Ethanol (E) was prepared by the procedure described by Murr,[18] with modifications by Buddenbaum.[19]

2,2,2-Trifluoroethanol (TFE or T) was prepared by the procedure described by Shiner et al.[20]

Ethanol-Water. These volume percent solutions were prepared by weight with densities and buoyancy corrections as described by Murr.[18]

2,2,2-Trifluoroethanol-Water. These solutions were prepared as weight percent solutions by the method described by Shiner et al.[20]

Conductance Kinetics Procedure. Conductance measurements were made by using a bipolar pulsed conductance apparatus built in this laboratory based on the design of Caserta and Enke.[21] This instrument was calibrated by comparing the experiment resistances with known fixed resistors and fitting the curve to an eight-parameter equation with the simplex method.[22] Conductivity cells used were made in this laboratory based on the design of Murr,[18] by using modifications by Dowd,[23] Tomasik,[24] and Wilgis.[25] The data were recorded by a TI 980 computer with programs developed by McMullen[26] and Tomasik.[24] The instrument was later changed to an IBM PC with use of control and data acquisition programs written by Ensinger and Russ.[27] The data were analyzed on an IBM PC with a calibration program written by Tomasik[24] and Wilgis[25] and a nonlinear, doubly weighted least-squares program written by Buddenbaum,[19] with modifications by Vogel,[28] Pinnick,[29] Bowersox and Tomasik,[30] Wilgis,[25] Stoelting,[31] and Ensinger.[32]

Product Determination. Product studies by ^2H NMR spectroscopy were performed in the following manner. A 1.0-mL sample of reaction mixture (approximately 0.1 M in deuterium) was prepared in 1.0-mL volumetric flask with a molar excess of 2,6-lutedine. This was transferred to an NMR tube, sealed, and let react for more than 10 half-lives. The ^2H spectra were recorded by using a Nicolet 360 MHz spectrometer at 55.4 MHz. The Fourier transform NMR spectra were taken by using between 500 and 2000 scans. Product ratios were determined by integration of the peaks by using a curve fitting program on the Nicolet spectrometer. Comparison of this technique with the cut and weigh technique used in the past showed good agreement.

Deuterium chemical shifts varied slightly with solvent and are as follows in 97T (relative to external $CDCl_3$ at 7.2600 ppm): 4-(trimethylsilyl)-3-methyl-2-butyl-*2-d* trifluoroethyl ether, 3.645 ppm; 4-trimethyl-3-methyl-2-butanol-*2-d*, 3.841 ppm; *cis*-1,2-dimethylcyclopropane-1-*d*, 0.876 ppm; *trans*-1,2-dimethylcyclopropane-*1-d*, 0.546 ppm; 2-methyl-1-butene-*3-d*, or 4-(trimethylsilyl)-3-methyl-2-butanol-*3-d*, 2.383 (in 90E). The stereochemistry of the substitution reaction was determined by isolation of the product alcohol from the reaction of the threo isomer in 90E and the erythro isomer in 80E. For this determination the samples prepared from deuterium NMR analysis were opened, and the solvent was removed under high vacuum. The remaining material was filtered through a silica gel filter with diethyl ether rinses and separated by using high-pressure liquid chromatography on a prepacked 10 mm i.d. × 25 cm length silica gel column with 70% hexane-30% ethyl acetate as solvent. The peak corresponding to known 3,5,5-trimethyl-5-sila-2-hexanol was collected, and the high-resolution proton NMR spectra was recorded. Comparison of these spectra with known pure threo and erythro alcohols showed complete retention of configuration.

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Dimers, Trimers, and Tetramers of Cytosine with Guanine

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Abstract: Cytosine and guanine have been shown previously to form Watson-Crick type base pairs in nonaqueous solvents, suggesting that the monomers can be used to understand and possibly to predict structures of the polymeric nucleic acids. Yet, the poor solubility properties of cytosine and guanine (and their corresponding nucleosides) have limited the utility of the monomeric model of polymeric nucleic acids. The 2'-deoxynucleosides, which are substituted at both ribose hydroxyls with triisopropylsilyl groups, have high solubilities (greater than 200 mM) in nonpolar solvents such as chloroform-*d*. These substituted nucleosides are appropriate for detailed ^1H NMR study of hydrogen bonding between cytosine and guanine over a wide temperature range. In this DNA model system, cytosine and guanine form the stable Watson-Crick type dimer, as would be expected from previous studies of bases in higher dielectric solvents or at lower concentrations. We report here that the bases form such dimers and additional, more intricate hydrogen-bonded complexes. Cytosine and guanine monomers form both trimers (cytosine:guanine$_2$) and tetramers [(cytosine:guanine)$_2$] in low-dielectric solution. Thus, the interactions of monomers are consistent with formation of two-, three-, and four-stranded nucleic acid polymers.

Much of biological specificity is derived from hydrogen bonding. In nucleic acids, selective hydrogen bonds between complementary bases direct the fidelity of replication/transcription processes and also stabilize secondary and tertiary structures. Forces that stabilize double-stranded nucleic acids can be conceptually and experimentally decomposed into base-base hydrogen-bonding interactions (horizontal) and stacking interactions (vertical). The two types of interaction can be modeled by monomers in the appropriate environments. In relatively low dielectric solvents such as chloroform or even in dimethyl sulfoxide, the bases primarily form hydrogen bonds,[1-13] while in aqueous solution the bases

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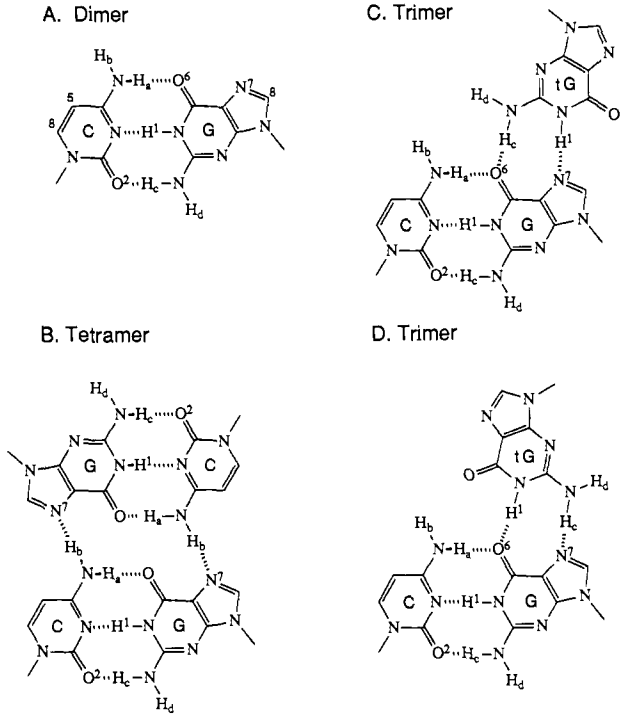

Figure 1. Structures: (A) C:G Watson–Crick base pair; (B) $(C:G)_2$ tetramer; (C) one possible conformation of the trimer, C:G:tG; (D) second possible conformation of the trimer, C:G:tG.

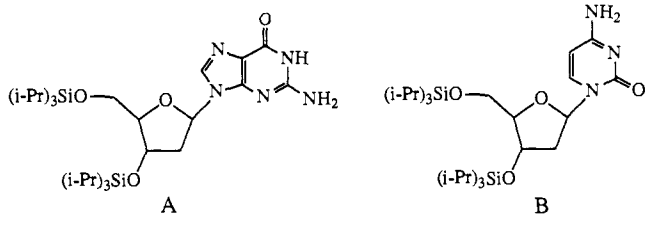

Figure 2. Two nucleoside derivatives used in this study: (A) 2′-deoxy-3′,5′-bis(triisopropylsilyl)guanosine; (B) 2′-deoxy-3′,5′-bis(triisopropylsilyl)cytidine.

stack.[14] Low-dielectric solvents mimic the dielectric environment of the interior of a nucleic acid double helix[15,16] and maximize hydrogen bonding.

Monomeric models have proven useful for studying polymeric nucleic acid structure and function. One important structural motif, the Hoogsteen base pair, was predicted from crystals of monomers[17] before being observed in yeast phenylalanine tRNA.[18] Another structure, the Watson–Crick base pair (Figure 1A), can form spontaneously between monomeric guanine and cytosine-.[1-10,19,20] This C:G dimer, stabilized by three hydrogen bonds, forms between monomers in nonaqueous solvents. However, the poor solubility properties of cytosine and guanine (and their corresponding nucleosides) had limited the usefulness of this monomeric model of DNA.

We have recently described[10,11] a well-behaved model system for studying the hydrogen-bonding interactions of these important biomolecules. Improved solubity of cytosine and guanine in chloroform-*d* and other low-dielectric solvents is achieved by addition of lipophilic triisopropylsilyl groups to both hydroxyls of the 2′-deoxynucleosides (Figure 2). Hence, hydrogen bonding of cytosine with guanine can be investigated over a wide temperature range up to relatively high concentrations (greater than 200 mM). In this DNA model system, ^{1}H NMR shows cytosine and guanine form a stable Watson–Crick type dimer, as would be expected from previous studies of bases in higher dielectric solvents or at lower concentrations. We report here that the bases form such dimers and additional, more intricate hydrogen-bonded complexes shown in Figure 1B–D. Cytosine and guanine monomers form both trimers (cytosine:guanine$_2$) and tetramers [(cytosine:guanine)$_2$] in low-dielectric solution.

Experimental Section

The 2-′deoxy-3′,5′-bis(triisopropylsilyl) derivatives of guanosine and cytidine were prepared and purified as described elsewhere.[10,11] Chloroform-*d* (Stohler) was distilled over P_2O_5 under argon just prior to sample preparation. NMR samples, in 5-mm tubes, were prepared under anhydrous conditions under argon. The solutions were degassed by bubbling argon or by at least three freeze/pump/thaw cycles.

Spectra were obtained on a Varian XL-300 spectrometer using 16K double-precision (32-bit) data points over a 5000-Hz spectral width. In the nuclear Overhauser experiments, for which an array of decoupler offsets was used, spectra were obtained in the interleave mode. For all other spectra, the decoupler was used to suppress the large triisopropyl peak at approximately 1.0 ppm. Deuteriochloroform was used as a lock, and the trace nondeuterated chloroform contaminant was used as a reference. Ethylene glycol was used for high-temperature calibration of the probe, and methanol was used for low-temperature calibration. Probe temperatures were reproducible to ±1 °C.

Results

We have previously reported the preparation and utility of 3′,5′-bis(triisopropylsilyl) derivatives of 2′-deoxycytidine and 2′-deoxyguanosine (Figure 2) for characterizing the thermodynamics of the C:G base pair with isoperibolic titration calorimetry.[10] The same nucleoside derivatives were used in this spectroscopic study. The lipophilic triisopropylsilyl groups enhance the solubilities of the nucleosides in nonaqueous solvents and prevent interference of hydrogen bonding by ribose hydroxyls. These nucleoside derivatives provide a sensitive system for studying hydrogen bonding exclusively between the bases. For simplicity of description, the 3′,5′-bis(triisopropylsilyl) derivatives of 2′-deoxyguanosine and 2′-deoxycytidine shall be referred to as G and C, respectively.

The nuclear Overhauser effect (NOE) was used here for structural elucidation and to aid in resonance assignment. Initially, we were somewhat surprised that in this model system the NOE's are exclusively negative. Negative NOE's were observed between hydrogens (such as between H1′ and H^8 of G, not shown) where exchange, scalar coupling, and the intermediacy of a third spin can be excluded as causes for a decrease in signal intensity. The negative sign of the NOE's indicates that these mononucleosides are not in the extreme narrowing limit (where $\tau_c\omega \ll 1$) but instead are behaving like much larger molecules. As described below, the formation of hydrogen-bonded and possibly stacked complexes explains the negative sign of the observed NOE's. In these experiments the decrease in N–H resonance intensities caused by selective saturation of other N–H resonances can result from two

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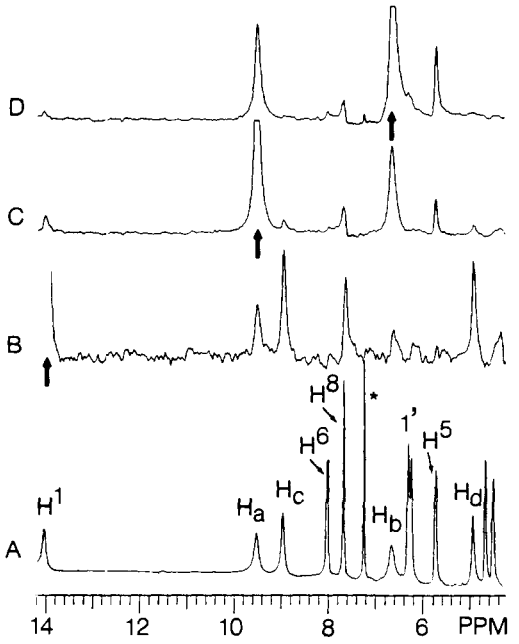

Figure 3. Spectra: (A) partial 300-MHz ^{1}H NMR spectrum of 30 mM 2′-deoxy-3′,5′-bis(triisopropylsilyl)guanosine plus 30 mM 2′-deoxy-3′,5′-bis(triisopropylsilyl)cytidine at −57 °C in chloroform-*d*, off-resonance selective irradiation; (B–D) difference spectra, arrows indicating sites of pre-steady-state selective irradiation, (B) selective irradiation of H^1 of G, (C) selective irradiation of H_a of C, (D) selective irradiation of H_b of C. The resonance marked with an asterisk at 7.24 ppm is the nondeuterated chloroform contaminant.

different processes: dynamic polarization via dipolar coupling (i.e., NOE) and chemical exchange. The specific mechanism in each case (dipolar coupling or exchange) was determined by varying temperature (a paper describing the dynamics of this system is in preparation).

1. Base Pairs. With the system described here, it has been possible with ^{1}H NMR to investigate the C:G base pair over a temperature range of 123 °C. When equimolar amounts of C and G are mixed in chloroform-*d*, the resonances of the protons involved in Watson–Crick hydrogen bonding move downfield as expected from previous studies in dimethyl sulfoxide.[1-3] In chloroform-*d*, each of the five nitrogen-bound protons of the C:G base pair is clearly resolved at low temperatures (<−25 °C, 30 mM of each species). In this temperature range, amino groups of both C and G are in slow rotation on the NMR time scale. As described in the following sections, the simultaneous observation of all five N–H resonances of the base pair allows us to determine which hydrogen-bonded conformations exist in solution.

In Figure 3A (at −57 °C), the three downfield ^{1}H NMR resonances correspond to the three N–H protons involved in hydrogen bonding (see Figure 1 for labeling scheme). The resonance of the imino proton of G (H^1) is observed at 14.04 ppm. The resonances at 9.52 and 8.97 ppm have been assigned (see below) to the hydrogen-bonded amino protons, H_a of C and H_c of G, respectively. The two non-hydrogen-bonded amino proton resonances, H_b of C and H_d of G, are observed at 6.73 and 4.94 ppm, respectively.

As can be seen in the difference spectrum in Figure 3B, selective saturation of the H^1 resonance caused the intensities of three of the four remaining N–H resonances to decrease (the apparent change in intensity of H^8 is an instrumental artifact). Decreases in the intensities of H_a and H_c from selective saturation of H^1 are NOE's and are not caused by chemical exchange. In a three-spin effect, polarization of H_c was transferred to H_d via chemical exchange (by rotation about the amino bond of G).

The NOE from H^1 of G to H_a of C confirms formation of Watson–Crick type dimers. Further support for dimer formation was provided by selective saturation of H_a of C (Figure 3C). The NOE's between H_a of C and H^1 of G are expected from the proximity of these two protons in the dimer. In addition, there

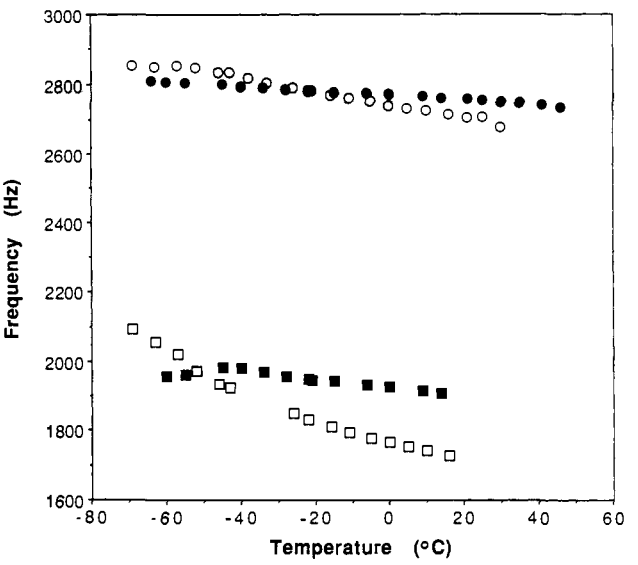

Figure 4. Plot of frequency versus temperature of the H_a (circles) and H_b (squares) resonances of C in 1:1 mixtures (hollow) and 1:2 mixtures (full) of C and G.

was an intramolecular NOE from H_a and H_b and a smaller, three-spin effect through H_b to H^5. Selective saturation of H_b (Figure 3D) caused NOE's to H^5 and H_a.

The difference in the resonance frequencies of H_c and H_d (over 1100 Hz at 300 MHz) is large considering that these two protons are covalently bound to the same nitrogen. The chemical environments of these two protons are radically different due to hydrogen bonding of H_c to O^2 of C and steric protection of H_d by the substituted ribose moieties, which form an equivalent of the DNA minor groove.

The NOE data shown here confirm both the formation of the C:G dimer in chloroform-*d* and the resonance assignments of Figure 3A. Inter- and intramolecular NOE's were observed between protons expected to be located nearby in space in the base pair. However, as described in the following section, we have observed convincing evidence that hydrogen-bonded structures in addition to the C:G base pair form readily in mixtures of C and G in chloroform-*d*.

2. Tetramers. The complementary arrangement of hydrogen bond donors (H_b of C) and acceptors (N7 of G) suggests that pairs of Watson–Crick type dimers can associate to form tetramers (Figure 1B). Such hydrogen-bonded tetramers have been observed previously in crystals[19] obtained from 1:1 mixtures of G and C. Here, we report chemical shift evidence that C and G can also form tetramers in chloroform-*d* solution.

In 1:1 mixtures of C and G (30 mM C plus 30 mM G), the H_b proton of C appears to be involved in hydrogen bonding. As the temperature decreases from +16 to −69 °C, this resonance moves downfield by over 350 Hz (over 1.2 ppm at 300 MHz; Figure 4). The temperature dependence of the H_b resonance frequency is consistent with increasing tetramer formation as temperature decreases. Similarly, the concentration dependence of H_b resonance frequency is consistent with increasing tetramer formation as the total concentration increases (data not shown). The involvement of the H_b proton in hydrogen bonding supports formation of $(C:G)_2$ tetramers. This phenomenon cannot be explained by dimer formation alone.

3. Trimers. The complementary arrangement of hydrogen bond donors (H^1 and H_c of G) and acceptors (O^6 and N7 of G) suggests that a second G can associate with a Watson–Crick C:G base pair to form a trimer of C:G:tG (tG refers to the guanine in the extra-Watson–Crick orientation). The two possible conformations of C:G:tG are shown in parts C and D in Figure 1. Formation of either C:G:tG conformation would be favored in 1:2 mixtures of C and G. Trimer formation would exclude $(C:G)_2$ tetramer formation (Figure 1B). Hence, certain conditions should promote tetramer formation (i.e., 1:1 mixtures, above), and certain con-

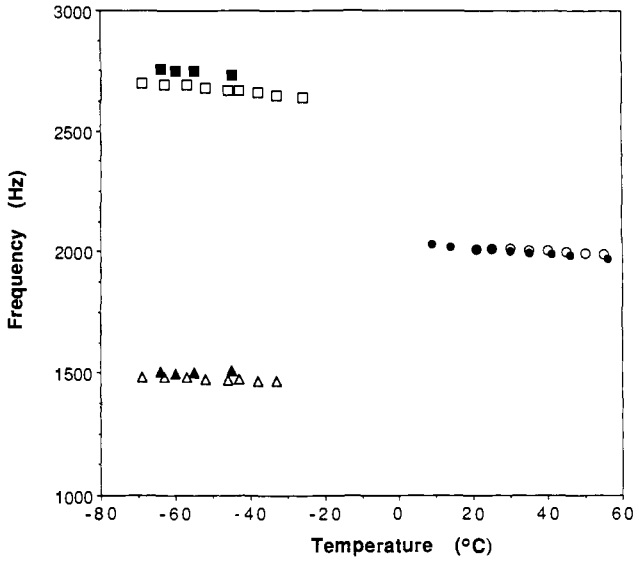

Figure 5. Plot of frequency versus temperature of the coalesced amino resonances of G (circles) and the distinct H_c (squares) and H_d (triangles) resonances in 1:1 mixtures (hollow) and 1:2 mixtures (full) of C and G.

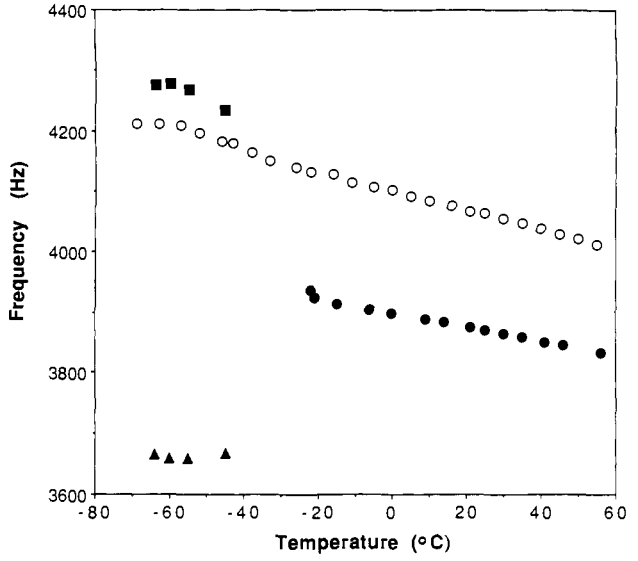

Figure 6. Plot of frequency versus temperature of the H^1 resonances of G in 1:1 mixtures (open circles), G in 1:2 mixtures (full circles), and the distinct H^1 resonances of G (squares) and the tG (triangles) resonances.

ditions should exclude tetramers and promote trimers (i.e., 1:2 mixtures of C and G). Therefore, to study C:G:tG, we combined C and G in a 1:2 ratio (30 mM C plus 60 mM G in chloroform-*d*).

Due to tetramer formation as described above, the frequency of the H_b resonance was sensitive to temperature in 1:1 mixtures of C and G. In contrast, under conditions expected to promote trimer formation and exclude tetramer formation (i.e., 1:2 mixtures of C and G), the H_b resonance of C was relatively insensitive to temperature. As the temperature decreases from +14.0 to −64 °C, the frequency of the H_b resonance does not change significantly (Figure 4). This lack of change in frequency with temperature in 1:2 mixtures is expected if formation of trimers precludes formation of tetramers. The protection of the H_b proton from hydrogen bonding in 1:2 mixtures is consistent with formation of C:G:tG in 1:2 mixtures and $(C:G)_2$ in 1:1 mixtures.

The chemical shift data can be used to infer if one of the orientations of C:G:tG (Figure 1C,D) predominates in solution. As described below, the results suggest that one conformation is favored.

In the experiments with 1:1 mixtures, the exchange of H_c and H_d is a two-site process. Above 21 °C, the amino bond of G is in fast rotation on the NMR time scale and a single amino resonance (Figure 5) is observed for H_c and H_d. Below −26 °C, the amino bond of G is in slow rotation on the NMR time scale such that H_c and H_d are observed as distinct resonances (Figure 5). The lack of temperature effect on the frequencies of these resonances (except for the effects of exchange) indicates 100% base pairing, even at high temperatures. Comparing the temperature effect (in 1:1 mixtures) on the amino group of G (Figure 5) with the effect on the amino group of C (Figure 4) illustrates the essentially complete dimer formation at all temperatures (invariant chemical shifts of G) whereas tetramer formation is temperature dependent (variable chemical shifts of C).

In the experiments with 1:2 mixtures, exchange of the four amino protons (of G and tG) is a four-site process. The first process involves rotations about the amino bonds (of G and tG) such that each H_c exchanges with the adjacent H_d. The second process involves exchange of G with tG. The exchange H_c of G with H_c of tG is always fast on the NMR time scale, and distinct H_c resonances of integral 1 were not observed. Above 9 °C, at least one of the amino bonds (of G or Gt) is in fast rotation on the NMR time scale and a single resonance (of integral 4, Figure 5) is observed for H_c and H_d. The single amino peak in this temperature realm is the resonance of four rapidly exchanging protons: H_c and H_d of G; H_c and H_d of tG. However, below −45 °C, both amino groups of G and tG appear to be in slow rotation on the NMR time scale such that H_c and H_d are distinct resonances even though G to tG exchange is fast. In this slow-rotation realm, the observed resonance frequency of H_c (Figure 5) should be the average of the frequencies of H_c of G and of H_c of tG. The frequency of H_d (Figure 5) is also an average.

The two hydrogen bond acceptors for H_c in C:G:tG are chemically similar. In 1:1 mixtures, essentially 100% of H_c protons will bond with O^2 (of C) hydrogen bond acceptors. In 1:2 mixtures, only 50% of the H_c protons will bond to O^2. The remaining 50% (i.e., the H_c protons of tG) bond to either O^6 or N^7 of G. However, the three limiting frequencies (of the coalesced amino group, H_c, and H_d) show striking similarities in 1:1 compared to in 1:2 mixtures (Figure 5). Although in C:G:tG the tG amino resonances cannot be observed independently of those of G, we can infer that the resonance frequency of H_c is nearly the same in G as in tG and the frequency of H_d is also nearly the same in G and as in tG. This analysis suggests that the chemical environment of the amino group of tG is very similar to that of G. In Figure 1C, both H_c's form hydrogen bonds to a carbonyl oxygen (O^2 of C and O^6 of G). Further, both N^1's form hydrogen bonds to basic nitrogens and both H_d's are free from hydrogen-bonding interactions. The resonance frequencies of the amino protons of G are most consistent with formation of C:G:tG as shown in Figure 1C.

In addition to the amino exchange process, in 1:2 mixtures the H^1 proton of G exchanges between two sites as G exchanges with tG. This exchange is fast on the NMR time scale above −22 °C and is slow on the NMR time scale below −40 °C (Figure 6). Thus, in 1:2 mixtures above −22 °C, the observed frequency of the H^1 resonance is the average of the frequencies of H^1 of G and H^1 of tG. However, below −40 °C, the frequency of H^1 at each of the two sites can be determined.

As shown in Figure 6, the downfield site resonates at a frequency near the frequency of H^1 in 1:1 mixtures. Thus, it is very likely that the downfield resonance in 1:2 mixtures is H^1 of G while the upfield resonance is the H^1 of tG. The large difference in the frequency of H^1 of G versus that of tG (over 500 Hz) suggests some basic difference in chemical environment of the two H^1 protons of G:G:tG. As can be seen in parts C and D in Figure 1, one significant difference between G and tG (in either conformation of C:G:tG) is the absence of the hydrogen bond to the O^6 of tG.

Additional support for formation of C:G:tG in solution has been provided by our previous calorimetric studies,[10] indicating that heats of dimerization when [G] > [C] were not consistent with heats of dimerization when [G] < [C], and by ^1H NMR continuous variation plots, suggesting formation of $C:G_{n>1}$ complexes,[10] although there the stoichiometry of interaction was am-

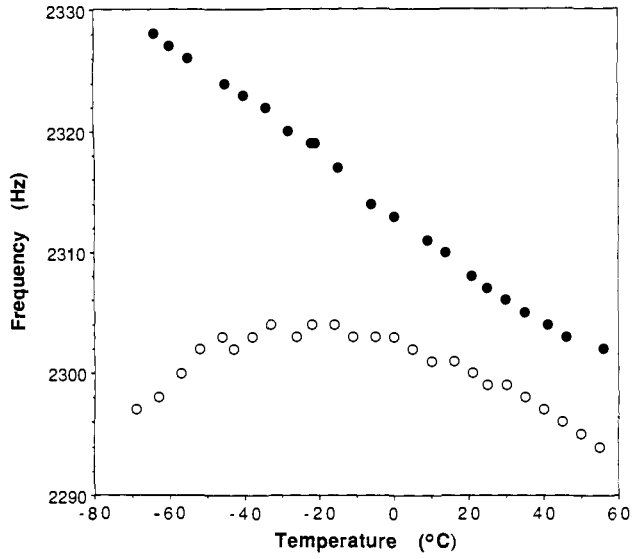

Figure 7. Plot of frequency versus temperature of the H^8 resonances of G in 1:1 mixtures (hollow circles) and 1:2 mixtures (full circles of C and G.

biguous. It is not possible to definitively determine whether one conformation (Figure 1C,D) is favored over the other. However, the data presented here support formation of C:G:tG as shown in Figure 1C.

4. Stacking. Vertical stacking interactions predominate in water,[14] and to our knowledge, there have been no previous reports that nucleosides stack in nonaqueous solvents. However, in the experiments described here, some evidence for stacking at low temperatures in chloroform-*d* was observed. In 1:1 mixtures C and G, the H^8 proton shows a biphasic change in frequency with temperature (Figure 7). In the lower temperature realm (below −46 °C), the H^8 moves to lower frequency as temperature decreases. This observation is in analogy with the extensive shielding of H^8 observed previously from stacking in aqueous solution.[14] The shielding of H^8 observed here may suggest a slight amount of stacking of the bases in chloroform-*d*.

Discussion

The Watson–Crick base pairs remain a most intriguing example of hydrogen bonding. Recognition of intact base pairs by external agents is thought to involve hydrogen bonding at non-Watson–Crick sites that are accessible from the major groove of double-stranded DNA.[21] Thus, proteins can recognize DNA sequence by specific hydrogen bonding of amino acid side chains to sites in the major groove of DNA.[22] Single- and possibly double-stranded nucleic acids also form hydrogen bonds to the same groove, resulting in sequence specific three- and four-stranded structures.[23-27]

The interactions stabilizing such large and complex biological structures have an accessible physical and chemical basis. Indeed, we have observed three types of hydrogen-bonded complexes between monomers in chloroform-*d* solution: (a) the previously observed *dimer*, the C:G Watson–Crick base pair as shown in Figure 1,[1-10] (b) a *tetramer* (formed by two base pairs) stabilized by two additional hydrogen bonds in a 2-fold symmetric pair of C:G base pairs (shown in Figure 1B), and (c) a *trimer*, (C:G:tG) stabilized by two hydrogen bonds between a G which is in a Watson–Crick base pair and a second G in a Hoogsteen-like orientation (shown in Figure 1C,D). Our results suggest that the conformation of Figure 1C may be favored over that of Figure 1D.

In the simple model system described here, hydrogen bonds are observed (to sites on Watson–Crick base pairs) that would stabilize specific three- and four-stranded nucleic acid complexes. The trimer (C:G:tG) observed in chloroform-*d* is analogous to that proposed for the triple-stranded rC·rG·rG[25] and observed crystallographically in yeast phenylalanine tRNA.[18] The tetramer has been proposed previously to explain DNA–DNA recognition processes.[27,28] With the model system described here, the behavior of monomers can thus be used to understand, and hopefully to predict, that of biopolymers.

The patterns of hydrogen bonding in dimers, trimers, and tetramers formed between nucleic acid monomers in solution here appear to correspond with patterns of hydrogen bonding in nucleic acid polymers. This correspondence of monomeric interactions with those in polymers underscores the importance of hydrogen bonding in the specificity and stability of a variety of DNA secondary structures.

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