarity with the d(CGCCGTAGC) hairpin. We have direct experimental evidence that (i) in both hairpins G2·C9 and C3·G8 form the stem and C4, G5, C6/T6, and A7 form the loop (from the results of Figure 1) and (ii) in both of them all the nine residues (with average C2′-endo,anti geometry) in d(CGCCGCAGC) maintain the identical spatial relation with their neighbors as in d(CGCCGTAGC)—and it is at this level we made the assumption that the ith nucleotide in d(CGCCGCAGC) maintains the same spatial relation with the

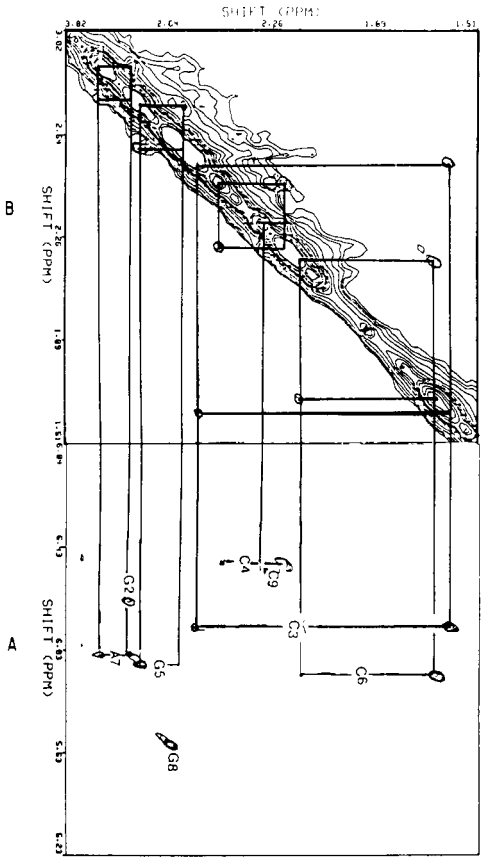


FIGURE 7: COSY experiment on the d(CGCCGCAGC) hairpin in D_2O was conducted under the same condition as in Figures 5 and 6; NS = 64; RD = 1.5 s. Thus, combining the results of Figures 5–7, one arrives at the assignment of H8/H6, H1′, H2′, and H2″ of nine residues in the d(CGCCGCAGC) hairpin. (A) COSY cross section showing the H1′---H2′,H2″ connectivity in d(CGCCGCAGC) sugar, $J_{1'2'} \sim 10$ Hz and $J_{1'2''} \sim 5$ Hz (Sarma, 1980). Thus, a strong COSY cross-peak is expected for H1′---H2′ and a weaker one for H1′---H2″. (B) COSY cross-section showing the H2′---H2″ connectivity ($J_{2'2''} \sim 15$ Hz). Note that H2″ which shows a weak/no peak in (A) appears here as a H2′---H2″ cross-peak.

(i − 1)th and (i + 1)th residues as the ith residue in d(CGCCGTAGC) does with the (i − 1)th and (i + 1)th residues.

A Working Model for the Hairpins

We believe that the hairpins d(CGCCGTAGC) and d(CGCCGCAGC) are very similar in overall structure; i.e., in both of them the double-helical stem contains G2·C9 and C3·G8 Watson–Crick pairs with C4, G5, C6/T6, and A7 in the loop, and all the residues in the hairpin display an average C2′-endo,anti conformation. In order to arrive at a working model of the hairpin, we have utilized the NOE data on the d(CGCCGTAGC) hairpin at τ_m = 500, 200, and 100 ms.

Figure 2 indicated that the residues in the d(CGCCGTAGC) hairpin belonged to the C2′-endo,anti domain. For a nucleotide in C2′-endo,anti conformation, the intranucleotide primary NOEs are as follows: H8/H6(i)---H2′(i), distance ~2.3 Å; H1′(i)---H2″(i), distance ~2.4 Å; H1′(i)---H2′(i), distance ~3.1 Å [see Gupta et al. (1985, 1986) and Sarma et al. (1986a)]. Figure 8 shows the NOESY spectrum for the d(CGCCGTAGC) hairpin at τ_m = 100 ms. The cross section (A) involving H8/H6---H2′/H2″ shows a strong intranucleotide NOE for H8/H6(i)---H2′(i) for all residues (except the terminal C1). The cross section (C) involving H1′---H2′/H2″ cross-peaks shows strong intranucleotide NOEs for H1′(i)---H2″(i)—the intranucleotide NOEs for H1′(i)---H2′(i) are absent for all the residues

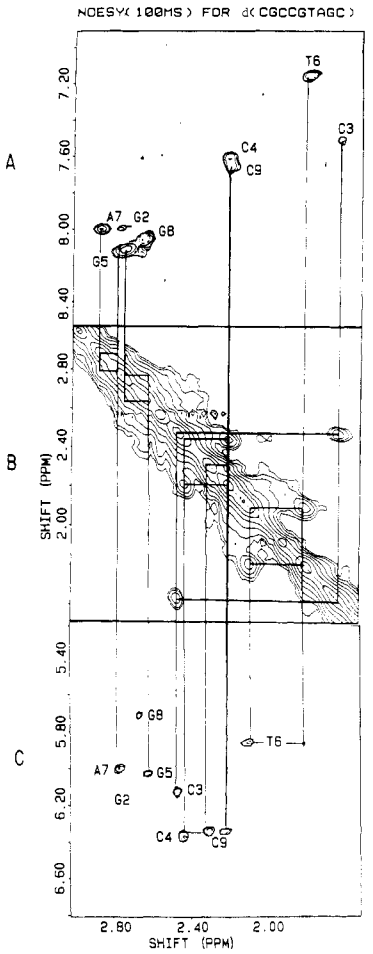


FIGURE 8: Three NOESY cross sections for the d(CGCCGTAGC) hairpin in D_2O at 15 °C; NS = 128; RD = 1.5 s; τ_m = 100 ms; 512 × 512 data matrix. In the cross section involving H8/H6---H2′/H2″ cross-peaks (A), strong intranucleotide NOEs are observed between H8/H6(i) and H2′(i)—except for the terminal C1. Notice that for τ_m = 100 ms, only primary NOEs for distances ≤2.6 Å are observed, and the distance estimate from NOE assuming a two-spin system is justified. By comparing the NOEs H8/H6(i)---H2′(i) and H6-(i)---H5(i) (fixed distance of 2.5 Å), we estimate that the distances H8/H6(i)---H2′(i) = 2.6 Å. This along with the fact that the intranucleotide NOEs H8/H6(i)---H3′(i) were either weak/absent (at τ_m = 100 ms) suggested that the residue in the d(CGCCGTAGC) hairpin adopted an average C2′-endo,anti conformation (Gupta et al., 1985, 1986; Sarma et al., 1986). The cross section involving H2′---H2″ cross-peaks (B) shows strong intranucleotide NOEs for H2′(i)---H2″(i) (fixed distance of 1.8 Å). In the cross section involving H1′---H2′/H2″ cross-peaks (C), strong intranucleotide NOEs are observed for H1′(i)---H2″(i) for all the residues except for the terminal C1; only one NOE is observed for H1′(i)---H2′(i), i.e., for T6. This indicates that at τ_m = 100 ms, we are essentially observing primary NOEs and secondary NOEs; H1′(i) → H2″(i)–H2′(i) are almost eliminated. For a C2′-endo sugar, H1′---H2″ ~ 2.4 Å and H1′---H2′ ~ 3.1 Å. Assuming a two-spin system, the distance estimate from NOE indicates that H1′---H2″ = 2.4–2.5 Å for all the residues in the d(CGCCGTAGC) hairpin. Notice that from the base protons H8/H6(i) the primary sites of NOE are H2′(i) (A) while from the sugar protons H1′(i) the primary sites of NOE are H2″(i). This is consistent with the fact that all the residues in the d(CGCCGTAGC) hairpin belong to C2′-endo,anti domain (Gupta et al., 1985, 1986; Sarma et al., 1986a).

[except for weak H1′(T6)---H2′(T6)]. The data in Figure 8 indicate that at τ_m = 100 ms only the primary NOEs are observed. Thus, at τ_m = 100 ms, the estimate of pairwise interproton distances from the NOE data on the basis of a two spin system is justified. By comparing the NOEs for H6-(i)---H5(i) for four C's and those for H8/H6(i)---H5(i) and H1′(i)---H2″(i), the following estimates of intranucleotide distances were obtained: H8/H6(i)---H2′(i), 2.3 Å; H1′-(i)---H2′(i), ~2.4 Å. This is consistent with the fact that the residues in the d(CGCCGCAGC) hairpin belong to the C2′-endo,anti domain (Gupta et al., 1985, 1986; Sarma et al. 1986). When C2′-endo,anti nucleotides are stacked in a right-handed stacking arrangement, the internucleotide distance H2″(i)---H8/H6 (i + 1) could be 2.0–3.2 Å. As stated earlier, the NOESY data at τ_m = 200 ms (Figure 2B) and at τ_m = 100 ms (Figure 8A) essentially embody the primary NOEs in the hairpins. From Figure 2B (τ_m = 200 ms) we observe two internucleotide NOEs, i.e., for H2″(G5)---H6-(T6) and H2″(G8)---H6(C9). In Figure 8A (τ_m = 100 ms) only an internucleotide NOE is seen for H2″(G8)---H6(C9), although all the intranucleotide NOEs for H2′(i)---H8/H6(i) in Figure 2B (τ_m = 200 ms) and Figure 8A (τ_m = 100 ms) are very similar. At this stage, it may be pointed out that the NOEs observed for a pair of intra- or internucleotide protons could be different even for the same distance. The reason is as follows: As we know (Redfield & Gupta, 1972) for $\omega\tau_c \gg 1$, the NOE for a pair of protons at a distance r is proportional to $\tau_c r^{-6}$, where τ_c is the effective correlation time. For a pair of intranucleotide protons [viz., H2′/H2″(i)---H8/H6(i)] τ_c is dependent upon the restricted motion in the sugar ring and the motion around the glycosylic bond, which may also be restricted especially in the stem of the hairpin. However, for a pair of internucleotide protons [viz., H2″-(i)---H8/H6(i + 1)], in addition to the motion of the sugar ring and glycosyl torsion, six freely rotating single bonds that connect the nucleotides i and i + 1 also affect the τ_c. In other words, the NOE observed for a pair of intranucleotide protons [viz., H2′(i)---H8/H6(i)] for a distance r could be stronger than the NOE observed for a pair of internucleotide protons [viz., H2″(i)---H8/H6(i + 1) especially in the loop region of the hairpin] for the same r—because in latter case the effective τ_c could be much shorter. For a pair of intranucleotide protons [viz., H2′(i)---H8/H6(i)], the distance estimate from the NOE could be made by comparison with the intranucleotide NOE H6---H5 (a fixed distance of 2.5 Å). But for a pair of internucleotide protons [viz., H2″(i)---H8/H6(i + 1)] there is no NOE for a pair of protons at a fixed distance to compare with. Hence, the following distance estimates are made on a very qualitative basis: (i) because H2″(G8)---H6(C9) NOEs are observed at τ_m = 200 ms (Figure 2B) and 100 ms (Figure 8A), the corresponding distance is taken to be below 2.5 Å, and (ii) because H2″-(G5)---H6(T6) NOE is not observed at 100 ms (Figure 8A) but at 200 ms (Figure 2B), the corresponding distance is taken as a little over but close to 2.5 Å. Other internucleotide distances H2″(i)---H8/H6(i + 1) are taken as ≥3.0 Å.

For the d(CGCCGTAGC) hairpin no NOESY cross-peaks were observed for H1′---H8/H6 at τ_m = 100 and 200 ms (data not shown). For C2-endo,anti conformations of residues in the hairpin, H1′(i)---H8/H6 = 3.5–3.8 Å and H1′(i)---H8/H6(i + 1) = 3.0–3.8 Å (for the residues in the stem). Intranucleotide H1′(i)---H8/H6(i) NOEs were observed for the d(CGCCGTAGC) hairpin at τ_m = 500 ms (data not shown). This observation supported that the residues belong to the C2′-endo,anti domain in which H1′(i)---H8/H6(i) = 3.5–3.8 Å and ruled out a C2′-endo/C3′-endo,syn conformation in which H1′(i)---H8/H6(i) = 2.1–2.5 Å and which could have produced observable NOEs at 200 and 100 ms (Sarma et al., 1986a,b).

In addition to the distance criteria from NOE data for the hairpin structure (Figures 2–8), 1D NMR spectra of d-(CGCCGCAGC) and d(CGCCGTAGC) in water (Figure 1) also revealed a very important structural parameter; i.e., the

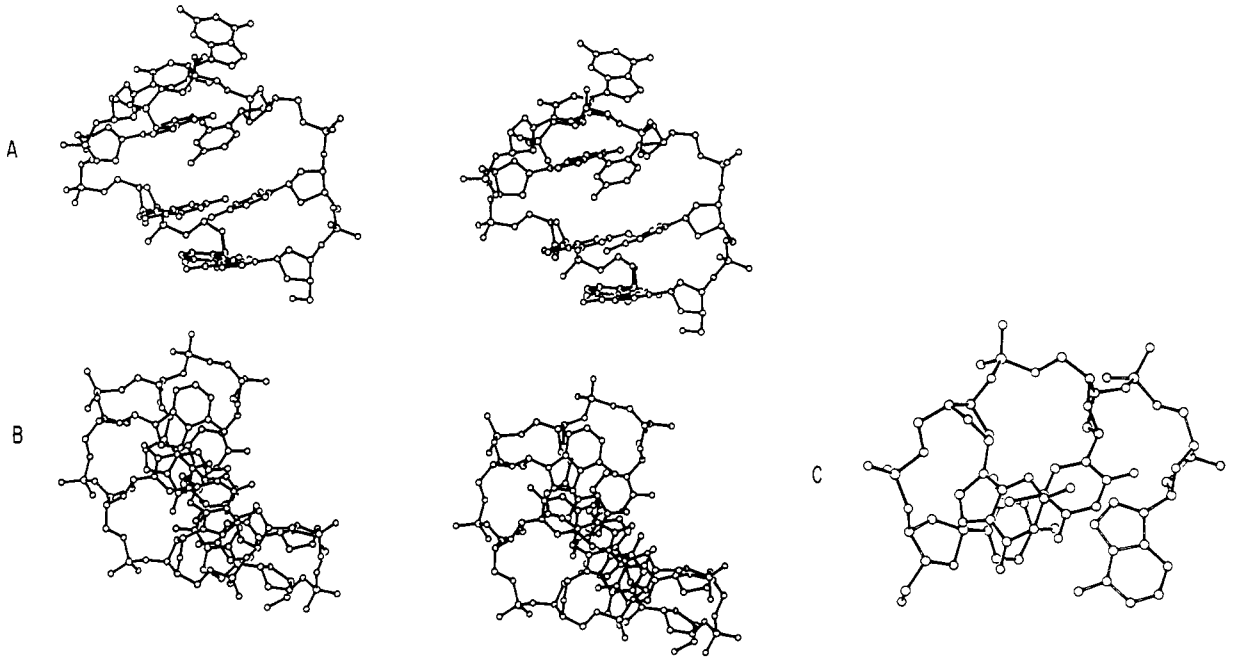


FIGURE 9: (A) Stereo pair of the hairpin structure of d(G2-C3-C4-G5-C6/T6-A7-G8-C9) as viewed along the helix axis of the double-helical stem. The terminal C1 is not included. The same molecular structure is proposed for the two oligonucleotides studied—the stereo diagram is for d(G2-C3-C4-G5-T6-A7-G8-C9). The torsion angles for the model are listed in Table II. Notice that adjustments in the P–O torsions (α, ξ) allow a smooth folding in the loop segment even though all the residues belong to an average C2′-endo,anti conformation (Table II). It may be mentioned that d(CGCCGCAGC) and d(CGCCGTAGC) have the same average hairpin structure, but local mobility of the residues in the loop regions of the two hairpin structures are different (see text). (B) A stereo pair of the hairpin model of d(C2-G3-C4-G5-T6-A7-G8-C9) as viewed down the helix axis of the double-helical stem. Notice that the bases (in the stem and the loop) from an inner hydrophobic core of the structure. (C) A down view of the bases in the loop segment of the hairpin model of d(C2-G3-C4-G5-T6-A7-G8-C9). The bases in the loop (i.e., C4, G5, T6, and A7) are favorably stacked with the CH_3 group of T6 right in the middle of the stacked array. Residues G5 and T6 are designated by solid bonds.

N1-H of G5 and N3-H of T6 are not completely exposed to the solvent.

Stereochemically allowed models of hairpin structures generated are subject to the following criteria: (i) interproton distance criteria from the NOE data; (ii) solvent accessibility criteria of N1-H of G and N3-H of T. That is, N1-H of G2 is a part of G2-C3, N1-H of G8 is a part of C3-G8, and N1-H of G5 is in the loop but partly buried and so is N3-H of T6 in the loop (see later).

Table II lists the torsion angles (in degrees) of a stereochemically acceptable hairpin model for d(CGCCGCAGC)/d(CGCCGTAGC) that agrees with the NMR data. The structural features of the model are as summarized:

(i) Watson–Crick pairs G2-C9 and C3-G8 form the double-helical stem while C4, G5, C6/T6, and A7 constitute the loop segment of the hairpin structure.

(ii) We have computed the solvent accessibility of the NH protons in the model following the method of Richmond (1984). We observed that N1-H of G8 (in C3-G8) was the least accessible (i.e., 0% in the scale of accessibility with N1-H of G in a free G as 100% accessible), N1-H of G2 (in G2-C9) was 30% exposed, N1-H of G5 (in the loop) was 67%, and N3-H of T6 (in the loop) was 50% accessible.

(iii) All the intranucleotide H8/H6---H2′ distances were 2.2–2.5 Å, i.e., all the nucleotides belonging to average C2-endo,anti domain.

(iv) The prominent base–sugar connectivities involving two neighboring nucleotides with distances ≤2.5% Å are H2″G5---H6T6 = 2.5 Å and H2″G8---H6C9 = 2.2 Å. Other internucleotide H2″(i)---H8/H6 (i + 1) distances were ≥3.0 Å.

Figure 9A shows a stereo pair of the hairpin structure of d(CGCCGCAGC)/d(CGCCGTAGC) as viewed along the helix axis of the double-helical stem: notice that even though all the nucleotides belong to C2′-endo,anti domain, stereochemically allowed variations in the P–O torsions (α and ξ in Table II) resulted in a smooth folding in the loop segment of the hairpin structure. Figure 9B shows the down view of the hairpin structure, which clearly demonstrates that bases in the stem along with those in the loop form a hydrophobic core of the molecule.

Figure 9C shows the stacking arrangement of the bases in the loop segment of the hairpin structure of d(CGCCGTAGC). Notice that the CH_3 group resides right in the middle of the stacked array of bases (G5, T6, and A7). The presence of a bulky CH_3 group can in principle restrict the mobility of neighboring bases (G5 and A7) in the loop, thereby offering rigidity to the loop segment and to the overll hairpin structure.

Recently, it has been shown that under a wide range of

Table II: Torsion Angles (deg) of the Model of the Hairpin Structure of d(C2-G3-C4-G5-C6/T6-A7-G8-C9)[a]

residue	α	β	γ	δ	ϵ	ξ	χ
G2			55	150	220	215	270
C3	300	155	40	140	190	260	250
C4	180	204	71	120	175	235	210
G5	218	184	47	156	217	260	245
C6/T6	267	166	50	131	220	238	240
A7	307	158	38	121	188	300	230
G8	309	168	63	140	222	215	260
C9	298	152	42	135			245

[a] The backbone and glycosyl torsion are as defined by Saenger (1984); the atomic coordinates of the hairpin model can be obtained from the authors upon request. It should be emphasized that the hairpin structure proposed here only serves as a working model to explain the NMR data and is by no means a description of the structure on the same footing as can be derived from the single-crystal X-ray data.

solution conditions the DNA oligomer d-(CGCGCGTTTTCGCGCG) adopts a monomeric hairpin in solution (Ikuta et al., 1986). A structural model has been proposed for a similar hairpin with the sequence d-(CGCGTTTTCGCG) by Hare and Reid (1986). This model, although it contains some forbidden torsion angles, is derived by constrained minimization, i.e., energy minimization of the hairpin structure with interproton distances from the NOE data as constraints. It may be pointed out that even though our working model for the DNA oligomers d(CGCCGCAGC) and d(CGCCGTAGC) satisfies the NOE distance and solvent accessibility criteria and the model is free of any steric compression, we have not performed any energy minimization on the model. The refined model of the hairpin could be obtained by simulation of theoretical NOESY data for a given model and comparison with the experimental NOESY data at various mixing times. Efforts in this direction are currently under way in our and other laboratories.

A referee has suggested that the atomic coordinates of our hairpin model be deposited with Brookhaven Data Bank. Even though these coordinates can be obtained by writing to the authors, we believe that it is premature to deposit them in the Data Bank. This is because the model proposed is only a semiquantitative working model and we are in the process of refining the model by computer simulation of the NOESY data at various mixing times. When this is successfully completed and a quantitative model is derived, the atomic coordinates will be deposited at the Data Bank.

Discussion

Haasnoot et al. (1983, 1986) did an extensive study to explore the structural stabilities of hairpin structures with varying lengths of the loop segment. Essentially by introducing variable $(dT)_n$ in the loop, they concluded that an optimum length of $d(T)_{4-5}$ in the loop is required to stabilize the hairpin structure. They also observed that under certain conditions even a self-complementary DNA can form a hairpin structure provided there are at least four bases in the loop (Haasnoot et al., 1983, 1986). Very recently Orbons et al. demonstrated that a hairpin structure is formed for d(*CG*CG*TG*CG) in solution (for a low concentration of salt and DNA and within a critical temperature range). Orbons et al. (1986) also suggested that introduction of the non-self-complementary G·T pair lowers the stability of the duplex and enhances the probability of a hairpin structure. Our choice of the DNA oligomers, to form and characterize hairpin structure, was prompted by the same hypothesis; i.e., in the d-(CGCCGCAGC) duplex formation requires two mismatch A·C pairs and in d(CGCCGTAGC) duplex formation requires two A·C and two G·T pairs—therefore, for a critical salt and DNA concentration two oligomers are expected to exhibit hairpin structures over a range of temperature. This is exactly what we observed for d(CGCCGCAGC) and d-(CGCCGTAGC) in solution from our NMR studies.

We have shown elsewhere that one of the two oligomers discussed here can form a double-helical structure under conditions of high salt and high DNA concentration and at a very low temperature (Sarma et al., 1987). In the duplex the presence of A·C pairs is also implicated.

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