Structural Studies of a Stable Parallel-Stranded DNA Duplex Incorporating Isoguanine:Cytosine and Isocytosine:Guanine Basepairs by Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT Isoguanine (2-hydroxyladenine) is a product of oxidative damage to DNA and has been shown to cause mutation. It is also a potent inducer of parallel-stranded DNA duplex structure. The structure of the parallel-stranded DNA duplex (PS-duplex) 5′-d(TiGiCAiCiGiGAiCT) + 5′-d(ACGTGCCTGA), containing the isoguanine (iG) and 5-methyl-isocytosine (iC) bases, has been determined by NMR refinement. All imino protons associated with the iG:C, G:iC, and A:T (except the two terminal A:T) basepairs are observed at 2°C, consistent with the formation of a stable duplex suggested by the earlier T_m measurements [Sugiyama, H., S. Ikeda, and I. Saito. 1996. *J. Am. Chem. Soc.* 118:9994–9995]. All basepairs are in the reverse Watson-Crick configuration. The structural characteristics of the refined PS-duplex are different from those of B-DNA. The PS duplex has two grooves with similar width (7.0 Å) and depth (7.7 Å), in contrast to the two distinct grooves (major groove width 11.7 Å, depth 8.5 Å, and minor groove width 5.7 Å, depth 7.5 Å) of B-DNA. The resonances of the amino protons of iG and C are clearly resolved and observable, but those of the G and iC are very broad and difficult to observe. Several intercalators with different complexities, including ethidium, daunorubicin, and nogalamycin, have been used to probe the flexibility of the backbone of the (iG, iC)-containing PS-duplex. All of them produce drug-induced UV/vis spectra identical to their respective spectra when bound to B-DNA, suggesting that those drugs bind to the (iG, iC)-containing PS-duplex using similar intercalation processes. The results may be useful in the design of intercalator-conjugated oligonucleotides for antisense applications. The study presented in this paper augments our understanding of a growing number of parallel-stranded DNA structures, including the G-quartet, the i-motif, and the unusual homo basepaired parallel-stranded double helix. Their possible relevance is discussed.

INTRODUCTION

The right-handed B-DNA is presumed to be the predominant conformation of DNA in vivo. However, other alternative stable conformations are also known. A-DNA, another right-handed duplex conformation, is favored by a low-humidity environment. A more dramatic departure from A- and B-form DNA is the left-handed Z-DNA, which is favored by stretches of alternating purine-pyrimidine sequences like $(CG)_n$ or $(TG)_n$ (Wang et al., 1979; Rich et al., 1984; Palecek, 1991). All three major duplex structures consist of two anti-parallel stranded sugar-phosphate backbones with Watson-Crick basepairs.

Interestingly, some repetitive sequences like $(TTGGGG)_n$, which is found in certain eukaryotic telomere DNA (Blackburn, 1991, 1992; Sen and Gilbert, 1992); $(GGAAT)_n$, which is found in centromere DNA (Grady et al., 1992); and $(CCG)_n$, which is found in the X-chromosome (responsible for the fragile-X syndrome) (Kremer et al., 1991; Oberle et al., 1991); have been implicated in various biological functions and are associated with specific conformations. The telomere sequence $(TTGGGG)_n$ can adopt either parallel or anti-parallel quadruplex structures using novel G-G basepairs (Kang et al., 1992; Smith and Feigon, 1992; Laughlan et al., 1994; Phillips et al., 1997). Under low pH condition, $(C)_n$, part of the complementary sequence of $(TTGGGG)_n$, adopts a novel parallel quadruplex structure (called i-motif) with the cytosine bases intercalated with one another from the neighboring strands (Gehring et al., 1993; Chen et al., 1994).

Therefore, it is clear that DNA adopts different conformations using not only novel basepairs, but also different chain polarities. Among the parallel DNA structures mentioned above, all involve more than two strands of DNA. The question of whether a stable parallel DNA duplex exists has been addressed previously by Jovin and colleagues (reviewed in Rippe and Jovin, 1992). A series of A, T-containing DNA sequences was designed to form parallel-stranded (PS) duplexes using the reversed Watson-Crick basepairs. The stability of those PS-duplexes is modest; for example, the T_m of a 21-mer PS-duplex is 15°C lower than that of the corresponding antiparallel duplex (Ramsing and Jovin, 1988).

An important requirement for a PS-duplex is that the two glycosyl bonds within a basepair have to come from the opposite direction because of the identical chain polarity. For the normal nucleic acid bases, this can be accomplished using the reverse Watson-Crick basepair conformation.

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However, A-T and G-C basepairs in a reverse Watson-Crick conformation are not isostructural due to their hydrogen bonding restrictions. Therefore, it has not been easy to design a stable PS-duplex in which all four bases can be incorporated in random order.

This problem has been overcome by using alternative nucleosides, 2′-deoxyisoguanosine (iG) and 2′-deoxy-5-methyl-isocytosine (iC), which can form stable reverse Watson-Crick basepairs with the normal 2′-deoxycytosine (C), and 2′-deoxyguanosine (G), respectively (Fig. 1). It was noted by Seela and colleagues that iG is a potent inducer for the formation of parallel DNA duplex structure (Seela et al., 1996; Seela and Wei, 1997). Significantly, it has been shown by some of us that oligodeoxynucleotides containing iG and iC can form remarkably stable parallel-stranded duplexes with the complementary (G, C)-containing DNA or RNA strands. The T_m of the PS-duplex is comparable with that of the corresponding antiparallel duplex even for a 10-mer (Sugiyama et al., 1996). More recently, DNA molecules containing a string of iGs have been demonstrated to form very stable parallel tetraplex structure (Seela et al., 1996; Roberts et al., 1997).

Parenthetically, it may be useful to note that iG is a product of oxidative damage to the adenine base in DNA. The addition of a hydroxyl group at the C2-position of adenine resulting from the oxidative damage process produces 2-hydroxyadenine, which is chemically identical to isoguanine. While the amount of 2-hydroxyadenine produced during the oxidative damage process is less than that of 8-oxoguanine, it is still a significant product. It is known that both 2-hydroxyadenine (i.e., iG) and 8-oxoguanine are mutagens. The repair mechanism of the lesions involving these two oxidized bases is under intense study (Tchou and Grollman, 1993; Kamiya and Kasai, 1996, 1997). Whereas the structural consequences due to the 8-oxoguanine incorporation have been studied extensively (Grollman et al., 1994; Lipscomb et al., 1995), little is known regarding the structural effective of 2-hydroxyadenine (i.e., iG) in DNA. Therefore it is desirable to gain insight to the properties associated with the modified base 2-hydroxyadenine (i.e., iG).

Finally, the ability for (iG, iC)-containing DNA oligomers to form specific stable parallel-stranded duplexes may offer new opportunities for designing useful probes for applications such as antisense or aptamer molecules. To date, only limited structural work on PS-duplexes has been carried out (Germann et al., 1989; Zhou et al., 1993). Our understanding of the PS-duplexes remains mostly at the level of stability from T_m measurements (Rippe and Jovin, 1992). Whether they have sequence-dependent conformations, as in B-DNA, or how they interact with other ligands (proteins or small molecules) remains largely unexplored. In this paper, we describe the structural analysis of the parallel-stranded DNA duplex 5′-d(TiGiCAiCiGiGAiCT) + 5′-d(ACGTGCCTGA) by NMR refinement. In addition, the flexibility of the backbone of the (iG, iC)-containing PS-duplex has been probed using several intercalators with different complexities, including ethidium, daunorubicin, and nogalamycin.

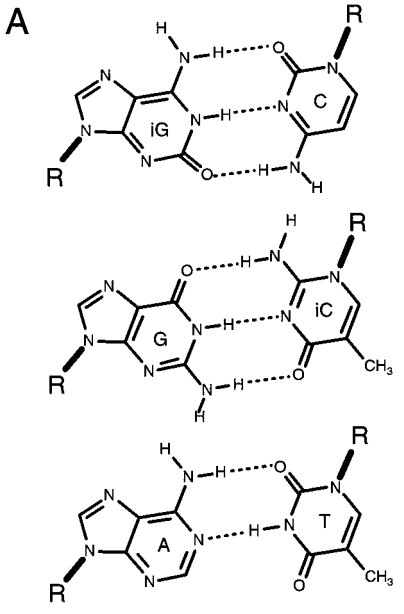

FIGURE 1 (*A*) Reverse Watson-Crick basepairing scheme of the isoguanine:cytosine, 5-methyl-isocytosine:guanine, and adenine:thymine basepairs. (*B*) Sequence and numbering of the PS-duplex.

MATERIALS AND METHODS

Phosphoramidite derivatives of 5-methylisocytosine and isoguanine, 5′-*O*-DMT-*N2*-[(di-*n*-butylamino)methylene]-5-methyl-2′-deoxyisocytidine β-cyanoethyl-phosphoramidite and 5′-*O*-DMT-*O2*-(*N,N*-diphenylcarbamoyl)-*N6*-[(di-*n*-butylamino)methylene]-2′-deoxyisoguanosine β-cyanoethyl-phosphoramidite were prepared as described previously (Sugiyama et al., 1996). Calf intestine alkaline phosphatase (AP) (1000 U/μl) and snake venom phosphodiesterase (s.v.PDE) (3 U/μl) were purchased from Boehringer Mannheim (Tokyo). Deoxyoligonucleotides were prepared by the β-cyanoethyl phosphoramidite method on controlled pore glass supports (1 μmol) by ABI381A DNA synthesizer using normal protocol except for an extended coupling time of 5 min. After automated synthesis, oligonucleotides were cleaved from the supports and deprotected by heating the concentrated ammonia solutions at 50°C for 12 h. Oligonucleotides were purified by reverse phase HPLC using a CHEMCOBOND 5-ODS-H column (10 mm × 150 mm) with a flow rate of 3.0 ml/min and a linear gradient of acetonitrile in 50 mM ammonium formate. Purity and concentration of oligonucleotides were determined by complete digestion with s.v.PDE and AP. For analysis of digested nucleosides, a cosmosil 5C-18AR column (4.6 mm × 150 mm) was used with a flow rate of 1.0 ml/min. The final product of the (iC, iG)-decamer has a measured mass of 3070.0 (expected 3070.1) by electrospray mass spectrometry.

Solutions of the DNA oligomer were prepared as described earlier (Robinson and Wang, 1992). Lyophilized powder (3.47 mg) of 5′-d(TiGiCAiCiGiGAiCT) was dissolved in 0.55 ml H_2O containing 20 mM phosphate buffer at pH 7.0, then another 3.47 mg lyophilized powder of 5′-d(ACGTGCCTGA) was added into it, resulting in a 1.91 mM duplex solution. NMR spectra were collected on a Varian VXR500 500 MHz and

Unity Inova750 750 MHz spectrometers, and the data were processed with FELIX v1.1 (Hare Research, Woodinville, WA). The nonexchangeable 2D NOE spectra in D_2O were collected at 2°C at a mixing time of 200 ms and a total recycle delay of 6.27 s, where the average T1 relaxation was 2.3 s. The data were collected by the States/TPPI technique (States et al., 1982) with 512 t_1 increments and 2048 t_2 complex points, each the average of 32 transients. TOCSY (total correlated spectroscopy) spectra were used together with the NOE spectra to derive the assignment using the standard sequential assignment procedure. The chemical shifts (in parts per million) were referenced to the HDO peak, which was calibrated to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at different temperatures. The chemical shifts of all assigned resonances are listed in Table 1.

The starting parallel-stranded decamer DNA model was built by taking the B-DNA duplex of 5′-d(TCAGGCACGT) + 5′-d(ACGTGCCTGA), and replacing the first strand with 5′-d(TiGiCAiCiGiGAiCT). The reverse Watson-Crick basepair conformations of Fig. 1 were imposed in the model. The model was energy-minimized by conjugate gradient minimization using X-PLOR (Brünger, 1993). The structure refinement of the starting model was carried out by the SPEDREF procedures (Robinson and Wang, 1992). The procedure incorporates the full-matrix relaxation theory [of MORASS (Post et al., 1990)] using all measurable nuclear Overhauser effect crosspeak intensities in a quantitative manner. The use of the full-matrix relaxation theory, which takes into consideration spin diffusion, reduces the need to collect multiple 2D-NOESY data at different mixing times. The force field parameters of iG and iC were generated through a standard database in X-PLOR. X-PLOR's all atom force-field for DNA was used with explicit hydrogen bond potentials. Minimization of the residual errors is performed by conjugate gradient minimization with the NOE-constraints within the program X-PLOR (Brünger, 1993) using 60 cycles of SPEDREF refinement. The isotropic correlation time (t_c) for each refinement was empirically determined to be 4 ns. The NMR *R*-factor is defined as $R = \Sigma|No - Nc|\Sigma No$, where *No* and *Nc* are the experimental and calculated NOE crosspeak intensity, respectively. The atomic coordinates of the refined structure of the PS-duplex have been deposited in the Brookhaven Protein Databank (accession number 1be5 for coordinates and r1be5mr for NMR restraint).

The exchangeable 2D NOESY spectra at 2°C in 90% H_2O/10% D_2O were collected using the 1-1 pulse sequence as the read pulse (Hore, 1983; Sklenar et al., 1987). Thirty-two transients were averaged with a mixing time of 100 ms. The excitation offset was set to one-quarter of the spectral bandwidth, which was set to 12,000 Hz so that the imino resonances around 13 ppm were maximally excited.

UV/vis spectra were recorded on an HP 8453 UV-vis spectrophotometer. The concentration of the sample was 60 μM intercalators with varying concentrations of the DNA duplex in 100 mM phosphate buffer at pH 7.

Thermal denaturation profiles of oligonucleotides solution (3.5 μM duplex in 200 mM phosphate buffer, pH 6.0) were obtained with a Beckman DU-7000 spectrophotometer using a 1-cm path-length cell. Absorbance of the samples was monitored at 260 nm from 5°C to 75°C with a heating rate of 1°C/min. Melting temperatures were determined as the maximum in a plot ΔAbs260/Δtemp versus temperature.

RESULTS AND DISCUSSION

NMR analysis

The (iG, iC)-duplex exhibited a well-resolved 1D-NMR spectrum with the linewidth of the resonances comparable to that of other B-DNA decamers (data not shown). The temperature-dependent study suggested a remarkable stability of the structure, as the spectra change very little up to 40°C (data not shown). 2D-NOESY (Fig. 2, *left panel*) and TOCSY data were collected for resonance assignments and structural refinement. All resonances (except for some base amino protons) were assigned in a straightforward manner starting with a sequential assignment of the crosspeaks between H1′ to aromatic protons (Fig. 2). Most of the NOE crosspeaks are well-dispersed, making the measurement of the NOE intensities reliable (Robinson and Wang, 1992). The NOE crosspeak of A11H8-A11H1′ is stronger than other H8-H1′ crosspeaks of other purine nucleotides, suggesting that A11 may adopt a *syn* conformation. However, inspection of other NOE crosspeaks associated with the A11 nucleotide indicates that the *anti* conformation is also present. For example, we observed NOE crosspeaks between both A11H8 and A11H2 resonances to T10 methyl resonance (data not shown). The A11H8-T10-methyl crosspeak could be detected when T10 is basepaired with A11 in *syn* conformation. Such observations are consistent with the

TABLE 1 Chemical shifts (ppm) for the PS-duplex at 2°C

	H5/H2/Me	H6/H8	H1′	H2′/H2″	H3′	H4′	H5′/H5″	H1/H3	H2a/4a	H2b/4b
T1	1.60	7.43	5.87	2.32/2.05	4.67	4.06	3.66/3.66		na	na
iG2		7.96	6.11	2.71/2.63	4.92	4.37	4.03/3.97	12.78	8.98	7.12
iC3	1.76	7.21	5.28	2.29/1.82	4.82	4.09	4.32/4.32		na	na
A4	8.08	8.27	6.05	2.43/2.75	4.94	4.45	4.07/4.01		6.81	6.12
iC5	1.74	7.12	5.35	2.14/1.71	4.83	4.01	4.25/4.03		na	na
iG6		7.93	4.70	2.35/2.60	4.81	4.23	3.97/3.87	13.38	9.41	6.76
iG7		7.84	5.07	2.53/2.60	4.94	4.27	3.88/3.88	13.32	9.03	6.80
A8	8.08	8.32	6.27	2.78/2.85	5.04	4.47	4.14/4.07		6.56	6.23
iC9	1.67	7.27	5.68	2.62/2.16	4.83	4.20	4.38/4.38		na	na
T10	1.65	7.42	6.28	2.19/2.25	4.59	4.01	4.20/4.20		na	na
A11	7.95	8.06	6.20	2.69/2.52	4.76	4.25	3.70/3.70		na	na
C12	5.69	7.48	5.64	2.46/2.12	4.83	4.13	4.04/4.04		8.68	6.88
G13		7.87	5.99	2.65/2.65	4.90	4.43	4.10/4.10	12.78	na	na
T14	1.64	7.22	5.73	2.47/2.10	4.87	4.19	4.14/4.14	13.86	na	na
G15		7.83	5.99	2.74/2.65	4.96	4.43	4.30/4.14	12.50	na	na
C16	5.41	7.41	5.93	2.58/2.16	4.78	4.25	4.14/4.14		8.70	6.70
C17	5.57	7.53	5.87	2.48/2.00	4.78	4.08	4.17/4.17		8.88	7.07
T18	1.66	7.28	5.63	2.21/1.91	4.85	4.02	4.10/4.10	14.11	na	na
G19		7.89	5.33	2.62/2.62	4.94	4.27	4.03/3.93	12.57	na	na
A20	8.04	8.25	6.38	2.39/2.71	4.71	4.27	4.13/4.13		na	na

H2/4(a) are basepair hydrogen-bonded amino protons; H2/4(b) are not.

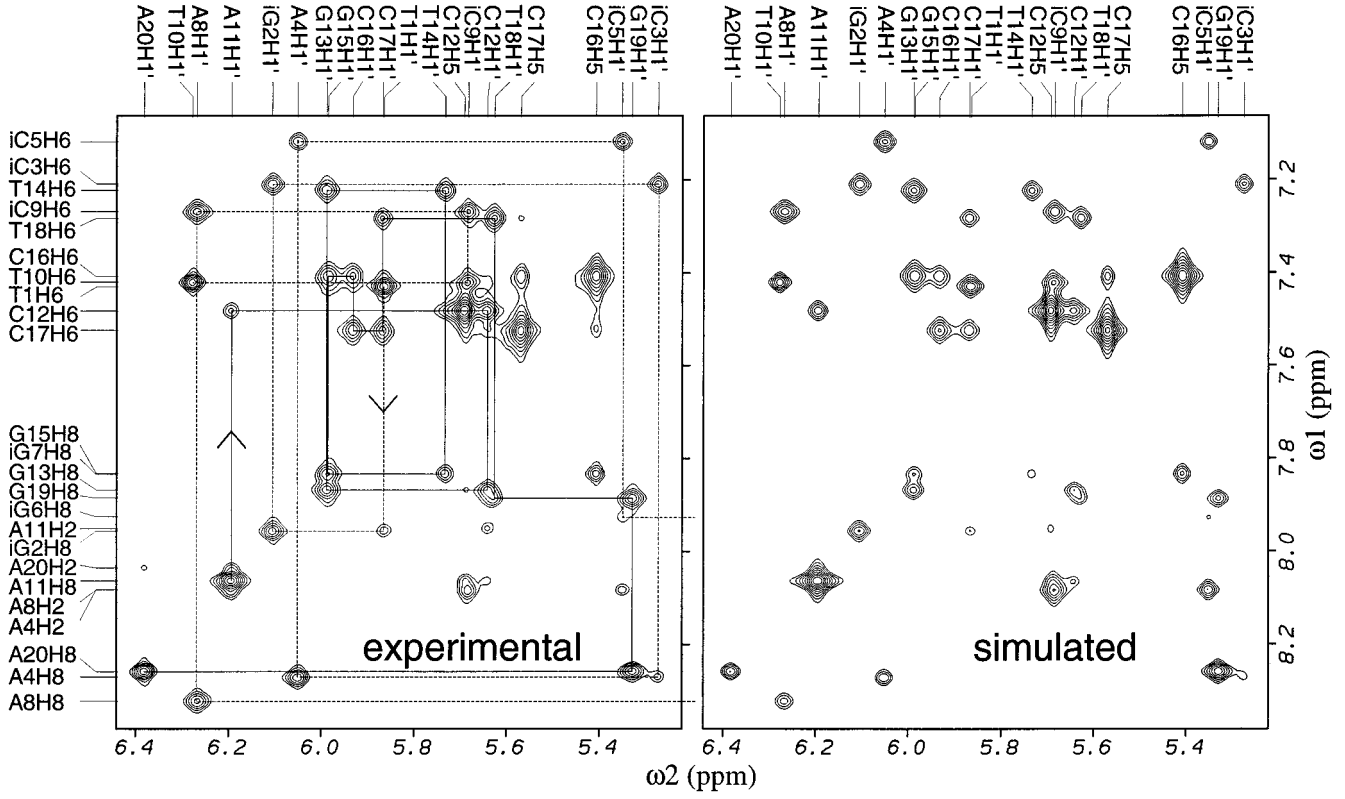

FIGURE 2 *Left panel:* aromatic to H1′/H5 region of the nonexchangeable proton phase-sensitive 2D-NOESY spectra of the parallel-stranded DNA duplex 5′-d(TiGiCAiCiGiGAiCT) + 5′-d(ACGTGCCTGA) at pH 7.0 showing the internucleotide connectivity. It provides key structural information including the glycosyl conformation, sugar puckers, and base-base stack. *Right panel:* simulated NOE spectra of the same region based on the refined PS-DNA model (*R*-factor = 23.7%).

interpretation that A11 is in fast equilibrium between the *syn* and *anti* conformations on the NMR time scale at 750 MHz.

There are several indications that the two strands form a duplex. First, the H8/H6-H1′ sequential assignment pathways in both strands are continuous and consistent with a right-handed helical geometry. Second, many NOE crosspeaks associated with the intra-stranded base-base stackings, e.g., G15H8-C16H5, C16H6-C17H5 in the normal strand and iG2H8-iC3M5, A4H8-iC4M5 in the (iG, iC)-strand, are observed.

However, more definitive proof that a duplex is formed comes from the exchangeable proton 2D-NOESY data. Fig. 3 shows portions of the 2D-NOESY spectrum of PS-decamer in 90% H_2O/10% D_2O at 2°C. Most of the exchangeable (amino and imino) proton resonances were detected and they have been unambiguously assigned. All imino protons from the inner eight basepairs of the duplex are observed at 2°C. It is interesting to note that the amino protons of the iG bases are clearly visible, whereas those from the iC bases are not. Such observations are opposite to those of the normal G and C bases. The rate of rotation of the N6-C6 bond in iG is presumably slower than that of the N2-C2 bond in G, so that the two amino protons (NHa and NHb) of iG are resolved. The reverse situation happens in iC and C.

NOE crosspeaks, many of them noted in the schematic drawing of Fig. 4, were used for SPEDREF/X-PLOR refinement. The structure of the refined PS-DNA helix (Fig. 5 *A*) shows that the two *sugar-phosphate backbones* of the new helix have an approximate twofold symmetry coinciding with the helix axis. The PS-duplex has two grooves with similar width (7.0 Å) and depth (7.7 Å), in contrast to the two distinct grooves (major groove width 11.7 Å, depth 8.5 Å, and minor groove width 5.7 Å, depth 7.5 Å) of B-DNA (Saenger, 1984). All nucleotides (except A11) are in the *anti* conformations and most of them have C2′-endo conformations. This new PS structure is favored by the stable iG:C and iC:G basepairs in the reverse Watson-Crick configuration. Since all basepairs (including A-T) are in the same configuration, the diameter of the helix, determined by the C1′-C1′ distance of ~11 Å, remains uniform throughout the helix. Note that because of the nonstandard basepairing scheme and the opposite strand alignment, the disposition of various atoms on the surface of the grooves is completely different from B-DNA. The contrast between the PS-DNA and B-DNA is better seen in their van der Waals drawings (Fig. 5, *B* and *C*).

The observed NOE crosspeaks are consistent with the reverse Watson-Crick configuration, which would place the N6-amino groups of iG close to the H1′ of the nucleotide next to the basepaired C on the 3′-side. For example, the

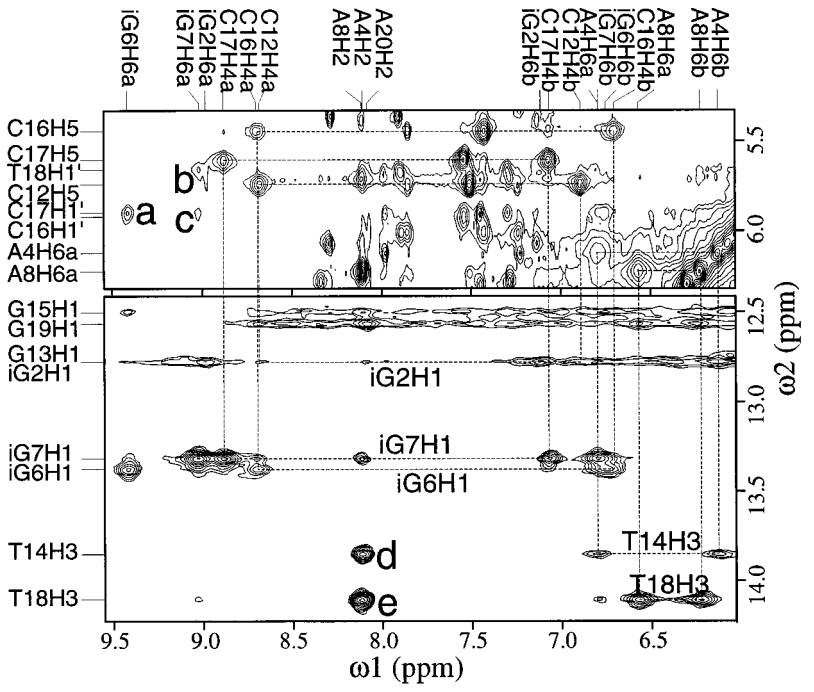

FIGURE 3 Imino-to-amino and amino-to-H1′/H5 regions in the 2D-NOESY exchangeable spectra of the PS-duplex in 90% H_2O/10% D_2O, pH 7.0, at 2°C. These spectra provide evidence for the reverse Watson-Crick basepairs and the assignment of the exchangeable proton resonances. Some of the interstrand crosspeaks are labeled: (*a*) iG6H6a-C17H1′, (*b*) iG7H6a-T18H1′, (*c*) iG7H6a-C17H1′, (*d*) A4H2-T14H3, (*e*) A8H2-T18H3. The NOE crosspeak intensities between the exchangeable to the exchangeable, as well as the exchangeable to the nonexchangeable, protons agree with the calculated distances from the refined structure.

iG6NH6a-C17H1′ and iG7NH6a-T18H1′ NOE crosspeaks are clearly detectable (peak a and peak b, respectively, in Fig. 3). In addition, interstrand NOE crosspeaks between the AH2 protons and the TMe protons for each of the four A-T basepairs are observed (data not shown). This observation is consistent only with the reverse Watson-Crick basepairing between A and T (Zhou et al., 1993). The consistency of the exchangeable-proton NOE data, which are independent of the refinement process, with the refined structure lend further credence to the validity of the structure.

The T1IR measurement of the new helix indicated that their average relaxation time (2.3 s) is comparable to that of the B-DNA (2.0 s). This could be interpreted to mean the backbone is not inflexible, as in Z-DNA, which has an average relaxation time of 2.8 s (Yang and Wang, 1997).

Interactions between intercalators and PS-duplex

DNA oligonucleotides conjugated with intercalator have been shown to be effective antisense molecules. Helene and colleagues prepared end-modified oligonucleotides targeted to Ha-ras oncogene and showed that the oligonucleotides carrying an acridine tail not only protected the modified oligonucleotides from nucleases, but also enhanced the RNaseH cleavage of the target (Saison-Behmoaras et al., 1997). For possible applications of the (iG, iC)-containing oligonucleotide as antisense molecules, such a strategy involving intercalator end-modifications may be used. However, it has not been demonstrated that duplex derived from (iG, iC)-containing oligonucleotide is capable of binding intercalators. Therefore, the backbone flexibility of the (iG, iC)-containing duplex was probed by the ability of the new PS-helix to bind intercalators. The intercalation process requires that the adjacent basepairs are separated to 6.8 Å with a concomitant extension of the backbone.

Fig. 6 shows the titration of three different intercalators, ethidium, daunorubicin, and nogalamycin, with PS-helix and B-DNA (using CGTACG). These three intercalators represent different degrees of complexity in their intercalation processes. Ethidium is a "simple" intercalator that binds to DNA without pronounced sequence preference (Sobell et al., 1978). Daunorubicin prefers a 5′-CG(A/T) sequence by using its O9 hydroxyl group to hydrogen-bond to the N2 and N3 of a guanine for recognition (Wang et al., 1987). The daunosamine sugar of the daunorubicin drug occupies the minor groove of DNA. Finally, nogalamycin binds to the CpG sequence with its two sugars simultaneously occupying the minor and major grooves (Liaw et al., 1989).

Although Jovin and colleagues have shown that ethidium binds readily to parallel-stranded DNA duplex using fluorescence measurements (van de Sande et al., 1988; Germann et al., 1989), it is interesting to note that the three intercalators we used produce nearly identical UV spectra in the presence of the (iG, iC)-containing PS-duplex and the B-DNA of CGTACG (Fig. 6). Not only do they have the same hypochromic red-shifts, they also have nearly identical isosbestic points. These unexpected results have several implications. First, it further proves that the PS-helix is as flexible as B-DNA. Second, the chromophore of intercalators is embedded in similar intercalation cavities, despite the different basepairing schemes.

The very similar UV spectra of intercalators in the presence of the (iG, iC)-containing PS-duplex and the B-DNA of CGTACG raise an interesting question of whether the (iG, iC)-containing PS-duplex may be converted to B-DNA conformation by intercalators, reminiscent of those found in

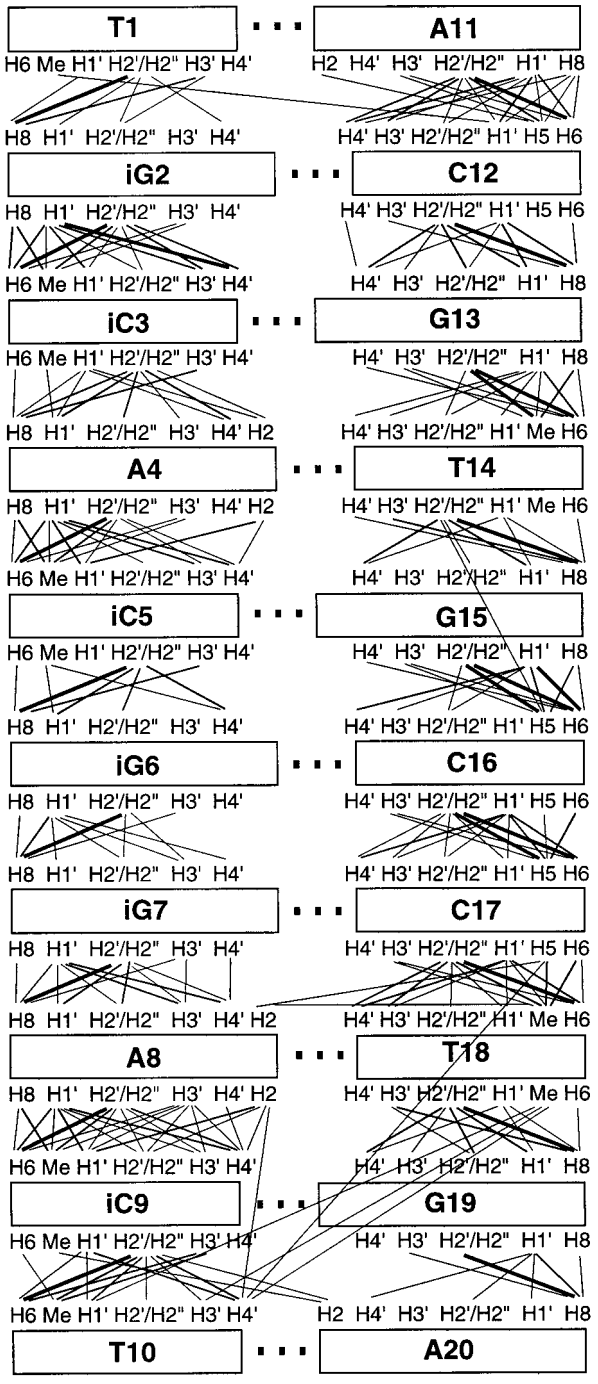

FIGURE 4 Schematic diagram showing the internucleotide crosspeaks observed in the experimental nonexchangeable 2D-NOESY spectra. The NOE intensities are represented by straight lines of varying thickness, denoting strong, medium, and weak NOE crosspeaks.

the Z- to B-DNA conversion induced by ethidium (Walker et al., 1985a) or actinomycin D (Walker et al., 1985b). We believe that such conversion is highly unlikely in our system, where the two strands, i.e., the (iG, iC)-strand and the normal strand, can form only the parallel duplex because of their unique basepairing schemes. Neither can each individual strand form any stable B-DNA structure. The Z- to

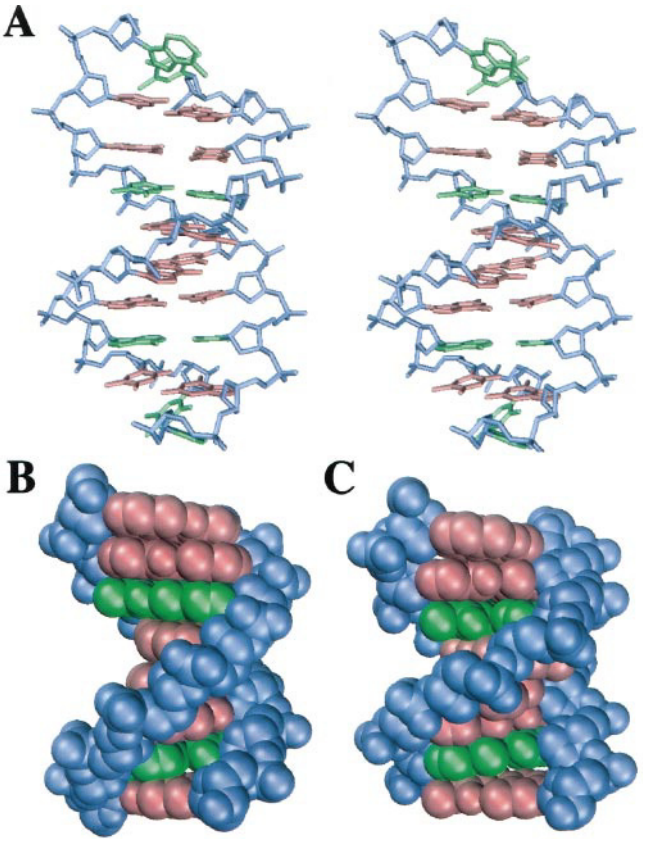

FIGURE 5 (*A*) Stereoscopic model of the refined PS-duplex structure. (*B*) Van der Waals molecular model of the regular B-DNA 5′-d(CGTGCCTG) + 5′-d(CAGGCACG) structure. (*C*) Van der Waals molecular model of the PS-duplex 5′-d(CGTGCCTG) + 5′-d(iGiCAiCiGiGAiC) structure. The terminal basepairs are not shown in the refined PS decamer due to fraying or multiple conformations. iG:C and iC:G or G:C basepairs are pink, and A:T basepairs are green.

B-DNA transition of poly(dG-dC), induced by the intercalator binding, can happen because the sequence (self-complementary alternating dC and dG sequence) allows the backbone handiness to change from left-handed (Z) to right-handed (B) without changing the Watson-Crick G-C basepairing scheme. Therefore, we conclude that the spectra we observe in Fig. 6 are due to the binding of intercalators with PS-duplex.

The use of alternative bases to generate new types of duplex structures adds to the repertoire of chemical modifications that have been used in the design of antisense probes (Freier and Altmann, 1997). Our results unequivocally showed that (iG, iC)-containing oligonucleotides conjugated with intercalators may be used to bind to target mRNA molecules in a unique parallel-stranded conformation. Such (iG, iC)-containing oligonucleotides conjugated with intercalators may possess desirable properties for antisense applications.

Comparison with other PS-duplexes

As discussed before, there are other types of parallel-stranded DNA structures, including G-quartet (Kang et al.,

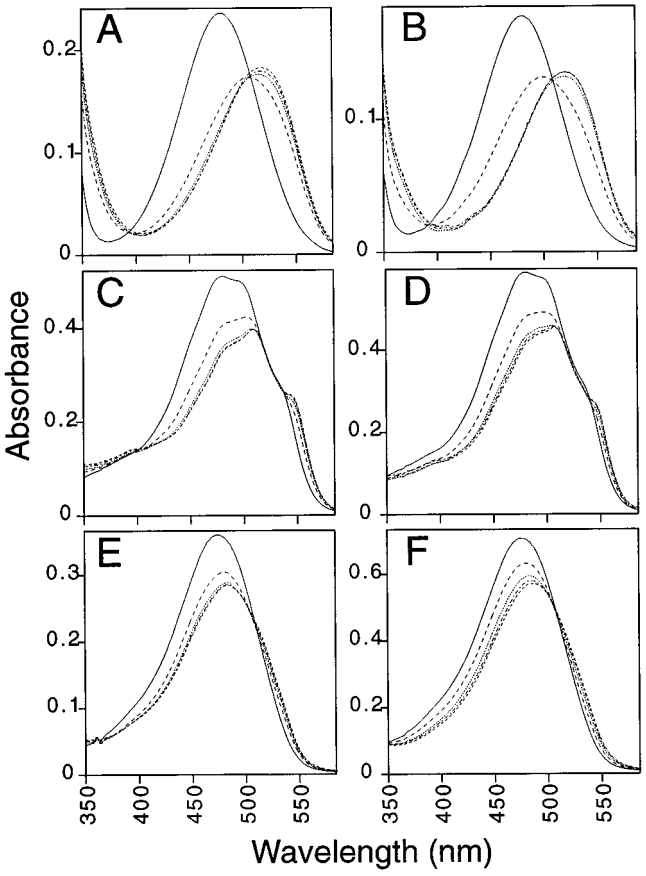

FIGURE 6 UV-vis spectra from the titration of three different intercalators with the PS-duplex and B-DNA d(CGTACG). (*A*) ethidium + B-DNA; (*B*) ethidium + PS-duplex; (*C*) daunorubicin + B-DNA; (*D*) daunorubicin + PS-duplex; (*E*) nogalamycin + B-DNA; (*F*) nogalamycin + PS-duplex. (—), 60 μM drug only; (- -), 60 μM drug + 30 μM DNA duplex; (· · ·), 60 μM drug + 60 μM DNA duplex; (· - · -), 60 μM drug + 90 μM DNA duplex; (---), 60 μM drug + 120 μM DNA duplex.)

1992; Smith and Feigon, 1992; Laughlan et al., 1994; Phillips et al., 1997), i-motif (Gehring et al., 1993; Chen et al., 1994), and Π-DNA (Robinson et al., 1992, 1994; Robinson and Wang, 1993; Wang and Patel, 1994). It remains to be answered whether those parallel-stranded nucleic acid structures are compatible with one another. For example, can the sequence of 5′-d(TiGiCAiCiGiGAiCTCGACGACGA), which has the CGA sequence favoring Π-DNA, form a stable parallel duplex with 5′-d(ACGTGCCTGACGACGACGA) at pH 6.0? Our data of the T_m values of 5′-d(TiGiCAiCiGiGAiCTCGACGACGA), 5′-d(TiGiCAiCiGiGAiCT), and 5′-d(CGACGACGA) paired with their respective complementary parallel strands of 5′-d(ACGTGCCTGACGACGACGA), 5′-d(ACGTGCCTGA), and 5′-d(CGACGACGA) at pH 6.0 were 61, 35, and 27°C, respectively. These results clearly demonstrate the compatibility of these two types of parallel duplexes, which increase the combinations of nucleotide sequences in designing antisense molecules.

The finding that (iG, iC)-containing oligonucleotides form a highly stable parallel-stranded helix with normal nucleotides significantly increases the potential utility of parallel-stranded helix structures. No longer is the PS-duplex restricted to (A, T)-containing oligonucleotides (Rippe and Jovin, 1992), which have relatively low stability of the duplexes. Moreover, the (iG, iC)-containing PS-duplex binds intercalators as well as the B-DNA does.

These new types of stable PS-helices are completely different from B-DNA. Would proteins (like RNaseH) still recognize the new structures? Is the PS-duplex molecule sensitive toward nuclease and chemical agents including alkylating agents? The chemical reactivity of the A, T-containing PS-duplex has been studied previously (Klysik et al., 1990). In our iG, iC-containing PS-duplex we noted that the resonances of the two N6 amino protons of iG are clearly resolved, but those of the N2 amino protons of iC are not, opposite to those found for normal G and C. It is likely that those amino groups have different chemical reactivities with respect to those of G and C. Inspection of the chemical structures of those bases shows that the amino groups of G and iC are part of the guanidine system, whereas those from the A and iG bases are part of the amidine system. It is well-established that the N2 amino group of G is particularly susceptible to nucleophilic reactions with many compounds, including anticancer drugs (e.g., ecteinascidin; reviewed in Guan et al., 1993). It is possible that amino groups of iC, but not iG, in DNA may mimic that of G toward chemical agents. This hypothesis can be tested. Answers to many of those questions will help clarify the possible roles of this unique family of nucleic acid structures.

CONCLUSION

In conclusion, we have shown that (iG, iC)-containing oligonucleotides formed stable PS-duplexes and provided a three-dimensional structure by NMR refinement. In addition, the (iG, iC)-containing PS-duplex is capable of binding intercalators, and such a property may be useful in the design of antisense oligonucleotides. Recently we have also shown that iG can pair with thymine in either the Watson-Crick basepairing or the reverse wobble basepairing schemes, depending on the local environments (e.g., dielectric constant) (Robinson et al., 1998). That study may allow us to understand the role of oxidative damage of adenine, which produces 2-hydroxyadenine. The study here and elsewhere by others (Seela et al., 1997; Roberts et al., 1997) should be useful in generating new interest in the study of DNA molecules containing novel bases like iG and iC.

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REFERENCES

Blackburn, E. H. 1991. Structure and function of telomeres. *Nature*. 350:569–573.

Blackburn, E. H. 1992. Telomerases. *Annu. Rev. Biochem.* 61:113–129.

Brünger, A. X-PLOR (version 3.1). 1993. The Howard Hughes Medical Institute and Yale University, New Haven, CT.

Chen, L., L. Cai, X. Zhang, and A. Rich. 1994. Crystal structure of a four-stranded intercalated DNA: d(C4). *Biochemistry*. 33:13540–13546.

Freier, S. M., and K.-H. Altmann. 1997. The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically modified DNA:RNA duplexes. *Nucleic Acids Res.* 25:4429–4443.

Gehring, K., J. L. Leroy, and M. Gueron. 1993. A tetrameric DNA structure with protonated cytosine:cytosine basepairs. *Nature*. 363: 561–565.

Germann, M. W., H. J. Vogel, R. T. Pon, and J. H. van de Sande. 1989. Characterization of a parallel-stranded DNA hairpin. *Biochemistry*. 28: 6220–6228.

Grady, D. L., R. L. Ratliff, D. L. Robinson, E. C. McCanlies, J. Meyne, and R. K. Moyzis. 1992. Highly conserved repetitive DNA sequences are present at human centromeres. *Proc. Natl. Acad. Sci. USA*. 89: 1695–1699.

Grollman, A. P., F. Johnson, J. Tchou, and M. Eisenberg. 1994. Recognition and repair of 8-oxoguanine and formamidopyrimidine lesions in DNA. *Ann. N.Y. Acad. Sci.* 726:208–213.

Guan, Y., R. Sakai, K. L. Rinehart, and A. H.-J. Wang. 1993. Molecular and crystal structures of ecteinascidins: potent antitumor compounds from the Caribbean tunicate *Ecteinascidia turbinata*. *J. Biomol. Struct. & Dyn.* 10:793–818.

Hore, P. J. 1983. A new method for water suppression in the proton NMR spectra of aqueous solutions. *J. Magn. Reson*. 54:539–542.

Kamiya, H., and H. Kasai. 1996. Effect of sequence contexts on misincorporation of nucleotides opposite 2-hydroxyadenine. *FEBS Lett.* 391: 113–116.

Kamiya, H., and H. Kasai. 1997. Mutations induced by 2-hydroxyadenine on a shuttle vector during leading and lagging strand syntheses in mammalian cells. *Biochemistry*. 36:11125–11130.

Kang, C. H., X. Zhang, R. Ratliff, R. Moyzis, and A. Rich. 1992. Crystal structure of four-stranded Oxytricha telomeric DNA. *Nature*. 356: 126–131.

Klysik, J., K. Rippe, and T. M. Jovin. 1990. Reactivity of parallel-stranded DNA to chemical modification reagents. *Biochemistry*. 29:9831–9839.

Kremer, E. J., M. Pritchard, M. Lynch, S. Yu, K. Holman, E. Baker, S. T. Warren, D. Schlessinger, G. R. Sutherland, and R. I. Richards. 1991. Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence $p(CCG)_n$. *Science*. 252:1711–1714.

Laughlan, G., A. I. Murchie, D. G. Norman, M. H. Moore, P. C. Moody, D. M. Lilley, and B. Luisi. 1994. The high-resolution crystal structure of a parallel-stranded guanine tetraplex. *Science*. 265:520–524.

Liaw, Y. C., Y. G. Gao, H. Robinson, G. A. van der Marel, J. H. van Boom, and A. H.-J. Wang. 1989. Antitumor drug nogalamycin binds DNA in both grooves simultaneously: molecular structure of nogalamycin-DNA complex. *Biochemistry*. 28:9913–9918.

Lipscomb, L. A., M. E. Peek, M. L. Morningstar, S. M. Verghis, E. M. Miller, A. Rich, J. M. Essigmann, and L. D. Williams. 1995. X-ray structure of a DNA decamer containing 7,8-dihydro-8-oxoguanine. *Proc. Natl. Acad. Sci. USA*. 92:719–723.

Oberle, I., F. Rousseau, D. Heitz, C. Kretz, D. Devys, A. Hanauer, J. Boue, M. F. Bertheas, and J. L. Mandel. 1991. Instability of a 550-basepair DNA segment and abnormal methylation in fragile X syndrome. *Science*. 252:1097–1102.

Palecek, E. 1991. Local supercoil-stabilized DNA structures. *Crit. Rev. Biochem. Mol. Biol.* 26:151–226.

Phillips, K., Z. Dauter, A. I. H. Murchie, D. M. J. Lilley, and B. Luisi. 1997. The crystal structure of a parallel-stranded guanine tetraplex at 0.95 Å resolution. *J. Mol. Biol.* 273:171–182.

Post, C. B., R. P. Meadows, and D. G. Gorenstein. 1990. On the evaluation of interproton distances for three-dimensional structure determination by NMR using a relaxation rate matrix analysis. *J. Am. Chem. Soc.* 112: 6796–6803.

Ramsing, N. B., and T. M. Jovin. 1988. Parallel stranded duplex DNA. *Nucleic Acids Res.* 16:6659–6676.

Rich, A., A. Nordheim, and A. H.-J. Wang. 1984. The chemistry and biology of left-handed Z-DNA. *Annu. Rev. Biochem.* 53:791–846.

Rippe, K., and T. M. Jovin. 1992. Parallel-stranded duplex DNA. *Methods Enzymol.* 211:199–220.

Roberts, C., J. C. Chaput, and C. Switzer. 1997. Beyond guanine quartets: cation-induced formation of homogenous and chimeric DNA tetraplexes incorporating iso-guanine and guanine. *Chem. Biol.* 4:899–908.

Robinson, H., G. A. van der Marel, J. H. van Boom, and A. H.-J. Wang. 1992. Unusual DNA conformation at low pH revealed by NMR: parallel-stranded DNA duplex with homo basepairs. *Biochemistry*. 31: 10510–10517.

Robinson, H., J. H. van Boom, and A. H.-J. Wang. 1994. 5′-CGA motif induces other sequences to form homo base-paired parallel-stranded DNA duplex: the structure of (G-A)n derived from four DNA oligomers containing $(G\text{-}A)_3$ sequence. *J. Am. Chem. Soc.* 116:1565–1566.

Robinson, H., and A. H.-J. Wang. 1992. A simple spectral-driven procedure for the refinement of DNA structures by NMR spectroscopy. *Biochemistry*. 31:3524–3533.

Robinson, H., and A. H.-J. Wang. 1993. 5′-CGA sequence is a strong motif for homo base-paired parallel-stranded DNA duplex as revealed by NMR analysis. *Proc. Natl. Acad. Sci. USA*. 90:5224–5228.

Robinson, H., Y.-G. Gao, C. Bauer, C. Roberts, C. Switzer, and A. H.-J. Wang. 1998. 2′-Deoxy-isoguanosine adopts more than one tautomer to base pair with thymidine observed by high resolution crystal structure analysis. *Biochemistry*. (in press).

Saenger, W. 1984. Principles of Nucleic Acid Structure. Springer-Verlag, New York.

Saison-Behmoaras, T. E., I. Duroux, T. T. Nguyen, U. Asseline, and C. Helene. 1997. Antisense properties of end-modified oligonucleotides targeted to Ha-ras oncogene. *Antisense Nucleic Acid Drug Dev.* 7:361–368.

Seela, F., Y. Chen, A. Melenewski, H. Rosemeyer, and C. Wei. 1996. Synthesis and application of novel nucleoside phosphonates and phosphoramidites modified at the base moiety. *Acta Biochim. Pol.* 43:45–52.

Seela, F., and C. F. Wei. 1997. Oligonucleotides containing consecutive 2′-deoxyisoguanosine residues: synthesis, duplexes with parallel chain orientation, and aggregation. *Helvetica Chimica Acta.* 80:73–85.

Sen, D., and W. Gilbert. 1992. Novel DNA superstructures formed by telomere-like oligomers. *Biochemistry*. 31:65–70.

Sklenar, V., B. R. Brooks, G. Zon, and A. Bax. 1987. Absorption mode two-dimensional NOE spectroscopy of exchangeable protons in oligonucleotides. *FEBS Lett.* 216:249–252.

Smith, F. W., and J. Feigon. 1992. Quadruplex structure of Oxytricha telomeric DNA oligonucleotides. *Nature*. 356:164–168.

Sobell, H. M., B. S. Reddy, K. K. Bhandary, S. C. Jain, T. D. Sakore, and T. P. Seshadri. 1978. Conformational flexibility in DNA structure as revealed by structural studies of drug intercalation and its broader implications in understanding the organization of DNA in chromatin. *Cold Spring Harbor Symp. Quant. Biol*. 42:87–102.

States, D. J., R. A. Haberkorn, and D. J. Ruben. 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson*. 48:286–292.

Sugiyama, H., S. Ikeda, and I. Saito. 1996. Remarkably stable parallel-stranded oligonucleotides containing 5-methylisocytosine and isoguanine. *J. Am. Chem. Soc.* 118:9994–9995.

Tchou, J., and A. P. Grollman. 1993. Repair of DNA containing the oxidatively damaged base, 8-oxoguanine. *Mutat. Res.* 299:277–287.

van de Sande, J. H., N. B. Ramsing, M. W. Germann, W. Elhorst, B. W. Kalisch, E. von Kitzing, R. T. Pon, R. M. Clegg, and T. M. Jovin. 1988. Parallel stranded DNA. *Science*. 241:551–557.

Walker, G. T., M. P. Stone, and T. Krugh. 1985a. Ethidium binding to left-handed (Z) DNAs results in regions of right-handed DNA at the intercalation site. *Biochemistry*. 24:7462–7471.

Walker, G. T., M. P. Stone, and T. Krugh. 1985b. Interactions of drugs with Z-DNA: cooperative binding of actinomycin D or actinomine to the left-handed forms of poly(dG-dC):poly(dG-dC) and poly(dG-m^5dC):poly(dG-m^5dC) reverses the conformation of the helix. *Biochemistry*. 24:7471–7479.

Wang, Y., and D. J. Patel. 1994. Solution structure of the d(T-C-G-A) duplex at acidic pH. A parallel-stranded helix containing C+.C, G.G and A.A pairs. *J. Mol. Biol.* 242:508–526.

Wang, A. H.-J., G. J. Quigley, F. J. Kolpak, J. L. Crawford, J. H. van Boom, G. A. van der Marel, and A. Rich. 1979. Molecular structure of a left-handed double helical DNA fragment at atomic resolution. *Nature*. 282:680–686.

Wang, A. H.-J., G. Ughetto, G. J. Quigley, and A. Rich. 1987. Interactions between an anthracycline antibiotic and DNA: molecular structure of daunomycin complexed to d(CpGpTpApCpG) at 1.2-A resolution. *Biochemistry*. 26:1152–1163.

Yang, X.-L., and A. H.-J. Wang. 1997. Structural analysis of Z-Z DNA junctions with A:A and T:T mismatched basepairs by NMR. *Biochemistry*. 36:4258–4267.

Zhou, N., M. W. Germann, J. H. van de Sande, N. Pattabiraman, and H. J. Vogel. 1993. Solution structure of the parallel-stranded hairpin d(T8<>C4A8) as determined by two-dimensional NMR. *Biochemistry*. 32:646–656.