Structure and Dynamics of Interstrand Guanine Association in Quadruplex Telomeric DNA†

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ABSTRACT: Exchanges of the amino and imino protons in guanine quartets of telomeric DNA have been time-resolved by laser Raman spectroscopy. The Raman dynamic probe has been applied to parallel and antiparallel quadruplexes formed by the telomeric repeat of *Oxytricha nova*, $d(T_4G_4)_4$, and to the highly thermostable parallel quadruplex formed by $d(G_{12})$. Time-dependent Raman spectra of the $d(G_{12})$ quadruplex reveal two characteristic exchange reactions for guanine N2 amino groups. At 10 °C, the pseudo-first-order exchange rates are $k_{N2H}(10\ °C) = 5.7 \times 10^{-3}$ and $k'_{N2H'}(10\ °C) = 1.2 \times 10^{-2}$ min^{-1}, assignable to Hoogsteen-hydrogen-bonded and non-hydrogen-bonded N2 protons, respectively. These measurements provide the first quantitative determination of two kinetically distinct N2 amino proton exchange reactions in the guanine quartet and demonstrate that amino group rotation about the C2−N2 bond is highly restricted in the quadruplex. No exchange of guanine N1 imino sites occurs in $d(G_{12})$ at 10 °C, and N1 exchange remains slow even at 95 °C [$k_{N1H}(95\ °C) = 2.7 \times 10^{-2}$ min^{-1}], indicating severe suppression of imino exchange in guanine quartets. For both parallel and antiparallel quadruplexes of $d(T_4G_4)_4$, proton exchange rates decrease in the order thymine N3 imino > guanine N2 amino > guanine N1 imino. The rapid exchange of thymine N3 imino sites indicates that thymine quartets are not stabilized in *Oxytricha* quadruplexes. The protium → deuterium exchange experiments also establish new guanine Raman band assignments. Importantly, the 1603 cm^{-1} band is due to in-plane bending of N1−H, while the 1644 cm^{-1} band involves scissoring of the N2 amino group. Accordingly, the 1603 and 1644 cm^{-1} bands are potentially valuable markers of hydrogen-bonding interactions specific to guanine imino and amino sites, respectively. The present findings also show that guanine hydrogen bonding and exchange dynamics are not interrelated in a simple manner. Despite extraordinary retardation of N1 imino proton exchange, Raman markers suggest that Hoogsteen-type guanine−guanine hydrogen bonding (N1−H···O=C6) is comparable in strength to hydrogen bonding of N1−H with water and, surprisingly, much weaker than hydrogen bonding between N1−H and the cytosine N3 acceptor of Watson−Crick B DNA.

Telomeres, the termini of linear eukaryotic chromosomes, are characterized by tandem repeats of guanine-rich sequences which extend beyond the 5′ end of the complementary DNA strand. The 3′ overhang, or telomere tail, is capable of forming a four-stranded (quadruplex) structure in which guanine quartets are linked by Hoogsteen hydrogen bonding as shown in Figure 1. Stacked arrays of guanine quartets are strongly stabilized by favorable π-electron interactions and coordination of metal ions with exocyclic carbonyls. A particularly striking stabilization effect has been noted for the potassium ion. This is demonstrated by the ability of K^+ at physiological concentration to disproportionate the Watson−Crick duplex, $d(C_4A_4C_4)$·$d(G_4T_4G_4)$, into a parallel-stranded quadruplex of $d(G_4T_4G_4)$ plus single-stranded $d(C_4A_4C_4)$ (Miura & Thomas, 1994) and also by the efficacy of K^+ in promoting interquadruplex conversions (Miura et al., 1995).

A widely studied telomeric sequence is that of the ciliate *Oxytricha nova*, $d(T_4G_4)$. In the tandem repeat $d(T_4G_4)_4$ (Oxy-4),[1] the thymine tracts facilitate formation of an antiparallel fold-back quadruplex at appropriate experimental conditions [reviewed by Williamson (1994)]. Polymorphism in telomeric DNA and particularly the interconversion between antiparallel and extended (parallel) configurations is governed by the type and concentration of alkali metal ion (Sen & Gilbert, 1988; Miura et al., 1995). Additional structural details of *Oxytricha* telomeric DNA have been

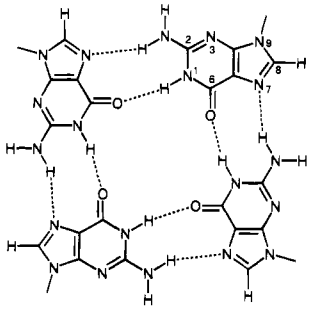

FIGURE 1: Structure of the guanine quartet stabilized by N1−H···O=C6 and N2−H···N7 Hoogsteen hydrogen bonds.

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[1] Abbreviations: D, ^{2}H or deuterium isotope of hydrogen; $d(G_{12})$, quadruplex structure of deoxyguanylate dodecamer; dGMP, deoxyguanosine 5′-monophosphate; dCMP, deoxycytidine 5′-monophosphate; Oxy-4, $d(T_4G_4)_4$; rGMP, guanosine 5′-monophosphate; UVRR, ultraviolet resonance Raman.

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revealed by methods of X-ray crystallography (Kang et al., 1992; Laughlan et al., 1994), NMR spectroscopy (Smith & Feigon, 1993; Wang & Patel, 1993; Wang et al., 1994; Aboul-ela et al., 1994), and Raman spectroscopy (Miura & Thomas, 1994).

In the present work, we apply Raman spectroscopy as a probe of the dynamics of telomeric DNA by monitoring the hydrogen isotope exchange kinetics of guanine amino and imino groups in the Hoogsteen-hydrogen-bonded quartet (Figure 1). Measurement of the kinetics of protium−deuterium (H−D) exchanges can provide valuable insights into the hydrogen-bonding states of nucleic acid bases in solution (Englander & Kallenbach, 1984). Although Raman spectroscopy is generally well suited to such measurements (Reilly & Thomas, 1994), it has not been feasible previously to monitor the separate exchanges of guanine N1 imino and N2 amino protons, owing to the rapidity of the exchanges and the time resolution limits of conventional Raman instrumentation. However, the extraordinary rigidity of the DNA quadruplex and recent improvements in experimental design provide a situation amenable to time resolution of the guanine imino and amino exchanges by Raman spectroscopy.

The Raman dynamic probe is applied here to investigate both H → D and D → H exchange reactions of dG residues in the antiparallel and parallel quadruplex structures of Oxy-4 and in the parallel quadruplex structure of $d(G_{12})$. We have also investigated related model systems as controls to assist in the interpretation of the static and dynamic Raman results. The spectroscopic data are interpreted in terms of the structures and exchanges of N1 imino (dG), N2 amino (dG), and N3 imino (dT) groups of telomeric DNA. The present findings provide new information on the hydrogen-bonding states of guanine and thymine residues in telomeric DNA quadruplexes. The results also define specific Raman marker bands which can be exploited for the characterization of guanine imino and amino interactions in other DNA assemblies. Additionally, we have identified several Raman bands of the dG and dT nucleosides which had not been assigned previously but which should be valuable as nucleoside conformation markers in structural studies of nucleic acids by Raman spectroscopy.

MATERIALS AND METHODS

Synthesis and Purification of DNA. The oligodeoxynucleotides $d(G_{12})$ (MW 3890) and $d(T_4G_4)_4$ (Oxy-4; MW 10 050) were synthesized on an Applied Biosystems Model 381A DNA synthesizer. Purification of DNA was performed on an ISCO Model 2350 HPLC system, employing a Hamilton PRP-1 (250 × 4.1 mm) reversed-phase column. The instrument was equipped with an ISCO V^4 variable wavelength absorbance detector, a Model 2360/2361 gradient programmer and a 250-mL injection loop, and it was controlled with ISCO Chemsearch chromatographic data management software. The triethylamine acetate−acetonitrile buffer was prepared by using a ternary gradient in which the mobile phase contained 0.2 M triethylamine acetate (TEA-Ac) at pH 7.0, distilled water, and 99.9% acetonitrile. A flow rate of 1.0 mL/min was used. The column was heated at 90 °C to ensure elimination of DNA secondary structure during separation. The eluted peaks were monitored by UV absorbance at 297 nm. Appropriate peak fractions were pooled, frozen, and lyophilized. The purified oligonucleotides were desalted on Sephadex G-25 gel filtration columns (Pharmacia, NAP-5) or by dialysis.

Poly(dT) was purchased from Sigma Chemical Co. (St. Louis, MO) and was used without further purification.

Sample Preparation and Monitoring of H−D Exchange Reactions. An aqueous solution of $d(G_{12})$ (0.2 mM ≈ 0.78 mg/mL, pH 7.0) was incubated at 95 °C for 30 min to generate the parallel quadruplex structure (Miura & Thomas, 1994). A 20-μL aliquot of the solution was introduced into a glass capillary tube and lyophilized. The time course of H → D exchange was initiated upon mixing the lyophilized powder of $d(G_{12})$ in the capillary tube with 2 μL of 100 mM KCl/D_2O at 10 °C.

Oxy-4 solution (2 mM ≈ 20 mg/mL, pH 7.0) in either 100 mM KCl or 100 mM NaCl was incubated at 95 °C for 30 min followed by slow cooling to generate, respectively, either the parallel extended quadruplex or an antiparallel fold-back quadruplex structure, as described (Miura et al., 1995). A 2-μL aliquot of each solution was introduced into a glass capillary tube, lyophilized, and redissolved in 2 μL D_2O.

Each H−D exchange reaction at 10 °C was performed in the sample illuminator of the Raman spectrometer concurrent with collection of the Raman spectrum. Glass capillary tubes containing D_2O solutions of oligonucleotides were thermostated using a temperature-controlled block designed for the 90° scattering geometry (Thomas & Barylski, 1970). On the other hand, the H−D exchange reaction at 95 °C was performed in a water bath prior to Raman measurements. The sample solutions were cooled slowly and Raman spectra were collected at 10 °C.

Raman Spectroscopy. Raman spectra were excited with the 514.5-nm line of an argon ion laser (Innova-70, Coherent Inc., Santa Clara, CA) using 250 mW of radiant power at the sample. The spectra were collected on a triple spectrograph (Triplemate Model 1877, SPEX Ind., Metuchen, NJ) equipped with a liquid nitrogen cooled, charge coupled device detector (Model LN-CCD-1152UV, Princeton Instruments, Princeton, NJ) of 1152 × 298 pixels operating under microcomputer control. The effective spectral slit width was 5 cm^{-1}.

Raman frequencies were calibrated using the spectrum of liquid indene. The frequencies cited are believed accurate to within ±0.5 cm^{-1} for sharp bands and ±2 cm^{-1} for broad or poorly resolved bands.

RESULTS AND CONCLUSIONS

H−D Exchange Kinetics of $d(G_{12})$. The Raman spectrum of $d(G_{12})$ dissolved at 2 mM (7.8 mg/mL) in H_2O containing also 100 mM KCl is shown at the top of Figure 2. (Hereinafter, we employ the notation /H_2O or /D_2O to indicate either protiated or deuteriated water, respectively, as the aqueous solvent.) Several structural features of $d(G_{12})$/H_2O can be identified from this Raman spectrum: (i) The 1478 cm^{-1} band indicates that guanine N7 acceptor sites are involved in strong hydrogen-bonding interactions (Hoogsteen quartets). (ii) Deoxyguanosine residues of $d(G_{12})$ adopt the C2′-*endo*/*anti* conformation exclusively (685 and 1337 cm^{-1} marker bands). (iii) The backbone of $d(G_{12})$ exhibits phosphodiester geometry similar to that of B DNA (836 and 1090 cm^{-1} markers). The basis for each of these structural conclusions is as described previously (Miura &

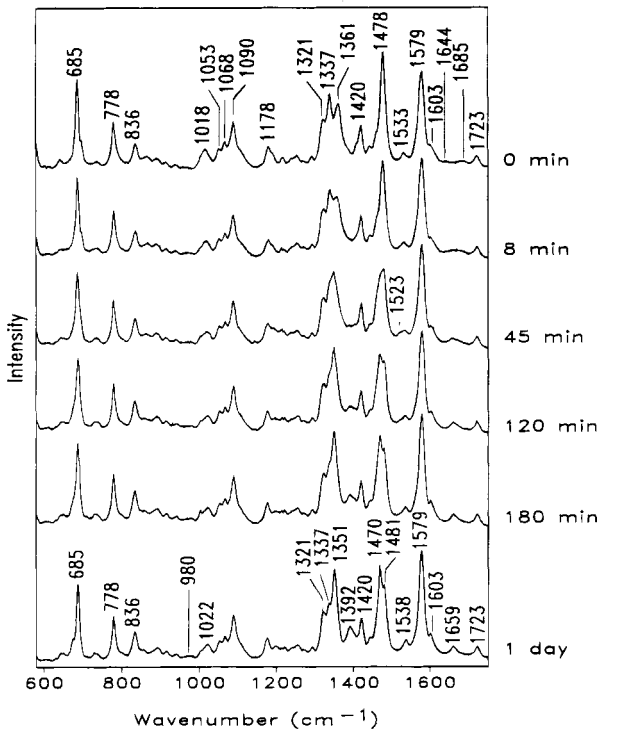

FIGURE 2: Time-resolved Raman spectra (580−1750 cm^{-1}) of the parallel quadruplex structure of $d(G_{12})$ in 100 mM KCl/D_2O at 10 °C (2 mM oligonucleotide, pD 7.0). The top spectrum (0 min) was recorded in H_2O solution; all other spectra were recorded in D_2O solution following the indicated period of H → D exchange at 10 °C. Each spectrum required a data accumulation protocol of 8 min, and the time designated at the right represents the midpoint of the measurement. Labels indicate frequencies of prominent Raman bands (in cm^{-1} units) which are discussed in the text.

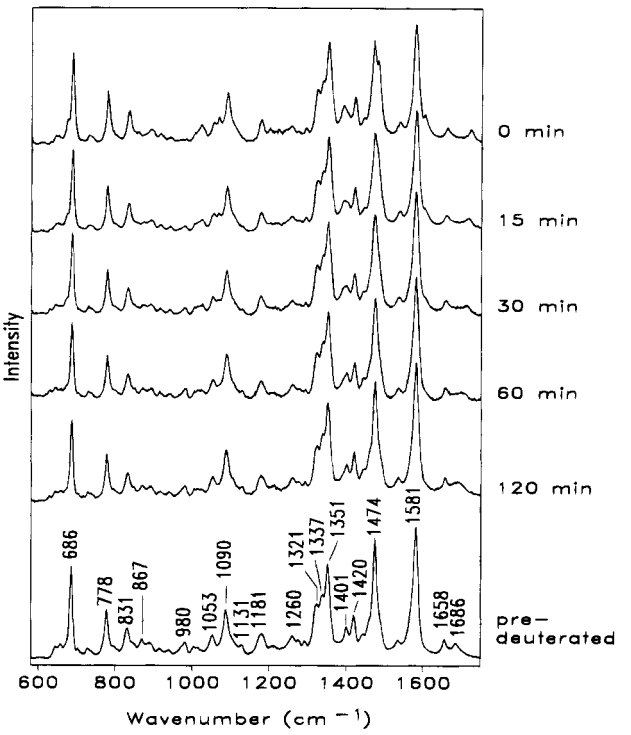

FIGURE 3: Time-resolved Raman spectra (580−1750 cm^{-1}) of the parallel quadruplex structure of $d(G_{12})$ in 100 mM KCl/D_2O solution at 95 °C (2 mM oligonucleotide, pD 7.0). The top spectrum (0 min) was obtained from $d(G_{12})$/D_2O that had not been heated to 95 °C, but had been preincubated for 48 h at 10 °C. The same sample was subsequently heated at 95 °C for 15, 30, 60, and 120 min, which in each case was followed by slow cooling and spectral measurement at 10 °C. The spectrum shown at the bottom was obtained from a sample which had been fully pre-deuteriated in pure D_2O and then lyophilized and redissolved in 100 mM KCl/D_2O.

Thomas, 1994). Collectively, these marker bands in the Raman signature of $d(G_{12})$ are characteristic of an *inter*strand parallel quadruplex, a structure which is clearly distinguished by Raman spectroscopy from an *intra*strand antiparallel fold-back quadruplex (Miura & Thomas, 1994).

The parallel quadruplex of $d(G_{12})$ is remarkably thermostable. No significant Raman band shifts, peak intensity changes, or band broadening could be detected for the $d(G_{12})$/H_2O spectrum (Figure 2, top) at temperatures up to 95 °C.

Figure 2 illustrates use of the Raman spectrum to monitor the H → D exchange reactions of guanine bases in the parallel quadruplex structure of $d(G_{12})/D_2O$ at 10 °C. Figure 3 shows subsequent monitoring of exchange in $d(G_{12})/D_2O$ at 95 °C. The observed spectral changes reflect deuterium substitution of *three* distinct classes of guanine protons, namely, the hydrogen-bonded N1 imino proton, the hydrogen-bonded N2 amino proton (hereinafter N2H), and the non-hydrogen-bonded N2 amino proton (N2H′) (Figure 1). As shown below, no detectable H → D exchange occurs for the guanine C8 proton, even at 95 °C, during the time course of the imino and amino exchange reactions of Figures 2 and 3.

At 10 °C, two kinetically distinct H → D exchange reactions are resolved by the time dependence of the Raman spectrum of $d(G_{12})/D_2O$ shown in Figure 2. The faster process, which is largely complete after 120 min of exposure to D_2O, is characterized by the dramatic Raman band frequency shift from 1361 to 1351 cm^{-1} and splitting of the band at 1478 cm^{-1} into components of greater and lesser intensity at 1470 and 1481 cm^{-1}. This is the most rapid exchange process which can be detected for the $d(G_{12})$ parallel quadruplex. It is ascribed to deuteriation of the exposed amino proton of guanine, i.e., to the exchange N2H′ → N2D′. Plots of the accompanying Raman intensity changes versus time yield a first-order rate constant at 10 °C of $k'_{N2H'}$(10 °C) = 1.2 × 10^{-2} min^{-1} (Table 1). The slower kinetic process revealed in Figure 2 is characterized by the growth of the band at 1392 cm^{-1}. The exchange to which this corresponds is substantially complete after 180 min. It is ascribed to deuteriation of the hydrogen-bonded amino proton (N2H → N2D), for which we obtain k_{N2H}(10 °C) = 5.7 × 10^{-3} min^{-1} (Table 1).

Table 1: Rate Constants for Deuterium Exchange of Amino and Imino Guanine Sites in the $d(G_{12})$ Quadruplex

temp (°C)	k (min^{-1})	exchange site	Raman marker (cm^{-1})
10	1.24 × 10^{-2}	N2H′	1361
10	5.71 × 10^{-3}	N2H	1392
95	2.63 × 10^{-2}	N1H	1723
95	2.73 × 10^{-2}	N1H	980
10	1.5 × 10^{-6}	N1H	*a*

[a] Calculated value assuming an Arrhenius activation energy of 23.6 kcal/mol (Benevides et al., 1984).

In the ^{1}H-NMR spectrum of the parallel quadruplex structure of d(TTAGGG), both the N2H and N2H′ protons of a guanine base give characteristic NMR resonances separated by about 2.4−3.5 ppm (Wang & Patel, 1992), consistent with their different hydrogen-bonding environments; i.e., N2H is involved in guanine−guanine Hoogsteen

hydrogen bonding (N2−H···N7), whereas N2H′ is exposed to solvent. Although the appearance of two proton resonances is suggestive of amino group rotation slower than the NMR time scale of microseconds, different H−D exchange rates for the two amino protons have not been measured previously in telomeric quadruplexes. In the present Raman spectroscopic study, significantly different exchange rates, 5.7×10^{-3} min^{-1} for N2H and 1.2×10^{-2} min^{-1} for N2H′, are detected at 10 °C for the parallel quadruplex structure of d(G_{12}), indicating essentially no rotation of amino groups in this structure.

The Raman spectrum recorded 48 h after initiation of H → D exchange at 10 °C (Figure 3, top trace) is virtually identical to that after 24 h (Figure 2, bottom trace), demonstrating no further H → D exchange of d(G_{12}) at 10 °C. However, additional changes in the Raman spectrum of d(G_{12}) are detected after heating the sample to 95 °C (middle four traces of Figure 3), signifying further H → D exchange of guanine at the elevated temperature. It should be noted that all of the spectra of Figure 3 were recorded at 10 °C, following the 95 °C heating cycle described in the figure caption. The diagnostic spectral effects include several shifts of Raman band frequencies (674 → 659, 1392 → 1401, and 1723 → 1686 cm^{-1}), various intensity changes (980, 1068, and 1131 cm^{-1}), and fusion of the 1470/1481 cm^{-1} doublet into a single band at 1474 cm^{-1}. Despite the high temperature, all of the deuteriation effects of Figure 3 proceed slowly and are attributed to the reaction N1H → N1D. The computed rate constant is k_{N1H}(95 °C) = 2.7×10^{-2} min^{-1} (Table 1). For comparison with the amino exchanges measured at 10 °C, we estimate a value for k_{N1H} at 10 °C of 1.5×10^{-6} min^{-1} (Table 1). This estimate is based upon the assumption that the Arrhenius activation energy for the imino exchange is similar to the value of 23.6 kcal/mol measured for other nucleotide exchanges (Benevides et al., 1984).

Very slow imino proton exchange has been reported in recent ^{1}H-NMR studies of guanine quadruplexes (Wang & Patel, 1992; Smith & Feigon, 1992; Gupta et al., 1993). For example, imino proton signals from internal bases of the guanine cluster in the parallel quadruplex structure of d(T_2G_4) do not disappear after 2 days in D_2O at 5 °C, in sharp contrast to the very rapid disappearance of all amino proton signals (Wang & Patel, 1992). Even at 60 °C, imino proton exchanges are slow in the parallel quadruplex of d(T_4G_4)$_4$ (Gupta et al., 1993).

Slow deuteriation of the guanine C8H site is also possible in D_2O solutions of the oligonucleotides examined here and requires consideration at elevated temperatures. Since Raman markers of the C8D isotopomer of dG are well known (Lane & Thomas, 1979; Benevides et al., 1984), we can exclude by the data of Figure 3 any appreciable C8 deuteriation in d(G_{12}). Thus, both the sample heated for 120 min at 95 °C and the pre-deuteriated control lack the strong marker band expected at 1460 cm^{-1} for the C8D form of guanine (Lane & Thomas, 1979). The present data are consistent with C8H exchange studies of other N7 coordinated guanine and adenine complexes (Benevides & Thomas, 1985) which show resistance of C8H to deuteriation whenever N7 is involved in strong and specific hydrogen bonding. The apparent retardation of exchange of C8H protons in d(G_{12}) is consistent with a quadruplex structure in which guanine−guanine hydrogen bonds are of the Hoogsteen type.

Table 2: Deuterium Isotope Shifts of Raman Marker Bands of Guanine in the d(G_{12}) Quadruplex Structure

Raman marker (cm^{-1}) G-d_0	isotope shift (cm^{-1}) G-d_1	G-d_2	G-d_3
1723	−37	0	−37
1644	+4	−252	−243
1603	−458	0	−472
1579	0	0	+2
1533	+4	+5	+2
1478	−1	+3, −8	−4
1420	0	0	0
1361	−1	−10	−10
1337	0	0	0
1321	0	0	0
1255	−4	0	+5
1178	+10	0	+2
1068	*a*	0	−88
1053	0	0	0
697	−27	−23	−38
685	+5	0	+1

[a] No band observed at 1068 cm^{-1} in G-d_1.

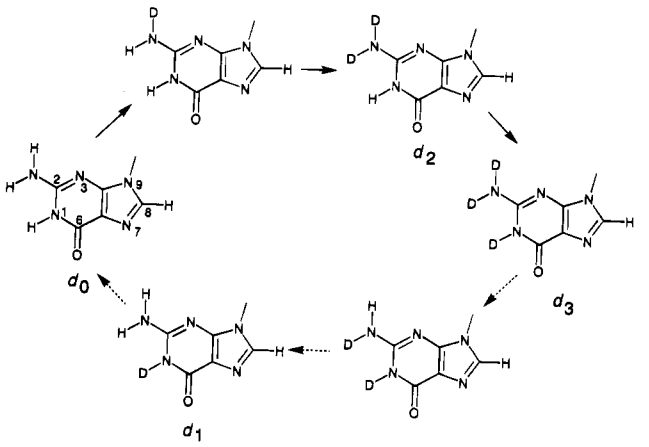

FIGURE 4: H → D (solid arrows) and D → H (dashed arrows) exchange schemes for guanine bases in the quadruplex structure of d(G_{12}).

A tabulation of deuteriation-sensitive Raman bands of d(G_{12}) is given in Table 2, and more specific assignments for these bands are given in the Discussion section below.

Pathway of Hydrogen Isotope Exchange in the Guanine Quartet. The d(G_{12}) quadruplex, containing fully deuteriated N1 imino and N2 amino sites, was lyophilized and redissolved in H_2O solution to promote D → H reverse exchange reactions. These back-exchanges were also monitored by Raman spectroscopy (data not shown) using procedures similar to those described in the preceding section. The observed spectral changes indicate D → H substitutions in the following kinetic order: non-hydrogen-bonded amino (N2D′ → N2H′) > hydrogen-bonded amino (N2D → N2H) > hydrogen-bonded imino (N1D → N1H), consistent with the observed H → D exchange kinetics. Accordingly, the overall scheme for H → D and D → H exchange reactions of the guanine quartet can be depicted as shown in Figure 4.

In Figure 5 we compare Raman spectra of d(G_{12}) isotopomers consisting of the following site-specific guanine deuterioisomers: G-d_0 in which the imino and both amino sites are protiated, G-d_1 in which only N1 is deuteriated, G-d_2 in which both N2 amino sites are deuteriated, and G-d_3 in which the imino and both amino sites are deuteriated. The

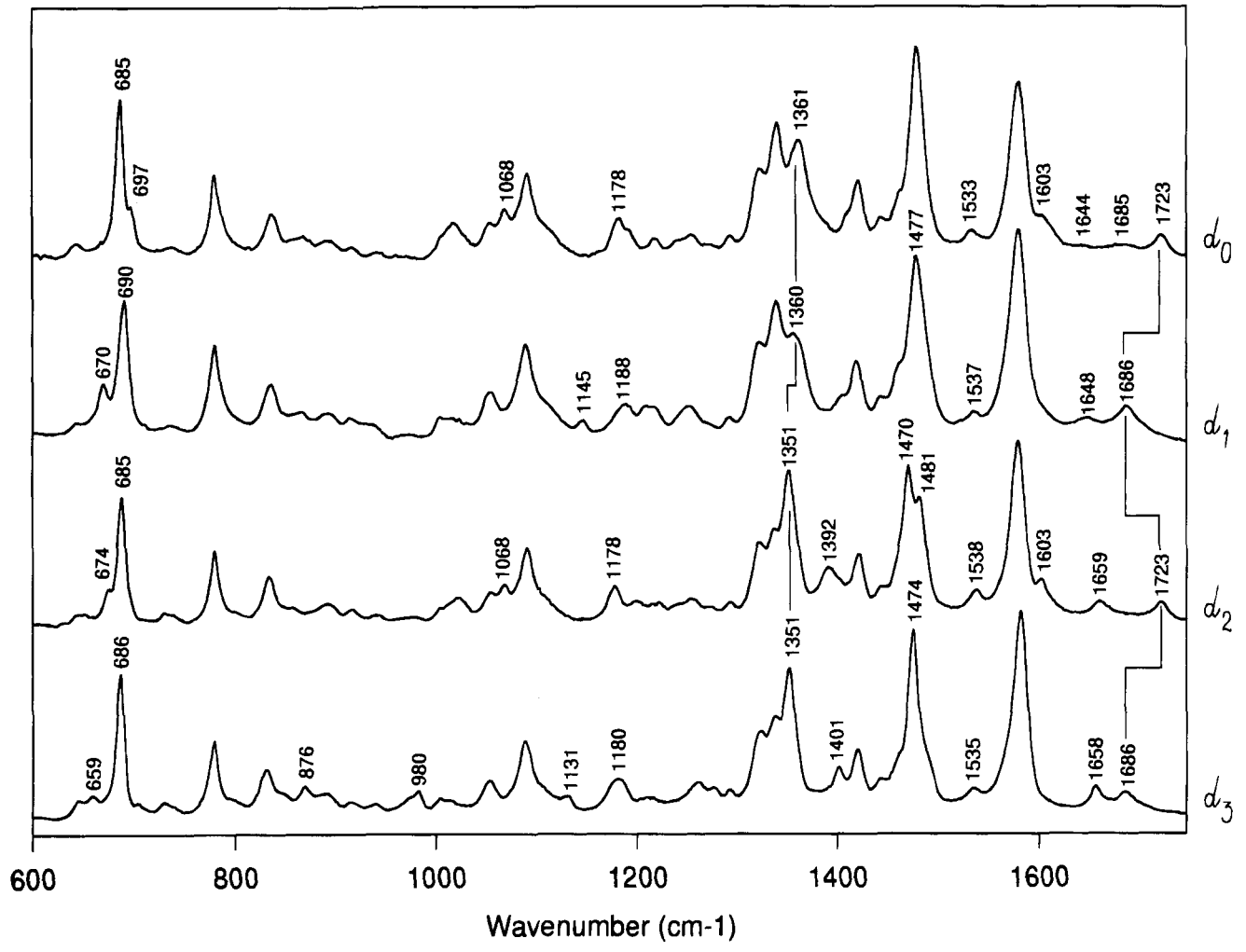

FIGURE 5: Raman spectra of quadruplex structures of d(G_{12}) in which the guanine residues contain no deuterium label (d_0), only N1 imino deuteriation (d_1), only N2 amino deuteriation (d_2), or both N1 imino and N2 amino deuteriations (d_3).

diagnostic Raman band shifts accompanying these site-specific isotope substitutions are summarized in Table 2.

Striking features of Figure 5 are the invariance of the 1723 cm^{-1} band to amino deuteriation in N1H isotopomers and the invariance of the 1686 cm^{-1} band to amino deuteriation in N1D isotopomers. Thus, the 1723 cm^{-1} band represents a *coupled* vibration involving the carbonyl bond (C6=O) stretching and imino (N1H) in-plane bending, but not involving the exocyclic amino group scissoring. Accordingly, the 1723 cm^{-1} band should serve as a valuable marker of the Hoogsteen N1—H···O=C6 hydrogen bonds which constitute the inner shell of hydrogen bonds in the quartet of Figure 1.

H—D Exchange Kinetics of the Parallel Quadruplex Structure of Oxy-4. The Raman spectrum of Oxy-4/H_2O containing 100 mM KCl is shown in the top trace of Figure 6. At these conditions Oxy-4 forms an extended quadruplex structure through parallel association of four oligonucleotide strands (Miura & Thomas, 1994). The Raman indicators of exclusively C2′-*endo*/*anti* dG conformers (684 and 1336 cm^{-1} markers) and B-type backbone phosphodiester conformation (835 and 1091 cm^{-1} markers) are consistent with recent X-ray crystallographic results on the parallel quadruplex structure of d(TG_4T) (Laughlan et al., 1994). Although Hoogsteen hydrogen bonding at guanine N7 sites is indicated by the 1481 cm^{-1} marker, the strength of hydrogen bonding may be somewhat weaker than that occurring in the parallel quadruplex of d(G_{12}), which exhibits a 1478 cm^{-1} marker (Figure 2, top trace). Raman bands at 666, 748, 1182, 1238, 1372, and 1663 cm^{-1} are all assigned to dT residues.

H → D exchange reactions of Oxy-4 were monitored initially at 10 °C (Figure 6) and subsequently at 70 °C (Figure 7). We consider first the exchange of thymine imino sites of Oxy-4, by reference to the model compound poly(dT). Poly(dT) exhibits clear-cut Raman frequency shifts of 666 → 652, 748 → 736, and 1185 → 1155 cm^{-1}, as well as large intensity increases at 1268 and 1306 cm^{-1} upon N3H → N3D exchange (spectra not shown). Corresponding changes in the Raman spectrum of the parallel Oxy-4 structure proceed rapidly at 10 °C. Figure 8 shows that the Oxy-4 difference spectrum following a 15-min exchange interval (top trace) is similar to the difference spectrum of poly(dT)/D_2O minus poly(dT)/H_2O (bottom trace), although the former contains additional difference bands in the 1350—1600 cm^{-1} region indicative of simultaneous guanine amino exchange. On the other hand, the Oxy-4 difference spectrum corresponding to the 45-min exchange interval between 60 and 15 min (Figure 8, middle trace) lacks any markers of dT imino exchange. Therefore, thymine N3H → N3D exchanges in the parallel quadruplex are complete within 15 min at 10 °C. The precise exchange rate for dT residues is not time-resolved by the

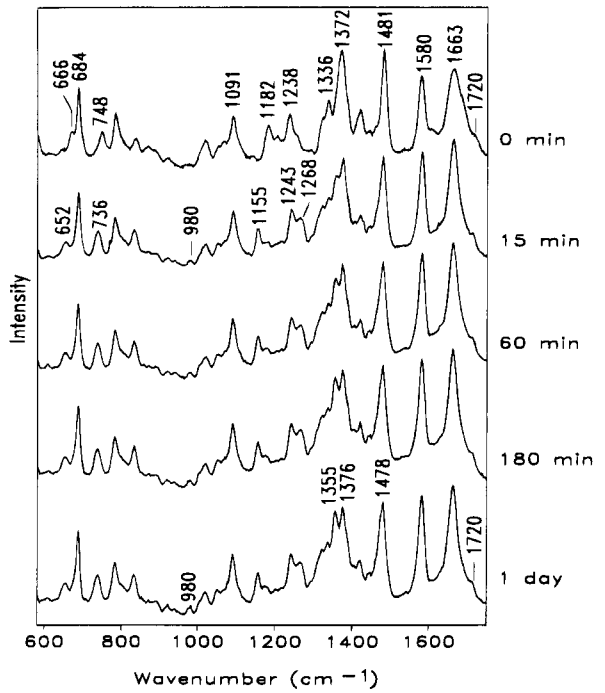

FIGURE 6: Time-resolved Raman spectra of the parallel quadruplex structure of Oxy-4 in 100 mM KCl/D_2O at 10 °C (2 mM oligonucleotide, pD 7.0).

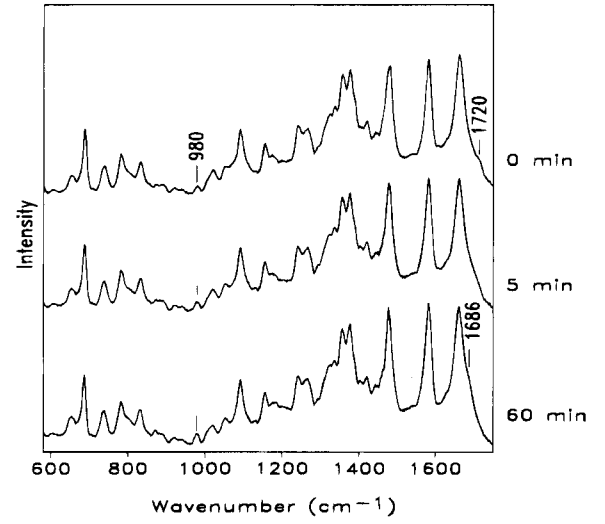

FIGURE 7: Time-resolved Raman spectra of the parallel quadruplex structure of Oxy-4 in 100 mM KCl/D_2O at 70 °C (2 mM oligonucleotide, pD 7.0). The top spectrum (0 min) was obtained from Oxy-4/D_2O that had not been heated to 70 °C but had been preincubated for 24 h at 10 °C. The same sample was subsequently heated at 70 °C for 5 and 60 min, which in each case was followed by slow cooling and spectral measurement at 10 °C.

method adopted in this study, which is limited to exchanges slower than 30 s.

With respect to guanine exchanges in the Oxy-4 parallel quadruplex, we use Raman marker bands of N1 imino and N2 amino deuteriations described in the previous section to reach the following conclusions from time-dependent spectra of Oxy-4 recorded at 10 °C (Figure 6) and 70 °C (Figure 7).

First, exchanges of guanine N2 amino protons are slow compared with thymine N3 imino exchange and proceed overall at rates comparable to those of $d(G_{12})$ (Table 1). Quantitative measurements are complicated by the fact that the marker of N2 amino deuteriation expected near 1363

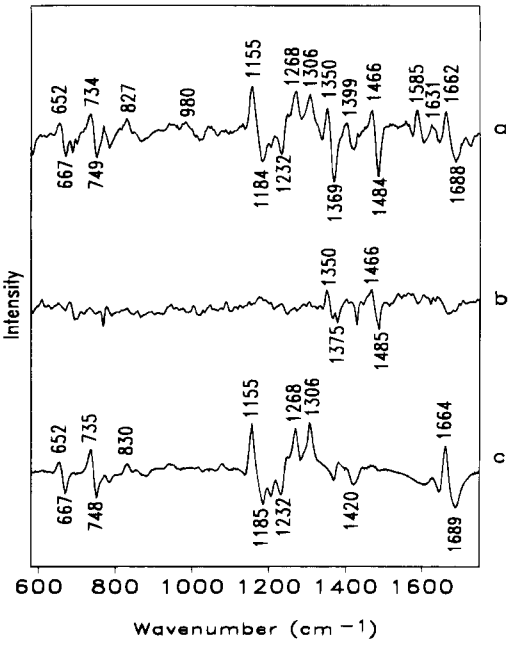

FIGURE 8: Difference Raman spectra of (a) Oxy-4/D_2O (15 min) minus Oxy-4/H_2O, from data of Figure 6; (b) Oxy-4/D_2O (60 min) minus Oxy-4/D_2O (15 min); and (c) poly(dT)/D_2O minus poly(dT)/H_2O. Constituent spectra for traces a and b are from Figure 6, and those for trace c are from unpublished spectra of the authors.

cm^{-1} is overlapped partially by the 1375 cm^{-1} band of dT in Oxy-4/H_2O (Figure 6, top trace). Nevertheless, in Oxy-4/D_2O, the band exhibits the anticipated shift to lower frequency, as is reflected by the gradual intensity increase at 1355 cm^{-1} with amino deuteriation. The pseudo-first-order exchange rate estimated from the 1355 cm^{-1} band increase is 3.0×10^{-3} min^{-1}, consistent with the mean value for amino exchanges of $d(G_{12})$ (Table 1). Isotopic shifts of guanine modes at 1363 and 1481 cm^{-1} are confirmed in the difference spectrum corresponding to 60 minus 15 min in D_2O (Figure 8, middle trace).

Second, exchange of guanine N1 imino protons proceeds in two distinguishable phases. At 10 °C, the 980 cm^{-1} band, which is a marker of both imino- and amino-deuteriated dG (Figure 5), is clearly observed in the Raman spectrum of Oxy-4 after 15 min in D_2O, and the intensity remains essentially constant thereafter (Figure 6). However, the 1720 cm^{-1} band, which should disappear upon complete N1 imino deuteriation, still exhibits considerable intensity after 1 day in D_2O. When Oxy-4/D_2O is heated to 70 °C, the intensity increase of the 980 cm^{-1} band resumes, and concomitantly, the 1720 cm^{-1} band is completely replaced by the band at 1686 cm^{-1} (Figure 7). Assuming that the intensity of the 980 cm^{-1} band is roughly proportional to the number of N1 and N2 deuteriated guanine residues (i.e., the mole fraction of G-d_3), we estimate that guanine N1H → N1D exchange is half complete after 15 min in D_2O ("facile exchange" fraction), while the remaining half of the imino sites ("protected" fraction) are resistant to further exchange at 10 but not at 70 °C.

H−D Exchange Kinetics of the Antiparallel Quadruplex Structure of Oxy-4. Oxy-4 forms an *intra*molecular (fold-back) antiparallel quadruplex in the presence of 100 mM NaCl, in contrast to the *inter*molecular (extended) parallel quadruplex formed in 100 mM KCl (Miura & Thomas, 1994). As in the case of the parallel structure, the antiparallel

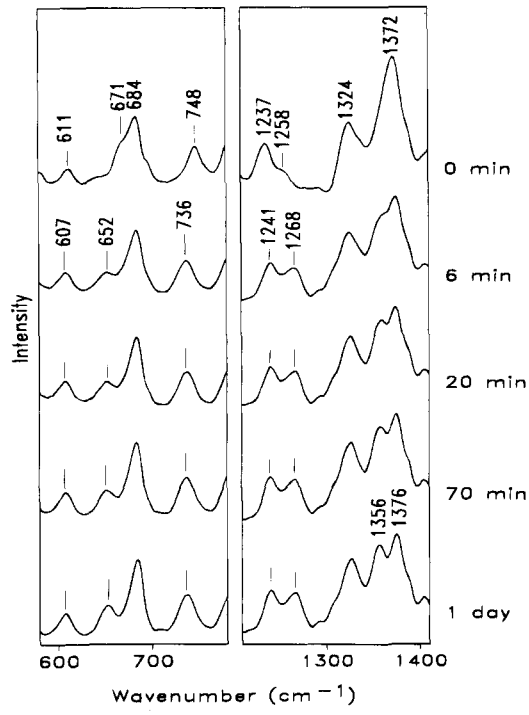

FIGURE 9: Time-resolved Raman spectra (580−780 and 1210−1410 cm^{-1}) of the antiparallel quadruplex structure of Oxy-4 in 100 mM NaCl/D_2O at 10 °C (2 mM oligonucleotide, pD 7.0).

structure exhibits Raman markers at 835 and 1481 cm^{-1} indicative, respectively, of Hoogsteen hydrogen bonding at N7 sites of dG and backbone torsions similar to those of B DNA. On the other hand, Raman markers diagnostic of dG nucleoside conformation are very different in the antiparallel and parallel quadruplexes. The most striking differences for spectral intervals 580−780 and 1210−1410 cm^{-1} are depicted in Figure 9. The top trace shows that the antiparallel structure contains the C2′-*endo*/*syn* dG conformer (671 cm^{-1} marker) in addition to the C2′-*endo*/*anti* dG conformer (684 cm^{-1} marker). This confirms previous Raman results (Miura & Thomas, 1994) and is consistent with alternating *anti* and *syn* dG conformers along each strand of the antiparallel quadruplex as proposed for X-ray crystal (Kang et al., 1992) and NMR solution structures (Smith & Feigon, 1992, 1993; Wang et al., 1994).

H → D exchange kinetics for the antiparallel quadruplex are similar to those of the parallel quadruplex described above. As shown in Figure 9, deuteriation of the N3 imino proton of dT is complete within 6 min in D_2O at 10 °C (748 → 736 cm^{-1}). Note also that the dT residues of Oxy-4 generate a band at 666 cm^{-1} which underlies the 671 cm^{-1} marker of C2′-*endo*/*syn* dG conformers and that this dT component shifts to 652 cm^{-1} within 6 min of exposure to D_2O. The gradual intensity increase of the 1356 cm^{-1} band establishes much slower N2 amino exchange of dG. Deuteriation of the facile exchange fraction of N1 imino protons occurs at 10 °C, whereas the protected fraction is largely nonexchanged. Interestingly, the C2′-*endo*/*syn* dG marker at 1324 cm^{-1}, which is not present in Raman spectra of parallel quadruplexes (all *anti* dG), shows no significant isotope shift with deuterium substitutions at either N1 or N2 sites.

The 611 cm^{-1} band (Figure 9, top trace) is a unique indicator of the antiparallel quadruplex structure and has not been detected in Raman spectra of other nucleic acid structures. This band was tentatively assigned to C2′-*endo*/*syn* dG (Miura & Thomas, 1994), on the basis of its significant intensity decay accompanying the antiparallel → parallel structure transformation of Oxy-4 and its absence from Raman spectra of poly(dT). On the basis of the present H → D exchange kinetics, however, the 611 cm^{-1} band can be confidently assigned to dT residues of Oxy-4. As shown in Figure 9, the frequency shift from 611 to 607 cm^{-1} is complete within 6 min at 10 °C. This rapid deuteriation effect cannot be explained by exchanges of N1 imino or N2 amino sites of dG and is consistent only with N3 imino proton exchange of dT residues.

DISCUSSION

Novel Assignments and Key Guanine Markers in Raman Spectra of DNA Quadruplexes. (A) Raman Bands Sensitive to N1 Imino Deuteriation. The Raman spectrum of dGMP/H_2O exhibits a band near 1603 cm^{-1} which is absent from the spectrum of dGMP/D_2O. On the basis of this deuteriation shift, the 1603 cm^{-1} band was assigned previously to scissoring of the N2 amino group (Fodor et al., 1985). However, as shown clearly in Figure 5, the 1603 cm^{-1} band of d(G_{12}) is not affected by deuteriation of the N2 amino group; rather, the band disappears upon N1 imino deuteriation, demonstrating its origin not in NH_2 scissoring but in N1−H in-plane bending.

The band at 1068 cm^{-1} is also observed only in spectra of N1 imino protiated forms (G-d_0 and G-d_2, Figure 5) and must therefore represent a vibration involving the N1H moiety.

As noted above (Results and Conclusions), the d(G_{12}) quadruplex exhibits a Raman band at 1723 cm^{-1} characteristic of G-d_0 and G-d_2 forms of the guanine residue. The 1723 cm^{-1} marker is shifted to 1686 cm^{-1} upon N1 imino deuteriation, but is insensitive to N2 amino deuteriation (Figures 2, 3, and 5), indicating coupling of the C6=O stretching mode with in-plane N1−H bending, but not with NH_2 scissoring.

We find also a very weak and broad band at 1685 cm^{-1} in the G-d_0 quartet (Figure 2, top trace) which undergoes an apparent shift to 1659 cm^{-1} upon N2 amino deuteriation (Figure 2, bottom trace). However, the assignment of the mode is not straightforward. One possibility is to attribute the very weak 1685 cm^{-1} band to a complex vibration involving both double bond stretching of the G-d_0 form of the guanine ring and N2 amino scissoring. Previous workers have also wrestled with the complexity of the guanine C6=O bond stretching assignment. For example, in the absence of interbase associations, rGMP/H_2O generates a single band near 1680 cm^{-1} which shifts to 1670 cm^{-1} in rGMP/D_2O (Lord & Thomas, 1967). However, the mode is not a pure carbonyl group stretch and gives evidence of extensive coupling with endocyclic double bond stretching vibrations as well as with N1−H bending and $N2H_2$ scissoring. For example, the 1680 cm^{-1} band of rGMP/H_2O is shifted to 1675 cm^{-1} solely as a result of C8H deuteriation (Lane & Thomas, 1979). In the quadruplex structure of poly(rG)/H_2O, two distinct bands appear at 1690 and 1720 cm^{-1}, and these are shifted to 1670 and 1700 cm^{-1} in poly(rG)/D_2O (Rice et al., 1973; Simard & Savoie, 1994). Doublets are also observed for poly(rI) (Chou et al., 1977; Simard &

Savoie, 1994) and tetrameric rGMP (Audet et al., 1991). If the hydrogen-bonded carbonyls are chemically equivalent in each quadruplex structure, some special situation such as interbase vibrational coupling or Fermi resonance must be invoked to account for the doublet. Savoie and co-workers (Savoie et al., 1978; Audet et al., 1991) have postulated that the four carbonyls in a square planar array (C_{4h} symmetry) are coupled with one another to generate two distinct Raman-active vibrations, of symmetry species A_g and B_g.

In the Raman spectrum of $d(G_{12})/H_2O$, the corresponding doublet is observed at 1685 and 1723 cm^{-1} (Figure 2, top trace). Although both bands shift to lower frequency in $d(G_{12})/D_2O$, we have demonstrated that the two shifts (1685 → 1659 and 1723 → 1686 cm^{-1}) are independent of one another. At 10 °C, the 1685 cm^{-1} band shifts to 1659 cm^{-1}, whereas the 1723 cm^{-1} band exhibits no frequency shift at the same temperature (Figure 2). On the other hand, the shift of the 1723 cm^{-1} band to 1686 cm^{-1} occurs at 95 °C without a significant effect upon the 1659 cm^{-1} band (Figure 3). Accordingly, neither interbase vibrational coupling nor Fermi resonance is sufficient to account for the observed stepwise shift of the two bands. Although elevation of the frequency of the carbonyl stretching vibration upon quadruplex formation can be attributed to interbase vibrational coupling, the lower frequency component cannot be assigned reasonably to a carbonyl stretching mode of B_g symmetry in the guanine quartet. An alternate proposal for assignment of the lower frequency "carbonyl" band (1685 cm^{-1}) of the guanine quartet is given below (section B under Quadruplex Structure and Dynamics).

(B) Raman Bands Sensitive to N2 Amino Deuteriation. As shown in Figure 5, the 1644 cm^{-1} band of G-d_0 disappears upon N2 amino deuteriation (G-d_2 and G-d_3), whereas N1 imino deuteriation apparently causes only a minor shift (+4 cm^{-1}, G-d_1) (Table 2.) This demonstrates significant involvement of the exocyclic amino moiety in the 1644 cm^{-1} mode, most likely NH_2 scissoring. Accordingly, the band at 1392 cm^{-1} (G-d_2) is assigned to the corresponding ND_2 mode. We note that the 1644 cm^{-1} band loses intensity very quickly in D_2O solution and is completely eliminated within 45 min at 10 °C (Figure 2). The rate of intensity decay is comparable to that of the 1361 cm^{-1} band (1.2×10^{-2} min^{-1}), which is also a marker of amino deuteriation (see below). These observations are consistent with the NH_2 scissoring assignment. Interestingly, the 1644 cm^{-1} band decay is clearly faster than the growth of the ND_2 scissoring band at 1392 cm^{-1} (5.7×10^{-3} min^{-1}). The observed time lag presumably reflects production of the transient hemideuteriated amino group (NHD) along the pathway to full amino deuteriation. Thus, scissoring of the NHD isotopomer is assigned to the transient 1523 cm^{-1} band which appears only at early stages of amino exchange and then disappears. The 1523 cm^{-1} band reaches maximum intensity after about 45 min at 10 °C (Figure 2). Therefore, Raman markers for three distinctive N2 amino forms of a guanine quartet are as follows:

Isotopomer:	NH_2 form (G-d_0)	NHD form (transient)	ND_2 form (G-d_2)
Raman marker:	1644 cm^{-1}	1523 cm^{-1}	1392 cm^{-1}

The foregoing assignments apply to quartets containing nonexchanged (i.e., protiated) N1H imino sites and account satisfactorily for both the structure and the dynamics of the Hoogsteen-hydrogen-bonded guanine quartet.

The 1363 cm^{-1} band has been assigned to a guanine imidazole ring mode on the basis of a large shift to lower frequency (−11 cm^{-1}) in $^{15}N7$ and $^{15}N9$ isotopomers and a relatively small shift (−3 cm^{-1}) in $^{15}N1$, $^{15}N2$, and $^{15}N3$ isotopomers (Delabar & Guschlbauer, 1979). On the other hand, Fodor et al. (1985) have assigned this band to a mode of the C2=N3—C4=C5—N7=C8 triene moiety, on the basis of the ultraviolet resonance Raman (UVRR) excitation profile. Our results show that the 1363 cm^{-1} band exhibits a large frequency shift (−12 cm^{-1}) with N2 amino deuteriation but is insensitive to N1 deuteriation, consistent with the assignment by Fodor et al. (1985).

The 1478 cm^{-1} band has been assigned to a vibration involving a large displacement of N7 and C8 atoms, and the frequency is sensitive to the N7 environment (Nishimura et al., 1985; Benevides et al., 1991) and C8 deuteriation (Lane & Thomas, 1979). Consistent with previous work, a significant frequency shift is not observed with N1 imino deuteriation. Upon N2 amino deuteriation, however, the band is replaced by a doublet at 1470 and 1481 cm^{-1}. The doublet is not an artifact of stepwise amino deuteriation, since both components are distinct from markers of G-d_0 (1474 cm^{-1}) and G-d_3 (1478 cm^{-1}). Presumably, the 1470/1481 cm^{-1} doublet is due to Fermi resonance between the 1478 cm^{-1} fundamental and an overtone or combination mode which is sensitive to N2 amino deuteriation.

(C) Raman Bands Sensitive to Both N1 and N2 Deuteriations. Raman bands at 867 and 980 cm^{-1} are necessary and sufficient markers of simultaneous deuteriation of both imino and amino sites of the guanine quartet (Figures 3 and 5). The 1579 cm^{-1} band also exhibits a small (+2 cm^{-1}) frequency shift with both imino and amino deuteriations, whereas no significant frequency shift results from deuteriation of only imino or only amino sites (Table 2). The intensity of the 1578 cm^{-1} band is also increased by the deuteriations: +11% with N1H → N1D, +20% with $N2H_2$ → $N2D_2$, and +38% with both. (Peak intensities are measured versus the 1090 cm^{-1} band.)

Quadruplex Structure and Dynamics. (A) dG Residues. The frequency of the 1603 cm^{-1} band is a potentially valuable indicator of guanine N1H hydrogen bonding, owing to its origin in N1−H in-plane bending. The band is strongly enhanced in UVRR spectra over a wide range of excitation wavelengths (200−270 nm) (Fodor et al., 1985), and sensitivity to environment has been proposed (Fodor & Spiro, 1986). The 1603 cm^{-1} band is prominent in 240-nm UVRR spectra of $dGMP/H_2O$ and equimolar mixtures of dGMP and dCMP (Fodor et al., 1985). However, the frequency appears to be shifted to 1620 cm^{-1} in poly(dG-dC)·poly(dG-dC) (Fodor & Spiro, 1986), which suggests that the interbase N1−H···N3 hydrogen bonds in Watson−Crick GC pairs are stronger than those between N1H and solvent water molecules, thus accounting for the high-frequency shift of the N1−H bending mode. However, the present results on $d(G_{12})$ show that there is no high-frequency shift of the N1−H in-plane bending vibration with quadruplex formation (Figure 2), despite the implication for strong hydrogen bonding (i.e., virtually complete protection from H → D exchange). This is evident even at 95 °C (Figure 3). Surprisingly, although imino protons of the quadruplex exist in extraordinarily rigid (nonexchangable) structures, the

interbase hydrogen bonding is presumably weaker (1603 cm^{-1} marker) than in the Watson–Crick GC duplex (1620 cm^{-1} marker).

The scissoring vibration of the guanine amino group near 1644 cm^{-1} is expected to be an indicator of the relative strength of hydrogen bond donation by the N2H proton. In solutions containing relatively weak N2H hydrogen bonds, e.g., for dGMP in H_2O at 75 °C (Audet et al., 1991) and GpG in H_2O at 80 °C (unpublished results of T. Miura and G. J. Thomas, Jr.), the scissoring mode occurs at 1640 ± 1 cm^{-1}. In the d(G_{12}) quadruplex (Figure 2), the frequency is elevated to 1644 cm^{-1}, suggesting that N2–H···N7 hydrogen bonding is stronger than for the solvated amino group. This is consistent with the strong hydrogen bond acceptor role of N7 which is evidenced by the 1478 cm^{-1} marker (Figure 2).

As described above, full deuteriation of N2 amino groups in the d(G_{12})/D_2O quadruplex structure is complete within 24 h at 10 °C, while N1 imino exchange requires much more prolonged exposure and heating to 95 °C. Thus, in the absence of heating, the 980 cm^{-1} marker does not achieve prominence in the spectrum of amino-deuteriated d(G_{12}) (Figure 2, bottom trace). Conversely, in both the parallel and antiparallel quadruplexes of Oxy-4, the 980 cm^{-1} band is more clearly discernible after 24 h at 10 °C (cf. Figures 2 and 6), indicating that combined deuteriation of both amino and imino protons is more complete. Accordingly, time resolution of N2 amino and N1 imino exchanges is more feasible for the d(G_{12}) quadruplex. Nevertheless, we observe a very small intensity increase at 980 cm^{-1} in d(G_{12}) during the time frame of Figure 2. We estimate that the 980 cm^{-1} intensity after 24 h at 10 °C (Figure 2, bottom) is about 17% of that of fully deuteriated d(G_{12}) (Figure 3, bottom). Assuming that the 980 cm^{-1} intensity is proportional to the number of G-d_3 residues, N1 imino exchange is estimated to occur in 2 out of 12 dG residues at 10 °C. We speculate that the appearance of *two carbonyl stretching bands at 1723 and 1685 cm^{-1} in the Raman spectrum of d(G_{12}) is due to two inequivalent dG environments in this structure.* The 1685 cm^{-1} band is assigned to the dG residues of peripheral quartets [at the 5′ and 3′ termini of d(G_{12})], and the 1723 cm^{-1} band is assigned to the 10 inner quartets. Since the former guanines are involved in relatively flexible or possibly distorted quartets, their guanine imino exchanges may proceed more quickly. The latter guanines are presumed to form more highly ordered and thermostable quartets, and thus their imino exchanges are extraordinarily slow.

Recent NMR studies of telomeric DNA quadruplexes consisting of d($T_{2-4}G_4$) repeats show indeed that N1 imino proton exchanges of guanines in the outer two quartets proceed more quickly than those of the inner quartets (Wang & Patel, 1992; Smith & Feigon, 1992; Gupta et al., 1993). The present results and the previous NMR results indicate that guanines in the terminal layers of stacked quartets are characterized by greater distortion and/or flexibility than those of the inner layers.

(B) dT Residues. A previous NMR study (Cheong & Moore, 1992) has shown that uracil quartets are formed in the parallel RNA quadruplex structure of r(UG_4U) in the presence of 60–100 mM K^+. In the r(UG_4U) quadruplex, H → D exchange of the N3 imino proton of rU is significantly retarded even at 65 °C due to stable N3–H···O4 uracil–uracil hydrogen bonding. Conversely, no retardation of N3 imino exchange is observed in either the parallel or the antiparallel quadruplex of Oxy-4 at temperatures as low as 10 °C. Accordingly, stable thymine quartets probably do not occur in quadruplex structures of Oxy-4. Thymine quartets of square planar symmetry (C_{4h}) may be disfavored in relation to uracil quartets by steric clash between C2 carbonyl and C5 methyl groups.

Finally, we note that the weak 611 cm^{-1} band, assigned here to dT residues of the Oxy-4 antiparallel quadruplex on the basis of exchange characteristics illustrated in Figure 9, has counterparts in spectra of d($G_4T_4G_4$) and d($G_4T_4G_4T_4G_4T_4G_4$) (unpublished results of T. Miura and G. J. Thomas, Jr.). On the other hand, no band at 611 cm^{-1} appears in spectra of the parallel Oxy-4 quadruplex, or in duplex DNA of the B or A form, or in single-stranded poly(dT). *Therefore, the 611 cm^{-1} band identifies an unusual dT conformer, specific to the antiparallel quadruplex.* A likely candidate is a *syn* conformer of dT, as recently proposed to exist in the dT_4 loop regions of antiparallel quadruplexes (Wang et al., 1994). The Raman signature appears to provide a valuable and convenient indicator of the unusual (*syn*?) dT conformer present in antiparallel fold-back quadruplexes of telomeric DNA.

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Corrections

Enzymatic Synthesis of Octadecameric Saccharides of Multiply Branched Blood Group I-Type, Carrying Four Distal α1,3-Galactose or β1,3-GlcNAc Residues, by Antti Seppo, Leena Penttilä, Ritva Niemelä, Hannu Maaheimo, Ossi Renkonen,* and Anita Keane, Volume 34, Number 14, April 11, 1995, pages 4655−4661.

Page 4658. In column 1, line 26, the parenthetical expression should read 4.442 and 4.457 ppm, respectively.

BI955006T