Sr^{2+} Facilitates Intermolecular G-Quadruplex Formation of Telomeric Sequences†

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ABSTRACT: Electrophoretic and spectroscopic studies were made with the telomere-related sequences d-($G_4T_2G_4T_2G_4T_2G_4$) (T2) and d($G_4T_4G_4T_4G_4T_4G_4$) (T4) in the presence of Na^+, K^+, and Sr^{2+}. Electrophoretic evidence indicates that these two oligomers exist in multiconformational states in solutions. A band identified as that of intermolecular (tetramolecular) G-quadruplex is apparent in both T2 and T4, whereas a band identified as intramolecular (monomeric) G-quartet is only evident in T4. The remaining electrophoretic bands that exhibit mobilities intermediate of these two extremes are identified as those of hairpin-related duplexes and tetraplexes. In the presence of millimolar concentrations of Sr^{2+} and subsequent thermal treatment, the intensity corresponding to the band attributable to the intermolecular G-quadruplex is dramatically enhanced in T2 while those of the hairpin-related bands of intermediate mobility are greatly reduced. Similar but less dramatic enhancement of the intermolecular quadruplex band is also observed in T4. Although these effects can also be induced by K^+, orders of magnitude higher concentrations are needed. The intensity of the intramolecular G-quartet band, apparent in T4 but not in T2, appears to be relatively insensitive to the type of cation present in the solution. These results demonstrate that both Sr^{2+} and K^+ facilitate the intermolecular G-tetraplex formation, with the divalent cation being much more effective. Comparison with the corresponding CD spectral characteristics suggests that the electrophoretic intensity enhancement of the intermolecular G-quadruplex band is correlated to the intensity enhancement of the positive CD maximum at 265 nm. CD as well as absorbance melting studies indicate that the Sr^{2+}-induced intermolecular quadruplexes are thermally more stable than those of K^+, with melting temperatures of some complexes exceeding 95 °C.

Telomeres are specialized DNA–protein structures at the termini of chromosomes which have been shown to be important for their stability and accurate replication. Telomeric DNAs of eukaryotes consist of simple repetitive guanine-rich sequences having the general formula $(T/A)_{1-4}G_{1-8}$ (Blackburn & Szostak, 1984; Blackburn, 1990). The most common units include three or four guanine nucleotides, and the more extensively studied sequences include those of $d(TTAGGG)_n$ (humans), $d(TTGGGG)_n$ (*Tetrahymena*), and $d(TTTTGGGG)_n$ (*Oxytricha*).

The structural properties of these G-rich sequences are of current intense interest, as their characteristics may be intimately related to the telomeric functions. Several laboratories have recently studied the possible structures of these G-rich clusters using various chemical and physical techniques. Principal strategies include (1) specific base modifications by dimethyl sulfate (DMS) to test the accessibility of guanine N7 for distinguishing between paired and unpaired bases and (2) the replacement of guanosine by inosine to see whether the 2-amino group is engaged in base pairing.

Henderson et al. (1987) demonstrated that these dG-rich telomeric sequences have the unusual ability to form stable single- or double-haipin conformations via G·G base pairings to result in conformations which migrate faster than the original single strands. Their NMR studies on $d(TTGGGG)_4$ revealed that at least 14 imino protons are involved in hydrogen bonding and NOESY experiments indicated that 4–6 G residues are in a syn conformation while the others are in the anti conformation, in conformity with the antiparallel strand orientations. Oka and Thomas (1987) studied DNA fragments containing *Oxytricha* sequence and showed that at high DNA concentrations they can cohere in an interstrand manner to form an unusual structure which is stabilized by K^+ and exhibits a melting temperature about 25 °C higher than that of Na^+. The cohered telomeres appear to be relatively poor substrates for nucleases.

Studies on synthetic oligonucleotides mimicking the dG-rich immunoglobulin switch regions led Sen and Gilbert (1988) to discover that under physiological salt conditions these oligomers migrated much slower than the single strands in nondenaturing gels. DMS treatment of these oligomers demonstrated that all N7 positions of dG were protected from methylation and it was concluded that the dG residues were paired in a Hoogsteen manner, but in four-stranded parallel structures, similar to those proposed for GMP gel (Gellert et al., 1962) and poly(G) (Zimmerrman et al., 1975). They speculated that this self-recognition of guanine-rich motifs of DNA serves to bring together the four chromatids during meiosis.

Williamson et al. (1989) investigated the structures formed by oligonucleotides composed of two or four repeats of the telomeric sequences from *Oxytricha* and *Tetrahymena* and found that the sequence $d(TTTTGGGG)_4$ forms structures with increased electrophoretic mobility in gels containing Na^+, K^+, or Cs^+, but not in gels containing Li^+. On the basis of these results and the thymidine cross-linking results, which were found to be concentration independent, they concluded that intramolecular quadruplex species were formed in which the strands folded back on each other. An intramolecular G-quartet model in which hydrogen-bonded structures formed from four guanosine residues in square-planar arrangement was proposed to account for these results. The strands are pairwise antiparallel, and two of the four dG residues are in

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the syn conformation. The central cavity of the G-quartet allows the medium-sized alkali cations to form bonds with the four keto groups.

Model telomeric DNA complexes that contained the 3′-d-$(T_2G_4)_2$ overhang were studied by Sundquist and Klug (1989), and it was discovered that the molecules dimerize in a Na^+- or K^+-dependent manner, producing complexes in which all N7 atoms of dG are inaccessible to DMS modification. It was proposed that telomeric DNA dimerizes by hydrogen bonding between two intramolecular G-hairpins to form antiparallel quadruplexes containing cyclic guanine base tetrads.

Henderson et al. (1990) found differential DMS reactivities at the G residues in some telomeric sequences which are dependent on ion type. By systematically substituting dI for dG in $d(TTGGGG)_4$, they identified N2 groups of guanine to be essential for the formation of G-structures. They also showed that dI-substituted molecules that cannot form the G-DNA structures nonetheless function as substrates for telomere repeat addition by telomerase in vitro. Panyutin et al. (1989, 1990) cloned $(dG)_n$ oligomers (n = 27 or 37) in a plasmid, and their DMS modification experiments suggested that the dG stretches adopt a complex intrastrand structure other than a simple hairpin. They proposed a model analogous to that of Williamson et al. (1989) in which the principal element consists of a quadruplex formed by pairwise antiparallel segments of the twice-folded dG stretch.

Sen and Gilbert (1990) extended their earlier studies to include telomeric sequences and demonstrated that the formation of the G-tetrads was strongly dependent on the Na^+/K^+ ratio. Raghuraman and Cech (1990) studied the rates of folding and unfolding of $d(TTTTGGGG)_4$ and estimated thermodynamic parameters of the folded form. They also showed that the fully-folded DNA is not recognized by the telomere binding protein. Spectroscopic and calorimetric studies (Jin et al., 1990) have also been made using a family of short, guanine-containing DNA single strands of the form d[GGTTXTTGG], where X = A, C, G, or T. In 1 M NaCl at low temperature, these molecules do not behave as single strands but rather exhibit properties consistent with tetraplex formation. Thermodynamic parameters for the formation of each tetraplex were obtained.

These studies have clearly demonstrated that telomeric sequences can form either intra- or intermolecular tetraplexes. The roles of cations are found to be particularly important for this structural formation. In our efforts to further elucidate the structural characteristics of the G-rich sequences, the telomere-related oligomers $d(G_4T_2G_4T_2G_4T_2G_4)$ (T2) and $d(G_4T_4G_4T_4G_4T_4G_4)$ (T4) were synthesized and studied. During our investigations of the differential cationic effects on the conformations of G-rich DNAs, it was found that Sr^{2+} can greatly enhance the intermolecular G-tetraplex formation. Results of our findings are detailed in this report.

Materials and Methods

Oligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer with use of β-cyanoethyl phosphoramidite chemistry. The crude oligomer was purified by a strong anion-exchange (SAX) HPLC and repurified by reverse-phase HPLC chromatography as detailed earlier (Chen, 1988). The purified and lyophilized oligomers were dissolved in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer of pH 7.3. Since oligomers rich in guanine are notorious in aggregate formation, these stock solutions were first heated in boiling water for about 5 min and then cooled back to ambient temperatures. Concentrations of these oligomers (per nucleotide) were determined with absorbances at 260 nm after melting, with use of extinction coefficients obtained through nearest-neighbor approximation with mono- and dinucleotide values tabulated in Fasman (1975). The desired cation concentrations were obtained by adding appropriate amounts of concentrated stock solutions.

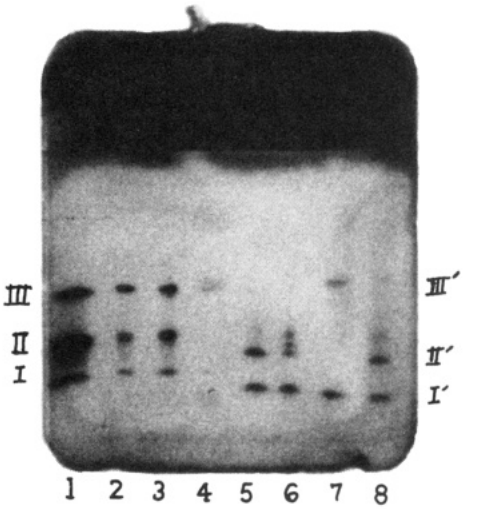

FIGURE 1: Comparison of electrophoretic mobilities of d-$(G_4T_2G_4T_2G_4T_2G_4)$ (lanes 1–4) and $d(G_4T_4G_4T_4G_4T_4G_4)$ (lanes 5–8) in the absence of added salt (lanes 1 and 8) and in the presence of 0.3 M NaCl (lanes 2 and 5), 0.3 M KCl (lanes 3 and 6), and 30 mM $SrCl_2$ (lanes 4 and 7) after a 5-min thermal treatment. Experiments were run on a 20% polyacrylamide PhastGel at 4 °C and 200 V with 75-Vh run time. PhastGel native buffer strips containing 0.25 M Tris at pH 8.8 were used, and the gels were prerun for 200 Vh prior to being loaded. The loading concentrations are in the range of 1–2 mM nucleotide. I, II, and III are band designations for T2 (lanes 1–4) with decreasing mobilities, and the corresponding I′, II′, and III′ are those of T4 (lanes 5–8).

Absorption spectra were measured by a Cary 210 spectrophotometric system. CD measurements were made by a Jasco J-500A recording spectropolarimeter at appropriate temperatures, with use of water-jacketed cylindrical cells. Thermal melting profiles of oligomers were carried out by monitoring absorbances at 275 nm and collecting data every 15 s with an Apple II microcomputer. A heating rate of 0.5 °C/min was maintained by a Neslab RTE-8 refrigerated circulating bath and an EPT-4RC temperature programmer. Numerical differentiations were performed to obtain differential melting profiles from which melting temperatures were deduced. Gel electrophoresis was run with a PhatSystem using 20% polyacrylamide gels and developed with silver staining.

Results

Observation of Multiconformational Electrophoretic Patterns. Electrophoretic patterns of T2 and T4 in the presence of three different cations, along with samples without added salts, are compared in Figure 1. These solutions have been placed in boiling water for about 5 min and subsequently cooled back to room temperature (henceforth referred to as "thermal treatment" in the text). Multiconformational electrophoretic patterns are evident in both T2 (lanes 1–4 with band designations I, II, and III) and T4 (lanes 5–8 with band designations I′, II′, and III′) solutions. In T2, the patterns in the presence of Na^+ and K^+ (lanes 2 and 3, respectively) do not appear to be greatly different from each other and from that of the absence of added salt (lane 1). In T4, some differences (note the appearance of three weak bands in the intermediate mobility region) are observed in the presence of K^+ (lane 6) as opposed to the presence of Na^+ (lane 5) and the absence of added salt (lane 8). Most interestingly, however, is the fact that Sr^{2+} induces significant alterations in the

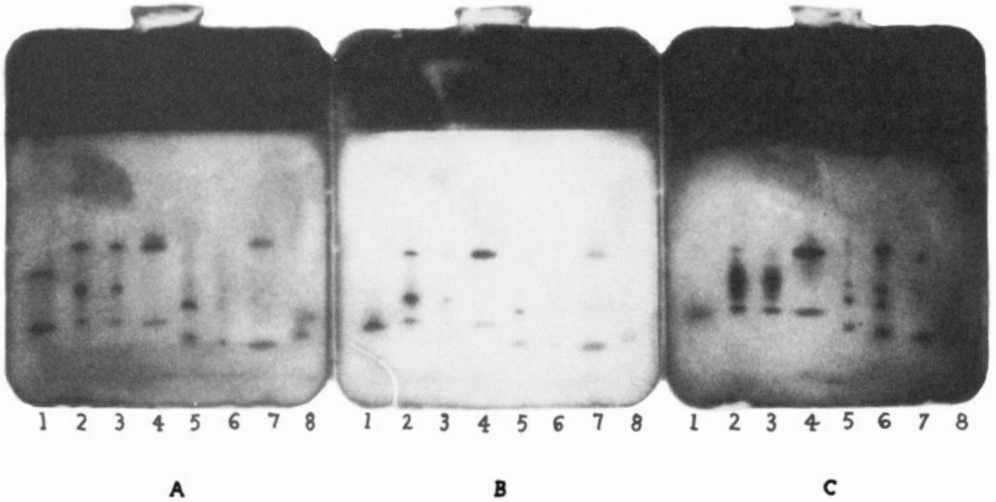

FIGURE 2: Comparison of electrophoretic mobilities of d($G_4T_2G_4T_2G_4T_2G_4$) (lanes 2–4) and d($G_4T_4G_4T_4G_4T_4G_4$) (lanes 5–7) with markers (lanes 1 and 8) at 4 (panel A), 14 (panel B), and 24 °C (panel C). Lanes 1 and 8 are 22-mer d(TGCA-TTT-TGCATGCA-TTT-TGCA) and 18-mer d(GGGG-TTT-GGGG-TTT-CCCC), respectively, containing 0.1 M NaCl and serving as markers. Lanes 2 and 5 contain 0.2 M NaCl, lanes 3 and 6 contain 0.2 M KCl, and lanes 4 and 7 contain 5 mM $SrCl_2$. Gel running conditions are identical to those of Figure 1. The DNA concentration in lane 6 of panel C has been enhanced via multiple sample applications prior to being loaded.

mobility pattern for both T2 (lane 4) and T4 (lane 7). These can be seen more clearly in Figure 2 where the electrophoretic patterns are compared at three different temperatures with reference markers.

Sr^{2+} Facilitates Intermolecular Quadruplex Formation. Lanes 1 and 8 in Figure 2 are markers corresponding, respectively, to a nearly self-complementary 22-mer d(TGCA-TTT-TGCATGCA-TTT-TGCA), capable of forming either a dimeric duplex with TTT bulges or a fold-back double-hairpin with an 8 bp stem and two three-base loops, and an 18-mer d(GGGG-TTT-GGGG-TTT-CCCC), capable of forming intramolecular triplex of the G·G·C type (Chen, 1991) via double-hairpin formation. At 4 °C (panel A), two bands are clearly evident in lane 1, with the faster moving band being that of the fold-back double-hairpin and the slow mobility band corresponding to that of the pseudodimeric duplex of the 22-mer. The faster moving band in lane 8 is that of the intramolecular triplex of the 18-mer.

At least three bands are now clearly apparent in T2 (lanes 2–4 of panel A). The fastest moving bands (I, see Figure 1 for the band designation) exhibit electrophoretic mobilities only slightly slower than those of double-hairpin species of the 22-mer (lane 1). The bands with intermediate mobilities (bands II) move considerably slower than the double-hairpin band but significantly faster than the 22 bp dimeric duplex. The slowest moving bands (III), on the other hand, are much slower than the dimeric duplex of the 22-mer. As the concentrations of the solutions can vary considerably, intensity comparison of different lanes should be made with caution. Comparison of the band intensity distribution in the same lane suggests that in T2, band II makes the greatest contribution to the Na^+-containing solution (lane 2). Of particular interest is the dominance of band III with simultaneous disappearance of band II in the presence of Sr^{2+} (lane 4).

For the T4 oligomer (lanes 5–7), the electrophoretic patterns are significantly different from those of T2 and vary considerably among cations. The most mobile component (band I′) of T4 is apparent in all three cations and migrates significantly faster than band I of T2 as well as the double-hairpin form of the 22-mer (lane 1). The slowest band (III′) of T4, however, becomes prominent only in the Sr^{2+} sample (lane 7) and exhibits a mobility nearly identical to band III of T2. An intermediate band (II′) is clearly visible in the Na^+ solution of T4 (lane 5), having a mobility significantly faster than band II of T2 and situated roughly midway between bands I and II.

Aside from the smaller band separation between I and II (lanes 2 and 3) of T2, the electrophoretic pattern of 14 °C (panel B) is not greatly different from that of 4 °C. At 24 °C (panel C), the diminution of band III with accompanied smearing of band II in the Na^+ and K^+ samples of T2, but no apparent alteration for the Sr^{2+} solution, is apparent, suggestive of the differential cationic thermal stability of the band III complexes. The prominent presence of band III′ of T4 in a K^+ sample with higher DNA concentration (lane 6 of panel C) is consistent with the intermolecular nature of this band. It is also interesting to note the apparent disappearance of the dimeric band of the 22-mer marker (lane 1) at higher temperatures, a consequence of the less favorable thermal stability for dimeric duplexes with large bulges as compared to the monomeric hairpin structures.

With mobilities comparable to the intramolecular triplex of the 18-mer (lane 8), band I′ of T4 can reasonably be identified as the intramolecular (monomeric) G-quartet, whereas bands III and III′ of T2 and T4, having mobilities considerably slower than the dimeric form of the 22-mer, may be attributed to that of the intermolecular (tetramolecular) quadruplex. As band I of T2 migrates roughly at the same rate as that of the fold-back double-hairpin of the 22-mer, it may be associated with that of G-hairpins and band II be correlated to those of G-quadruplexes formed by dimerization of these hairpins.

The striking enhancement of band III with concomitant intensity reduction of band II in T2 due to the presence of Sr^{2+} (lane 4 of Figure 2A), and to a lesser extent in T4 (lane 7), suggests that this divalent cation facilitates the intermolecular G-quadruplex formation in these sequences. Another interesting observation is the presence of the intramolecular G-quartet (band I′) in T4 (lanes 5–7) and its absence in T2 (lanes 2–4). Its intensity, however, does not appear to be greatly affected even by the presence of Sr^{2+}. This result suggests that although a significant presence of intramolecular G-quartet in T4 is apparent, its contribution is relatively insensitive to the cationic type.

K^+ Also Facilitates Intermolecular G-Quadruplex Formation and the Importance of Thermal Treatments. It should

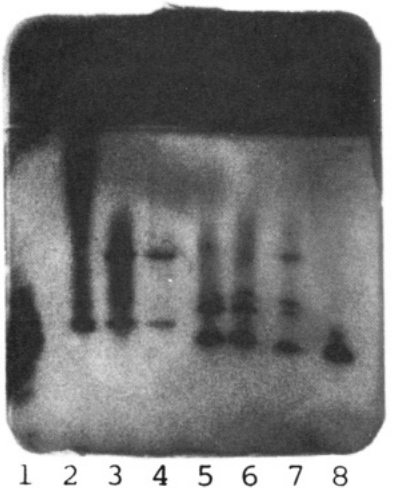

FIGURE 3: Electrophoretic patterns of d($G_4T_2G_4T_2G_4T_2G_4$) (lanes 2–4) and d($G_4T_4G_4T_4G_4T_4G_4$) (lanes 5–7) after an additional thermal treatment. Except for the extra 10-min thermal treatment and the running temperature of 14 °C, sample conditions in lanes 2–7 are identical to those of the corresponding lanes in Figure 1. Lanes 1 and 8 are the markers d(TGCA-TTT-TGCATGCA-TTT-TGCA) and d(GGGG-TTT-GGGG-TTT-CCCC), respectively.

be noted in passing that in the absence of thermal treatments after salt additions, both T2 and T4 exhibit somewhat diffuse electrophoretic patterns and appear to be relatively insensitive to the types of cation present (not shown). Significant sharpening of the electrophoretic images as well as the differential cationic effect is only evident upon thermal treatments. The importance of thermal treatments is further illustrated by Figure 3 in which samples have been subjected to additional 10-min heating in boiling water. The prominent presence of band III in the K^+ sample (lane 3), in contrast to that of Na^+ (lane 2), is clearly evident. Note also the clear presence of band III′ of T4 in the K^+ sample at higher DNA concentration (lane 6 of Figure 2C). These results suggest that K^+ also facilitates the formation of intermolecular G-quadruplex formation, albeit not as efficient as that of Sr^{2+}. The need for thermal treatments for observing differential cationic effects is consistent with the fact that these G-rich oligomers in solutions form tight self-structures which prevent significant internal interactions with cations unless these structures are thermally disrupted. This also underscores the difficulties in comparing results from various laboratories, as the nature and history of sample preparations can significantly alter the experimental outcome.

Correlation with CD Spectral Characteristics. The effect of Sr^{2+} on the CD spectra of T2 is shown in Figure 4. In the absence of $SrCl_2$, the CD spectrum of T2 at 20 °C (dotted curve) exhibits a positive maximum at 293 nm and a shoulder around 265 nm. The relative intensities of these two bands are somewhat variable but usually are comparable and depend on the solution conditions and history. An enhancement of the 265-nm band accompanied by a concomitant intensity reduction and a slight bathochromic shift of the 293-nm band is seen upon addition of 2 mM $SrCl_2$. The intensities of these two bands, however, remain relatively comparable. Thermal treatment resulted in a dramatic intensity enhancement of the 265-nm band and a concomitant demise of the positive intensity at 293 nm to result in a weak negative band (solid curve). A moderate negative band at 240 nm is also to be noted. The resulting conformation appears to be thermally very stable, as is indicated by the temperature-dependent CD spectra shown in Figure 4B. As is apparent, the spectra at 58 and 77 °C are not greatly different from that of 20 °C

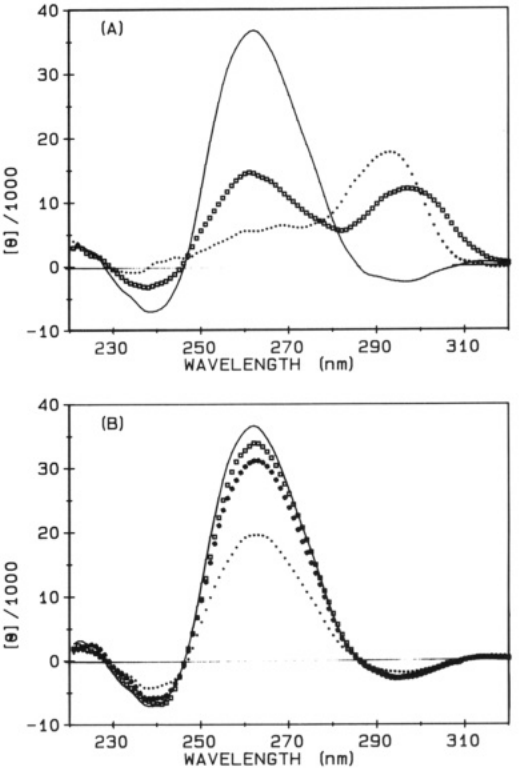

FIGURE 4: (A) Room temperature CD spectra of d($G_4T_2G_4T_2G_4T_2G_4$) in solutions containing 0.1 M NaCl (dotted curve), 2 mM $SrCl_2$ without thermal treatment (squares), and 2 mM $SrCl_2$ after thermal treatment (solid curve). (B) CD spectra of thermally treated d-($G_4T_2G_4T_2G_4T_2G_4$) containing 2 mM $SrCl_2$ at some representative temperatures: 20 °C (solid curve); 58 °C (open squares); 77 °C (+); 95 °C (dotted curve). A water-jacketed cylindrical cell of 2-cm path length and 40 μM nucleotide concentration were used in all CD measurements.

(solid curve), and more than half of the CD intensity is still retained even at 95 °C (dotted curve), suggesting that the melting temperature is slightly above this value. The correlation with the electrophoretic results indicates that the CD characteristics of an intense positive maximum at 265 nm flanked by moderate and weak negative bands at 240 and 293 nm, respectively, are those of intermolecular G-quadruplex.

The effect of K^+ on the CD spectral characteristics is shown in Figure 5A. The CD spectrum in the presence of 0.1 M NaCl after thermal treatment is characterized by the presence of two comparable positive bands around 293 and 265 nm (dotted curve). The addition of 0.1 M KCl and subsequent heating and cooling resulted in the enhancement of both bands, with the 265-nm band exhibiting a greater effect. Further additions of KCl to 0.3 M and two subsequent thermal treatments resulted in further enhancements of the 265-nm band and concomitant reductions of the 293-nm band (solid curve). The presence of isoelliptic points around 280 and 250 nm suggests a two-state transition. These observations are consistent with the increased contributions of the intermolecular G-quadruplex as the K^+ ion is increased. Temperature-dependent CD spectra of T2 in the presence of 0.3 M KCl are shown in Figure 5B. The slightly lower intensity of the 265-nm band as compared to the $SrCl_2$ solution and the presence of a significant shoulder at 293 nm in the room temperature spectrum suggest that there are significant contributions from conformations other than intermolecular G4-DNA, in conformity with the prominent presence of the hairpin-related electrophoretic bands in the K^+-containing solution. Substantial but less than half of the CD intensities are still retained at 95 °C (dotted curve), with the melting

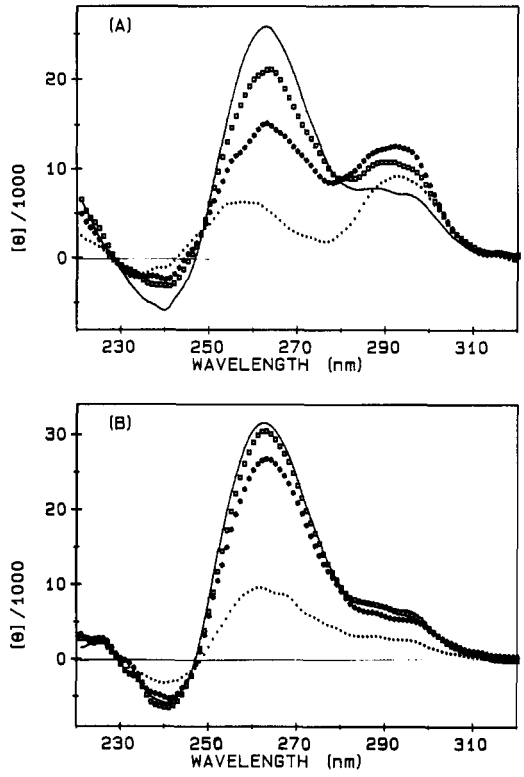

FIGURE 5: (A) CD spectra of $d(G_4T_2G_4T_2G_4T_2G_4)$ at room temperature in the presence of 0.1 M NaCl (dotted curve), with 0.1 M KCl added and thermally treated (+), and with 0.3 M KCl added and after second (open squares) and third (solid curve) thermal treatments. (B) CD spectra of thermally treated $d(G_4T_2G_4T_2G_4T_2G_4)$ containing 0.3 M KCl at some representative temperatures: 20 °C (solid curve); 58 °C (open squares); 77 °C (+); 95 °C (dotted curve).

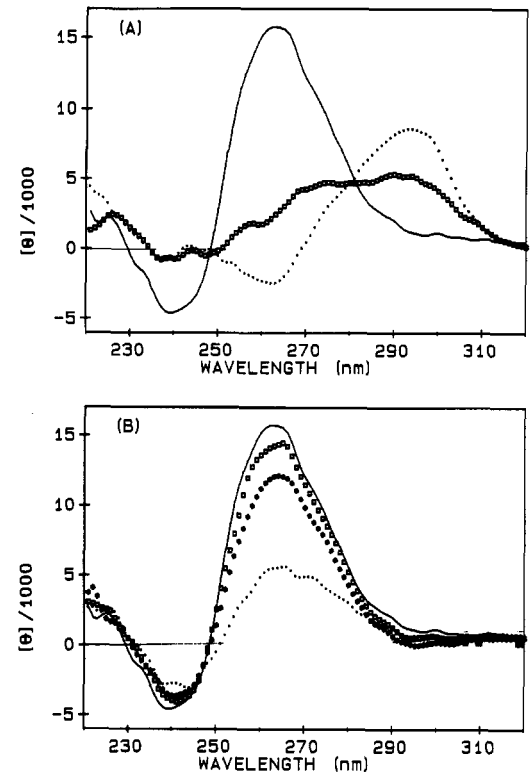

FIGURE 6: (A) CD spectra of $d(G_4T_4G_4T_4G_4T_4G_4)$ at room temperature in solutions containing 0.1 M NaCl (dotted curve), 2 mM $SrCl_2$ without thermal treatment (squares), and 2 mM $SrCl_2$ after thermal treatment (solid curve). (B) CD spectra of $d(G_4T_4G_4T_4G_4T_4G_4)$ in the presence of 2 mM $SrCl_2$ at some representative temperatures: 20 °C (solid curve); 58 °C (open squares); 77 °C (+), 95 °C (dotted curve).

temperature estimated to be slightly below this value.

The patterns of CD alterations induced by Sr^{2+} on T4 are quite similar but less dramatic (Figure 6A and compare the scale with Figure 4). In the absence of this divalent cation, the spectrum exhibits a positive CD maximum at 293 nm but a weak negative maximum at 265nm (dotted curve). The negligible contribution from the intermolecular G-quadruplex, as evidenced from electrophoresis (see lane 8 of Figure 1), suggests that these CD characteristics are those of hairpin-related structures, intramolecular quadruplex (a double-hairpin), or simple fold-back hairpins and their dimers. Additions of $SrCl_2$ to 2 mM resulted in the appearance of a positive shoulder in the 265-nm region and a simultaneous reduction of the 293-nm intensity. Subsequent thermal treatment resulted in the disappearance of this long-wavelength band and the conversion of the 265-nm shoulder into a sizable positive band (solid curve). The intensity, however, is not as great as that of T2, suggesting that the transformation to the intermolecular G4 complex in T4 is not as complete as that of T2. These observations are consistent with the evidence from the electrophoretic results, indicating less dramatic intensity enhancement of band III′ (see lane 7 in Figures 1 and 2). Again, the complex is extremely stable and melts slightly below 95 °C (Figure 6B).

In contrast to T2, the presence of K^+ in the T4 solution does not have the same effect as that of Sr^{2+}. No appreciable alteration is seen upon thermal treatments, an observation consistent with the near-absence of band III′ in dilute T4 solutions containing K^+.

Thermal Denaturation via Absorbance Monitoring. Representative thermal melting profiles for T2 and T4 are shown in Figure 7. Although the melting of T2 in the presence of 0.1 M NaCl is not monophasic, the dominant transition clearly occurs around 62 °C (Figure 7A). Addition of 0.1 M KCl increases this melting transition by about 20 °C to around 81 °C. Further increase in the KCl concentration to 0.3 M raises the melting temperature to 92 °C (not shown). In the presence of 2.3 mM $SrCl_2$, the melting of T2 is not complete even at 99 °C (curve 3).

In the presence of 0.1 M NaCl, the T4 oligomer exhibits a very broad and apparently multiphasic melting profile with a transition around 63 °C which appears to be somewhat cooperative (Figure 7B). A similar broad denaturation curve is also observed in the 0.1 M KCl solution, except now the discernible melting temperatures are found around 45 and 85 °C. The high melting temperature moves to around 95 °C in the 2.3 mM $SrCl_2$ solution (curve 3). The melting temperatures deduced from the absorbance monitoring are consistent with those obtained in the temperature-dependent CD studies and demonstrate the extreme thermal stability of the Sr^{2+}- and K^+-induced conformations. The differential melting profiles of 0.1 M KCl solutions are compared in Figure 7C and Figure 7D to highlight the approximate monophasic nature of T2 and the multiphasic nature of T4, respectively.

Discussion

Our electrophoretic results revealed that the telomere-related sequences $d(G_4T_2G_4T_2G_4T_2G_4)$ (T2) and $d(G_4T_4G_4T_4G_4T_4G_4)$ (T4) exist in multiconformational states in solutions. A band with mobility comparable to that of an intramolecular triplex of 4-base-triad stem and two 3-base loops formed via double-hairpin formation is apparent in T4 and is likely that of intramolecular (monomeric) G-quartet. On the other hand, the slowest band which migrates considerably slower than the

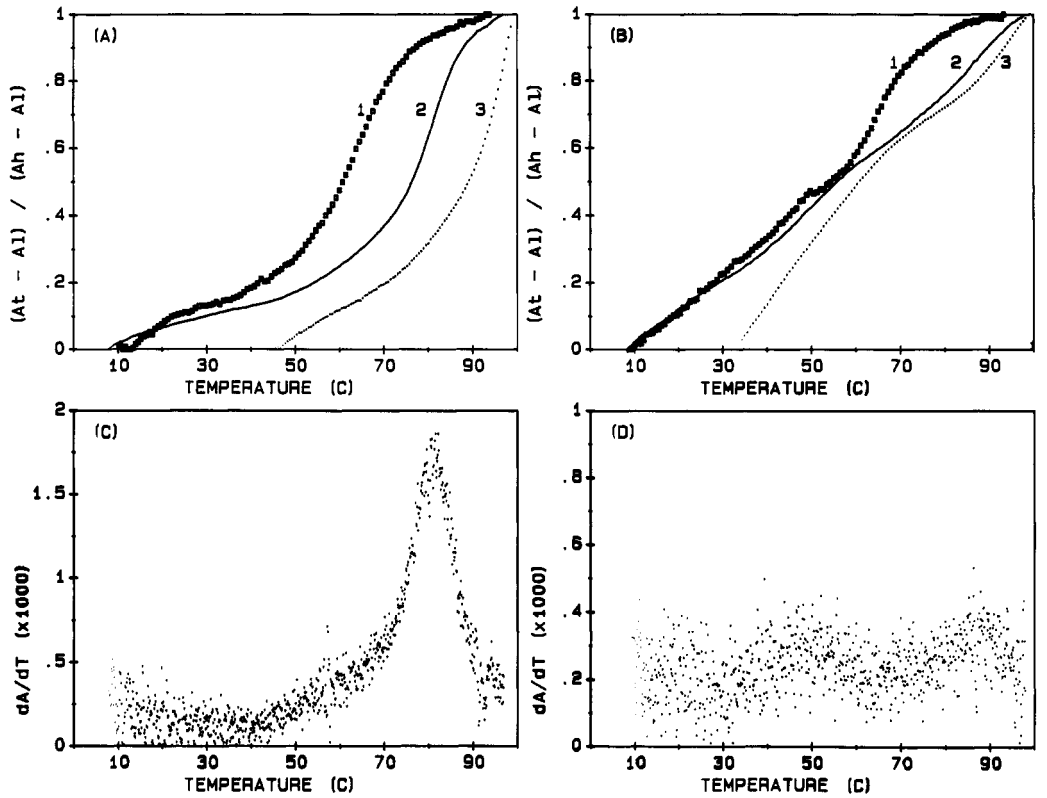

FIGURE 7: (A) Thermal melting profiles of 40 μM d($G_4T_2G_4T_2G_4T_2G_4$) in the presence of (1) 0.1 M NaCl, (2) 0.1 M KCl, and (3) 2 mM $SrCl_2$. (B) Thermal melting profiles of 20 μM d($G_4T_4G_4T_4G_4T_4G_4$) in the presence of (1) 0.1 M NaCl, (2) 0.1 M KCl, and (3) 2 mM $SrCl_2$. *Al*, *Ah*, and *At* are the 275-nm absorbances at the lowest, highest, and *t* temperatures measured, respectively. (C) Differential melting profiles of 40 μM d($G_4T_2G_4T_2G_4T_2G_4$) with 0.1 M KCl. (D) Differential melting profile of 20 μM d($G_4T_4G_4T_4G_4T_4G_4$) with 0.1 M KCl.

22 bp duplex marker, evident in T2 as well as in T4, may be identified as that of intermolecular (tetramolecular) G-quadruplex. The rest of the electrophoretic bands lie somewhere between these two extremes and are likely those of G-hairpins and their dimerized tetraplexes.

The electrophoretic patterns are not greatly altered by simply adding KCl or $SrCl_2$ to the solutions at room temperatures. In the presence of millimolar concentrations of Sr^{2+} and subsequent thermal treatment, the intensity corresponding to the band attributable to the intermolecular G-quadruplex is dramatically enhanced while those of intermediate mobility bands are greatly reduced in T2. Similar but less dramatic enhancement of the intermolecular quadruplex band is also observed in T4. Although these effects can also be induced by K^+, additional thermal treatments and orders of magnitude higher concentrations are needed. The intensity of the intramolecular G-quartet band which is apparent in T4, but not in T2, appears to be relatively insensitive to the type of cation present in the solutions. These results demonstrate that both Sr^{2+} and K^+ facilitate the intermolecular G-tetraplex formation, with the divalent cation being much more effective.

Comparison with the corresponding CD spectral characteristics suggests that the intensity enhancement of the intermolecular G-quadruplex is correlated to the CD intensity enhancement of the 265-nm band. A strong positive CD band at 265 nm flanked by respective moderate and weak negative CD bands at 240 and 293 nm characterizes such an intermolecular G-quadruplex whereas the intramolecular G-quartet, G-hairpins, and the G-quadruplex formed by dimerization of hairpins most likely are correlated to a positive CD band at 293 nm and a weak negative band at 265 nm. CD as well as absorbance melting studies indicate that the Sr^{2+}-induced intermolecular quadruplexes are thermally more stable than those of K^+ which in turn are more stable than the Na^+ complexes, with melting temperatures of some Sr^{2+} complexes exceeding 95 °C.

The finding that both K^+ and Sr^{2+} facilitate intermolecular G-quadruplex formation in telomeric sequences is not totally unexpected, as Chantot and Guschlbauer (1969) had earlier noted that certain cations, in particular these same two ions, stabilize gel formation of 8-Br-Guo. The ability of these two ions to facilitate guanosine gel formation as well as intermolecular G-quadruplex formation is apparently correlated to their approximately 1.3-Å ionic radii which enable them to be sandwiched snugly between the tetramers forming the cavity (Guschlbauer et al., 1990). When two G-quartets are on adjacent planes separated by the pitch of Watson–Crick (WC) DNA, the geometry is such that the carbonyl oxygen–K^+ distance is 2.8 Å (Sundquist & Klug, 1989), in good agreement with the value observed in a number of polyether·K^+ cryptand complexes (2.78 Å). In contrast, the value of typical Na^+ chelation complexes is 2.4 Å, significantly smaller than the cavity formed by adjacent G-quartets.

Gray and Bollum (1974) studied $d(G)_5$ and found that it can exist in self-complexed forms as well as in single-stranded forms. The CD of the self-complexed forms is characterized by a strong positive maximum at 263 nm and a weaker negative band at 240 nm, whereas that of the single strands is characterized by weak negative and positive bands at 275 and 255 nm, respectively. The CD characteristics of T2 in the presence of Sr^{2+} are almost identical to those of self-complexed forms of $d(G)_5$ which in turn are remarkably similar to those of poly(G) (Howard et al., 1977) and poly(dG). Since these polynucleotides have been shown to form parallel tetraplexes, these observations suggest that the intermolecular quadruplex structures induced by Sr^{2+} in T2 and T4 most likely also possess parallel strand orientations. Parallel tetraplex structure has also been proposed by Sen and Gilbert (1988) to exist in

the dG-rich immunoglobulin switch regions.

If the strand orientation in the intermolecular G-quadruplex is indeed parallel, it clearly contrasts with the antiparallel nature of the structures formed via hairpin formation (including the intramolecular G-quartet). The distinctly opposite CD spectral characteristics observed in our study may, thus, be the consequences of parallel vs antiparallel strand orientation and/or anti vs alternating anti-syn base conformations. If such correlations are valid, the CD spectrum can be a useful tool for determining if these structures are present in a sample.

As to the chirality of the Sr^{2+}-induced intermolecular tetraplex, it is interesting to note a recently published report on the liquid-crystal formation of four-stranded aggregates of oligodeoxyguanylates (Bonazzi et al., 1991). Aqueous solutions of guanosine derivatives $d(G)_n$, where $n = 1, 2, 3, 4$, or 6, were investigated by X-ray diffraction, optical microscopy, and circular dichroism and found to give rise to cholesteric and hexagonal mesophases at high concentrations. The building block of these phases is a chiral rod-shaped aggregate composed of a stacked array of Hoogsteen-base-paired guanosine tetramers. Our CD spectra of thermally treated T2 in the presence of Sr^{2+} are nearly identical to those of $d(G)_3$, $d(G)_4$, and $d(G)_6$ in dilute solutions whose chiralities were identified to be right-handed. Furthermore, our correlation of such CD characteristics to those of intermolecular G-quadruplex appears to be greatly strengthened, as these oligomers are too short to form intramolecular G-quartets or hairpins.

On the basis of calorimetric results, Jin et al. (1990) concluded that a family of guanine-containing DNA of the form d[GGTTXTTGG] exists as intermolecular (tetramolecular) quadruplexes. It is interesting to note, however, that their CD spectra exhibit positive and negative maxima at 293 and 265 nm, respectively. Assuming our structure–CD correlations are correct, such a CD characteristic is more in line with a quadruplex formed via dimerization of G-hairpins than with an intermolecular one. This conjecture seems intuitively reasonable, as hairpin formation with a five-base loop (and subsequent dimerization to tetraplex) is expected to be energetically more favorable than dimeric formation with a large bulge (and subsequent dimerization to tetraplex) in dilute solutions.

Two papers dealing with telomeric DNAs have just appeared in print: one studies the monovalent cation induced structural transitions in $d(T_2G_4)_4$ (Hardin et al., 1991) whereas the other investigates the coherence of synthetic telomeric DNA containing a $d(T_4G_4T_4G_4)$ overhang (Acevedo et al., 1991).

Hardin et al. (1991) found that changing the counterion from sodium to potassium specifically induces conformational transitions in $d(T_2G_4)_4$ which resulted in a change from the intramolecular species to an apparent multistranded structure, accompanied by an increase in the melting temperature by more than 25 °C. Their results suggest that K^+ and NH_4^+ favor intermolecular quadruplex formation whereas Na^+ facilitates the structures formed via hairpin formation. They proposed a model in which duplex or hairpin forms of G-DNA are folding intermediates in the formation of either one-, two-, or four-stranded quadruplex structures. The mechanisms for complex formation involve two different modes of ion binding: (i) pairing of individual strands to form non-WC duplex or hairpin structures driven by counterion condensation and hydrophobic effects or (ii) pairing of non-WC duplexes to form quadruplexes driven by formation of octahedral coordination complexes by monovalent cations caged within two stacked G-quartets and their carbonyl groups. Na^+ and K^+ are essentially equally effective in driving step i, whereas step ii will

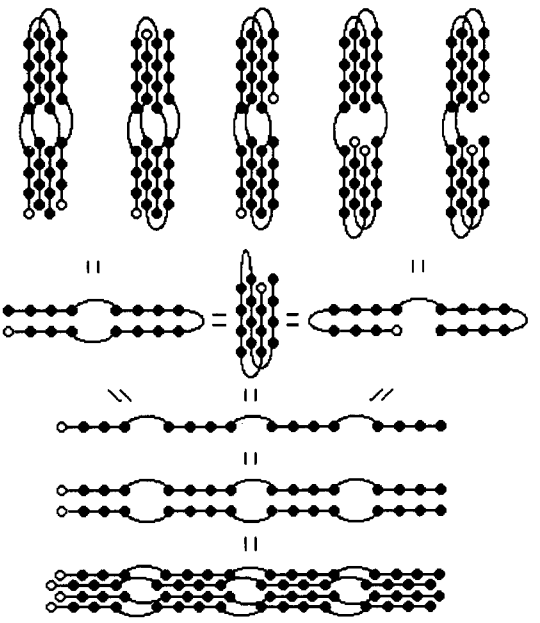

FIGURE 8: Possible conformations and formation pathways for $d(G_4T_2G_4T_2G_4T_2G_4)$ and $d(G_4T_4G_4T_4G_4T_4G_4)$. Curved lines represent dT_n stretches, and circles represent dG, with the open circles designating guanosines at the 5′-end. Only parallel orientations are shown for the intermolecular conformations and antiparallel orientations for the hairpin-related structures.

be preferentially driven by K^+ due to its optimal ionic radius. Our results and interpretations on the facilitation of intermolecular G-quadruplex formation by K^+ are consistent with this model and further indicate that Sr^{2+} is a very efficient octahedral coordinator for the carbonyl groups in a cage formed by guanine tetrads. Since the two ions are of approximate size, our finding that the divalent cation is more effective in inducing intermolecular quadruplex formation may be the consequence of enhanced electrostatic interactions in the cavity due to the increased surface charge density of the cationic sphere, with the enthalpy effect dominating over the possible compensating entropy effect (Inoue & Hakushi, 1985). Indeed, stability constants in water for Sr^{2+} have been found to be greater than those for K^+ in some cryptand complexes such as [2.2.2] and [3.2.2] (Kauffmann et al., 1976). Hardin et al. (1991) further suggested that the strand orientations are parallel in the intermolecular G-quadruplex, a notion we also share.

Some possible conformations and formation pathways, very similar to those proposed by Hardin et al. (1991) but based on parallel orientations for interstrand interactions and antiparallel orientations for the formation of hairpins and their dimerizations, are shown in Figure 8. The intermolecular complexes are depicted below the single-stranded form, whereas the hairpin-related species appear in the upper half. The appearance of the multiconformational electrophoretic patterns is thus to be expected and is entirely consistent with the coexistence of some or all of these forms. Indeed, the presence of three discernible electrophoretic bands in the intermediate mobility region in some samples (for example, lane 6 of Figure 1) is likely the manifestation of different forms of quadruplexes formed via dimerization of G-hairpins.

Acevedo et al. (1991) presented evidence to demonstrate that the telomeres of *Oxytricha* cohere to form dimers of at least five different shapes having different electrophoretic mobilities in the presence of K^+ and exhibit enhanced thermal stability. Dimerization experiments using a variety of synthetic constructs and probing with restriction endonucleases indicate

that the cohered structure only involves the $d(T_4G_4T_4G_4)$ tail from each telomere. Oligomers containing two T_4G_4 motifs protected their N7 positions of dG by forming dimers; those with five T_4G_4 could do so by internal folding, but the 3′-terminal group of G_4 was left unprotected. Our observations of the simultaneous presence of inter-, intra-, and hairpin-molecular forms in T4 and the role of K^+ are consistent with their experimental results. Our results of the significant presence of intramolecular G-quartet in T4 but not in T2 would indicate that the loop size may be important in such structural formation. Although hairpins with two-base loops have been found to exist in WC base pairing [see Chen (1989) and the references cited therein], the loops may be too short for intramolecular G-quartet formation. It is to be noted, however, that an intramolecular quadruplex structure consisting of -TTG- loops had earlier been proposed to exist in $d(T_2G_4)_4$ (Williamson et al., 1989). In contrast to our observation of multiconformational electrophoretic patterns in both T2 and T4 and that band I of T2 moves significantly slower than band I′ of T4, their electrophoretic result of $d(T_2G_4)_4$ consists of a single band which is considerably more mobile than that of $d(T_4G_4)_4$. It seems rather unlikely that the presence of an additional T_n stretch at the 5′-end in their oligomers can result in such contrasting experimental results. We have no ready explanation for these puzzling discrepancies except to point out the orders of magnitude lower DNA concentrations, which should favor the monomeric intramolecular species, and radiolabeling in their gel runs. An intriguing alternate interpretation of our results cannot be discounted: that not only band I′ of T4 but also band I of T2 corresponds to intramolecular G-quartets, with the structure formed by T4 having a more compact form but for some reason not being detected by radiolabel. One possible structure may be an intramolecular quartet formed via pseudoknot formation (Henderson et al., 1987) in T4, with the loops of T2 likely being too short for such a folding.

Finally, our preliminary investigations on the binding of drugs to the G-rich sequences have indicated that T2 and T4 are poor receptors for actinomycin D, ethidium bromide, and chromomycin A_3. It is interesting to note, however, that cyclic ribodiguanylic acid, c(GpGp), can associate with itself to form a self-intercalated dimer and can interact with planar organic intercalator molecules in ways similar to double-helical DNA (Liaw et al., 1990). The authors proposed a cagelike model consisting of a tetrameric c(GpGp) aggregate in which a large cavity is generated to afford a binding site for certain planar intercalators.

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