Thermally Induced DNA·RNA Hybrid to G-Quadruplex Transitions: Possible Implications for Telomere Synthesis by Telomerase†

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ABSTRACT: Telomerase is a specialized reverse transcriptase that contains its own RNA template for synthesis of telomeric DNA [Greider, C. W., & Blackburn, E. H. (1989) *Nature 337*, 331−337; Shippen-Lentz, D., & Blackburn, E. H. (1990) *Science 247*, 546−552]. The activity of this ribonucleoprotein enzyme has been associated with cancer cells [Kim et al. (1994) *Science 266*, 2011−2015] and is thus a potential target for anticancer chemotherapy. Telomeric DNA·RNA hybrids are important intermediates in telomerase function and form after extension of the growing telomere on the telomerase RNA template. Translocation is a critical step in telomerase function and consists of unwinding of the telomeric DNA·telomerase RNA hybrid followed by repositioning of the 3′-end of the extended telomere. A central question in telomerase function is how translocation of the extended telomere occurs in the absence of ATP or GTP. It has been hypothesized that unwinding of the telomeric hybrid may be facilitated by the formation of stable hairpins or G-quadruplexes by the telomere product (i.e., a hybrid to G-quadruplex transition) and that this may provide at least part of the driving force for translocation [Shippen-Lentz & Blackburn, 1990; Zahler et al. (1991) *Nature 350*, 718−720]. However, so far there has been no effort aimed at examining the possibility that a hybrid/G-quadruplex equilibrium can occur and to what extent this equilibrium depends on buffer and concentration conditions. Examination of these transitions may provide insight into telomerase function and may also provide clues for the development of anti-telomerase agents. Using a model system consisting of the DNA·RNA hybrid d(GGTTAGGGTTAG)·r(cuaacccuaacc), we present evidence that a thermally induced transition of telomeric DNA·RNA hybrid to G-quadruplex can occur under certain conditions. These results provide support for the hypothesis that G-quadruplex formation by the telomere product may in fact regulate telomerase function at the translocation step (Zahler et al., 1991) and suggest an Achilles' heel for indirectly targeting telomerase. Thus, on the basis of the insight gained from the present studies and the results of Zahler et al. (1991), we propose that ligands that selectively bind or cleave G-quadruplex structures may modulate telomerase processivity.

Telomeres consist of characteristic tandem repeats (TTAGGG in humans) found at the ends of most eukaryotic chromosomes (Blackburn, 1991). The stability and integrity of eukaryotic chromosomes depend on these genetic elements, which are synthesized by the ribonucleoprotein enzyme telomerase. Recently, telomerase has been associated with cancer cells (Kim et al., 1994), and its activity may be essential to their survival. Thus, telomerase may be a potential chemotherapeutic target for anticancer agents. A mechanism for telomere synthesis by telomerase has been proposed by Blackburn and co-workers (Greider & Blackburn, 1989; Shippen-Lentz & Blackburn, 1990). In this mechanism, the processivity of telomere synthesis depends critically on translocation of the growing telomere. Although the exact mechanism of translocation is not yet well understood, this step must involve unwinding of the DNA·RNA hybrid formed by the extended telomere and the telomerase RNA template, followed by repositioning of the 3′-end of the extended telomere at the start site on the template. Since translocation can occur in the absence of a high-energy cofactor, it has been proposed that the formation of either G·G hairpin or G-quadruplex structures by the telomere product may provide part of the driving force for translocation (Shippen-Lentz & Blackburn, 1990; Zahler et al., 1991).

The mechanisms proposed by Shippen-Lentz and Blackburn (1990) and Zahler and co-workers (1991) call for telomeric DNA·RNA hybrid to G·G hairpin or G-quadruplex transitions. However, although DNA hairpin or DNA duplex to G-quadruplex transitions have been observed previously (Hardin et al., 1992; Miura & Thomas, 1994), to our knowledge, the theoretically important DNA·RNA hybrid to G·G hairpin or G-quadruplex transitions have never been reported. Here we present nuclear magnetic resonance (NMR)[1] and UV thermal denaturation analyses of the dodecamer hybrid d(GGTTAGGGTTAG)·r(cuaacccuaacc) (where lower case letters represent RNA) containing the predicted human telomerase RNA template 5′-cuaacccuaac-

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[1] Abbreviations: NMR, nuclear magnetic resonance; UV, ultraviolet; HPLC, high-performance liquid chromatography; ppm, parts per million.

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3′ (Feng et al., 1995). This DNA·RNA hybrid duplex represents a model system for the hybrid that may form after extension of the telomere primer on the telomerase RNA template. The NMR and UV thermal denaturation data indicate that, under certain conditions, DNA·RNA hybrid to G-quadruplex transitions occur readily and point to the possibility that G-quadruplex formation may promote dissociation of the hybrid. The results support the hypothesis made by Zahler and co-workers (1991) that G-quadruplex structures may regulate telomere length *in vivo*. Most importantly, their results and the data presented here suggest an Achilles' heel for targeting telomerase. Thus, given that G-quadruplex formation may promote unwinding of the bound telomere repeat, we propose that compounds that selectively bind to these structures or prevent their formation may inhibit telomerase processivity.

EXPERIMENTAL PROCEDURES

Sample Preparation. Ten micromole syntheses of the DNA and the RNA strands were carried out separately on an automated DNA synthesizer (Applied Biosystems 381A). Solid supports and phosphoramidites were purchased from Biogenex, Glen Research, and Applied Biosystems. DNA was deprotected in concentrated NH_4OH at 55 °C overnight, purified by reverse-phase HPLC on a C18 column (Dynamax-300A), and then dialyzed extensively against deionized water. Solid-phase RNA synthesis was carried out using base-protected 5′-DMT 3′-(cyanoethyl-*N*,*N*-diisopropyl) phosphoramidites protected with *tert*-butyldimethylsilyl (TBDMS) groups at the 2′-position. RNA deprotection and cleavage from the support were carried out with anhydrous 2 M methanolic ammonia at room temperature for 18−24 h. The solution was then dried by rotary evaporation, and the TBDMS protecting groups were removed by adding 5−6 mL of neat triethylamine trihydrofluoride (TEA 3HF) to the reaction vessel and letting the solution stir overnight at room temperature. Neat TEA 3HF is a more reliable desilylating agent than solutions of tetrabutylammonium fluoride because TEA 3HF is less sensitive to water contamination, does not lead to major phosphodiester linkage migration, and results in a product that is easier to purify (Westman & Strömberg, 1994). Thus, following TBDMS deprotection, major impurities were easily removed from the RNA by ethanol precipitation; the RNA was further purified by reverse-phase HPLC and then desalted by extensive dialysis against deionized water. In the preparation of the telomeric DNA·RNA hybrid, the purified DNA was dissolved in ca. 5 mL of deionized water and then gradually added to a 2 mL solution of the complementary RNA in deionized water. Under these conditions, the formation of G-quadruplex structures was minimized, and the major product formed was the DNA·RNA hybrid. Single-stranded material and any G-quadruplex that may have formed were then removed by hydroxylapatite column chromatography with a phosphate gradient. The DNA·RNA hybrid was subsequently desalted by extensive dialysis against deionized water.

NMR Spectroscopy. All NMR experiments were carried out at the specified temperature on a Bruker AMX 500 MHz spectrometer. Imino proton spectra were acquired in 90% H_2O/10% D_2O using a 1−1 echo pulse sequence with maximum excitation centered at 12.0 ppm. The variable-temperature imino NMR spectra were obtained with 128 scans and a 2-s relaxation delay. At least 10 min between temperature changes was allowed before acquiring each of the spectra. The data were processed with an exponential window function using 2 Hz of line broadening.

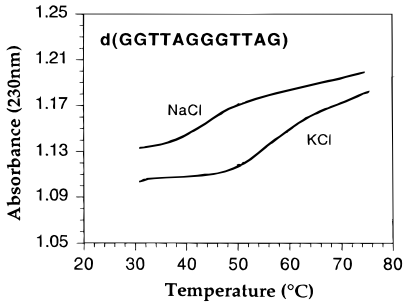

FIGURE 1: UV thermal denaturation profiles of the telomeric DNA strand d(GGTTAGGGTTAG) in 100 mM KCl/50 mM KH_2PO_4 and 100 mM NaCl/50 mM NaH_2PO_4, pH 7.0, buffers at DNA strand concentrations of ca. 20 μM.

UV Thermal Denaturation Studies. The UV thermal denaturation data were determined on a Gilford Model 2600 spectrometer interfaced to a Gilford Model 2527 thermoprogrammer. The final data were transferred to a personal computer for processing. The melting experiments were conducted in the appropriate buffers with heating rates of 0.5 °C/min. The absorbance was monitored at a wavelength of 230 nm (due to the relatively high oligonucleotide concentrations). The total sample concentrations were estimated using the calculated values of the extinction coefficients derived from nearest-neighbor approximations (Fasman, 1975).

RESULTS AND DISCUSSION

Hybrid to G-Quadruplex Transitions Are Promoted by K^+. G-Quadruplex structures consist of guanine tetrads held together by reverse Hoogsteen base pairs and display a higher stability in solutions containing potassium cations versus sodium cations [reviewed in Williamson (1993)]. As a basis for the study of DNA·RNA hybrid to G-quadruplex transitions, we first wanted to examine the relative stability of the G-quadruplex structure formed by the DNA strand of the DNA·RNA hybrid d(GGTTAGGGTTAG)·r(cuaacccuaacc) in K^+- and Na^+-containing buffers. Figure 1 shows the thermal denaturation profiles of the DNA strand d(GGTTAGGGTTAG) in 100 mM KCl/50 mM KH_2PO_4, pH 7.0, and 100 mM NaCl/50 mM NaH_2PO_4, pH 7.0, at a concentration of ca. 20 μM. The first derivatives of these curves (not shown) indicate that under these conditions the G-quadruplex is ca. 11 °C more stable in the presence of K^+ that in the presence of Na^+.

In order to examine the possibility of thermally induced hybrid to G-quadruplex transitions, the purified d(GGTTAGGGTTAG)·r(cuaacccuaacc) hybrid was prepared as described under Experimental Procedures and then divided into two samples of equal concentration (ca. 5 mM duplex concentration each). The two separate samples were then dissolved in pH 7.0 buffers containing either 100 mM NaCl/1 mM EDTA/50 mM NaH_2PO_4 or 100 mM KCl/1 mM EDTA/50 mM KH_2PO_4. Figure 2 shows the imino proton region of the NMR spectra of this hybrid obtained in 90% H_2O/10% D_2O at 5, 27, 50, and 75 °C in either Na^+- or K^+-containing buffers. Very strong signals in the 12−14 ppm region, due to imino protons engaged in Watson−Crick

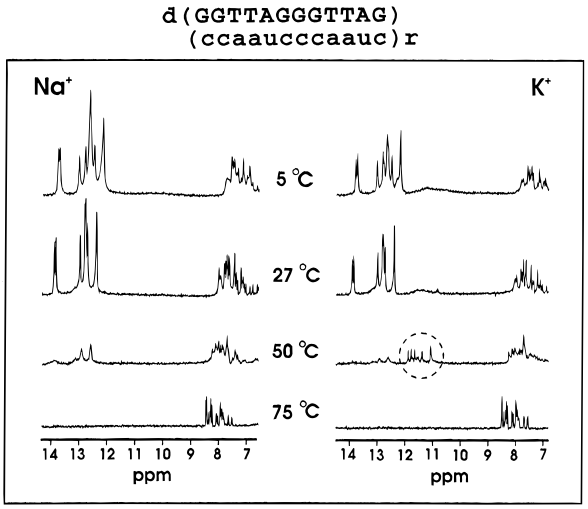

FIGURE 2: Imino proton region of the 500 MHz NMR spectra of the telomeric DNA·RNA hybrid d(GGTTAGGGTTAG)·r-(cuaacccuaacc) at a total concentration of ca. 5 mM in pH 7.0 buffers consisting of 100 mM NaCl/50 mM NaH_2PO_4/1 mM EDTA (left column) or 100 mM KCl/50 mM KH_2PO_4/1 mM EDTA (right column) and at four different temperatures. The peaks centered at ca. 11.7 ppm in the 50 °C spectrum of the hybrid in the K^+ buffer (broken circle) are diagnostic of G-quadruplex structures.

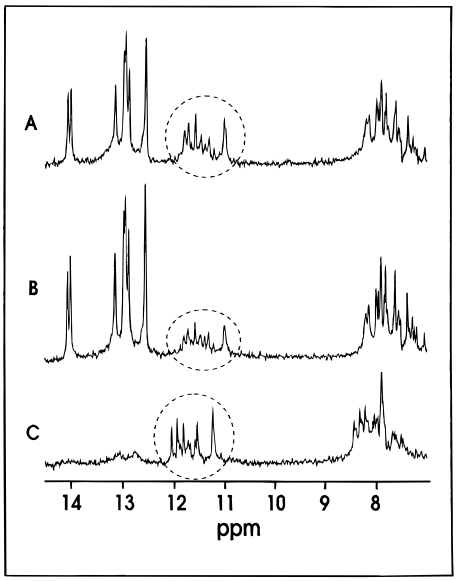

FIGURE 3: Imino proton region of the 500 MHz NMR spectra of the telomeric DNA·RNA hybrid d(GGTTAGGGTTAG)·r-(cuaacccuaacc) in 100 mM KCl/50 mM KH_2PO_4/1 mM EDTA, pH 7.0, buffer. The same sample as in Figure 2 is shown immediately after cooling the sample to room temperature (A), after standing at room temperature for 4 days (B), and back to 50 °C (C). The sample temperatures are 27 °C in Figure 3A,B. The broken circles denote the peaks arising from G-quadruplex formation.

hydrogen bonding, indicate the formation of a DNA·RNA hybrid duplex in both buffers. These signals are quite strong in the solution containing Na^+ at 5 and 27 °C, but at 50 °C base-pair-opening results in imino proton exchange with solvent, and only two weak imino proton signals remain at this point. Further heating to 75 °C entirely denatures the hybrid, and only the nonexchangeable signals due to single-stranded material can be observed in the 7−9 ppm region of the NMR spectra. In contrast, the NMR signals due to Watson−Crick hydrogen-bonded imino protons are much weaker in the K^+-containing sample, and in addition, weak and broad signals can be observed in the 10−12 ppm region at 5 and 27 °C. Upon heating the sample to 50 °C, a sharp new set of peaks appeared in the 11−12 ppm region, and only two extremely weak peaks from the imino protons of Watson−Crick base pairs could be observed at this temperature. The sample became completely single stranded at 75 °C.

The spectra in the presence of K^+ in Figure 2 indicate the formation of G-quadruplex species by the DNA strand of the hybrid sample that are clearly detectable at 50 °C. For this particular sequence, and at the high oligonucleotide concentrations used, the G-quadruplex species formed at 50 °C are most likely of the parallel type, as indicated by the six sharp (two overlapped) peaks in the 10−12 ppm region of the NMR spectra. The NMR spectra were examined up to 16 ppm for the presence of $C{\cdot}C^+$ base pairs that might be formed by the C-rich RNA strand. However, at the neutral pH used, no peaks were observed in this region of the NMR spectra. Furthermore, there was no NMR evidence for the formation of a G-quadruplex structure in Na^+-containing solutions over the temperature range of 5−75 °C (data not shown).

At the DNA·RNA hybrid concentrations used for the NMR experiments, the G-quadruplex that forms in the solution containing K^+ melts at ca. 60 °C (data not shown). This melting point is higher than that for the heteroduplex, and consequently, stable G-quadruplex formation can be readily observed. On the other hand, based on the relative stabilities of the G-quadruplex structures in Na^+- versus K^+-containing buffers (*vide supra*), the G-quadruplex in the presence of Na^+ would be expected to melt at ca. 50 °C at the hybrid concentrations used. However, Figure 2 shows that this temperature is below the melting point of the heteroduplex. Thus, for this particular sequence, and at the hybrid and Na^+ concentrations used, no DNA·RNA hybrid to G-quadruplex transition is observed in Na^+ due to the higher stability of the DNA·RNA hybrid relative to the G-quadruplex.

Much weaker signals from imino protons of Watson−Crick base pairs were observed in K^+- versus Na^+-containing buffers at the same relative oligonucleotide concentrations. This is probably due to aggregation of the DNA strand into high molecular weight slipped-linked G-quadruplex structures of the type described by Dai et al. (1995). This leads to partial sequestering of the DNA, effectively lowering the hybrid concentration in K^+-containing buffer relative to the sample containing Na^+. Thus, at millimolar oligonucleotide concentrations and low temperatures, K^+ may allow the formation of these high molecular weight species, giving rise to the broad peak observed at ca. 11 ppm at 5 and 27 °C. Therefore, even at low temperatures, a hybrid to G-quadruplex transition may have already occurred in K^+-containing buffers, but the subsequent equilibrium that is established appears to favor the hybrid duplex. Further heating to 50 °C then shifts the equilibrium to favor formation of the G-quadruplex.

One aspect of the DNA·RNA hybrid to DNA G-quadruplex transition observed at 50 °C is that the DNA is not fully converted to G-quadruplex, even after extended heating above the melting point of the hybrid. Also, Figure 3 shows that in K^+-containing buffers an equilibrium is subsequently established between hybrid and G-quadruplex that is only partially reversible. Thus, heating the sample to 50 °C promotes the formation of G-quadruplex, but a large fraction

Scheme 1

of the DNA·RNA hybrid then re-forms upon cooling the sample to room temperature (Figure 3A). The data in Figures 2 and 3 indicate that, in K^+-containing buffer and at the oligonucleotide concentrations used, an equilibrium between the hybrid and the G-quadruplex is established (shown in Scheme 1) subsequent to the thermally induced transition from DNA·RNA hybrid to G-quadruplex.

The ability of complementary C-rich DNA strands to unfold G-quadruplex structures formed by G-rich DNA has been studied previously (Sen & Gilbert, 1988; Raghuraman & Cech, 1990; Hardin et al., 1991). In all cases studied, the DNA quadruplex unfolds and rehybridizes with the complementary C-rich DNA strand more readily in Na^+-containing buffers. However, in K^+-containing buffers, the G-quadruplex is quite stable, and rehybridization with the complementary DNA strand occurs at a very slow rate. Here we observed a similar situation. The sharp peaks in Figure 3A indicate that the DNA·RNA hybrid and the G-quadruplex species are in slow exchange at room temperature. However, the relative populations of the two species change over time, and the equilibrium shown in Scheme 1 is gradually established in which the DNA·RNA hybrid predominates. Figure 3B shows that when the sample was left standing at room temperature for ca. 4 days, the intensity of the G-quadruplex imino proton peaks decreased with a subsequent increase in the DNA·RNA hybrid imino proton peaks (Figure 3B). Heating to 50 °C led to recovery of the full intensity of the imino proton peaks for the G-quadruplex (Figure 3C).

Effect of K^+ Concentration and Effect of Mg^{2+} on DNA·RNA Hybrid to G-Quadruplex Transitions at Micromolar Oligonucleotide Concentrations. Since the type and stability of G-quadruplex structures depend strongly on the type and concentration of cation as well as the concentration of DNA (Sen & Gilbert, 1990; Hardin et al., 1991; Miura & Thomas, 1994; Miura et al., 1995), we reasoned that heteroduplex oligomer concentration as well as K^+ concentration might influence the observed DNA·RNA hybrid to G-quadruplex transitions. In addition, since Mg^{2+} is required for telomerase activity and because previous investigators have observed that Mg^{2+} can facilitate the formation of G-quadruplex structures (Schierer & Henderson, 1994), we also wanted to examine the effect of Mg^{2+} at micromolar hybrid concentrations.

Figure 4 shows a UV thermal denaturation study of the DNA·RNA hybrid d(GGTTAGGGTTAG)·r(cuaacccuaacc) at lower concentrations (ca. 40 μM) and at increasing K^+ concentrations, in the presence and absence of 15 mM Mg^{2+}. These melting profiles indicate that K^+ concentration and the addition of 15 mM Mg^{2+} have a significant effect on DNA·RNA hybrid to G-quadruplex transitions. Thus, Figure 4A shows that in the lowest K^+ concentration used (25 mM KCl/25 mM KH_2PO_4, pH 7.0, buffer), two major melting transitions can be observed irrespective of the presence or absence of Mg^{2+}, with the first transition corresponding to melting of the DNA·RNA hybrid duplex and the second transition corresponding to melting of the G-quadruplex. Here, Mg^{2+} does not appear to influence hybrid to G-quadruplex transitions since, except for a slight change in the melting point of the G-quadruplex, the addition of 15 mM Mg^{2+} did not change the overall shape of the melting curve. On the other hand, at intermediate K^+ concentrations (50 mM KCl/50 mM KH_2PO_4, pH 7.0, buffer) and in the absence of Mg^{2+} (Figure 4B), the shape of the melting curve changed significantly. In this case, only a single broad transition was observed. It is not clear what gives rise to this broad melting transition, but it is possible that at the K^+ and oligomer concentrations used the broad transition arises from an overlap of the melting points for both the DNA·RNA hybrid duplex and the G-quadruplex. In contrast, addition of 15 mM Mg^{2+} gave rise to two well-resolved transitions, indicating that the addition of Mg^{2+} stabilizes G-quadruplex formation [as observed previously by Schierer and Henderson (1994)]. Finally, Figure 4C shows that at higher concentrations of K^+ (100 mM KCl/50 mM KH_2PO_4, pH 7.0), only one main high-temperature transition is observed irrespective of the presence or absence of Mg^{2+}. This single high-temperature transition corresponds to the melting point of the G-quadruplex formed by the DNA of the hybrid duplex

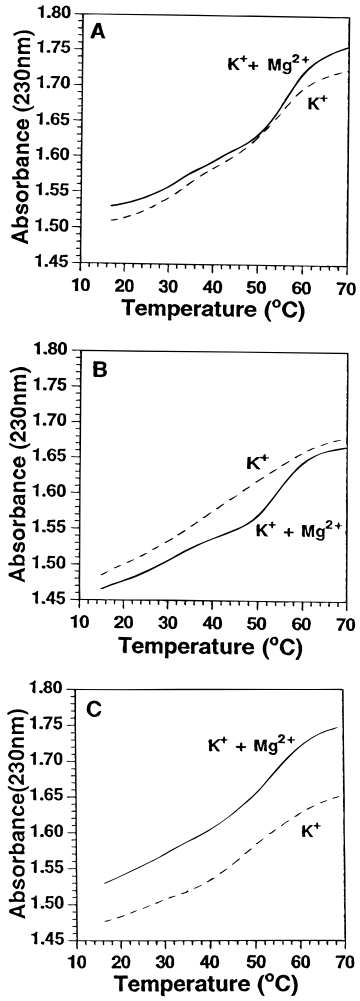

FIGURE 4: UV thermal denaturation profiles of the DNA·RNA hybrid d(GGTTAGGGTTAG)·r(cuaacccuaacc) showing the effect of increasing K^+ concentration and addition of 15 mM Mg^{2+} on the DNA·RNA hybrid to G-quadruplex transitions. The hybrid concentrations were ca. 40 μM, and the buffers used were as follows: (A) 25 mM KCl/25 mM KH_2PO_4, pH 7.0; (B) 50 mM KCl/50 mM KH_2PO_4, pH 7.0; and (B) 100 mM KCl/50 mM KH_2PO_4, pH 7.0.

and indicates that high K^+ concentration affects the equilibrium shown in Scheme 1 by favoring G-quadruplex formation.

CONCLUSION

G-Rich telomeric DNA is unique in that it tends to fold into a variety of G-quadruplex structures, depending on buffer conditions, sequence context, and DNA concentration [reviewed in Rhodes and Giraldo (1995)]. Hardin et al. (1992) have observed that in the G-rich DNA oligonucleotide d(CGCG$_3$GCG) added K^+ and high oligonucleotide concentrations favor the formation of the G-quadruplex over the hairpin. Similarly, Miura and Thomas (1994) have found that in the G-rich telomeric DNA duplex d($G_4T_4G_4$)·d($C_4A_4C_4$) G-quadruplex formation from duplex is favored in K^+. Here we find that the tendency of telomeric DNA to form G-quadruplex structures allows for an equilibrium between DNA·RNA hybrid and G-quadruplex to be established. The position of this equilibrium is affected by temperature, cation type and concentration, and oligonucleotide concentration.

The above observations may have potentially practical applications with regard to the manipulation of primers used in telomerase assays. Typically, short telomeric DNA primers consisting of 1−4 telomeric repeats are used to assay telomerase activity [see, for example, Greider and Blackburn (1987) and Morin (1989)]. The experiment in Figure 3 indicates that RNA does not hybridize readily with DNA that forms stable G-quadruplex structures. Also, the experiment in Figure 4C indicates that physiological concentrations of K^+ may shift the equilibrium shown in Scheme 1 to favor formation of G-quadruplex species. Thus, it is possible that under the appropriate microenviromental conditions, short telomeric primers might also form stable G-quadruplex structures. Since an essential requirement for primer extension is binding of the primer to the telomerase RNA template, the formation of stable G-quadruplex structures would preclude their use as primers by telomerase. This effect has been shown by Zahler et al. (1991) recently.

DNA·RNA hybrid to G-quadruplex transitions and the observed equilibrium may also be relevant to telomerase processivity. The telomere repeat ladders commonly observed in telomerase assays (Greider & Blackburn, 1985, 1987) are an indication that telomeric DNA (the original primer or partially extended primer) is in an equilibrium between the hybridized and the unhybridized state. In effect, an equilibrium such as that shown in Scheme 1 may be at play where the telomeric DNA in the unhybridized state may actually exist as G-quadruplex species. Thus, at any one time telomeric DNA can hybridize to the telomerase RNA template and be subsequently extended. Under the appropriate microenvironmental conditions, the extended primer may translocate and be extended further or may dissociate from the RNA template, a process that may be governed by an equilibrium such as that shown in Scheme 1.

Morin (1989) has observed that predominantly shorter products are produced with increased primer concentration. This effect may be explained, at least partly, by the equilibrium shown in Scheme 1. Assuming that Scheme 1 applies, Le Châtelier's principle dictates that increased primer concentration should lead to the formation of G-quadruplex species. Experimentally, at micromolar hybrid concentrations and in the presence of 50 mM K^+, the equilibrium shown in Scheme 1 is easily driven toward the right by the addition of ca. 70% excess DNA (data not shown). Thus, it is quite possible that in the appropriate microenvironment, increased amounts of telomeric primer may facilitate the formation of G-quadruplex structures not only by free primer DNA but also by any DNA primer already bound to the RNA template. This would decrease the availability of the primer for extension by telomerase and also would lead to shorter products.

Zahler et al. (1991) have hypothesized that the formation of G-quadruplex species by the telomere product may also regulate telomerase function by facilitating the translocation step in the telomerase reaction. Since prior to translocation the bound repeat must dissociate from the RNA template, it is possible that DNA·RNA hybrid to G-quadruplex transitions may play a role in the translocation step. However, other factors, such as the anchor binding site (Collins & Greider, 1993), telomere binding proteins (Fang & Cech, 1993; Giraldo et al., 1994; Schierer & Henderson, 1994), and telomerase RNA (Lingner et al., 1994; Gilley et al., 1995), may also influence translocation.

Finally, it should be pointed out that hypothetical G-quadruplex formation by the extended telomere [which may influence translocation (Zahler et al., 1991)] would be of the intramolecular type. However, the system in the present study is only relevant to intermolecular G-quadruplex formation, and entropic effects due to intramolecular G-quadruplex formation in the telomerase reaction cannot be addressed at this time. Thus, we caution against overinterpreting the present results with regard to telomerase processivity. Nevertheless, our observations suggest a potential Achilles' heel that may be exploited to target telomerase indirectly. Therefore, on the basis of the assumption that hybrid to G-quadruplex transitions are important for telomerase activity, we propose that telomerase processivity may be modulated indirectly either at the initial primer binding step or at the translocation step by ligands that selectively bind G-quadruplex species or by ligands that selectively cleave these types of nucleic acid structures.

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