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# Induction of parallel human telomeric G-quadruplex structures by $Sr^{2+}$

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**Abstract**

Human telomeric DNA forms G-quadruplex secondary structures, which can inhibit telomerase activity and are targets for anti-cancer drugs. Here we show that $Sr^{2+}$ can induce human telomeric DNA to form both inter- and intramolecular structures having characteristics consistent with G-quadruplexes. Unlike $Na^+$ or $K^+$, $Sr^{2+}$ facilitated intermolecular structure formation for oligonucleotides with 2 to 5 5′-d(TTAGGG)-3′ repeats. Longer 5′-d(TTAGGG)-3′ oligonucleotides formed exclusively intramolecular structures. Altering the 5′-d(TTAGGG)-3′ to 5′-d(TTAGAG)-3′ in the 1st, 3rd, or 4th repeats of 5′-d(TTAGGG)$_4$-3′ stabilized the formation of intermolecular structures. However, a more compact, intramolecular structure was still observed when the 2nd repeat was altered. Circular dichroism spectroscopy results suggest that the structures were parallel-stranded, distinguishing them from similar DNA sequences in $Na^+$ and $K^+$. This study shows that $Sr^{2+}$, promotes parallel-stranded, inter- and intramolecular G-quadruplexes that can serve as models to study DNA substrate recognition by telomerase.


*Keywords:* G-quadruplex; G-tetraplex; Telomere; G4 DNA

Telomere DNA assembles with specific proteins to form complexes that protect the ends of eukaryotic chromosomes. The guanine-rich sequences of telomeres are capable of adopting a planar, G-tetrad arrangement by reverse Hoogsteen-like base-pairing between four guanines (Fig. 1A). More than one G-tetrad from an oligonucleotide strand(s) are stacked together form a G-quadruplex (Fig. 1B and C) which is stabilized by coordination of a cation with guanine O6 [1].

There is growing interest in G-quadruplexes because stabilization of these structures may have chemotherapeutic potential. Telomeres are maintained in the majority of cancer cells by the activity of the enzyme telomerase that uses an internal RNA template to direct addition of telomeric sequence onto the 3′ end of the telomere [2]. Telomerase is inhibited by G-quadruplexes formed by the intramolecular folding of a single telomeric DNA strand (Fig. 1B and C) [3–6]. However, telomerase from either *Tetrahymena* or *Euplotes* efficiently recognizes G-quadruplexes formed by association of 4 strands in a parallel-stranded configuration [6]. In addition, telomestatin, a small molecule that stabilizes an intramolecular G-quadruplex [7], preferentially affects the growth of telomerase positive cancer cells [8]. This compound also causes telomere shortening and reduction of the telomeric G-strand overhang [9]. Higher doses disrupt the telomeric nucleoprotein complex [10,11]. A pentacyclic acridine compound, RHPS4, stabilizes 4-stranded quadruplexes and inhibits growth in both telomerase positive and negative cancer cells [12] without reducing telomere length [13].

The coordinating cation can stabilize one form of G-quadruplex over another. Physiological concentrations of $Na^+$ have been shown to stabilize anti-parallel quadruplexes, while $K^+$ can stabilize parallel and anti-parallel quadruplexes [1]. Divalent cations can induce both intra- and intermolecular quadruplexes that are generally highly stabile. For example, the thermostability of a quadruplex complex is increased by $Sr^{2+}$ more than by $Na^+$ or $K^+$, with some melting temperatures surpassing 95 °C [14].

Here, we use native polyacrylamide gel analysis, UV absorption and circular dichroism spectroscopy to analyze

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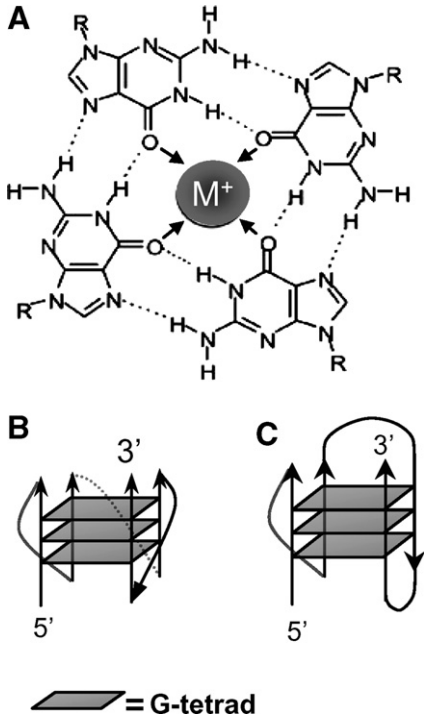


Fig. 1. (A) G-tetrad with the metal cation ($M^+$) coordinating with O6 of the guanines within the tetrad (arrows). (B) Parallel-stranded intramolecular G-quadruplex with external "propeller" loops. (C) Mixed parallel/anti-parallel-stranded G-quadruplex with "propeller" and lateral loops.

the types of G-quadruplexes induced by $Sr^{2+}$ when human telomeric DNA sequence and length are manipulated. We find that $Sr^{2+}$ preferentially stabilizes parallel-stranded, intermolecular G-quadruplexes for DNA oligonucleotides previously shown to form mixed parallel/anti-parallel, intramolecular structures in $K^+$.

## Materials and methods

*Telomeric oligonucleotides.* HPLC purified oligonucleotides composed of normal or altered human telomeric repeats were ordered from Sigma Genosys. Telomeric oligonucleotides (Tn) consisted of $n = 2$–9, 5′-dTTAGGG-3′ repeats, while Tn-all had $n^* = 2$–9, 5′-dTTAGAG-3′ repeats. Tn-X oligonucleotides had a single or multiple 5′-dTTAGGG-3′ repeat(s) converted to 5′-dTTAGAG-3′ in the 5′ → 3′ direction (X).

*Native polyacrylamide gel electrophoresis.* Oligonucleotides (2 μM) prepared in $SrCl_2$ (0 or 100 mM), and TE (100 mM Tris, pH 8.0, 10 mM $Na_2EDTA$) were heated for 5 min at 95 °C, then cooled slowly to room temperature for overnight incubation. Samples were electrophoresed on native 15% polyacrylamide gels in tris–taurine EDTA ± 10 mM $SrCl_2$ at 4.2 V/cm for 16–24 h in the cold room (4 °C). Gels were stained with SYBR Gold after a 30 min wash in tris–borate EDTA.

*Temperature dependent rate of formation of intermolecular structures.* Oligonucleotide T4-1 (2 μM) was prepared in $SrCl_2$ (100 mM) and TE (100 mM Tris, pH 8.0, 10 mM $Na_2EDTA$). After 5 min at 95 °C, the sample was immediately transferred to 30, 40, 50, 60, or 70 °C for 0.25, 0.5, 1 or 2 h. Samples were loaded onto 15% native polyacrylamide gels in tris–borate $Na_2EDTA$ and run as described above. Band migration and intensities were determined using ImageQuant 5.2 software to obtain a plot of the intensity of the slower moving band relative to the total intensity of each lane.

*UV absorption spectroscopy.* Oligonucleotides (2 μM) in TE alone or with $SrCl_2$ (100 mM), were heated to 95 °C, cooled slowly and incubated overnight at room temperature. Absorption spectra (220–320 nm) of oligonucleotides were obtained in 0.1 nm increments using a Beckman Coulter DU-640 Spectrophotometer with a 100 μl cuvette, 1 cm pathlength. The difference spectra were calculated by subtracting the absorbance in TE alone from the absorbance in TE + $SrCl_2$.

*Circular dichroism spectroscopy.* Oligonucleotides (2 μM) prepared in $SrCl_2$ (100 mM) and $Na_2PO_4$ (1 mM), were heated for 5 min at 95 °C, cooled slowly and incubated overnight at room temperature. A Jasco J-720 spectropolarimeter was used to obtain CD spectra at wavelengths 200–320 nm. Samples were loaded in 200 μl cells with a 0.1 cm pathlength. Ten consecutively measured scans taken in 0.5 nm increments over 25 min were averaged for the final CD spectra.

## Results

To investigate the role of $Sr^{2+}$ in the formation of G-quadruplexes of different DNA length and sequence, oligonucleotides T2–T9 denoting the number of 5′-d(TTAGGG)-3′ repeats ranging from 2 to 9 were synthesized. Furthermore, many of the oligonucleotides had at least one of the 5′-d(TTAGGG)-3′ repeats altered to 5′-d(TTAGAG)-3′ to study the role of the middle guanine in formation of these DNA secondary structures.

### *Intramolecular and intermolecular structures were dependent on the size of the telomeric oligonucleotide*

To obtain $Sr^{2+}$-dependent changes in migration rates, samples for each experiment were electrophoresed in gels with double-stranded DNA size standards to compare relative mobilities. In the absence of $Sr^{2+}$, the migration rate of T2–T9 (5′-d(TTAGGG)$_{(2–9)}$-3′ repeats) in native polyacrylamide gels decreased as the size of the oligonucleotides increased, with oligonucleotide T4 running slightly faster than expected (Fig. 2A, no salt). The migrations of oligonucleotides with 2–9 5′-d(TTAGAG)-3′ repeats (T2*-all to T9*-all, highlighted by astericks in Fig. 2A) were very similar to those of T2–T9 except T4-all did not run as fast as T4.

To observe the effect of $Sr^{2+}$ on oligonucleotide structure, samples were first incubated in the presence of 100 mM $SrCl_2$ then subjected to electrophoresis in gels without $Sr^{2+}$ in the gel or running buffer (Fig. 2A, $Sr^{2+}$ in sample only) or in gels with $Sr^{2+}$ in buffer and gel (Fig. 2A, $Sr^{2+}$ sample + gel). $Sr^{2+}$ present in the sample, but not the gel, caused an increase in migration rate for oligonucleotides T4–T9 (Fig. 2A, $Sr^{2+}$ sample only) except T5. The fact that many of these $Sr^{2+}$-induced structures were retained in gels lacking $Sr^{2+}$ suggests tighter binding of the divalent cation compared to $K^+$ and $Na^+$.

The presence of $Sr^{2+}$ in the gels and running buffers stabilized additional structures (Fig. 2A, $Sr^{2+}$ sample+gel). T2, T3, T4, and T5 formed slower-moving structures. Unexpectedly, $Sr^{2+}$ caused Tn-all oligonucleotides with 7–9, 5′-d(TTAGAG)-3′ repeats to have slower migration rates and those with 2–5, 5′-d(TTAGAG)-3′ repeats to have faster migration rates.

To further investigate the role of each 5′-d(TTAGGG)-3′ repeat in the intramolecular T4 structure, oligonucleo-

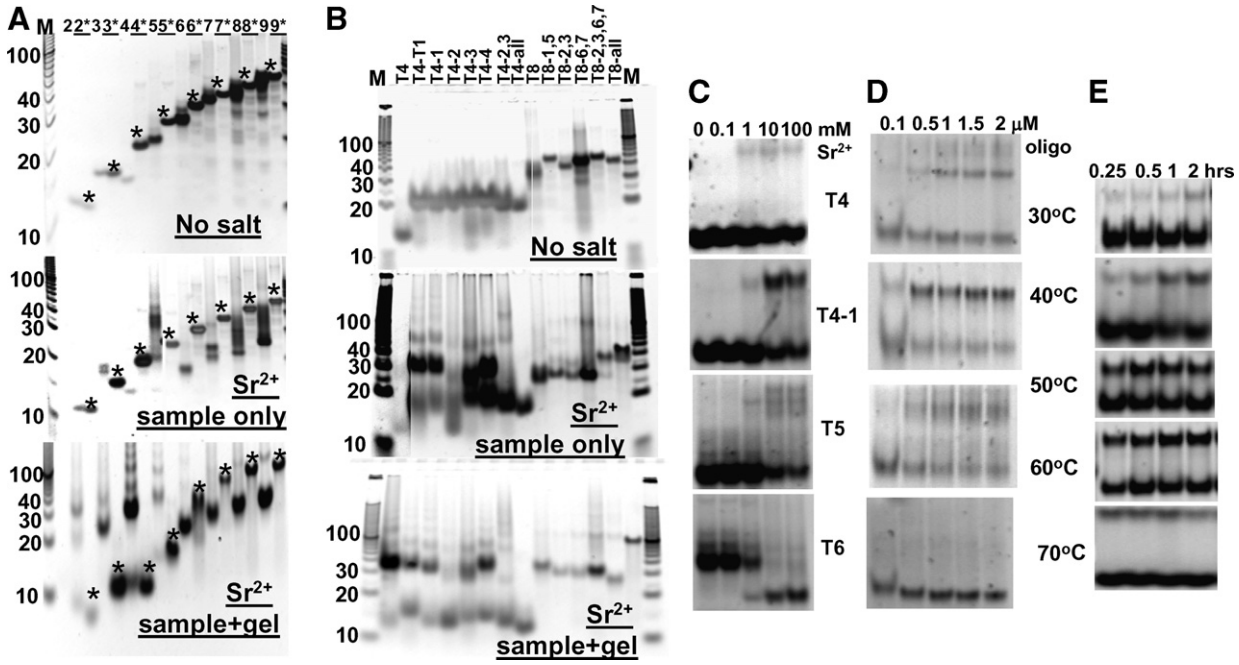


Fig. 2. Native gel electrophoresis of telomeric oligonucleotides in gels with (sample + gel) or without (no salt and sample only) 10 mM $SrCl_2$. Oligonucleotides (2 μM) were prepared ±100 mM $SrCl_2$ as described in Materials and methods. (A) Migration rates of different sized telomeric oligonucleotides with ($n$) number of telomeric repeats. Tn: [5′-d(TTAGGG)$_n$-3′] and numbers with astericks ($n^*$) refer to Tn*-all: [5′ – d(TTAG<u>A</u>G)$_{n^*}$ – 3′]. (B) Migration rates of T4-X and T8-X, where X denotes the repeats in the 5′ → 3′ direction that have been altered to 5′-d(TTAG<u>A</u>G)-3′, and T4–T1 [5′-dTTAG<u>T</u>G(TTAGGG)$_3$-3′]. M refers to a 10 bp DNA Ladder. (C–E) Effects of $Sr^{2+}$ and oligonucleotide concentration, time, and temperature on structure formation. Oligonucleotides (T4: [5′-d(TTAGGG)$_4$-3′], T5: [5′-d(TTAGGG)$_5$-3′], T6: [5′-d(TTAGGG)$_6$-3′], and T4-1: [5′-dTTAG<u>A</u>G(TTAGGG)$_3$-3′]) were prepared in TE with (C) 0–2 mM $SrCl_2$ concentrations at 2 μM oligonucleotide concentration and (D) 0.1–2 μM DNA concentrations in 100 mM $SrCl_2$. (E) Time course reactions with T4-1 (2 μM) in $SrCl_2$ (100 mM). Samples were treated according to Materials and methods at the indicated temperatures for the indicated times. Samples were electrophoresed on 15% native polyacrylamide gels according to Materials and methods. Bands were visualized using SYBR Gold.

tides were synthesized in which at least one 5′-dTTAGGG-3′ was converted to 5′-d(TTAG<u>A</u>G)-3′. In the absence of added cation, T4 and T8 had faster migration rates than all T4- and T8-derived oligonucleotides, respectively (Fig. 2B, no salt).

Alteration of the 5′-most, 5′-d(TTAGGG)-3′ repeat to 5′-d(TTAG<u>A</u>G)-3′ or 5′-d(TTAG<u>T</u>G)-3′ (Fig. 2B, T4-1 and T4–T1, $Sr^{2+}$ sample only) stabilized a slower-migrating species which most likely represents an intermolecular structure. Slower moving species were also present in T4-derived oligonucleotides with the 3rd and 4th repeats altered (T4-3 and T4-4). The mobility of T4-2,3 and T4-all were not substantially altered by $Sr^{2+}$ in the samples only. Interestingly, $Sr^{2+}$ caused T4-2 to form a more compact structure with an increased mobility similar to T4. However, T4-2 consistently formed a smeared band suggesting the existence of more than one structure or an unstable structure. $Sr^{2+}$ caused T8 and its derivatives to have greater migration rates with the exception of T8-2,3,6,7 and T8-all.

When $Sr^{2+}$ was present in both the samples and the gel (Fig. 2B, $Sr^{2+}$ sample+gel) T4 was converted to a slower moving structure with similar mobility to that of T4–T1, T4-1, T4-3, and T4-4. T4-2 still formed a more compact structure as did T4-2,3 and T4-all. In addition, T8-2,3,6,7 also formed a more compact structure with a similar mobility to T8 and the other T8-derivatives with the exception of T8-all. As shown in Fig. 2B, T8-all formed a structure that migrated significantly slower compared to that in the absence of salt.

To determine the amount of $Sr^{2+}$ required to cause structural changes, samples were incubated with increasing $SrCl_2$ and subjected to electrophoresis in gels lacking $Sr^{2+}$ (Fig. 2C). Between 1 and 10 mM $Sr^{2+}$ was required to induce structure formation regardless if the structures were larger (T4-1 and T5) or more compact (T6). Induction of slower-migrating structures by $Sr^{2+}$ was also dependent on the oligonucleotide concentration (Fig. 2D, T4, T4-1, and T5) suggesting that they were formed by association of more than one oligonucleotide strand. The $Sr^{2+}$-induced, faster-migrating structures formed by oligonucleotides such as T6 were not altered by oligonucleotide concentration consistent, with the formation of intramolecular G-quadruplexes.

Although intramolecular structures formed within a timescale of minutes (data not shown), intermolecular structures were much slower. At room temperature (data not shown) and 30 °C, few intermolecular structures were formed by 2 h (Fig. 2E). However, heating the samples greatly sped up the reaction such that substantial intermolecular structures were present after 1 h incubation at 40 °C.

*UV absorption and CD spectroscopy suggests the formation of G-quadruplexes*

G-quadruplexes and other structures that rely on Hoogsteen-type base pairing have a signature hyperchromicity at 295 nm [15]. The UV difference absorption spectra ($+Sr^{2+}$ vs $-Sr^{2+}$) for some of the oligonucleotides in this study possess this characteristic. For example, both T4 (Fig. 3A) and T8 (Fig. 3B) have a $Sr^{2+}$-induced hyperchromicity at 295 nm with hypochromicity at ~270 nm. The majority of the other oligonucleotides had difference spectra similar to T4 and T8 except that the region of hyperchromicity was less defined and centered around 290 nm instead of 295 nm. The exceptions were T4-2,3, T4-all, and T8-all. T4-2,3 and T4-all (Fig. 3A) had nondescript difference spectra (Fig. 3A). This was also true for T3-all (data not shown). T8-all (Fig. 3B) and T7-all (data not shown) had characteristic spectra but the hyperchromicity was shifted to 285–290 nm and the hypochromicity was shifted to 260 nm.

Circular dichroism spectroscopy was used to characterize the nature of the G-quadruplexes (Fig. 3C and D). Parallel-stranded G-quadruplex structures with an *anti*-glycosidic bond conformation typically have a CD spectrum with a strong positive peak at 260–265 nm and a smaller negative signal at 240 nm [16]. Parallel-stranded, intramolecular G-quadruplexes with *anti*-glycosidic bonds and external or "propeller" loops (Figs. 1B and 3E) also have a peak at 260–265 nm with a shoulder around 290 nm [17]. Anti-parallel-stranded G-quadruplexes typically seen in $Na^+$ buffers with lateral and diagonal loops and a mixture of *anti*/*syn*-glycosidic bonds have a strong positive ellipticity at 295 nm, a weaker negative signal at 265 and a weak positive peak at 245 nm [18,19]. Hybrid G-quadruplex structures have mixed anti-parallel/parallel strands and external (propeller) plus lateral loops with CD spectra having a strong positive peak at 290 nm, a shoulder at ~270 nm, and a small negative signal at ~240 nm [18,20–22].

The CD spectra generally mirrored the UV difference absorption spectra in that oligonucleotides with similar UV absorption spectra also had similar CD spectra. In the absence of salt, the CD spectra for all tested oligonucleotides were very similar with positive peaks around 250–260 nm (Fig. 3C and data not shown). In the presence of $K^+$, T4 (Fig. 3C) and T8 (data not shown) had spectra

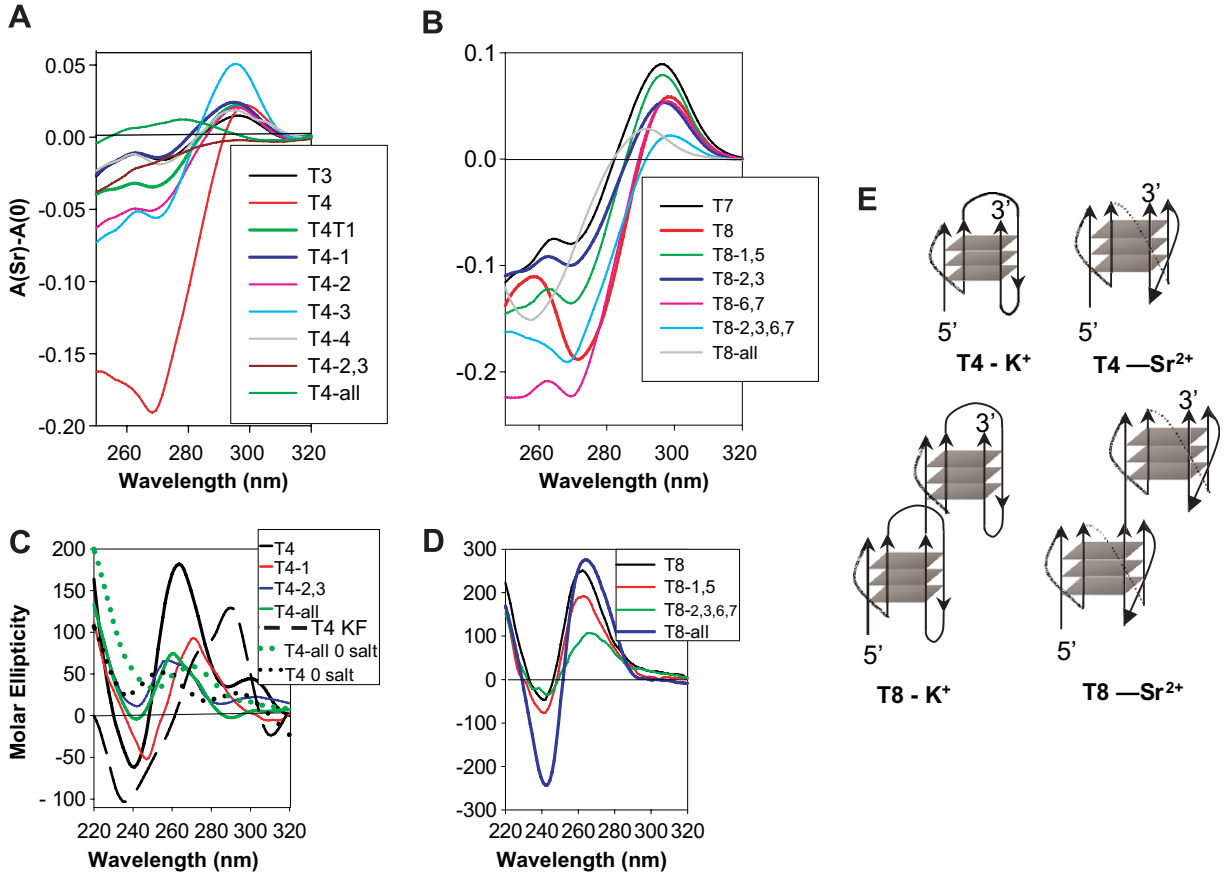


Fig. 3. UV difference absorption and CD spectroscopy of $SrCl_2$-induced structural formations of telomeric oligonucleotides, [Tn: 5′-d(TTAGGG)$_n$-3′, Tn*-all: 5′ – d(TTAGAG)$_{n^*}$ – 3′ and Tn-X, where X denotes the repeats in the 5′ → 3′ direction that have been altered to 5′-d(TTAGAG)-3′. (A,B) Difference in UV absorption wavelength scans of oligonucleotides incubated overnight in 100mM $SrCl_2$ (A(S)) and TE alone (A(0)). (C,D) CD spectroscopy of oligonucleotides incubated overnight in $Na_2PO_4$ and either 100 mM $SrCl_2$ (default), 100 mM KF or no additional salt (0 salt). Molar ellipticity is in deg * cm$^2$/dmol of bases. (E) Putative intramolecular structures formed by oligonucleotides T4 and T8 with mixed parallel/anti-parallel strand orientation in $K^+$ or all parallel-strand orientation in $Sr^{2+}$.

indicative of hybrid, parallel/anti-parallel G-quadruplexes. Unlike $K^+$, addition of $Sr^{2+}$ created T4 and T8 structures with strong positive peaks at 265 nm (Fig. 3C and D) indicating parallel-stranded forms of G-quadruplex (Fig. 3E).

The spectrum for T4-1 was slightly different in that the positive ellipticity centered around 270–275 nm instead of 265 nm. In addition, the negative signal at 240 nm moved to 250 nm. As with the UV difference absorption spectra, the CD spectra for T4-2, T4-3, and T4-4 were very similar to that of T4-1 (data not shown). Also like the UV difference absorption spectra, the CD spectra for T4-2,3 and T4-all were different than the rest of the T4-derived oligonucleotides with a positive ellipticity at 255–260 nm.

The CD spectra for the T8-derivatives were nearly identical to T8 (Fig. 3D and data not shown) with the exception of T8-all. As with the UV absorption difference spectrum, the CD spectrum of T8-all was slightly different. The negative signal at 240 nm was similar to that of T8. However, instead of a positive peak at 265 nm, the peak for T8-all centered around 270 nm. The T7-all spectrum (data not shown) was identical to that of T8-all.

## Discussion

The results from this study emphasize the diversity of G-quadruplex structures formed by human telomeric DNA. Although $Na^+$ typically stabilizes anti-parallel structures and $K^+$ stabilizes mixed parallel/anti-parallel structures for sequences similar to our T4 and T8, $Sr^{2+}$ appears to stabilize exclusively parallel-stranded structures. This has also been shown for the sequence 5′-d($G_4T_4G_4T_4G_4T_4G_4$)-3′ in the presence 2 mM $Sr^{2+}$ [14] and 5′-d($G_4T_4G_4$)-3′ in $Ca^{2+}$ [23]. However, $Sr^{2+}$ does not exclusively stabilize parallel-stranded G-quadruplexes for all G-rich DNA since the thrombin binding aptamer (5′-d(GGTTGGTGTGGTTG G)-3′) in $Sr^{2+}$ forms an anti-parallel G-quadruplex [24].

In addition to inducing parallel-stranded structures, our data show that the oligonucleotides T4 and T5, previously shown to form intramolecular quadruplexes in $K^+$ [21,22], can also form intermolecular structures in $Sr^{2+}$. This has been observed for the oligonucleotides 5′-d($G_4T_2G_4T_2G_4T_2G_4$)-3′ and 5′-d($G_4T_4G_4T_4G_4T_4G_4$)-3′ [14]. However, the longer oligonucleotides, T6–T9 in our study, form only intramolecular structures.

Intermolecular structures are also observed when the middle guanine in any of the 5′-d(TTAGGG)-3′ repeats of T4 was altered to an adenine or thymine. Both the UV difference absorption and CD spectra for these structures were only slightly different from those of T4 and T8 suggesting that they are G-quadruplexes.

Unlike the T4-derivatives, altering a middle guanine in two of the 5′-d(TTAGGG)-3′ repeats of T8 resulted in structures that appear to be very similar to T8 even though a stretch of 4, 5′-d(TTAGGG)-3′ repeats are disrupted. However, if all the repeats are altered (T7-all, T8-all, and T9-all) the oligonucleotides adopt structures with distinct electrophoretic mobilities, UV absorption and CD spectra. It is not clear at this time whether some of these structures with distinct spectra are G-quadruplexes.

G-quadruplex architecture is modified by different cations because of the various coordination complexes formed with guanines in the stacking G-tetrads. $Na^+$ ions have been found to be located within the plane of a G-tetrad [25]. However $K^+$ [26,27] and $Sr^{2+}$ [28,29] are sandwiched between two G-tetrads, coordinating with the carbonyls of eight guanines in a square anti-prism configuration. In the case of $K^+$ complexes, each carbonyl coordinates with a $K^+$ above and below each tetrad [27] and it has been proposed that this arrangement can have properties of ion channels [30]. Unlike $K^+$, $Sr^{2+}$ and other divalent cations [28,29,31,32], are only found between alternating G-tetrad planes because each carbonyl only interacts with one cation. In addition, the divalent cation-carbonyl bond lengths are shorter [31] resulting in a more compressed coordination sphere between the cation and the eight guanines. To accommodate these $Sr^{2+}$-guanine coordination complexes, glycosidic bond angles and consequently strand orientations will likely be different than in $K^+$ complexes.

These studies add to the growing literature demonstrating the high degree of structural polymorphism of telomeric G-quadruplexes. The finding that telomerase preferentially acts upon a parallel-stranded over an anti-parallel-stranded quadruplex [6] suggests that the enzyme may recognize specific telomeric DNA secondary structures. The structures in this study can be used to ascertain telomerase-DNA substrate interactions and the mechanisms of telomere maintenance disruption caused by G-quadruplex-stabilizing, small molecules.

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