Effect of DNA Secondary Structure on Human Telomerase Activity†

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ABSTRACT: Telomeres are specialized DNA−protein complexes located at the chromosome ends. The guanine-rich telomeric sequences have the ability to form G-quadruplex structures under physiological ionic conditions in vitro. Human telomeres are maintained through addition of TTAGGG repeats by the enzyme telomerase. To determine a correlation between DNA secondary structure and human telomerase, telomerase activity in the presence of various metal cations was monitored. Telomerase synthesized a larger proportion of products corresponding to four, five, eight, and nine full repeats of TTAGGG in 100 mM K^+ and to a lesser extent in 100 mM Na^+ when a $d(TTAGGG)_3$ input primer was used. A more even product distribution was observed when the reaction mixture contained no added Na^+ or K^+. Increasing concentrations of Cs^+ resulted in a loss of processivity but not in the distinct manner observed in K^+. When the input primer contained 7-deaza-dG, the product distribution resembled that of reactions without K^+ even in the presence of 100 mM K^+. Native polyacrylamide gel electrophoresis indicated that $d(TTAGGG)_4$, $d(TTAGGG)_5$, $d(TTAGGG)_8$, and $d(TTAGGG)_9$ formed compact structures in the presence of K^+. The oligonucleotide $d(TTAGGG)_4$ had a UV spectrum characteristic of that of the G-quadruplex only in the presence of K^+ and Na^+. A reasonable explanation for these results is that four, five, eight, and nine repeats of TTAGGG form DNA secondary structures which promote dissociation of the primer from telomerase. This suggests that telomerase activity in cells can be modulated by the secondary structure of the DNA template. These findings are of probable relevance to the concept of telomerase as a therapeutic target for drug design.

Telomeres are specialized DNA−protein complexes located at the ends of chromosomes. They are proposed to be responsible for chromosome stabilization (*1*) and to be involved in interactions with other chromosomes (*2, 3*). There is also evidence that telomeres are associated with the nuclear envelope and nuclear matrix (*4*−*6*). At least three separate structural domains are present in telomeres (depending on their length). In cells with long telomeres, the domain most distal to the end of the chromosome has a chromatin structure containing regularly-spaced nucleosomes (*7, 8*) and histone H1. Further towards the end, the chromatin structure is more heterogeneous and telomere binding proteins are present (*9*−*11*) forming a heterochromatic region termed the "telosome". Finally, a 3′ overhang of the guanine-rich strand exists which can interact specifically with single-stranded telomere binding proteins (*12*−*17*).

These single-stranded "tails" containing guanine-rich telomeric sequences from a variety of organisms including *Tetrahymena* (*18, 19*), *Oxytricha* (*20, 3*), *Saccharomyces* (*21*), and human (*22*−*24*) can form unique secondary structures, G-quadruplexes, based on the G-tetrad. The G-tetrad is formed by a Hoogsteen-type base pairing between four guanines and chelation of a metal cation. Consequently,

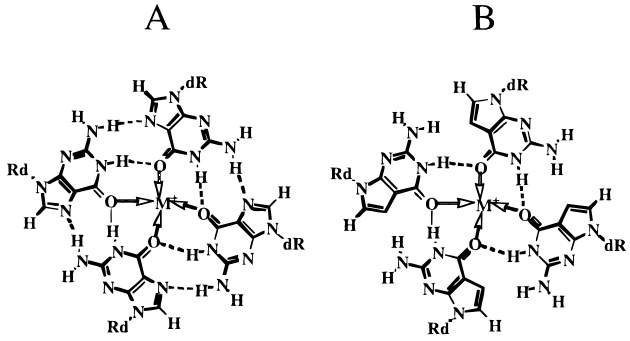

FIGURE 1: (A) Reverse Hoogsteen base-pairing in the G-tetrad. (B) Loss of hydrogen bonding when 7-deaza-2′-deoxyguanine replaces guanine. M^+ refers to the metal cation that coordinates with O6 of the guanine bases.

G-quadruplex formation is dependent both on DNA sequence and the cations present. For example, it is known that the sequence $d(TTAGGG)_4$ forms an intramolecular G-quadruplex structure in K^+ with N7 of the guanine base involved in reverse-Hoogsteen base pairing within each G-tetrad (Figure 1), whereas $d(TTAGGG)_3$ does not (*23*). This structure can be disrupted by the presence of 7-deaza-dG within the sequence (*23*). The stability of G-quadruplex structures is also sensitive to the type of cation present. Coordination of O6 of the guanine bases within the G-tetrad requires a cation with a specific ionic radius. Because of this, K^+ is more efficient at stabilizing the quadruplex than Na^+, which is more efficient than Cs^+. In addition, some proteins including RAP1 (*25*) and the β subunit of *Oxytricha* telomere binding protein (*26*) stabilize G-quadruplex structures.

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FIGURE 2: Telomerase time course reactions with either 0 (A1), 50 (B1), or 100 mM (C1) K^+. Reaction time points for lanes 1, 2, 3, and 4 were 10, 20, 30, and 60 min, respectively. The numbers (3), (4), etc., beside the gel refer to telomerase synthesizing $d(TTAGGG)_3ttag$ or three full repeats of TTAGGG, $d(TTAGGG)_4ttag$ or four full repeats of TTAGGG, etc. The 19-base marker added to the telomerase reactions and in the lane marked M consisted of a 5′ biotinylated $d(TTAGGG)_3$ that was 3′ labeled with [α-^{32}P]-cordycepin and terminal transferase. Telomerase reactions and sample processing were carried out according to procedures in Materials and Methods. Panels A2, B2, and C2 are the quantitation of A1, B1, and C1, respectively, showing the increase of (■) 4, (◆) 5, (□) 6, (◇) 7, (●) 8, (▲) 9, (○) 10, and (△) 11 TTAGGG repeats with time in 0 (A2), 50 (B2), and 100 mM K^+ (C2).

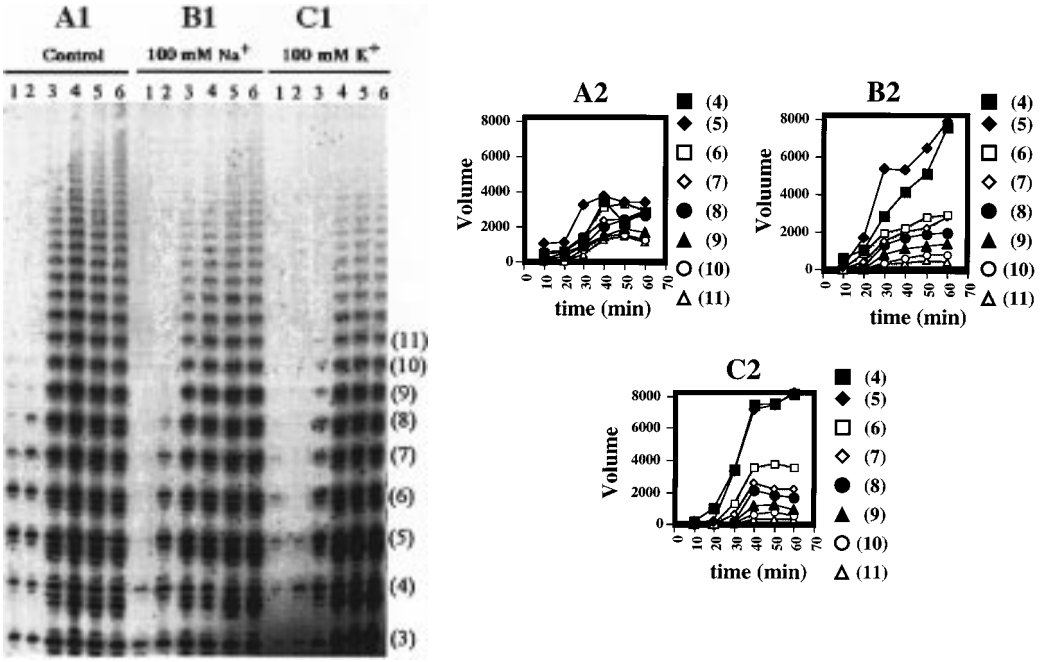

FIGURE 3: Telomerase time course reactions with no added monovalent cations (A1) and with 100 mM Na^+ (B1) and 100 mM K^+ (C1). Reaction time points for lanes 1, 2, 3, 4, 5, and 6 were 10, 20, 30, 40, 50, and 60 min, respectively. Panels A2, B2, and C2 are the quantitation of A1, B1, and C1 respectively, showing the increase of (■) 4, (◆) 5, (□) 6, (◇) 7, (●) 8, (▲) 9, (○) 10, and (△) 11 TTAGGG repeats with time with no added monovalent cations (A2) and with 100 mM Na^+ (B2) and 100 mM K^+ (C2). Numbers in parentheses at the right of the gel refer to *n*, the number of d(TTAGGG)$_n$ttag repeats.

Native Polyacrylamide Gel Electrophoresis. ^{32}P-5′-End-labeled oligonucleotides (10 μM) were incubated in the specified amount of KCl-, NaCl-, or CsCl/40 mM Tris-borate and 1 mM EDTA at pH 8 (TBE) solutions for 2 min at 90 °C. Samples were allowed to renature at 4 °C for 30 min before addition of 1 μL of loading dye (5% bromophenol blue, 5% xylene cyanol, and 60% sucrose) and loaded on the gels. The samples were run on 15% (29:1 acrylamide: bisacrylamide) native polyacrylamide gels at 7 V/cm (4 °C) until the bromophenol blue had run 30 cm in the gel (24−30 h). Before running, the gels were equilibrated for 30 min in the running buffers containing the appropriate monovalent cations.

Ultraviolet Spectroscopy. UV spectra and melting profiles were obtained using the Beckman DU 640 UV−visible spectrometer. Melting temperatures were determined by varying the temperature at a rate of 1 °C/min using a Peltier temperature controller with a six sample cell holder (Beckman). Samples contained 2 mM Tris-HCl, 0.1 mM Na_2-EDTA, 2−6 μM d(TTAGGG)$_4$ oligonucleotide, and 0 or 100 mM KCl, NaCl, or CsCl.

RESULTS

Effects of K^+ on Telomerase Activity. Since it is known that K^+ stabilizes G-quadruplex structures, the effect of K^+ on telomerase activity was addressed by observing a change in banding pattern in sequencing gels that correlates with particular sized telomerase synthesis products. The telomerase activity in the absence of added monovalent cations (Figure 2, A1 and A2) showed a ladder representing the products pertaining to additions of d(TTAGGG)$_n$ttag by telomerase onto the d(TTAGGG)$_3$ telomeric primer (*30*, *31*). These products increased with time at approximately the same rate regardless of the number of TTAGGG repeats added (Figure 2, A2). However, in the presence of 50 mM K^+, the rate at which 6 and 7 full TTAGGG repeat products were formed was reduced relative to that of 4 and 5 (Figure 2, B1 and B2). This effect was more pronounced when 100 mM K^+ was present in the reaction mixture (Figure 2, C1 and C2). In addition, the amount of products pertaining to 8 and 9 full TTAGGG repeats was slightly enhanced with a relative reduction in products pertaining to 10 and 11 full TTAGGG repeats. Interestingly, this effect was observed for telomerase products that have a difference in length of exactly 4 TTAGGG repeats.

Comparison of Telomerase Activity in K^+, Na^+, and Cs^+. To further investigate the effect of DNA structure on telomerase, telomerase activity was monitored in the presence of cations that stabilize G-quadruplex structures to varying degrees. In Figure 3, the effects of 0 and 100 mM K^+ and Na^+ on the telomerase ladder were compared. The presence of 100 mM Na^+ appeared to cause effects similar to those caused by K^+ (Figure 3, B1 and B2). However, in K^+, the increase in the 4 and 5 TTAGGG repeats appeared at earlier time points. Another monovalent cation, Cs^+, had a dramatic effect on the pattern of the telomerase ladder (Figure 4). In contrast to the distinct effects on the individual bands caused by the addition of K^+ and Na^+, Cs^+ caused an overall decrease in processivity with a profound enhancement of the early extensions. The striking reduction in telomerase processivity in the presence of increasing Cs^+ ions could be due to a DNA·RNA conformational change in the presence of Cs^+ that destabilizes binding of telomeric DNA within the telomerase active site. In this regard, it is known that Cs^+ can cause structural changes in duplex DNA (*32*−*34*).

Effects of K^+ on the Telomerase Ladder with a 7-Deaza-2′-deoxyguanine-Containing Oligonucleotide. To determine whether the changes in telomerase activity observed in the

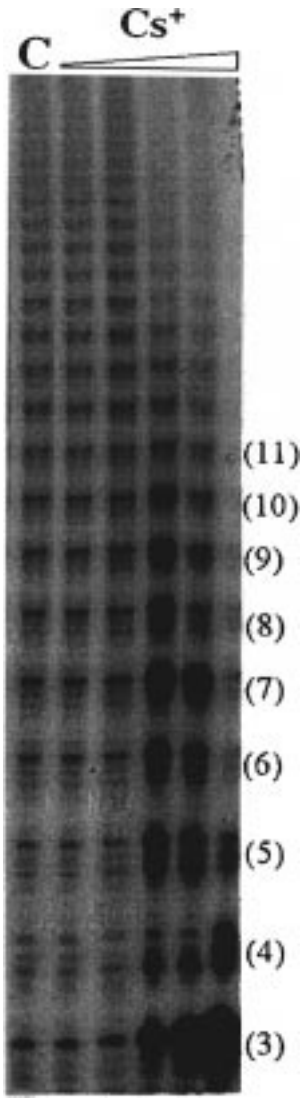

FIGURE 4: Effect of Cs^+ on telomerase activity. Increase in concentration of Cs^+ where lanes 1−6 refer to 0, 6.25, 12.5, 25, 50, and 100 mM respectively. Numbers in parentheses at the right of the gel refer to *n*, the number of d(TTAGGG)$_n$ttag repeats.

previous figures are truly due to secondary structure rather than cation effects, the telomerase activity in K^+ utilizing the primer, d(TTAGGGTTAGGGTTAGGG), where G is 7-deaza-dG, was determined. Both G-quadruplex and hairpin formation of guanine-rich oligonucleotides involve nitrogen 7 (N7) in reverse Hoogsteen base-pair hydrogen bonding. Replacement of N7 with a carbon greatly destabilizes secondary structure even in the presence of the appropriate cations. Figure 5 illustrates that there was a change in the telomerase ladder in the presence of 100 mM K^+. In general, more products were produced in the presence of K^+. However, the large drop in activity between products belonging to 5 and 6 TTAGGG repeats and between 9 and 10 TTAGGG repeats illustrated in Figures 2 and 3 was not observed.

Evaluation of Secondary Structure of TTAGGG Oligonucleotides with Native Polyacrylamide Gel Electrophoresis. Although the secondary structures of human telomeric sequences such as d(T_2AG_3)$_4$, d(G_3T_2A)$_3G_3$, d($G_3T_2AG_3$), (*22*) and d(AG_3(T_2AG_3)$_3$) (*24*) have been extensively characterized, the structures of longer human telomeric repeats

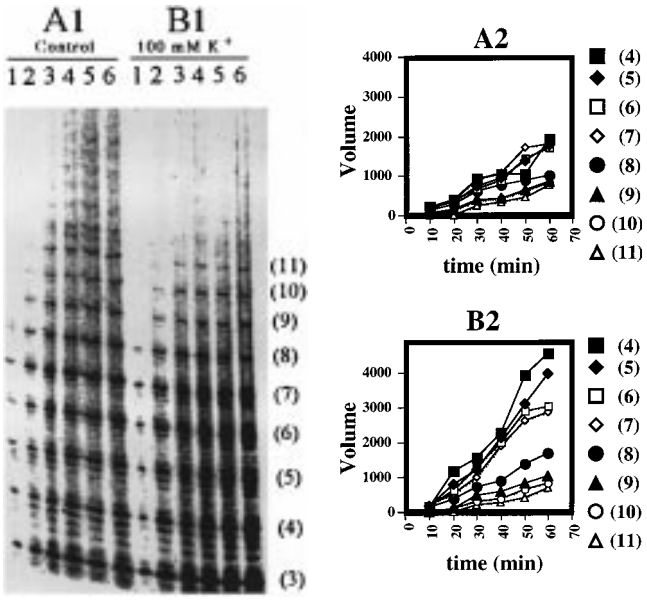

FIGURE 5: Telomerase time course reaction using a TTAGGGTTAGGGTTAGGG-containing input primer (where G = 7-deaza-dG) with no added monovalent cations (A1) and with 100 mM K^+ (B1). Incubation times for lanes 1−6 were 10, 20, 30, 40, 50, and 60 min. Numbers in parentheses on the right of the gel refer to *n*, the number of d(TTAGGG)$_n$ttag repeats. Panels A2 and B2 are the quantitation of A1 and B1, respectively, showing the increase of (■) 4, (◆) 5, (□) 6, (◇) 7, (●) 8, (▲) 9, (○) 10, and (△) 11 TTAGGG repeats with time with no added monovalent cations (A2) and with 100 mM K^+ (B2).

such as d(TTAGGG)$_n$, where $n \geq 5$, have yet to be determined. Since this is the size range of the telomeric products where we observed changes in the presence of K^+ and Na^+, it was essential that the secondary structure be analyzed. Thus, the secondary structure of d(TTAGGG)$_n$ oligonucleotides, where $n = 3-9$, was analyzed on the basis of the rates of migration in 15% non-denaturing polyacrylamide gels in the presence of TBE only, K^+, Na^+, and Cs^+, as illustrated in Figure 6, panels A, B, C, and D, respectively. In the gel containing no added cations, a line could be drawn that connected the oligonucleotides d(TTAGGG)$_3$ to d(TTAGGG)$_7$. The oligonucleotides d(TTAGGG)$_8$ and d(TTAGGG)$_9$ showed only a slight deviation from the line. Also, d(TTAGGG)$_4$ was composed of a species that ran along the line plus another species that ran slightly faster. However, in both 50 (Figure 6B) and 100 mM K^+ (data not shown), d(TTAGGG)$_4$ had a dramatically greater mobility that is suggestive of a more compact structure than single-stranded DNA. The d(TTAGGG)$_8$ oligonucleotide also had a significantly increased mobility in K^+. The oligonucleotides d(TTAGGG)$_5$ and d(TTAGGG)$_9$ also had increased mobilities relative to that with no monovalent cations but to a much lesser extent than what is observed for d(TTAGGG)$_4$ and d(TTAGGG)$_8$. This suggests that d(TTAGGG)$_5$ and d(TTAGGG)$_9$ form relatively less compact structures in the presence of K^+ or Na^+. Interestingly, a line could still be drawn through d(TTAGGG)$_3$, d(TTAGGG)$_6$, and d(TTAGGG)$_7$. The mobilities in the presence of Na^+ were similar to what was observed with K^+. In contrast, all oligonucleotides, with the exception of d(TTAGGG)$_4$, moved close to the diagnoal line in the presence of Cs^+, suggesting that in Cs^+, only d(TTAGGG)$_4$ could still form a secondary structure at 4 °C. The oligonucleotides d(T_2G_4)$_4$ and

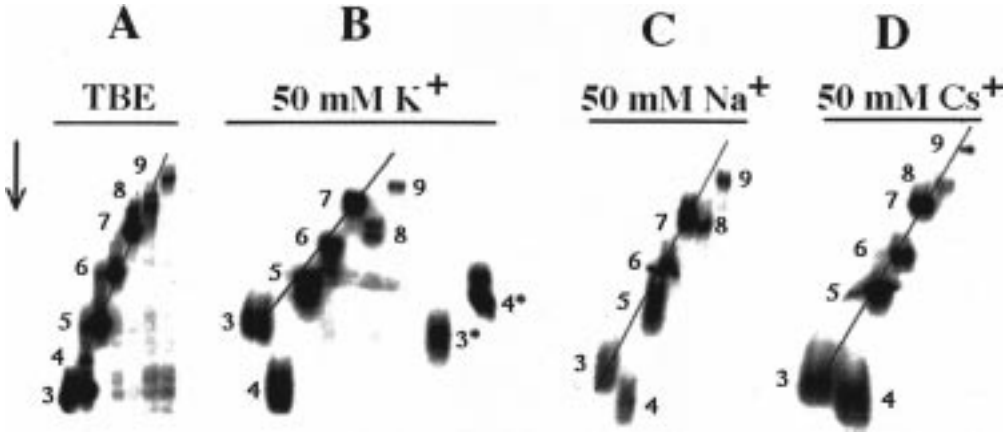

FIGURE 6: One-dimensional 15% native polyacrylamide gel electrophoresis with (A) no added monovalent cations or with (B) 50 mM K^+, (C) 50 mM Na^+, or (D) 50 mM Cs^+, containing 5′-^{32}P-labeled primers with 3, 4, 5, 6, 7, 8, and 9 TTAGGG repeats loaded, from left to right, for each gel. The numbers 3* and 4* in gel B refer to TTAGGGTTAGGGTTAGGG and TTAGGGTTAGGGTTAGGGTTAGGG, where G is 7-deaza-dG. The arrow indicates the direction of electrophoresis. Gel B was run for 24 h. Gels A, C, and D were run for 30 h.

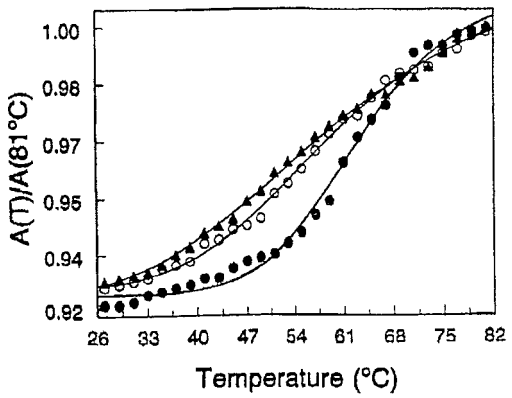

FIGURE 7: UV melting profile of $d(TTAGGG)_4$ monitored at 260 nm in K^+ (●), Na^+ (○), and Cs^+ (▲). Curves were plotted as the absorbance at various temperatures, *A*(*T*), divided by the absorbance at 81 °C, *A*(81 °C) vs temperature (°C).

$d(T_4G_4)_4$ also form compact structures in Cs^+ with greater mobilities in native polyacrylamide gels (*3*).

To investigate the requirement of N7 in the faster moving species formed by $d(TTAGGG)_4$ in K^+, oligonucleotide d(TTAGGGTTAGGGTTAGGGTTAGGG), where G is 7-deaza-dG, was also run in a native gel containing K^+ (Figure 6B, 4*). This oligonucleotide ran much slower than the $d(TTAGGG)_4$ in K^+. In fact, its migration is what would be expected for a single-stranded 24-base oligonucleotide lacking secondary structure.

Analysis of Secondary Structure through UV Spectroscopy. The melting profiles of $(TTAGGG)_4$ in each cation were decidedly different (Figure 7). The structure formed in K^+ melted at about 10 °C higher than that in Na^+, which melted almost 5 °C higher than that in Cs^+. In addition, the K^+ profile had a very sharp transition compared to both the Na^+ and the Cs^+ profile, which implies a greater enthalpic contribution to the stabilization of the structure(s) in K^+.

DISCUSSION

Using a modified primer extension assay, we have demonstrated for the first time that the concentration and type of monovalent cation can affect human telomerase in a distinct manner. In the presence of K^+ ions there was a reduction in activity in the formation of 6 and 7 full TTAGGG repeats in relation to an enhancement in 4 and 5 full TTAGGG repeats. Moreover, there was an equivalent loss in formation of 10 and 11 TTAGGG repeats. These results corresponded directly with the observation that $d(TTAGGG)_4$, $d(TTAGGG)_5$, $d(TTAGGG)_8$, and $d(TTAGGG)_9$ formed more compact structures in K^+-containing native polyacrylamide gels. Although $d(TTAGGG)_4$ also formed species with greater mobility in gels containing Na^+ and Cs^+, it is clear from the much lower melting temperatures that secondary structures formed in these salts are less stable than in K^+. A likely secondary structure that these sequences can form is the intramolecular G-quadruplex, a compact secondary structure that is sensitive to cation type. The formation of intramolecular G-quadruplex structures could explain the relatively slight increase in mobility of $d(TTAGGG)_5$ and $d(TTAGGG)_9$ in polyacrylamide gels upon addition of K^+ or Na^+. If four sets of TTAGGG are required for forming an intramolecular G-quadruplex, it is possible that $d(TTAGGG)_5$ and $d(TTAGGG)_9$ contain one set of TTAGGG that is single-stranded, resulting in less compact structures compared to $d(TTAGGG)_4$ and $d(TTAGGG)_8$. The combination of a dependence on cation type (Figures 3 and 4) and the requirement of N7 (Figure 5) for the telomerase banding patterns produced is suggestive of DNA secondary structure, most likely G-quadruplexes, modulating telomerase activity. Furthermore, G-quadruplex stabilizing compounds have been shown to cause changes in the human telomerase product distribution that greatly resemble those caused by K^+ (*28*).

It has been shown that the transition to the G-quadruplex is highly favored over the DNA·RNA hybrid, d(GGTTAGGGTTAG)·r(cuaacccuaacc), in the presence of K^+ (*35*). However, it is important to note that this study concerned an intermolecular G-quadruplex. Nevertheless, it is conceivable that when telomerase synthesizes 4, 5, 8, and 9 full repeats of TTAGGG, a transition from DNA·RNA hybrid to intramolecular G-quadruplex results in dissociation of the growing strand from the enzyme. This is consistent with the finding that G-quadruplex formation inhibits *Oxytricha* telomerase initiation (*27*). In this case, $d(TTTTGGGG)_4$ was a less efficient substrate in the presence of K^+ most likely because an intramolecular G-quadruplex structure was favored over the DNA·RNA duplex. Binding of telomeric DNA to the putative "anchor site" (*36*−*41*) may also be

effected by DNA secondary structure as suggested by Blackburn et al. (*42*). With the recent cloning of the catalytic unit of human telomerase (*43*, *44*), the effect of DNA structure on binding affinities of telomeric DNA to telomerase can be further explored.

In light of the finding that telomeres from a variety of human cell types have G-strand overhangs that are hundreds of bases in length (*45*), there is a strong possibility that G-quadruplex structures exist at the ends of human chromosomes. The results in this study suggest that one form of regulation of telomerase activity could be through the structures formed by the 3′ overhang of the telomere. In addition, proteins that bind to and stabilize the overhang in its single-stranded form such as cdc13 in yeast (*13*, *14*) and TEP in *Tetrahymena* (*17*) or *Oxytricha* (*15*, *16*, *46*) may actually promote telomerase activity by preventing G-quadruplex formation. With respect to *Oxytricha*, the presence of the telomere binding protein does not appear to prevent access of telomerase (*47*). Moreover, a G-quadruplex-specific nuclease has been discovered in yeast which, when deleted, causes telomere shortening and senescence (*48*, *49*). Conversely, RAP1 in *Saccharomyces cerevisiae* promotes G-quadruplex formation (*25*). Interestingly, the β subunit of the *Oxytricha* telomere protein alone also facilitates G-quadruplexes (*26*). Taken together, these studies suggest that G-quadruplexes play an active role in telomere maintenance and function. Small molecules that have a very strong affinity for G-quadruplexes over duplex or single-stranded DNA may both be useful as anticancer agents that target telomerase and provide further insight into the function of these unique structures *in vivo*.

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